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1 Pesticide Biochemistry and Physiology

2 Review

3

#### 4 **The global status of insect resistance to neonicotinoid insecticides**

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#### 10 **Abstract**

11 The first neonicotinoid insecticide, imidacloprid, was launched in 1991. Today this class of  
12 insecticides comprises at least seven major compounds with a market share of more than  
13 25% of total global insecticide sales. Neonicotinoid insecticides are highly selective agonists  
14 of insect nicotinic acetylcholine receptors and provide farmers with invaluable, highly  
15 effective tools against some of the world's most destructive crop pests. These include  
16 sucking pests such as aphids, whiteflies, and planthoppers, and also some coleopteran,  
17 dipteran and lepidopteran species. Although many insect species are still successfully  
18 controlled by neonicotinoids, their popularity has imposed a mounting selection pressure for  
19 resistance, and in several species resistance has now reached levels that compromise the  
20 efficacy of these insecticides. Research to understand the molecular basis of neonicotinoid  
21 resistance has revealed both target-site and metabolic mechanisms conferring resistance.  
22 For target-site resistance, field-evolved mutations have only been definitely characterized in  
23 two aphid species. Metabolic resistance appears much more common, with the enhanced  
24 expression of one or more cytochrome P450s frequently reported in resistant strains. Despite  
25 the current scale of resistance, neonicotinoids remain a major component of many pest  
26 control programmes. Resistance management strategies, based on mode of action rotation,  
27 are of crucial importance to preventing resistance becoming more widespread. In this review  
28 we summarize the current status of neonicotinoid resistance, the biochemical and molecular  
29 mechanisms involved, and the implications for resistance management.

#### 30 **Keywords:**

31 *Neonicotinoids, imidacloprid, nicotinic acetylcholine receptor, resistance management,*  
32 *resistance mechanisms, sucking pests*

## 33 **1. Neonicotinoid insecticides**

34 Neonicotinoids are one of the most important chemical classes of insecticides globally due to  
35 their high efficacy against a range of important insect pests and their versatility of use [1,2].  
36 They are registered in more than 120 countries worldwide [2] and are particularly active  
37 against numerous sucking pests, and also several coleopteran, dipteran, and lepidopteran  
38 pest species by foliar, soil and seed treatment applications [3]. Neonicotinoids are selective  
39 agonists of the insect nicotinic acetylcholine receptor (nAChR), a pentameric cys-loop ligand-  
40 gated ion channel located in the central nervous system of insects [1]. The mode of action  
41 classification scheme of the Insecticide Resistance Action Committee (IRAC) lists seven  
42 commercial neonicotinoids in Group 4A (nAChR agonists) (Sparks and Nauen, this issue).  
43 The first neonicotinoid launched was imidacloprid in 1991, followed by nitenpyram and  
44 acetamiprid in 1995, and others such as thiamethoxam in 1998 (Figure 1). Based on total  
45 global insecticide sales the market share of neonicotinoids was greater than 25% in 2014,  
46 with thiamethoxam, imidacloprid and clothianidin accounting for almost 85% of the total  
47 neonicotinoid sales in crop protection in 2012 (Figure 2). The main regions of neonicotinoid  
48 use are Latin America, Asia and North America (75%), with Europe accounting for 11% of  
49 total global sales (Figure 2). Increases in use have inevitably led to a mounting selection  
50 pressure for resistance to neonicotinoids. This review summarizes the global status of  
51 neonicotinoid resistance in a range of important insect pests with a particular focus on the  
52 biochemical and molecular mechanisms underlying resistance, and on information reported  
53 since the last comprehensive review of this subject published ten years ago [4].

54

## 55 **2. Neonicotinoid resistance: from mechanisms to field failure**

56 The first report of neonicotinoid resistance was published in 1996, describing low efficacy of  
57 imidacloprid against Spanish greenhouse populations of cotton whitefly, *Bemisia tabaci* [5].  
58 Since then more than 500 peer-reviewed papers have been published on neonicotinoid  
59 resistance issues (SciFinder® 2014, American Chemical Society) in different pest insects  
60 (Figure 3). A substantial proportion of these refer specifically to imidacloprid resistance. The  
61 Arthropod Pesticide Resistance Database (APRD) [6] lists more than 330 cases of  
62 imidacloprid resistance, followed by ca. 130 and 50 cases of thiamethoxam and acetamiprid  
63 resistance, respectively. Unsurprisingly the number of arthropod species with resistance to  
64 neonicotinoids has increased with time (Figure 4). However, most cases of neonicotinoid  
65 resistance (all compounds combined) concern *B. tabaci* followed by the green peach aphid,  
66 *Myzus persicae*, the cotton aphid, *Aphis gossypii* and the rice brown planthopper,  
67 *Nilaparvata lugens*. Other pests targeted by neonicotinoid insecticides with at least 10

68 assigned cases of resistance in the APRD are houseflies, *Musca domestica*, Colorado potato  
69 beetle, *Leptinotarsa decemlineata* and glasshouse whitefly, *Trialeurodes vaporariorum*  
70 (Figure 5). In the sections below we treat each of these seven species separately, but then  
71 combine others with fewer than 10 cases reported.

72

### 73 2.1 *Bemisia tabaci*

74 The cotton whitefly, *B. tabaci* (Gennadius) is a highly destructive and invasive sucking pest,  
75 damaging plants by direct feeding, honeydew excretion (as a nutritional source for sooty  
76 mold) and transmission of numerous plant viruses [7]. At least 24 cryptic and morphologically  
77 indistinguishable *B. tabaci* biotypes have been identified by recent phylogenetic comparisons  
78 based on DNA sequencing [8,9]. However, two widespread biotypes, the Middle East – Asia  
79 Minor 1 biotype (MEAM1, also referred to as biotype B) and the Mediterranean biotype  
80 (MED, also referred to as biotype Q) are of particular importance as crop pests [10]. Both  
81 biotypes have developed resistance to multiple classes of insecticide [11,12] including  
82 neonicotinoids [4]. Neonicotinoid resistance has been widely reported in both B and Q type  
83 *B. tabaci* from several geographic regions [4,12-19] particularly against imidacloprid.  
84 Resistance ratios for neonicotinoids in *B. tabaci* often exceed 1000-fold and lead to serious  
85 control failures [4].

86 Neonicotinoid resistance in *B. tabaci* is mainly conferred by enhanced detoxification  
87 by microsomal monooxygenases [17,20], and recently a single, constitutively overexpressed,  
88 cytochrome P450, CYP6CM1, was shown to be highly correlated with imidacloprid resistance  
89 in B- and Q-type whiteflies [21]. Functional expression of CYP6CM1 revealed its capacity to  
90 detoxify imidacloprid by hydroxylation of position 5 of the imidacloprid imidazolidine ring  
91 system [22], but also its inability to metabolise other neonicotinoids such as acetamiprid [23].  
92 Resistance to imidacloprid in cotton whiteflies was shown to be age-specific [24] and  
93 correlated with the expression of CYP6CM1 in different life stages [25]. Recently it was  
94 shown that CYP6CM1 also detoxifies pymetrozine by hydroxylation, an insecticide with a  
95 different mode of action and chemically very different from neonicotinoids [26]. These results  
96 provided the molecular basis for the observed cross-resistance between neonicotinoids and  
97 pymetrozine in *B. tabaci* [27]. Transgenic lines of *Drosophila melanogaster* expressing  
98 CYP6CM1 were shown to be less susceptible to imidacloprid, providing further functional  
99 evidence of its role in imidacloprid resistance in *B. tabaci* [28]. Next generation sequencing  
100 (RNAseq) has provided further insights into the diversity of detoxification genes over-  
101 expressed in a *B. tabaci* strain resistant to neonicotinoid insecticides such as thiamethoxam  
102 [29]. Another study on thiamethoxam resistance in *B. tabaci* also revealed stage-specific  
103 expression of CYP6CM1, but also other detoxification enzymes such as glutathione S-  
104 transferases [30]. Even though other cytochrome P450s such as CYP4C64 have been

105 reported to be over-expressed in neonicotinoid-resistant *B. tabaci*, the main P450 gene  
106 consistently over-expressed is *CYP6CM1* [31]. To date, no target-site mutations in *B. tabaci*  
107 nAChR subunits have been described.

108

## 109 2.2 *Myzus persicae*

110 The green peach aphid, *M. persicae* (Sulzer), is the most economically important aphid crop  
111 pest worldwide. Unlike other species in which differences in response to neonicotinoids  
112 emerged several years after first exposure to these compounds, low but statistically-  
113 significant variation in susceptibility to imidacloprid in *M. persicae* was reported in tandem  
114 with the first commercial releases of this insecticide [32,33]. Suspicions that such variation  
115 was a by-product of tolerance to nicotine, selected during the adaption of some populations  
116 of *M. persicae* (so-called *M. persicae* subsp. *nicotianae*) to feeding on tobacco, have been  
117 reinforced by research attributing resistance to over-production of a single P450 (CYP6CY3)  
118 [34,35]. Survival following exposure to discriminating concentrations of nicotine (and  
119 neonicotinoids) for a range of aphid clones from the UK, Greece, southern Africa and Japan  
120 was closely and positively correlated with levels of CYP6CY3 mRNA expression [34,35].  
121 Expression of recombinant CYP6CY3 enzyme in Sf9 insect cells showed it to be highly  
122 efficient at metabolizing nicotine and two neonicotinoids – imidacloprid and clothianidin – to  
123 less toxic metabolites [34]. Overexpression appears attributable both to a modification of the  
124 promoter region and to structural amplification of the CYP6CY3 gene, with some clones  
125 possessing up to 100 copies. Thus, in contrast to the usual case of resistance traits being  
126 selected *de novo* by chemicals used for aphid control, this appears to be a rare example of  
127 pre-selection resulting from host-plant adaptation and an expansion in host range [34]. At  
128 present it is unclear to what extent CYP6CY3-mediated resistance occurs in or has spread to  
129 non-tobacco-adapted *M. persicae* as a consequence of gene flow between races, or as a  
130 result of subsequent selection by neonicotinoids themselves.

131 The microarray study that initially implicated CYP6CY3 in resistance also showed a  
132 number of ESTs encoding cuticular proteins to be up-regulated in a resistant clone,  
133 suggesting that modified penetration through the cuticle might be operating in concert with  
134 enhanced detoxification to determine the resistance phenotype [35]. Further evidence for an  
135 additional mechanism in clones overexpressing CYP6CY3 came from incomplete  
136 suppression of resistance by enzyme inhibitors [36], the differential expression of resistance  
137 in feeding and contact bioassays [35], and *in vivo* penetration assays with radiolabelled  
138 imidacloprid [35]. However, without an unambiguous marker for a mechanism based on  
139 reduced penetration it has not been possible to quantify its importance and contribution to  
140 resistance, singly or alongside different levels of overexpression of CYP6CY3.

141 Receptor radioligand binding studies and nucleotide sequencing of nAChR subunit  
142 genes have also been undertaken to explore the possible occurrence of target-site  
143 resistance to neonicotinoids in *M. persicae*. These yielded negative results until a clone  
144 (termed FRC) was collected in 2009 from peach at a site experiencing a marked loss of  
145 control efficacy with neonicotinoids [37]. Resistance in FRC was markedly more resistant  
146 than any clone studied previously. In topical application bioassays with imidacloprid and  
147 thiamethoxam, resistance was impossible to quantify due to survival at the highest doses it  
148 was feasible to apply [37]. CYP6CY3 was overexpressed in FRC at levels similar to those in  
149 resistant clones studied previously, but in addition, sequencing of nAChR subunit genes  
150 identified a point mutation in the loop D region of the  $\beta$ 1 subunit that causes an arginine to  
151 threonine substitution (R81T). Loop D of  $\beta$ 1 has a known role in binding of the natural ligand  
152 acetylcholine and of synthetic neonicotinoids [38] and the R81 residue specifically has been  
153 shown through homology modelling to modulate neonicotinoid binding [39]. Indeed, the  
154 presence of threonine at this residue in most vertebrate receptors compared to the ubiquity  
155 of arginine in insects is considered a primary determinant of the selective toxicity of  
156 neonicotinoids. Hence it seems unequivocal that R81T is directly implicated in conferring a  
157 level of neonicotinoid resistance unrecorded previously in *M. persicae*. Its discovery  
158 represented the first proven case of a target-site modification leading to control failure with  
159 neonicotinoids under field conditions.

160 Using a PCR-based diagnostics the current distribution of the R81T mutation has  
161 been shown to extend in a band from southern Spain, through southern France to northern  
162 and Central Italy [40,41]. This distribution remains closely coincident with the cultivation of  
163 peach and closely-related crops. Extensive monitoring has failed to detect its presence  
164 further north in Europe despite continuing and extensive reliance on neonicotinoids for aphid  
165 control in countries such as the UK (S. Foster pers. comm. 2014). It seems likely that the  
166 transition from holocycly in the south of Europe to obligate anholocycly in the north is  
167 constraining the ability of the mutation to spread from its point of origin and/or establish in  
168 new localities. This is being investigated further.

169

### 170 2.3 *Aphis gossypii*

171 Like *M. persicae*, the cotton-melon aphid, *A. gossypii* (Glover) is highly polyphagous with a  
172 long history of resistance to insecticides. Its host plants, which include cucurbits, cotton and  
173 solanaceous crops, are often intensively treated with neonicotinoids and resistance to these  
174 products, although only confirmed relatively recently, now appears to be geographically  
175 widespread. Systematic monitoring of aphids on cotton in Australia and the USA has  
176 documented a temporal decline in sensitivity related to increased reliance on neonicotinoids  
177 as seed treatments and foliar sprays [42,43]. Discriminating concentration assays

178 complemented by full dose-response testing of insects from Australian cotton showed a  
179 gradual change from 2006-7 to 2008-9, with resistance factors in the latter season peaking at  
180 6.4-fold for acetamiprid, 22-fold for thiamethoxam and 6-fold for clothianidin, respectively  
181 [43]. This trend continued in 2009-2010 when 96% of samples contained resistant individuals  
182 [43]. To combat this trend there are recommendations to avoid foliar sprays of neonicotinoids  
183 against *A. gossypii* but these are compromised by the continuing importance of  
184 neonicotinoids for controlling other pests including whiteflies and mirids [43].

185         Monitoring of *A. gossypii* between 2008 and 2011 from cotton-growing regions of the  
186 southern USA that were reporting diminished efficacy of neonicotinoids showed a 48-fold  
187 range of LC<sub>50</sub> values for thiamethoxam across the four years, with resistance tending to be  
188 higher for fields that had received at least one foliar application of a neonicotinoid insecticide  
189 [42]. Interestingly, resistance factors were much higher after 48h exposure in a leaf-dip  
190 bioassay than after 72h, although the broad association between resistance and field  
191 treatment history was evident at both endpoints.

192         The mechanism(s) underpinning resistance in Australia and the USA remain to be  
193 elucidated, whereas in eastern Asia there is mounting evidence for the same target-site  
194 R81T amino acid substitution as found in *M. persicae*. Samples of *A. gossypii* collected from  
195 six sites in South Korea in 2012 gave maximum resistance of 1500-fold to imidacloprid,  
196 2600-fold to acetamiprid and 14,000-fold to clothianidin [44]. Even more remarkably,  
197 laboratory selection with imidacloprid of a strain (IMI-R) collected in 2011 led to resistance  
198 factors of 36,000 to imidacloprid, 69,000 to acetamiprid, and 285,000 to thiacloprid [44].  
199 Bioassays using synergists and enzyme assays yielded no evidence of enhanced  
200 detoxification in IMI-R compared to a susceptible strain, whereas full length cloning showed  
201 R81T to be present in the  $\beta$ 1 nAChR subunit of IMI-R and five of the field samples collected  
202 in 2012. Sixty generations of laboratory selection with imidacloprid of an originally  
203 susceptible strain collected in Shandong province in China in 2009 resulted in 66-fold  
204 resistance to this compound [45]. Cloning of six  $\alpha$  and the  $\beta$ 1 subunits again showed R81T to  
205 be present in the latter.

206         One notable discrepancy between these two studies suggesting R81T to be the  
207 primary sole cause of neonicotinoid resistance is in the magnitude of resistance factors: up to  
208 36,000-fold for imidacloprid in Korea but only 66-fold in the selected strain from China. One  
209 explanation might be the different bioassay methods utilized: dipping of leaves and apterous  
210 aphids in test solutions by Shi et al. [45], and placing untreated aphids on previously dipped  
211 and dried leaves by Koo et al. [44]. Side-by-side testing using both methods would be  
212 valuable for disclosing the importance of the route of exposure in influencing the phenotypic  
213 expression of resistance traits, as already documented when comparing systemic and topical  
214 application methods for *M. persicae* [46]. The parallel appearance of R81T in *M. persicae*

215 and *A. gossypii* is of evolutionary significance, highlighting again the limited scope for target-  
216 site mutations that confer appreciable resistance while retaining normal receptor function.

217

#### 218 2.4 *Nilaparvata lugens*

219 The brown planthopper, *N. lugens* (Stål), is the most economically significant pest of rice  
220 (*Oryza sativa* L.) throughout Asia, causing damage through direct feeding and the  
221 transmission of rice viruses [47]. The control of *N. lugens* has relied heavily on the use of  
222 synthetic insecticides with resistance developing to all of the older compounds used for  
223 control [48]. The first neonicotinoid, imidacloprid, was introduced against *N. lugens* in the  
224 early 1990's and because of its excellent efficacy and the fact that it was largely unaffected  
225 by resistance that had evolved to older compounds rapidly became a mainstay for control.  
226 After a decade of use populations of *N. lugens* were reported with reduced  
227 efficacy/resistance to imidacloprid, and resistance is now widespread in populations collected  
228 from across Asia with resistance factors of 600-800-fold recently described [48-52].

229 The first mechanism of resistance to neonicotinoids reported for *N. lugens* involved a  
230 target-site modification [53] with a strain of *N. lugens* selected with imidacloprid for 35  
231 generations exhibiting over 250-fold resistance compared to a lab susceptible strain in  
232 insecticide bioassays. Radioligand binding experiments to whole body membrane  
233 preparations revealed a significant lower level of [<sup>3</sup>H]imidacloprid-specific binding to  
234 preparations of the resistant strain suggesting a target-site resistance mechanism [53].  
235 Sequencing of nAChR subunit genes identified a single point mutation at a conserved  
236 position (Y151S) in two nAChR subunits, N1α1 and N1α3 with confirmation of the causative  
237 effect of these mutations coming from expression of hybrid nAChRs containing *N. lugens* α  
238 and rat β2 subunits, with the presence of Y151S associated with a substantial reduction in  
239 specific [<sup>3</sup>H]imidacloprid binding [53]. Surprisingly, since these findings were reported, this  
240 mechanism has never been identified in any field-collected population. Rather, several  
241 studies have provided both indirect and direct evidence that enhanced P450 activity  
242 contributes to the neonicotinoid resistance of field collected populations of *N. lugens*  
243 throughout Asia [4,54,55]. Use of the metabolic enzyme inhibitor piperonyl butoxide (PBO)  
244 and the model substrate 7-ethoxycoumarin were initially used to implicate P450-mediated  
245 detoxification in resistance [54,56]. However, more recently, molecular studies have  
246 identified the overexpression of two possible P450 enzymes with imidacloprid resistance in  
247 lab and field populations. The first of these, CYP6ER1, was identified as the only member of  
248 32 tentative unique P450s annotated from two recent sequencing projects as highly  
249 overexpressed (up to 40-fold) by quantitative RT-PCR in a range of resistant strains, with the  
250 level of expression observed in the different strains significantly correlated with the  
251 resistance phenotype [57]. The second P450, CYP6AY1, was one of six genes identified by



252 quantitative RT-PCR as significantly overexpressed (~18-fold) in a laboratory strain selected  
253 with imidacloprid for 40 generations [58]. This P450 was also overexpressed in four field  
254 strains (4-9-fold) compared to a susceptible strain [58]. This finding was surprising as  
255 CYP6AY1 was down-regulated (or neutrally expressed) in the resistant strains compared to  
256 the susceptible strain examined in the study by Bass et al. [57]. Nevertheless, functional  
257 expression of CYP6AY1 and RNAi experiments provided evidence that CYP6AY1 has the  
258 capacity to metabolise imidacloprid to 4/5-hydroxy-imidacloprid and confer resistance [58].  
259 More recently polymorphisms in the promoter of CYP6AY1 were identified between a  
260 resistant field-collected and lab susceptible strain that were shown to enhance promoter  
261 activity in reporter gene assays and may be acting as cis-acting factors to enhance the  
262 expression of CYP6AY1 [59]. Further work is required to elucidate the relative contribution of  
263 CYP6ER1 and CYP6AY1 in the imidacloprid resistance of *N. lugens* populations across Asia.

264

### 265 *2.5 Musca domestica*

266 The house fly, *M. domestica* L., is a passive vector for a range of debilitating human and  
267 animal diseases and is consequently an important pest on animal farms across the world.  
268 Like the other pest species highlighted in this review, effective control is often reliant on the  
269 use of pesticides and house flies have similarly proved highly adept at developing resistance,  
270 with reports of over 60 different compounds now listed in the APRD [6]. Neonicotinoids,  
271 primarily imidacloprid and thiamethoxam, are effective against a range of public hygiene  
272 pests and have been used as feeding baits and in spray applications to control house flies in  
273 animal facilities for a number of years [60]. Early studies showed good efficacy of  
274 imidacloprid against laboratory strains carrying resistance to other insecticide classes [61]  
275 and initial monitoring of field populations prior to the introduction of neonicotinoids for house  
276 fly control confirmed only limited variation in their response [62,63]. Recent studies have,  
277 however, revealed more significant resistance in field collected populations from several  
278 parts of the world, including the U.S. [64], Europe [65,66], Pakistan [67] and China [68], with  
279 further laboratory selection of these strains resulting in resistance factors for imidacloprid  
280 ranging from 100 fold [66] to over 2,000 fold [69].

281 Attempts to investigate the underlying mechanisms of resistance in these strains have  
282 implicated possible roles for both metabolic enzymes and target site modification, but have  
283 yet to unambiguously assign the metabolic activity to a specific enzyme or identify the exact  
284 target alteration(s) responsible. For example, both imidacloprid and thiamethoxam resistance  
285 in field-collected strains from Denmark was partly synergised by treatment with the  
286 cytochrome P450 inhibitor, PBO [66] and this was correlated with increased expression of  
287 several P450 genes (*CYP6A1*, *CYP6D1*, *CYP6D3*, *CYP6G4*) after neonicotinoid exposure  
288 [66,70]. However, as yet none of these genes have been functionally expressed and shown

289 conclusively to metabolise these compounds. The metabolic resistance was accompanied by  
290 an apparent 60% reduction in the expression level of the  $\alpha 2$  nicotinic acetylcholine receptor  
291 subunit (M $\alpha$ 2) in the same resistant strains and was suggested as a possible additional  
292 mechanism that contributes to their reduced sensitivity [71], although it should be pointed out  
293 that no other nicotinic subunits were investigated for either altered expression or target site  
294 modification in this study.

295 Interestingly, the high level of imidacloprid resistance (2,300 fold) selected from a  
296 Florida field strain was not synergisable by PBO [69], suggesting a possible target site  
297 alteration similar to that described in aphids. This resistance was mapped to autosomes 3  
298 and 4, both of which carry nicotinic acetylcholine receptor subunit genes, and would  
299 therefore seem to be a fruitful area for further investigation. The publication of a full genome  
300 sequence for *M. domestica* [72] offers new opportunities for a more detailed characterization  
301 of nAChR genes in this and other resistant strains, and should facilitate a clearer  
302 understanding of the molecular basis of resistance in this species.

303

#### 304 2.6 *Leptinotarsa decemlineata*

305 The Colorado potato beetle, *L. decemlineata* (Say), is a serious pest of potatoes and other  
306 solanaceous crops, particularly in North America and Europe. This species has gained  
307 notoriety for rapidly developing resistance to almost all of the insecticides used for its control  
308 [6]. The neonicotinoid imidacloprid was first introduced for *L. decemlineata* control in  
309 Northern America in 1995. Widespread monitoring of imidacloprid susceptibility in  
310 populations from North America and Europe collected over 1995-1998, revealed up to 29-  
311 fold variation in response [73]. Much of this variation was not a result of selection from  
312 imidacloprid use per se, as most of the populations assayed were never exposed to this  
313 compound, but was likely a consequence of cross-resistance from chemicals used earlier.  
314 The least sensitive strains described in this study came from Long Island, New York, an area  
315 with a history of intensive insecticide use against *L. decemlineata* [73]. In support of this  
316 finding a report published in the same year described 100-fold levels of resistance to  
317 imidacloprid in adults of an *L. decemlineata* population collected as early as 1997 from an  
318 imidacloprid-treated commercial potato field [74]. Subsequent monitoring of samples from  
319 Long Island has reported further increases in resistance to imidacloprid (309-fold) with lower  
320 levels of cross-resistance also observed to dinotefuran, clothianidin, acetamiprid, thiacloprid,  
321 thiamethoxam, and nitenpyram, despite these never having been used in the field up to this  
322 point [75].

323 The precise mechanism(s) underlying neonicotinoid resistance in *L. decemlineata*  
324 have not been fully characterized, however, several studies have advanced our  
325 understanding of the possible mechanisms involved. Two studies of resistant strains from

326 Long Island using insecticide synergists have suggested that P450-mediated detoxification  
327 plays a significant role in resistance, with esterases possibly also involved, however, the fact  
328 that enzyme inhibitors did not completely eliminate resistance in resistant strains suggests  
329 additional mechanisms may be involved [74,75]. In contrast to these findings  
330 pharmacokinetic experiments with other strains of *L. decemlineata* showed no significant  
331 difference in *in vivo* metabolism of radiolabelled imidacloprid [76]. The potential role of target-  
332 site modification in the neonicotinoid resistance of *L. decemlineata* has also been explored  
333 using binding assays with tritiated imidacloprid. Initial results failed to reveal differences in  
334 imidacloprid affinity to nAChRs from head membrane preparations of neonicotinoid-resistant  
335 and susceptible beetles (Nauen *et al.*, unpublished). Further work has compared the neural  
336 activity of imidacloprid on the spontaneous activity of a motor nerve leaving the isolated  
337 central nervous system of susceptible and resistant beetles [77]. Although no differences  
338 were seen in the sensitivity of the central nervous system of resistant and susceptible beetles  
339 to excitation by imidacloprid, significant reductions in the sensitivity of CNS preparations of  
340 the resistant strain to inhibition by imidacloprid were observed, suggestive of a possible  
341 change in the sensitivity of at least one subgroup of nAChRs [77]. Although the origin of the  
342 decreased sensitivity to block neural activity by imidacloprid in the resistant beetles requires  
343 further characterization, it is likely that it relates to the observed resistance to imidacloprid.

344

#### 345 2.7 *Trialeurodes vaporariorum*

346 The glasshouse whitefly, *T. vaporariorum* (Westwood) is an economically important pest of  
347 protected vegetable and ornamental crops in most temperate regions of the world. As for  
348 many of the other pests detailed in this review resistance of this species to a range of older  
349 insecticide classes, such as the pyrethroids and organophosphates [78], led to the increasing  
350 reliance on neonicotinoid insecticides for control after their introduction. The first cases of  
351 neonicotinoid resistance were reported in *T. vaporariorum* strains collected in 2004/2005  
352 from the United Kingdom the Netherlands and the U.S. [79,80]. More recent work has  
353 described neonicotinoid resistance in *T. vaporariorum* strains from the UK, Turkey, Spain,  
354 China, Germany [81] and Greece [82] with reduced susceptibility to imidacloprid also  
355 reported in strains from Finland [83]. Taken together these results suggest resistance to  
356 neonicotinoids in *T. vaporariorum* may now be widespread in global populations.

357 Interestingly, neonicotinoid resistance in *T. vaporariorum* shows several parallels with  
358 that of the tobacco whitefly *B. tabaci*. Cross-resistance bioassays and selection experiments  
359 revealed a clear correlation in the observed responses of *T. vaporariorum* to neonicotinoids  
360 and pymetrozine, strongly suggestive of cross-resistance between the two classes [81].  
361 Furthermore, resistance to the neonicotinoid imidacloprid and pymetrozine was shown to be  
362 age-specific, with resistance in nymphs failing to compromise recommended application

363 rates [81]. Taken together these results suggest a similar mechanism may underlie  
364 resistance in *B. tabaci* and *T. vaporariorum*. As detailed above, resistance to both  
365 imidacloprid and pymetrozine in *B. tabaci* results from enhanced expression of the P450  
366 CYP6CM1. Recent sequencing of the transcriptome of *T. vaporariorum* has allowed the  
367 identification of several P450 genes (*CYP6CM2*, *CYP6CM3*, *CYP6CM4*) that share  
368 significant homology with *B. tabaci* *CYP6CM1* and therefore represent candidates for a  
369 potential role in resistance in *T. vaporariorum* [84].

370

## 371 2.8 Other pests

372 Neonicotinoid resistance has also been reported in several other insect pest species in  
373 addition to those listed above and it is beyond the scope of this review to provide an  
374 exhaustive list, nevertheless, in some cases multiple reports of resistance have suggested a  
375 growing resistance problem for certain species and these are summarised below.

376 The white-backed planthopper, *Sogatella furcifera* (Horvath), and small brown  
377 planthopper *Laodelphax striatellus* (Fallén) are two important pests of rice in Asia. Screening  
378 for imidacloprid resistance in *S. furcifera* populations collected in 2006 from East and South-  
379 East Asia revealed that, in contrast to *N. lugens*, most populations displayed full sensitivity to  
380 this compound [85]. However, in the same study the first evidence of field resistance was  
381 detected in a single population from Japan. More recent monitoring of field populations of *S.*  
382 *furcifera* in China has suggested resistance has since become more widespread with ~30%  
383 of populations collected from 2010 to 2013 showing moderate resistance (<15-fold) to  
384 imidacloprid [86,87]. Despite these findings all populations tested remained susceptible to  
385 thiamethoxam [86,87]. Initial monitoring of the sensitivity of *L. striatellus* populations in China  
386 found high levels of resistance to imidacloprid in strains collected from Jiangsu province  
387 suggestive of a local hotspot of resistance [88]. However, more recent monitoring of  
388 populations in China (including from Jiangsu province) found all populations collected from  
389 2011-2013 were susceptible to both imidacloprid and thiamethoxam [87].

390 The Asian citrus psyllid, *Diaphorina citri* (Kuwayama), is one of the most economically  
391 important pests of citrus worldwide, primarily due to its status as a vector of citrus greening  
392 disease. Monitoring of populations of this pest in Florida collected in 2009/2010, where it is a  
393 significant problem to citrus growers, revealed reduced sensitivity in certain populations to  
394 imidacloprid and thiamethoxam, with 35- and 13-fold resistance to the two compounds  
395 respectively observed in the most resistant strain [89]. These findings suggested  
396 neonicotinoid/insecticide resistance may be becoming an emerging problem in this species in  
397 Florida, however, more recent monitoring has revealed, in contrast to other insecticide  
398 classes, a slight decrease in resistance to neonicotinoids [90]. Beyond Florida monitoring of  
399 *D. citri* populations collected from lime orchards in Central West Mexico has recently

400 revealed widespread, mostly moderate, resistance (<25-fold) to both imidacloprid and  
401 thiamethoxam [91]. However, a strain collected from one site (Apatzingan, Michoacan)  
402 displayed extremely high resistance to imidacloprid (>4000-fold) suggesting the emergence  
403 of more potent resistance in this area [91].

404 The codling moth, *Cydia pomonella* L., is a major pest of pome fruit worldwide. The N-  
405 cyano-imino neonicotinoids thiacloprid and acetamiprid, are relatively effective for codling  
406 moth control and have been widely adopted since their introduction. Resistance to both  
407 compounds has been reported in *C. pomonella* populations from Europe [92,93], the U.S.  
408 [94] and Argentina [95], with low level resistance to thiacloprid also reported in populations  
409 from Canada [96]. Surprisingly, resistance to thiacloprid in Europe has been observed in  
410 countries/regions prior to their use by growers and this is associated with cross-resistance  
411 with older compounds. A similar phenomenon has also been reported for acetamiprid with  
412 resistance to this compound correlated with levels of azinphos-methyl resistance in  
413 populations from the U.S. [94]. Both of these cases are suggestive of an underlying  
414 metabolic resistance mechanism that confers broad cross-resistance to a range of  
415 compounds. In relation to this several studies have also reported enhanced activity of  
416 detoxification enzymes, including P450s, glutathione-S-transferases and esterases to be  
417 correlated with resistance in biochemical assays [92,93,97]. However, to date, the precise  
418 enzymes involved in neonicotinoid resistance have not been characterized.

419 Western flower thrips, *Frankliniella occidentalis* (Pergande), is a major insect pest of  
420 several vegetable, fruit and ornamental crops. The first report of resistance of this species to  
421 neonicotinoids was in a laboratory strain originating from the United States which displayed  
422 moderate resistance to imidacloprid (RR 14-fold) [98]. Interestingly imidacloprid had not been  
423 used against this species at this time and therefore the observed resistance was almost  
424 certainly a result of cross-resistance from older insecticides [98]. More recent work has  
425 reported resistance to both imidacloprid and acetamiprid in strains of *F. occidentalis*  
426 originating from Japan and China [99]. Synergism bioassays using the metabolic enzyme  
427 inhibitor piperonyl butoxide (PBO) suggested that metabolism by P450s may be involved in  
428 acetamiprid resistance in these strains, and cloning and sequencing of nicotinic acetylcholine  
429 receptor (nAChR) subunits provided no evidence of a target-site mechanism [99]. Finally,  
430 modest levels of resistance to thiamethoxam (15-fold) were also recently reported in a strain  
431 of *F. occidentalis* selected in the laboratory with this compound for 55 generations [100].  
432 Interestingly this strain showed high levels of cross-resistance to the neonicotinoid  
433 imidaclothiz (392.1-fold) but no or very low cross-resistance to the neonicotinoids  
434 imidacloprid, acetamiprid, dinotefuran and nitenpyram. This finding might be explained by a  
435 metabolic resistance mechanism that exhibits substrate preference for chlorothiazolymethyl  
436 neonicotinoids such as thiamethoxam and imidaclothiz. In this regard thiamethoxam efficacy

437 against the resistant strains was synergized by PBO and triphenyl phosphate (TPP) and  
438 biochemical assays showed modest increased in monooxygenase and carboxylesterase  
439 activity suggesting a possible involvement of these enzyme systems in resistance [100].

440

### 441 **3. Implications and conclusions**

442

443 It is no coincidence that most species exhibiting economically-significant resistance to  
444 neonicotinoids are ones that have gained notoriety for resistance to a broad range of other  
445 insecticide groups. The same agronomic and biological traits that have predisposed them to  
446 resist older products must also underpin the evolution of resistance to neonicotinoids. This  
447 propensity for accumulating multiple resistance greatly constrains the implementation of  
448 approaches recommended for combating resistance in general [101] and to neonicotinoids  
449 specifically [5,102]. The most widely advocated tactic for managing resistance, other than the  
450 obvious one of minimizing reliance on chemicals per se, is the alternation of groups with  
451 different modes of action to avoid continuous selection for the same resistance  
452 mechanism(s). In the above cases, a lack of effective alternatives combined with the  
453 unprecedented versatility of neonicotinoids has led to intensive use of these compounds and  
454 enhanced the risk of resistance developing [4,103]. Bioassay results for several insecticides  
455 tested against a multi-resistant Spanish strain of the aphid *M. persicae* (Figure 6) exemplify  
456 well how the accumulation of resistance mechanisms can deplete the supply of compounds  
457 available for alternation schemes. The appearance of strong resistance to imidacloprid  
458 caused by the R81T target-site mutation (see above) in a genetic background already  
459 containing mechanisms conferring target-site insensitivity to the carbamate pirimicarb and  
460 synthetic pyrethroids [104] results in only two of the tested products (flonicamid and  
461 spirotetramat) retaining high levels of activity against this strain. Interestingly this field-  
462 collected strain also shows moderate resistance to pymetrozine (IRAC subgroup 9B), but not  
463 flonicamid (subgroup 9C). Both insecticides are known to act as modulators of chordotonal  
464 organs (IRAC main group 9), but are chemically different.

465 One of the major limitations to resistance management is the occurrence of cross-  
466 resistance. Insect pests very rarely resist just one compound; resistance mechanisms  
467 commonly encompass most or all chemicals within a particular mode-of-action group and  
468 can, much less predictably, affect other groups as well. The literature reviewed above  
469 contains numerous cases of resistance initially reported to one neonicotinoid being found  
470 through bioassays to extend to other compounds in this class. The magnitude of resistance  
471 factors to different molecules may vary considerably, presumably as a consequence of  
472 differences in the substrate specificity of detoxifying enzymes. However, based on the  
473 collective results of work so far it is impossible to identify consistent and exploitable patterns

474 of cross-resistance across commercially-available neonicotinoids. Recommendations  
475 advanced previously [102,103], reinforced by a common IRAC mode of action classification  
476 (Group 4A) (Sparks and Nauen, this issue), to treat the seven commercial neonicotinoids as  
477 a single group for resistance management purposes unquestionably remain appropriate  
478 when designing insecticide alternation strategies.

479 Interesting questions about cross-resistance arise with the introduction of new  
480 molecules targeting the same site as ones developed previously, but considered to display  
481 unique properties that distinguish them from predecessors. The sulfoximine, sulfoxaflor [105]  
482 and the butenolide, flupyradifurone [106] are unquestionably nAChR agonists but chemically  
483 different from neonicotinoids and thus have been placed in new subgroups (4C and  
484 4D, respectively) in the IRAC classification scheme. This distinction is supported by data  
485 showing that aphids and whiteflies with metabolic resistance to imidacloprid and other  
486 conventional neonicotinoids remain almost fully susceptible to sulfoxaflor and flupyradifurone  
487 [105-107]. However, a strain of *M. persicae* with the still geographically-restricted R81T  
488 mutation showed appreciable resistance to both of these new compounds (Figure 6). Thus,  
489 anticipating risks of cross-resistance involving novel members of a broad mode-of-action  
490 group requires caution as these risks can be mechanism-specific.

491 The predominance (so far) of enhanced metabolism, as opposed to target-site  
492 modification, as a cause of resistance to neonicotinoids increases the possibility of  
493 resistance extending to compounds with contrasting modes of action. The best documented  
494 example to date is cross-resistance between neonicotinoids and the azomethine pymetrozine  
495 in the whiteflies *B. tabaci* [27] and *T. vaporariorum* [81]. Examples of species showing  
496 variation in response to neonicotinoids at the time of their introduction can raise suspicions of  
497 resistance pre-selected by earlier used groups [73], although the exact nature of such cross-  
498 resistance remains to be investigated.

499 Since the last comprehensive review of this subject [4], there have been additional  
500 pest species acquiring neonicotinoid resistance, and changes in the extent and severity of  
501 cases of resistance already documented ten years ago. Most notably, there has been  
502 significant progress with characterizing the genetic and molecular basis of resistance  
503 mechanisms, providing exciting evolutionary insights and also techniques for rapid diagnosis  
504 and monitoring of resistance genotypes. These achievements can contribute not only to  
505 tracking and helping to contain known cases of resistance but also to anticipating the  
506 emergence and nature of new resistance outbreaks.

507

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513

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## 848 **Figure legends**

849

850 **Figure 1.** Important neonicotinoid insecticides (manufacturers) and year of market  
851 introduction.

852 **Figure 2.** Agricultural use by region and market share of individual neonicotinoids in percent  
853 (total market share 2012: 3.192bn US\$; Source: Wood Mackenzie). Abbreviations: TMX  
854 (thiamethoxam), IMD (imidacloprid), CLT (clothianidin), ACT (acetamiprid), TCP (thiacloprid),  
855 DNF (dinotefuran), NIT (nitenpyram).

856 **Figure 3.** Cumulative number of published peer-reviewed papers on resistance to  
857 neonicotinoids generally and to imidacloprid specifically.

858 **Figure 4.** Cumulative number of arthropod species with neonicotinoid resistance (Arthropod  
859 Pesticide Resistance Database, Michigan State University).

860 **Figure 5.** Number of reported cases of neonicotinoid resistance up to 2014 (Arthropod  
861 Pesticide Resistance Database, Michigan State University). Only those pests with >10  
862 reported cases are shown.

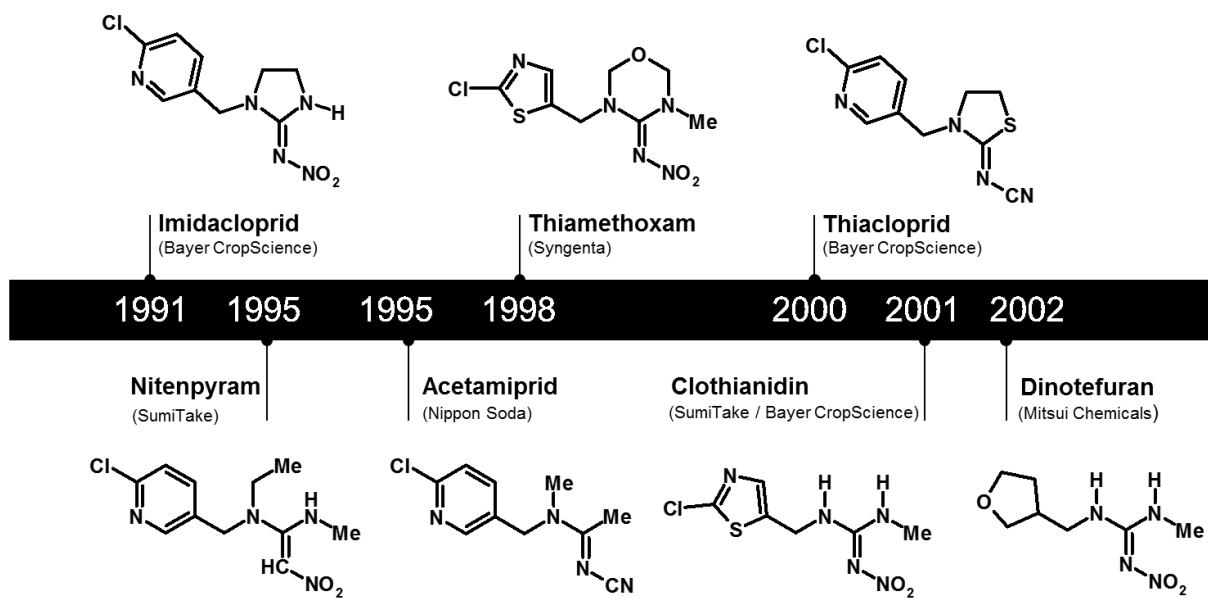
863 **Figure 6.** Dose response curves for different insecticides against 3rd instar nymphs of *Myzus*  
864 *persicae* in leaf-dip bioassays (72h). Strain HS is susceptible to insecticides, whereas clone  
865 E03-10 C2 is derived from a field strain collected in Spain in 2010 and homozygous for the  
866 R81T mutation in the  $\beta$ 1-subunit of the nAChR, conferring cross-resistance to neonicotinoids,  
867 sulfoxaflor and flupyradifurone. This clone also carries mutations in AChE (MACE) and  
868 voltage-gated sodium channel (kdr/skdr).

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870 **Figure 1**

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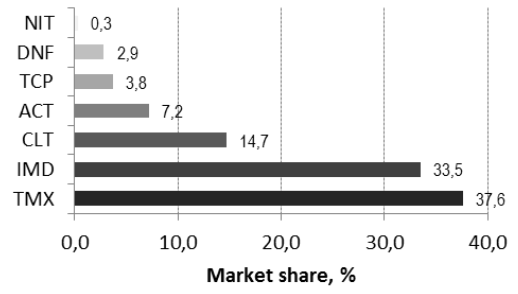
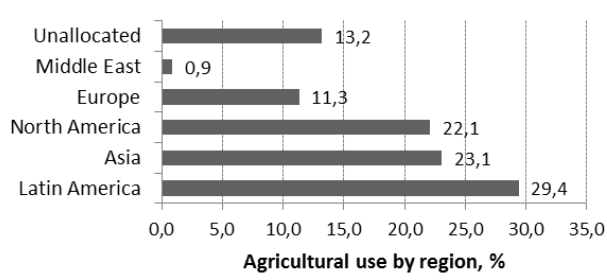
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875 **Figure 2**

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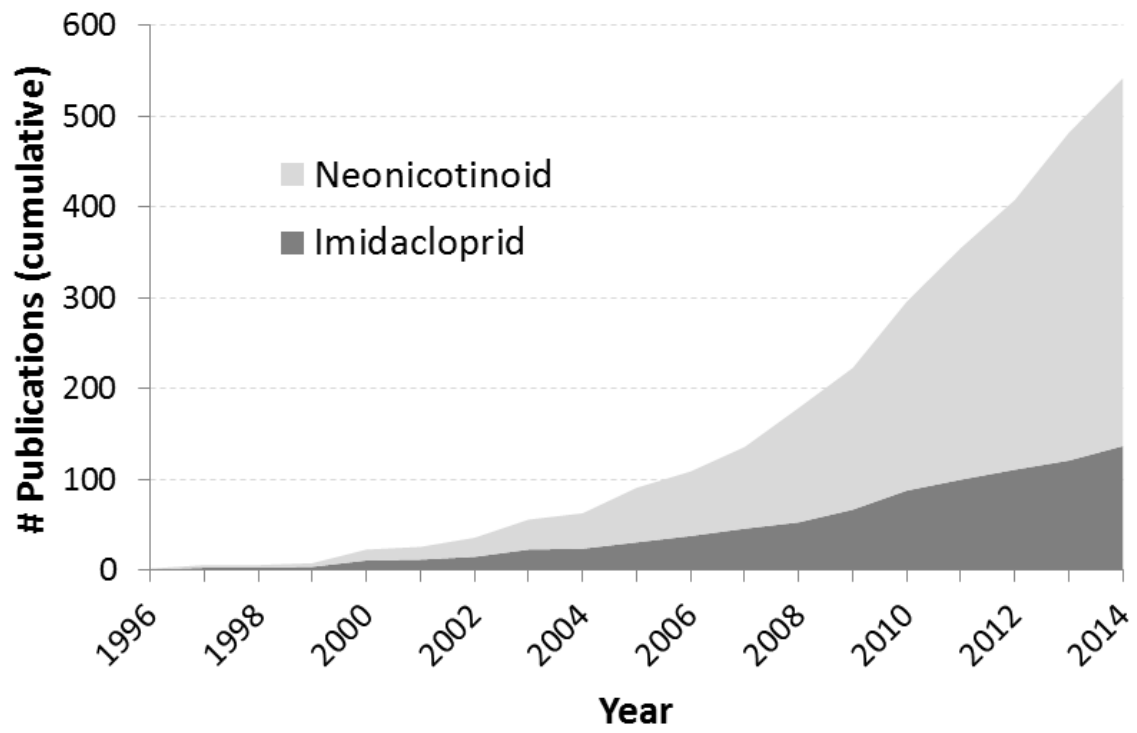
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880 **Figure 3**

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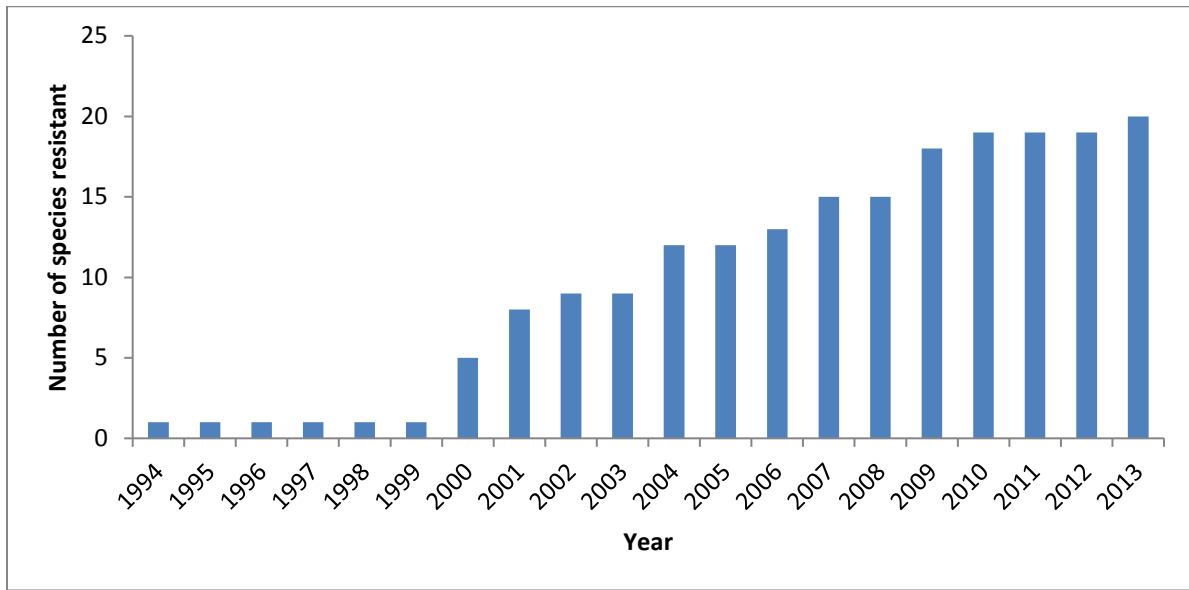
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885 **Figure 4**

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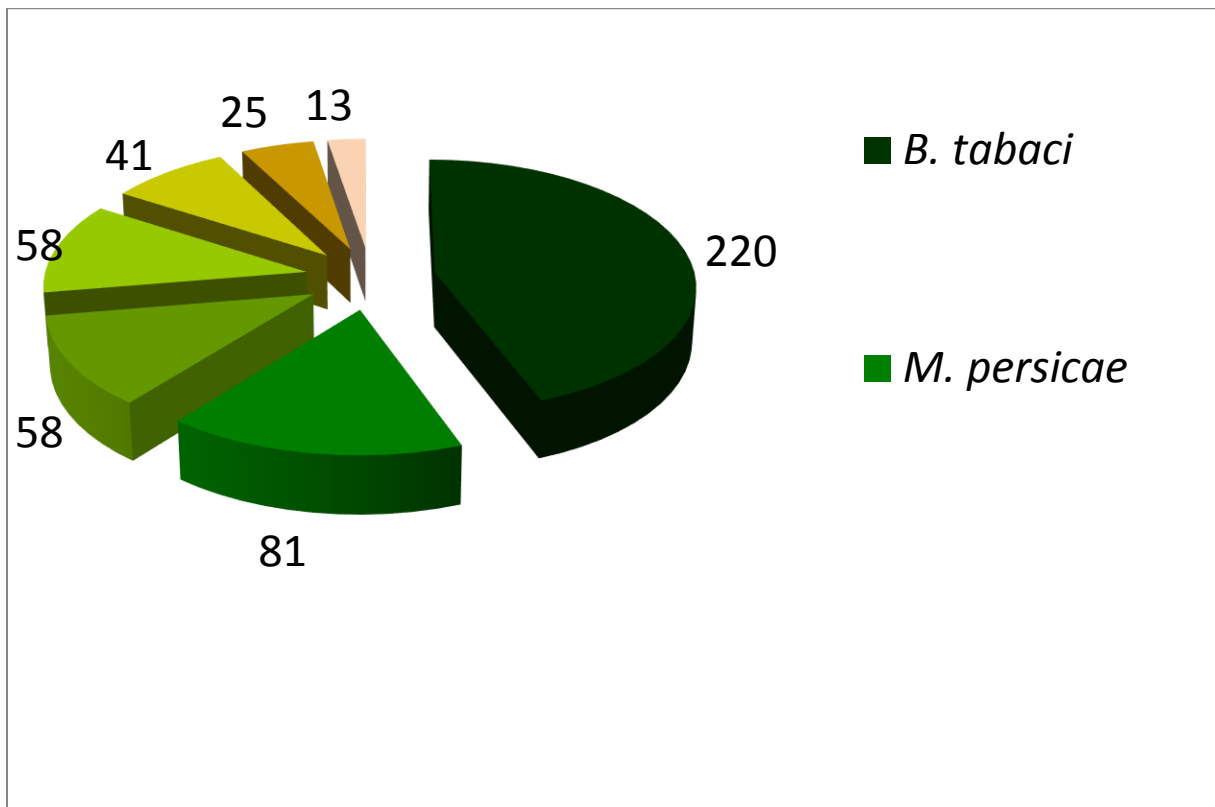
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890 **Figure 5**

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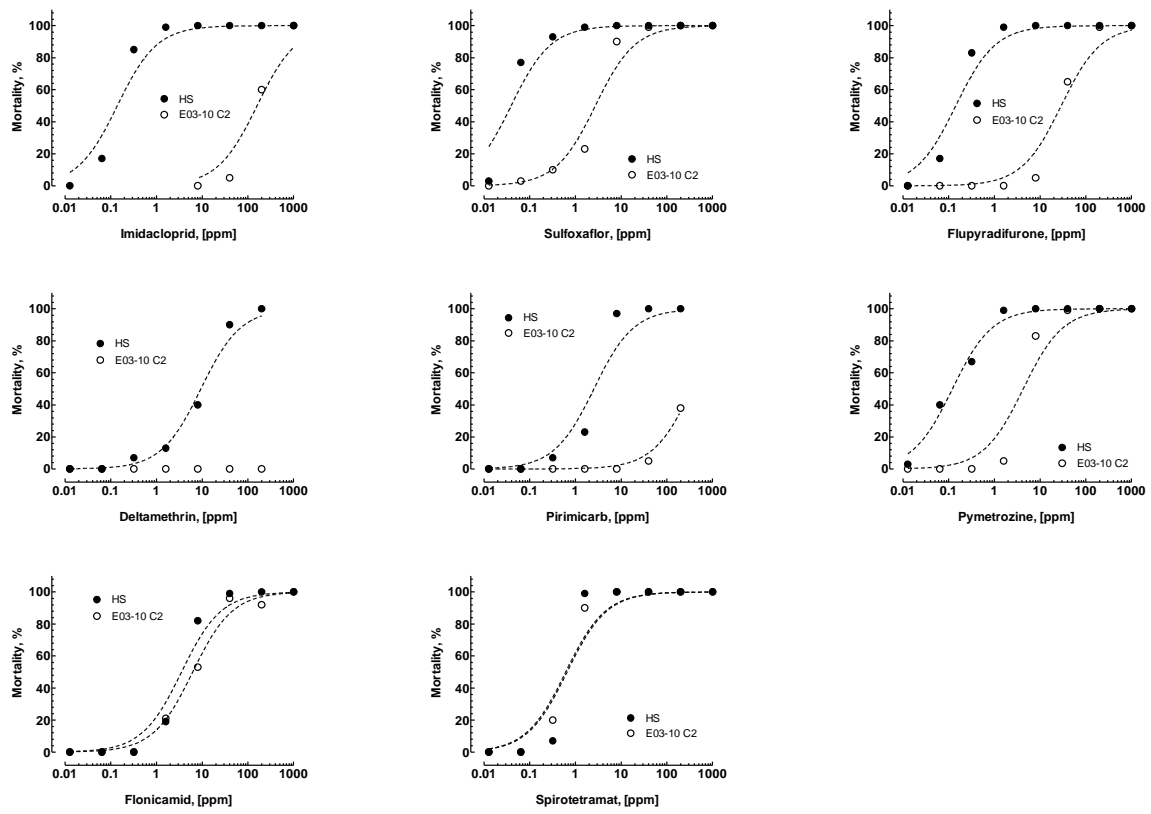


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895 **Figure 6**



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