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REVIEW

An overview of the main pathways of metabolic resistance in insects

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Abstract

Insecticides have played and still fulfil a very important role in sustainable production of food, animal feed and also as protection against disease vectors. They act to suppress insect populations and, as a consequence of their use, insecticide resistance has evolved. An overview of insecticide resistance mechanisms in insects is given, focusing on the metabolic systems involved in xenobiotic metabolism in the class Insecta. Several enzyme families (*e.g.*, esterases, mixed function oxidases, glutathione S-transferases) are involved in insecticide detoxification, sequestration and excretion and have differing relative importance within the various taxonomic groups. A brief discussion of their impact on control strategies is given.

Key Words: insecticide resistance; esterases; P450; glutathione-S-transferases; synergists

Introduction

Insect pests represent a serious threat to agricultural production and vector disease control. The use of insecticides fulfils an important role in controlling populations of insect pests, but as a result of their continued application several resistance mechanisms allowing survival have evolved. During the last 50 years, increased use, overuse and even misuse of pesticides has led to the selection of resistance in more than 500 arthropod pest species. Michigan State University developed an online database (APRD) (http://www.pesticideresistance.com) to list the resistant cases reported.

Following the first report of resistance at the beginning of the last century (Melander, 1914), the number of cases continued to grow, with an exponential increase during the late 1970s and early 1980s (Georghiou and Lagunes-Tejada, 1991). Today, the order with the highest number of resistant species is Diptera (27 %), followed by Lepidoptera (25 %), Hemiptera (17 %) and Coleoptera (10 %) (Whalon *et al.*, 2012). Diptera can have severe economic impact as many of them transmit diseases to humans and domestic animals, whilst others are pests of agricultural plants. In the other orders, many of the resistant species represent a serious threat for agricultural production and are responsible for important agricultural yield losses causing problems for future food security.

Development of resistance depends upon a variety of genetic, biochemical and ecological factors such as generation time, fecundity rate, dispersal ability or fitness costs, together with the frequency, the dosage or the persistence of insecticide applications (Brattsen *et al.*, 1986; Hemingway *et al.*, 2002; Kliot and Ghanim, 2012; Liu, 2015). The presence of different genotypes in a population can confer a selective advantage to some individuals and result in survival after insecticide exposure (Feyereisen *et al.*, 2015). As a result of continued insecticide application, the proportion of resistant insects increases compared to that of susceptible and the population becomes increasingly difficult to control (Nauen, 2007) (Fig. 1).

Insecticide resistance mechanisms

It has been shown that insecticide resistance evolves predominantly by two mechanisms: the enhanced production of metabolic enzymes, which sequester or detoxify the insecticide, and/or mutations of target proteins, which make them less sensitive to the insecticide (Fig. 2).

A number of subsidiary physiological mechanisms which contribute to reduce insecticidal effects have also been described, *e.g.*, a lower penetration of the chemicals or an increased excretion. A variety of different chemical classes, which act on different biological targets, have been

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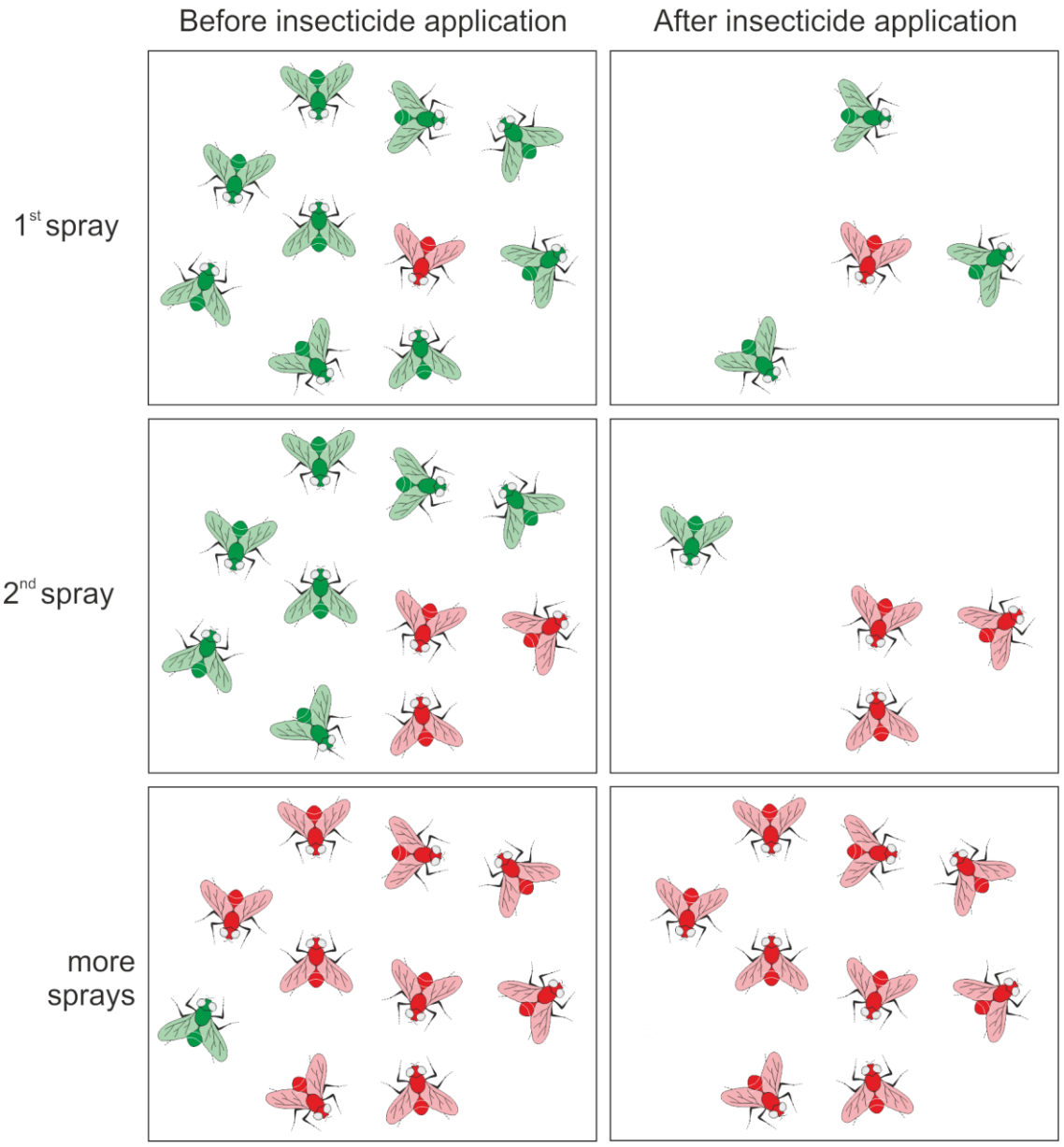

Fig. 1 Theoretical example illustrating the increase of insecticide resistance levels in a pest population. Some individuals (red) with genetic traits allowing them to survive insecticide applications can reproduce; if the selection pressure is frequent, they easily become the preponderant part of the population.

developed (http://www.irac-online.org/documents/moa-classification). However, many insect species have evolved mechanisms to overcome the toxicity of most classes of insecticide. Thus, the possibility that they could also evolve resistance against potential new products, with different modes of action, has to be considered.

Resistance mechanisms do not always occur in isolation but often interact to enhance the level of resistance.

The presence of combinations of different resistance mechanisms has been demonstrated in many insect populations and also in single individuals within a population. “Cross-resistance” occurs when a single defence mechanism against one insecticide also confers resistance to other insecticides, even if the insect has not previously been exposed to the latter product. This phenomenon can result from physical factors that can affect chemically unrelated compounds, or non-specific enzymes that attack functional groups of insecticides rather than specific molecules; indeed it is not only restricted to a specific chemical class but can involve insecticides with different modes of action. “Multiple-resistance” occurs when different resistance mechanisms co-exist and confer resistance to different insecticides to which the organism has been exposed (Oppenoorth and

Welling, 1976; Yu, 2008; Panini *et al.*, 2014). The occurrence of both cross-resistance and multiple-resistance is of particular importance, because they can create increased difficulty for pest control. Clearly, since pest insect populations are usually large in size and breed quickly, there is always a risk that insecticide resistance may evolve, especially when insecticides are misused or over-used (Soderlund and Bloomquist, 1990; Coleman *et al.*, 2006).

Metabolic resistance

Metabolic resistance is a common defence mechanism, based on enzymatic systems that protect the insect by detoxifying/sequestering insecticide molecules. The enzymes involved are those insects have developed as protection against naturally occurring plant toxins (allelochemicals) such as alkaloids, terpenes and phenols, in order to overcome the potential toxicity of the plants they feed on (Gatehouse, 2002; Despres *et al.*, 2007; War *et al.*, 2012; Heidel-Fischer and Vogel, 2015; Rane *et al.*, 2016). This could explain the rapid development of metabolic resistance against a very broad spectrum of insecticides that, in many cases, have direct or indirect botanical origin (Isman, 2006).

Enzymes can detoxify xenobiotics into a non-toxic compound and/or into a form more suitable for rapid elimination from the body. Resistant insects metabolise the insecticide faster because they possess forms of the enzyme with a higher catalytic rate, or higher quantities of the enzymes as a consequence of increased transcription or gene amplification. Detoxification can be divided into phase I (primary) processes, consisting of hydrolysis or oxidation, and phase II (secondary) processes, consisting of conjugation of phase I products with endogenous compounds, like glutathione, and their subsequent excretion from the body (Li *et al.*, 2007; Hollingworth and Dong, 2008; Yu, 2008; Berenbaum and Johnson, 2015).

In addition to these detoxification strategies that rely on enzymatic cleavage and excretion, sequestration is another important defence mechanism which several insects have evolved to tolerate xenobiotics. This is a common phenomenon among herbivorous insects, which involves the specific and selective uptake, transport and storage of plant secondary metabolites, preventing interference with the insects' physiological processes (Erb and Robert, 2016; Petschenka and Agrawal, 2016). Such behaviour was reported to be retained in mosquitoes whose haematophagy is probably a secondary adaptation to obtain high quality food needed for egg production (Moore, 2015).

The enzymes mainly involved in detoxification of xenobiotics in living organisms are transcribed by members of large multigene families of esterases, oxidases, and GSTs.

Esterases

Esterases are a large group of phase 1 metabolic enzymes that are able to metabolise a variety of exogenous and endogenous substrates. Their involvement in detoxifying insecticide molecules is well documented and it has been demonstrated that they can act against a broad range of chemical classes, including pyrethroids, organophosphates and carbamates (Hollingworth and Dong, 2008). Potential involvement in neonicotinoid resistance (Zhu and Luttrell, 2015) and even against Bt toxin (Gunning *et al.*, 2005) has also been reported.

Detoxification can occur through enzymatic cleavage or sequestration of the insecticide molecules. Esterases catalyse the hydrolysis of ester insecticides into their corresponding acid and alcohol compounds; this increases the polarity of the insecticidal metabolites which can then be excreted more readily from the insect body. Esterases can also sequester insecticides such that the toxic molecules are no longer available for interactions with target proteins (Devonshire and Moores, 1982; Oakeshott *et al.*, 2005; Wheelock *et al.*, 2005).

Esterases have been associated with insecticide resistance in many insect species as a consequence of quantitative and/or qualitative changes, resulting in the overproduction of the enzymes or in modifications of their structures (Li *et al.*, 2007).

Esterase overexpression can be due to either gene amplification or upregulation, or a combination of both. The most extensively studied example of insecticide detoxification by gene amplification is the overproduction of a specific carboxylesterase in the green peach aphid *Myzus persicae* (Hemiptera: Aphididae) (Field *et al.*, 1988; Bizzaro *et al.*, 2005; Rivi *et al.*, 2013; Bass *et al.*, 2014). Amplified esterases associated with insecticide resistance have also been found in mosquitoes of the *Culex* genus (Diptera: Culicidae) (Severini *et al.*, 1994; Raymond *et al.*, 1998; Hemingway *et al.*, 2004) and other species, for example the brown planthopper *Nilaparvata lugens* (Stal) (Hemiptera: Delphacidae) (Small and Hemingway, 2000). In some species, *e.g., Aphis gossypii* Glover (Hemiptera: Aphididae) or B-biotype *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), the increased expression of esterases results from increased transcription levels, due to upregulation of the corresponding gene (Alon *et al.*, 2008; Cao *et al.*, 2008).

Esterase-based resistance can also occur through changes of the enzyme structure, which confers an enhanced ability to metabolise the insecticide. This mechanism was first described in the housefly *Musca domestica* (Diptera: Muscidae) and became known as the "mutant ali-esterase theory" (Oppenoorth and van Asperen, 1960). The resistant insects showed an apparent decreased esterase activity compared to susceptibles, resulting from structural modifications of the enzyme that facilitated the hydrolysis of the insecticide but prevented or reduced the hydrolysis of the model substrates conventionally used to determine the esterase activity. Two amino-acid substitutions (Gly137Asp and Trp251Leu) were considered as the basis of this resistance mechanism in houseflies as well as in other insect species belonging to the order of Diptera (Campbell *et al.*, 1998; Claudianos *et al.*, 1999; Carvalho *et al.*, 2006).

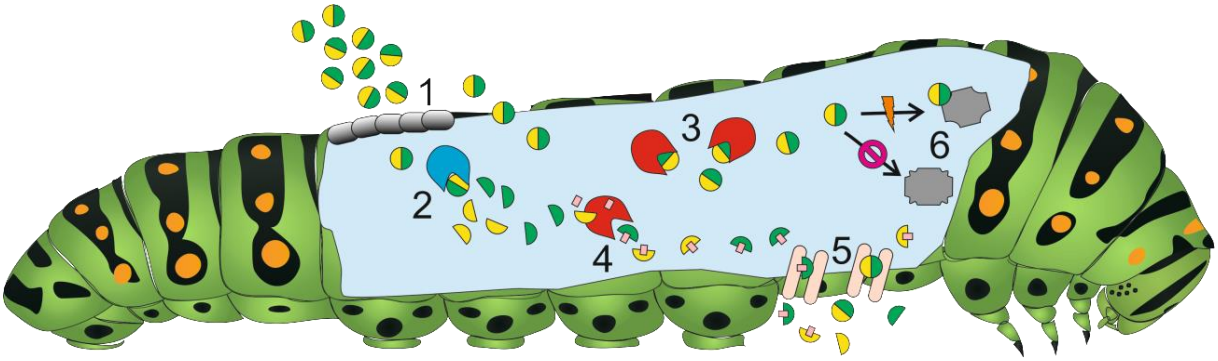

Fig. 2 Schematic illustration of different possible resistance mechanisms known in insects: 1) penetration resistance; 2) enzymatic cleavage; 3) sequestration; 4) conjugation; 5) excretion; 6) target-site modification.

Additional mechanisms can influence esterase overproduction, including demethylation or chromosomal rearrangements.

Demethylation mechanisms can cause gene silencing and consequent reduction of esterase levels in E4 populations (Field, 2000), but this is not true for the other esterase variant FE4, where no correlations were found between methylation levels and esterase activity (Bizzaro *et al.*, 2005).

Further differences were observed between E4 and FE4 *M. persicae* populations: amplification of isoform E4, leading to carbamate and OPs resistance, is closely linked to chromosomal translocation A1,3 (Field and Devonshire, 1997). The same autosomal rearrangement was recently reported for the other esterase isoform, FE4, in Italian populations of this aphid species, but only in populations with low esterase activity, meaning that translocation and esterase-based resistance are not always correlated. (Rivi *et al.*, 2012, 2013).

Monooxygenases

Mixed function oxidases (MFOs), or microsomal oxidases, are a large family of phase 1 enzymes involved not only in the detoxification of xenobiotics, but also in the metabolism of endogenous substances such as hormones, pheromones or fatty acids. They are able to convert lipophilic compounds into polar metabolites that can be easily eliminated from the body; for that reason, they are mainly located in the digestive system (Feyereisen 2005, 2015; Liu *et al.*, 2015).

Cytochrome P450 monooxygenases (P450s) are microsomal oxidases that belong to the group of heme thiolate proteins, so named because they show a characteristic absorbance peak at 450 nm (Soret peak) in their reduced form when complexed with carbon monoxide. They catalyse the transfer of one atom of molecular oxygen to a substrate and the reduction of the second atom of oxygen to form water; the process requires the transfer of two electrons provided by NADPH cytochrome P450 reductase (Feyereisen, 2005; Guengerich, 2008). The reaction is commonly described as:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

Due to the large number of enzymes and their substrate specificity, P450s are able to catalyse different reactions such as epoxidation, hydroxylation, N-dealkylation, O-dealkylation or desulphurisation; for that reason they play an important role in plant-insect interactions, as well as in the metabolism of many insecticide classes, including carbamates, organophosphates, pyrethroids and neonicotinoids (Despres *et al.*, 2007; Yu, 2008; Philippou *et al.*, 2010; Puinean, 2010; Alptekin *et al.*, 2016).

The nomenclature used is that a single P450 is named as CYP followed by an arabic numeral to designate the family, a capital letter to designate the subfamily and an arabic numeral to designate the individual protein; each form is coded by its own gene. To date, more than 600 insect P450 genes have been characterised and genes belonging to the families *CYP4*, *CYP6*, *CYP9* and *CYP12* have been associated with insecticide resistance (Feyereisen, 2005, 2015; Li *et al.*, 2007). The mixed-function oxidase system has been shown to occur in the fat body, Malpighian tubes, and the midgut. By far the most intensively studied mixed-function oxidase system is that of the house fly (Markussen *et al.*, 2010).

Because of the complexity of the P450 system and the difficulties in purifying these enzymes (due to their instability or problems in obtaining high yields), it is difficult to determine the mechanisms which induce resistance. However, it has been demonstrated that resistant insects can show increased levels of P450s and an enhanced monooxygenase activity. Many cases of resistance correlated to overexpression of P450 activity have been reported in the literature and it is commonly considered to be caused by gene upregulation, probably through changes in the regulatory elements (Feyereisen, 2005). Although this is the usual mechanism described, cases of gene amplification or qualitative changes have also been reported (Amichot *et al.*, 2004; Wondji *et al.*, 2009; Puinean *et al.*, 2010). Insect P450 enzymes may also activate certain types of insecticides, for instance the conversion of phosphorothioates (P=S) to phosphate (P=O). This can result in an increased

potency for inhibition of acetylcholinesterase by 3 or 4 orders of magnitude. P450s are also involved in the biosynthesis of ecdysone, juvenile hormone, and pheromone components (Feyereisen, 1995).

Glutathione-S-transferases

Glutathione transferases (GSTs) are a diverse family of enzymes found ubiquitously in aerobic organisms. They play a central role in the detoxification of both endogenous and xenobiotic compounds and are also involved in intracellular transport, biosynthesis of hormones and protection against oxidative stress (Ketterman *et al.*, 2011). Although GST enzymes can be involved in substrate sequestration, they usually catalyse the conjugation of reduced glutathione (GSH) with electrophilic substrates, converting those reactive molecules into more water-soluble and non-toxic conjugates that can be more readily excreted from the body. Specifically, they catalyse conjugations by facilitating nucleophilic attack of the sulphhydryl group of endogenous reduced glutathione (GSH) on electrophilic centres of a range of xenobiotic compounds, including insecticides or acaricides (Konanz and Nauen, 2004) and various plant toxins (Despres *et al.*, 2007). Thus the xenobiotics have increased solubility and are excreted from the insect system by the formation of mercapturic acid derivatives (Habig *et al.*, 1974; Enayati *et al.*, 2005). GSTs can also metabolise insecticides by facilitating their reductive dehydrochlorination or contribute to the removal of toxic oxygen free radical species produced through the action of pesticides (Hayes *et al.*, 2005).

Insect GSTs are divided into two different groups (microsomal and cytosolic) according to their location within the cell, but only the latter has been implicated in the metabolism of insecticides. Due to the broad range of substrates of the individual enzymes, they play an important role in resistance to different classes of insecticides, including organophosphates and pyrethroids. A DDT-dehydrochlorinase GST has also been reported as being responsible for DDT resistance in houseflies and mosquitoes (Enayati *et al.*, 2005).

Annotation of the *Anopheles gambiae* Giles and *Drosophila melanogaster* Meigen genomes has revealed the full extent of this enzyme family in insects (Enayati *et al.*, 2005). GST-based resistance is generally due to an increased amount of enzyme, resulting either from gene amplification or overexpression (Vontas *et al.*, 2002; Ranson and Hemingway, 2005). GSTs may also protect against pyrethroid toxicity in insects by sequestering the insecticide (Kostaropoulos *et al.*, 2001).

Additional metabolic resistance mechanisms: Pgp pumps

P-glycoprotein (Pgp) transporters are integral membrane proteins that belong to the ATP binding cassette (ABC) superfamily, which utilise the energy derived from ATP hydrolysis to translocate a variety of different metabolites and xenobiotics across cellular membranes (Hollenstein *et al.*, 2007). The action of Pgp pumps in removing a broad range of toxic compounds from cells is well established as a mechanism of antibiotic resistance in bacteria and fungicide resistance in fungi (Lage, 2003); in contrast very little is known about their physiological functions in insects. Only recently ABC transporters in insects have emerged as a putative mechanism which can contribute to resistance by facilitating efflux transport of insecticides and their metabolites derived from phase I and II reactions (O'Donnell, 2008). The involvement of Pgp pumps in insecticide resistance has been documented in several insect species and it has been correlated to increased expression of genes encoding ABC transporters (Porretta *et al.*, 2008; Aurade *et al.*, 2010; Bariami *et al.*, 2012). A survey of cases where the involvement of ABC transporters in insecticide resistance is suggested has recently been reviewed by Dermauw and Van Leeuwen (2014). ABC transporters have been associated with resistance to insecticides with different modes of action, based on the quantification of transcript or protein levels and by synergism studies using ABC inhibitors (Buss and Callaghan, 2008; Dermauw and Van Leeuwen, 2014).

Furthermore, in different lepidopteran species a mutant allele has been discovered which confers resistance to the pore-forming Cry1Ac toxin from *Bacillus thuringiensis* (Bt) by a mechanism that is not related to toxin extrusion, but because it causes the loss of Cry1Ac binding to membrane vesicles (Gahan *et al.*, 2010; Heckel, 2012).

Non-metabolic resistance mechanisms

Target-site resistance

Target site resistance is another important mechanism by which insecticide resistance is achieved and point mutations conferring insensitivity have been reported in many species. However, this is not a common mechanism by which insects become insensitive to plant toxins (Despres, 2007; Dobler *et al.*, 2015). Although mutations to the target protein of an insecticide can provide an insect with high levels of insensitivity, it would tend to be specific for a particular chemical class. Conversely, detoxification mechanisms have the potential to confer cross-resistance between plant toxins and insecticides and because of their capability to act against a broad range of molecules. As a consequence, metabolic resistance becomes more advantageous at the evolutionary scale, as it allows insects to survive in non-homogeneous environments, where plant toxins might be less uniformly distributed and the probability to encounter them could be reduced. In this situation, metabolic resistance is often less costly than target-site mutations, even if a real comparison of fitness costs among different populations can be very difficult or even impossible in field conditions (Kliot and Ghanim, 2012). In addition, there are several other mechanisms that may contribute at a more modest level. Although individually they may be only moderate in their impact, they can act as important intensifiers of resistance when combined with the major mechanisms in the same insect.

Penetration resistance

To reach its target, an insecticide must first penetrate the insect's cuticle. Penetration resistance occurs when insects have physico-chemical

alterations to the structure of their cuticle that results in a slower absorption of the chemicals or in a reduced amount of the insecticide passing through these physical barriers. This mechanism protects insects from a wide range of insecticides, but on its own confers low levels of resistance. Indeed, it is usually found in combination with other forms of resistance, enhancing their effects. For example, a delayed and slower penetration can provide more time for the detoxification of the insecticide by phase 1 enzymes (Oppenoorth and Welling, 1976; Scott, 1990; Strycharz *et al.*, 2013; Kasai *et al.*, 2014). Recently, indirect evidence of the importance of this mechanism has emerged in the green peach aphid, where several cuticular proteins were found to be differentially expressed in neonicotinoid resistant populations (Puinean *et al.*, 2010). Also, resistance to pyrethroid insecticides is associated with cuticle structure and composition in mosquitos (Fang *et al.*, 2015; Wood *et al.*, 2010).

Behavioural resistance

Behavioural resistance results from a change of insect behaviour in order to avoid the insecticide. This phenomenon is stimulus dependent and resistant insects can detect or recognise the danger and simply stop feeding or leave the treated area, walking or flying away (Gatton *et al.*, 2013). They can respond to lower concentrations of insecticide than normal insects, indicating the presence of receptors that allow development of the ability to detect the presence of insecticides (Sparks *et al.*, 1989; Yu, 2008). There are very few documented examples of behavioural resistance, one being the avoidance of malathion baits (Schmidt and LaBrecque, 1959).

Insecticide resistance management and metabolic resistance

One aim of resistance management is to delay the evolution of resistance in pests. The best way to achieve this is to minimize insecticide use, but the intrinsic difficulties of controlling a phenomenon driven by evolutionary forces must be considered (Hoy, 1998). One effective strategy involves the use of synergistic molecules in combination with insecticide products. Synergists are non-toxic compounds that enhance the efficacy of insecticides. They are capable of inhibiting the enzyme systems of insects that metabolise or sequester insecticide molecules. As a result, insect sensitivity increases, with the possibility to overcome metabolic resistance conferred by increased levels of detoxifying enzymes (Bernard and Philogene, 1993; Feyereisen, 2015). The best known synergist, piperonyl-butoxide (PBO), is widely used in household products against urban pests, but its application in agriculture remains limited to niche areas. Recent works have demonstrated *in vitro* the ability of PBO and its analogues to specifically interact with resistance-associated phase I metabolic enzymes of key insect pests, like *M. persicae* (Panini *et al.*, 2016) or *B. tabaci* (Panini *et al.*, submitted). In addition, Chen and Sun (1986) and Kumar *et al.* (2002) demonstrated that the use of PBO in combination with a synthetic pyrethroid can delay the development of resistance in Diamondback moth and mosquitoes, respectively. After several laboratory bioassays and field tests in these and other species, the concept of 'temporal synergism' was developed. If an insect is treated with a synergist a few hours prior to exposure to an insecticide, it allows time for the synergist to cross the insect cuticle and inhibit those enzymes involved in metabolic resistance, thus creating a sensitive or even hyper-sensitive insect and allowing a reduction of the insecticide dose to be subsequently applied (Moores *et al.*, 2005). Temporal synergism can be achieved not only with a split application of synergist and insecticide but also by using formulation technologies which allow a differential time release of synergist and insecticide, even when applied simultaneously (Bingham *et al.*, 2007; Mazzoni *et al.*, 2010).

Resistance management is a component of integrated pest management, which combines chemical and non-chemical controls to seek safe, economical, and sustainable suppression of pest populations. Alternatives to insecticides include biological control by predators, parasitoids, and pathogens (Lacey *et al.*, 2015). Also, valuable approaches include cultural practices (crop rotation, manipulation of planting dates to limit exposure to pests, and use of cultivars that tolerate pest damage), mechanical controls (exclusion by barriers and trapping) and behaviour manipulation (use of artificial signalling like mating disruption, false trail following or mass trapping) (Witzgall *et al.*, 2010). Since large-scale resistance experiments are expensive, time consuming, and might increase resistance problems, modelling has played a prominent role in devising tactics for resistance management. Although models have identified various strategies with the potential to delay resistance, practical successes have focused primarily on reducing the number of insecticide treatments, diversifying their types and, above all, the mode of action (MOA) of the insecticide employed.

Indeed, these strategies can be unsuccessful if the choice is made when the genetic background of the considered species and/or populations is unknown. Also, MOA rotating is effective only against target-site resistance mechanisms. Conversely, it can fail when multiple or cross resistance mechanisms are present, a situation that is common in many agricultural and urban pests (Bass *et al.,* 2014; Panini *et al.*, 2014, 2015; Abbas, 2015; Riveron *et al.*, 2015).

Only an in-depth knowledge of the genetic basis of insecticide resistance will allow a reduction of the environmental impact resulting from inadequate treatments. Molecular diagnostic tools can be important from this point of view, although they are generally utilised to detect target site resistance mechanisms (Cassanelli *et al.*, 2005; Bass *et al.*, 2007; Puinean *et al.*, 2013; Silva *et al.*, 2014; Chen *et al.*, 2015; Donnely *et al.*, 2016; Puggioni *et al.*, 2016;). Efforts should be made to improve the diagnosis of metabolic resistance allowing a reliable, fast and cost-effective tool. Furthermore, the information obtained from these studies would provide details essential for

the informed synthesis of effective and environmentally friendly actives.

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