

REVIEW

The potential immune alterations in insect pests and pollinators after insecticide exposure in agroecosystem**A Zibae^{1*}, D Malagoli²**¹Department of Plant Protection, Faculty of Agricultural Sciences, University of Guilan, Rasht, Iran²Department of Life Sciences, University of Modena and Reggio Emilia, Modena, Italy*This is an open access article published under the [CC BY license](https://creativecommons.org/licenses/by/4.0/)*

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Abstract

Agroecosystems are the habitat of pests and beneficial insects from different orders, which are exposed to agro-practices, especially treatments with chemicals. Insecticides are a wide group of chemicals used in agroecosystems that affect insect ecology and physiology in different ways. Among physiological components affected by insecticides, the immune system (IS) is an important one, enabling insects to resist against invading microorganisms and parasitoids thanks to the action of hemocytes and humoral components. So the determination of any immune alterations should be considered as a critical issue in insecticide application within agroecosystems. Insecticides of synthetic or natural origin, e.g. insect growth regulators (IGRs) and botanicals, are frequently cytotoxic and alter hemocyte morphology and number, impairing cellular-based immune responses in addition to humeral responses. Exposure of pollinators to neurotoxin insecticides like neonicotinoids may inhibit the immune-related transcription factor, NF- κ B, with a negative impact on the expression of antimicrobial peptides, melanization and clotting. In contrast, some IGRs may have enhancing effects on hemocyte spreading mainly plasmatocytes and cellular-based immune responses. Chemical insecticides have several impacts on the physiology of insects in which immune modulation is one of the most important cases because any alteration may alter their ability to respond toward invading pathogens and directly their survival. This is more severe once pollinators are in contact with chemicals because of the presence of several pathogenic agents that directly influence their performance.

Key Words: antimicrobial peptide; hemocyte; immune response; insecticide; pollinator**Introduction**

The widespread distribution of agricultural pests and climatic changes are the major threats to global food security, a topic that creates comprehensible concern in view of the growth of the human population. Nowadays agricultural pests are widespread in almost all agroecosystems, heavily influencing the economy and food resources worldwide. It has been estimated that more than 60000 species cause damages to agricultural products of which 50000 species are plant pathogens, 8000 species are weeds and 9000 species are insects and mites (Zhang *et al.*, 2011). The latter reduces the global harvest of about 14 %, while a further 26 % is lost in consequence of plant

pathogens and weeds (Pimentel, 2009a, b; Zhang *et al.*, 2011). Several control procedures try to limit the insect-driven damages, and among these chemical insecticides and biological control agents attract the attention of the experts. A loss of almost 78 % of fruit production, 54 % of vegetables and 32 % of cereals is estimated in absence of pesticides (Liu *et al.*, 2002; Cai, 2008; Zhang *et al.*, 2011; Zhang, 2018). Like other pesticides, also chemical insecticides negatively affect non-target organisms they leave polluting residues, display direct or indirect toxicity on humans, and pose the risk of a resurgence of resistant pests (Chandler *et al.*, 2011; Zhang, 2018). A common approach to reducing undesirable side effects of insecticides in agroecosystems is to use selective or low-persistent insecticides once the most sensitive developmental stage of target insect is available in the field (Dent, 2000).

The chemical insecticides most frequently distributed in agroecosystems have different modes

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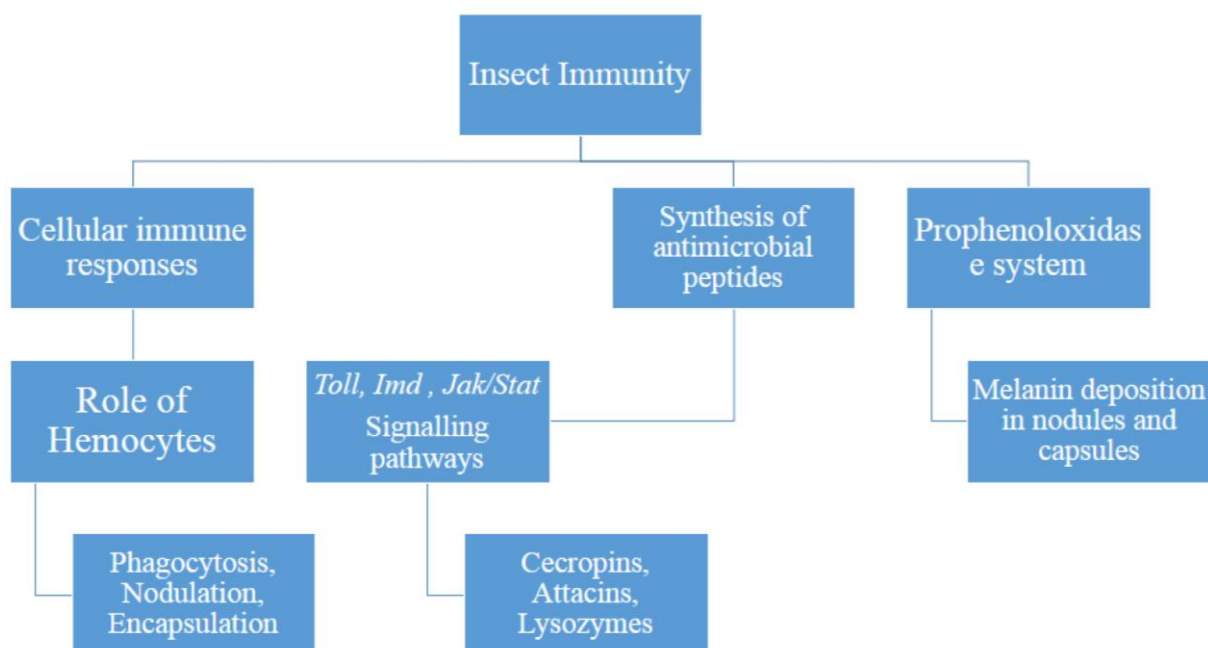


Fig. 1 An overview on the immune reactions of insects toward infectious objectives

of action. Organochlorine insecticides like cyclodienes as well as pyrethroids have neurotoxic and metabolic effects acting on the different ligand and voltage-gated ion channels (Yu, 2008; Timbrell, 2009). Organophosphates and carbamates, inhibitors of acetyl cholinesterase, slowly dispatched from the enzymes and cause repeated nervous pulses in the synaptic cleft. Nicotine and neonicotinoid insecticides block acetylcholine receptors on the post-synaptic membrane causing an influx of sodium ions and the generation of abnormal action potential. Insect growth regulators (IGRs) constitute a wide group of insecticides designed on growth-regulating hormones like juvenile hormone (JH), ecdysone and chitin synthesis inhibitors. IGRs disrupt insect development by intervening in molting, as well as on the synthesis of chitin, a major constituent of insect integument. Finally, plant-derived insecticides (Botanicals) may act as antifeedants, development and reproduction inhibitors (Yu, 2008).

Besides the most relevant and known effects, chemical insecticides may also cause widespread discrepancies on intermediary metabolism and immunity of insects (Zibae and Bandani, 2010; Zibae *et al.*, 2011; Zibae *et al.*, 2012; Mirhaghpars *et al.*, 2015a, b; Mirhaghpars *et al.*, 2016). Intermediary metabolism is referred to all biochemical and cellular processes which provide sufficient energy for the biological demands of insects (Klowden, 2007; Nation, 2008). Insect immune system (IIS) is a highly efficient component that confers protection against invading pathogens and tumors (Park and Lee, 2012). Once an invader passes non-specific defensive barriers like integument and alimentary canal, the immune

system raises several rapid and efficient responses (Nehme *et al.*, 2007; Tsaka and Marmaras, 2010). Cellular (e.g., phagocytosis, nodulation and encapsulation) and humoral (e.g., antimicrobial peptides, lectins, coagulation and melanization) reactions act synergistically to remove the infection from insect hemolymph and body cavity (Lavine and Strand, 2002; Nation, 2008; Park and Lee, 2012). Detailed immune reactions of insects toward infections have been illustrated in Figure 1.

In consideration of the concerns related to the usage of chemical pesticides, in the last four decades, there has been increasing attention towards the integrated pest management (IPM) to achieve sustainable agroecosystems. IPM aims to combine a responsible usage of chemicals, biological knowledge and cultural techniques in order to keep the populations of insect pests at acceptable levels. Efforts have been made in order to find the best synergistic combinations in the control of insect species. Insecticides and entomopathogen organisms are two elements that may prove highly efficient in IPM programs when used simultaneously (Zibae, 2019). Therefore, it becomes crucial to assess also in laboratory conditions the combined effects of different insecticides (e.g., synthetic, botanical or IGRs) and entomopathogens. Indeed, a pesticide alone could be ineffective in keeping the insect pest at an acceptable level, but it could prove highly efficient in weakening the IIS, acting as an enhancer for the entomopathogen organisms. Recently, this issue has been successfully addressed by exploring the fundamentals of *Bacillus thuringiensis* activity (Caccia *et al.*, 2016) and by testing the combination of an entomopathogenic fungus with a pesticide against

Leptinotarsa decemlineata (Coleoptera: Chrysomelidae) (Tomilova *et al.*, 2016). This review focuses on the effects of the different classes of insecticides on the IIS and provides a useful recapitulation of the insecticides more suitable for the combined use with entomopathogenic organisms.

Effects of synthetic conventional insecticides on IIS

As mentioned earlier, synthetic conventional insecticides (SI) from almost all classes impair nervous or hormonal systems leading to behavioral disability and subsequent death of insects (Chandler *et al.*, 2011). The connections between nervous and immune functions are well-known in all metazoans, including insects. For instance, after single or repeated pathogen exposures, reproducible changes of behavioral fever, illness-induced anorexia, decreased learning ability and increased egg-laying have been reported (Adamo, 2008). So, it is conceivable that the changes in insect neural function following sub-lethal treatment with SI may also affect IIS. Most frequently, insecticides play inhibitory effects on IIS, although the increased performance of IIS has been found in some cases (Zibae *et al.*, 2011; Zibae *et al.*, 2012; Mirhaghparast *et al.*, 2015a, b; Mirhaghparast *et al.*, 2016).

In order to infer how the insecticides could influence the IIS, Delpuech *et al.* (1996) designed an experiment to evaluate the effects of synthetic insecticides on host-parasitoid interactions. The authors treated topically the eggs of the susceptible and non-susceptible strains of *Drosophila melanogaster* (Diptera: Drosophilidae) with sub-lethal concentrations of chlordimeform, oxydemeton-methyl, propoxur, endosulfan, lindane, and dieldrin. The larvae hatched from these eggs were then reared on the media containing LC₃₀ of each insecticide till adult emergence. The eggs laid by insecticide-treated adults (Both sexes) were finally exposed to female parasitoid, *Leptopilina boulardi* (Hymenoptera, Eucoilidae). The results demonstrated that endosulfan and dieldrin significantly decreased the encapsulation rate in the treated embryos, while the total encapsulation rate decreased only after treatment by endosulfan. The other insecticides used in this study had no significant effects. The authors proposed that the adverse effects of endosulfan and dieldrin on *D. melanogaster* IS might be due to inhibition of monoamine oxidases responsible for melanization and, indirectly, for encapsulation efficiency (Delpuech *et al.*, 1996). A different point of view has been offered by studying the effect of SI, imidacloprid, on the immune response of the termite *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) against the entomopathogenic fungus *Beauveria bassiana* (Boucias *et al.*, 1996). The exposure to lethal and sub-lethal concentrations of imidacloprid did not modify the phagocytic competence of the treated termites. Further studies by using lipopolysaccharides, heat-killed or live bacteria revealed that *R. flavipes* may lack or have negligible cellular or humoral immune response in contrast to

other insects (Boucias *et al.*, 1996). This experiment suggests that while the IIS is a good target for control strategies, this might not hold true for all the species. A 10 to 20 day-treatment with monocrotophos, dimethoate, methylparathion, quinalphos or endosulfan increased the hemocyte count in the assassin bug *Rhynocoris kumarii* (Hemiptera: Reduviidae) (Caccia *et al.*, 2016). The results on endosulfan could have been related to its effects on insect diuresis and hemocyte dilution in the hemolymph. The increase in total hemocyte count corresponded to an unbalance in the hemocyte population, with a significant increase of granulocytes and a decrease of prohemocytes and plasmatocytes after 10 days of exposure (George and Ambrose, 2004). Recently, it has been observed that sublethal hexaflumuron exposure caused a concentration-dependent increase in plasmatocyte number, while the total hemocyte number and the phagocytic activity declined in the armyworm *Mythimna separate* (Lepidoptera: Noctuidae) (Huang *et al.*, 2016). Changes in the hemocyte population may not be directly linked to changes in the response versus a specific pathogen, but as different hemocytes play diverse roles, changes in cell population usually reflect changes in immune efficiency (Smith *et al.*, 2016). A gain in immune response was observed in the wax moth *Galleria mellonella* (Lepidoptera: Gelechiidae) and the potato beetle *L. decemlineata* after exposure to LC₅₀ or LC₃₀ of the phosphorothioate pirimiphos-methyl. The pesticide promoted a dose-dependent increase in the total hemocyte count and elevated the encapsulation rate toward nylon (Dubovskiy *et al.*, 2013). While the molecular basis for the effect is still unclear, the authors proposed that this organophosphate insecticide could suppress the neuro-hormonal stress reaction that in untreated control limited the immune response. As it will be further detailed below, in the section on the effects of insecticides on pollinators, in some cases the effects of SI on the IIS can be considered as an undesirable side-effect. In the silkworm *Philosamia ricini* (Lepidoptera: Saturniidae) the pyrethroid insecticide cypermethrin presented genotoxic effects and induced apoptosis in hemocytes at very low concentrations (Kalita *et al.*, 2017).

Effects of Insect growth regulators (IGRs) on IIS

Because of their structure, mimicking that of natural compounds, IGRs present more selective effects on insect pests than the previously mentioned SI (Merzendorfer, 2013). By regulating developmental and metabolic processes, the endocrine system has relevant interconnections with the immune response of insects, and hormones may act on both hemocytes and immune-related organs like the larval fat body (Adamo, 2008). On the other side, hemocytes directly intervene in tissue remodeling during molting and metamorphosis (Gillespie *et al.*, 1997; Zibae *et al.*, 2012). In these respects, Izzetoglu and Karacali (2003) made an *in vitro* experiment assessing the effects of a three-day treatment with different concentrations of the steroid hormone 20-hydroxyecdysone (20-E) on the total and

differentiated hemocyte counts in *G. mellonella* larvae. The authors reported an increase in total hemocyte count until the third day of 20-E treatment when the number of total hemocytes significantly decreased at all tested concentrations. A similar trend was observed for plasmatocytes and granulocytes, but the latter increased more significantly for low concentrations of 20-E (Izzetoglu and Karacali, 2003). In agreement with these observations, it has been observed in larvae of the grey flesh fly, *Neobellieria bullata* Parker (Diptera: Sarcophagidae) that 20-E and its synthetic agonists, RH2485, RH5849 and RH0345, increase laminarin-induced nodulation in a dose-dependent manner. The effects were observed either after injection or topical administration and were more evident for 20-E than its agonists. Conversely, juvenile hormone (JH) II and its analogs fenoxycarb and pyriproxyfen reduced laminarin-induced nodulation (Franssens *et al.*, 2006). The stimulatory action of 20-E and the inhibitory effect of JHI and II were observed also in the beet moth, where the response towards the plasmatocyte-spreading peptide was enhanced or inhibited by the two JHs, respectively (Kim *et al.*, 2008). Differently, form 20-E, the action of JH family members is mediated by extracellular receptors (Kim *et al.*, 2008). Several studies agreed on the hemocyte activating action of 20-E and the inhibiting activity of JH, which could play a physiological role in balancing the proliferation, differentiation and dispersal of hemocytes involved in immune competency (Rantala *et al.*, 2003; Sorrentino *et al.*, 2002). The IGRs tested against agricultural pests have shown effects on IIS partly similar to those described for 20-E and JH. Metryrapone, an IGRs and JHAs analog decreased the number of granulocytes but it increased plasmatocyte densities in the treated larvae of *Spodoptera littoralis* (Lepidoptera: Noctuidae) (Gelbič *et al.*, 2005).

Abd El-Aziz and Awad (2010a) demonstrated the effects of *B. thuringiensis* and Dimilin against the larvae of *Agrotis ipsilon* Hufnagel (Lepidoptera: Noctuidae). They reported significant increase in the numbers of plasmatocytes, granulocytes and spherulocytes but the number of prohemocytes showed a significant decrease in the treated larvae compared to control after 12 and 24 h. In another experiment, the authors found a significant decrease of phenoloxidase activity in the treated larvae compared to control in addition to changes in protein patterns of hemolymph in electrophoresis (Abd El-Aziz and Awad, 2010b).

Zibae *et al.* (2012) injected the adults of *Eurygaster integriceps* (Hemiptera: Scutelleridae) with methoxyfenozide, an activator of the ecdysteroid signaling cascade, or pyriproxyfen, four hours prior to injection of *B. bassiana* spores. Methoxyfenozide increased the number of total and differentiated hemocyte counts after 12 and 24 hours of treatment, whereas the opposite effects were observed with pyriproxyfen. Similar trends were observed for nodulation, especially at the highest concentrations used for pyriproxyfen (Zibae *et al.*, 2012). Similar effects on hemocytes but no effects on phenoloxidase were observed in the 4th instar larvae of *Ephesia kuehniella* Zeller

(Lepidoptera: Pyralidae) (Rahimi *et al.*, 2013). A course study of the effects of pyriproxyfen on the striped rice stemborer *Chilo suppressalis* (Lepidoptera: Crambidae) infected with *B. bassiana*, revealed a complex pattern of response, depending on both the time and the dosage of the pesticide (Mirhaghparast *et al.*, 2015a). In this case, hemocyte counts revealed a time-dependent fluctuation in plasmatocyte number and the increase of granulocytes only after 3 h incubation. Also, nodule formation was significantly increased at specific pyriproxyfen concentrations (Mirhaghparast *et al.*, 2015a). Similarly, the chitin-synthesis inhibitor hexaflumuron, increased hemocyte number and phenoloxidase activity of *C. suppressalis* larvae either alone or in combination with *B. bassiana* (Mirhaghparast *et al.*, 2015b). Shaurub and Sabbour (2017) determined the effects of pyriproxyfen and flufenoxuron on THC and DHC of *A. ipsilon* larvae. THC in the treated larvae significantly decreased compared to control. No significant changes were observed in the number of prohemocytes but pyriproxyfen significantly decreased the number of plasmatocytes. Both IGRs significantly decreased the number of granulocytes in the treated larvae. Yu *et al.* (2018) determined the effects of oral administration of tebufenozide on the immunity of the Asian corn borer, *Ostrinia furnacalis* (Lepidoptera: Crambidae). The IGR tebufenozide is an ecdysone agonist and belongs to diacylhydrazine molt-inducing insecticides which bind to ecdysteroid receptors into insect cells and mimics the effect of molting hormones. Tebufenozide increased both the total hemocyte count and the activity of phenoloxidase compared to control larvae. Moreover, the treated larvae displayed an enhanced ability to clear the bacterial infection from their hemolymph so that an increased survival rate was observed after 33 hours of bacterial challenge (Yu *et al.*, 2018).

Botanical insecticides (BI)

Plants developed various compounds during their evolutionary processes, in order to be well equipped against insect pests. These compounds are known as secondary metabolites and they may have several functions, including the defense against herbivore attacks (Wink, 2018). Due to the adverse effects connected with the usage of synthetic insecticides, the plants-derived compounds, further classified into the two categories of plant extracts and essential oils are promising candidates against insect pests. Today, botanical insecticides have shown significant effects with no or negligible destructive effects on the environment and less prone to be inactivated by resistant insects (Enane, 2001; Hardin *et al.*, 2009; Pavela and Benelli, 2016). As far as their target is concerned, botanical insecticides (BI) may affect digestion, reproduction, intermediary metabolism, oxidative stress, cell death, octopaminergic system and immune system. Recognized molecular targets include acetylcholine esterase, P450 cytochromes, Octopamine and gamma-aminobutyric acid (GABA) receptors (Abdelgaleil *et al.*, 2009; Zibae, 2011; Lukasik *et al.*, 2011; Senthil-Nathan, 2013;

Kumrungsee *et al.*, 2014; Wei *et al.*, 2015; Tak and Isman, 2016; Pavela and Benelli, 2016; Shahriari *et al.*, 2017; Shahriari *et al.*, 2018).

The observed effects of BI on IIS include abnormal hemocyte morphology, altered total and differentiated hemocyte counts, reduced phagocytosis, nodulation as well as inhibition of phenoloxidase (Zibae, 2011). Pioneer studies on the limonoid BI azadirachtin, revealed, besides the antifeedant effects, immune depression in the nymphs of the kissing bug *Rhodnius prolixus* L. (Hemiptera: Reduviidae) subjected to bacterial challenge. Effects of azadirachtin on the IIS involved hemocyte count, nodule formation and antibacterial and lysozyme activities. Interestingly, no effects were reported on the prophenoloxidase-activating system in the azadirachtin-fed nymphs (Azambuja *et al.*, 1991). Ayad *et al.* (2001) reported the higher THC and oenocytoid counts in the azadirachtin treated larvae of *Parasarcophaga surcoufi* (Diptera: Sarcophagidae) after 40 h while the numbers of plasmatocytes and granulocytes decreased after 10 and 40 h, respectively. Moreover, the authors recorded the significant hemocyte deformities as lysis of plasmatocytes and releasing of cytoplasmic components of granulocytes (Ayad *et al.*, 2001).

Relationships between BI effects and the IIS were observed also after treating target insects with Neem gold and *Artemisia calamus*. These vegetable extracts provoked significant damage in hemocytes, which resulted in almost disrupted by the treatment (Sharma *et al.*, 2003, 2008). After feeding *E. integriceps* with *Artemisia annua*, it was observed a significant decrease in phagocytic rate and nodule formation. At all-time intervals, the *A. annua* extract decreased the activity of phenoloxidase in the treated hemipterans and inhibited the enzyme activity by reducing V_{max} and increasing K_m . The authors concluded that *A. annua* may affect ligand-receptor complex formation on the plasma membrane of *E. integriceps* hemocytes, thus reducing or impairing immune reactions of phagocytosis and nodulation (Zibae and Bandani, 2010). Shaurub *et al.* (2014) found that the larvae of *S. littoralis* treated by azadirachtin had the less THC compared to control. Also, significant changes in morphology of plasmatocytes and granulocytes were observed as the presence of rough endoplasmic reticulum filled with fibrous materials, looped, vacuolated and swollen mitochondria with the specific appearance of autophagic lysosomes in granulocytes and ruptured cell membrane and folded nuclear envelope in plasmatocytes (Shaurub *et al.*, 2014).

Similar results were observed also in *G. mellonella* larvae treated with azadirachtin and in *S. littoralis* exposed to essential oils of castor and camphor (Er *et al.*, 2017). In contrast to the above-mentioned studies, Dhivya *et al.* (2018) reported an increase of total hemocyte counts and an augmented activity of phenoloxidase in *S. litura* larvae exposed to *Prosopis juliflora* extract. Individual and combined effects of a bacterium, *Micrococcus luteus*, and azadirachtin were evaluated on the immune system of *Sarcophaga argyrostoma* (Diptera: Sarcophagidae) larvae (Dorrah *et al.*, 2019). Azadirachtin decreased the

number of total hemocyte count 12 and 24 h post-treatment while a set of various changes were observed in differentiated hemocyte counts. Oenocytoids, spherulocytes, granulocytes and pro-hemocytes presented an irregular trend, with their number increased or decreased at different time points. Plasmatocytes significantly decreased at all time intervals after azadirachtin treatment except for 60 h when no significant difference was observed in comparison to control. The injection of larvae with bacterium and azadirachtin significantly reduced the numbers of formed nodules, phenoloxidase and lysozyme activity compared to individual bacterial injection. These effects were somehow similar in fat bodies, hemocyte and plasma preparations, and were attributed to a potential mutagenic action of azadirachtin (Dorrah *et al.*, 2019). However, the full explanation of these data has not been provided, yet.

Effects of insecticides on the immune system of pollinating bees

Pollinator insects are one of the most important organisms in agroecosystems as they engaged in pollinating about 240,000 species of flowering plants (McGregor, 1976; Dafni, 1992). These plants are not only directly fed by humans, but they are used also as food sources for livestock. Well-known estimates proposed that 30 – 50 % of total human food may directly or indirectly depend on pollinator insects as they influence the quality and quantity of agricultural products (McGregor, 1976). The agricultural techniques implemented in the last decades and the expansion of the food demand in the developing countries make it difficult to refine these estimates but still, the fundamental contribution of pollinators is out of the question (Aizen *et al.*, 2009). In addition to agricultural products and plant reproduction, the biodiversity of animals feeding on fruits and seeds also depends on pollinator activity, and in this sense the precise relevance of pollinators on the global ecosystem is hardly quantifiable (Eardley *et al.*, 2006).

Unfortunately, agrochemicals in general, and SI in particular, frequently affect life history, reproduction, motility, learning and physiological functions, including immunity, of pollinators beside those of insect pests (Desneux *et al.*, 2007; Domingues *et al.*, 2017) and sublethal doses of them can represent a chronic stress leading to colony failure of colonial pollinators such as bees, the principal pollinators in human agriculture (Brandt *et al.*, 2016). Several recent studies have highlighted the adverse effects of insecticides on immune responses of bees, especially the Western honeybee *Apis mellifera* (Hymenoptera: Apidae). In the case of neonicotinoids, it has been reported that these SIs up-regulate an inhibitor of the immune-related transcription factor, NF- κ B, thus paving the way to the replication of the deformed wing virus (Di Prisco *et al.*, 2013). Such inhibition could also suppress other forms of immune defenses, e.g., expressions of antimicrobial peptides, hemocyte clotting and melanization (Di Prisco *et al.*, 2013; Brandt *et al.*, 2016). Simultaneous exposure to a neonicotinoid and an immune challenge increases

the insecticide-mediated immune suppression, thus in open field, the action of sublethal doses of neonicotinoid or other pesticides could be even more aggressive than that measured in laboratory by exposing bees only to a pesticide (Lopez *et al.*, 2017; Walderdorff *et al.*, 2018). This observation agrees with previous results indicating that the co-exposure to the SI imidacloprid and the entomopathogenic microsporidia *Nosema apis* has a significant impact on mortality while leaving apparently unaffected hemocyte count and phenoloxidase activity (Alaux *et al.*, 2010). In these respects, the oral administration of a mixture containing the SI diazinon, malathion, profenofos and chlorpyrifos did not impact on bee survival after short-term exposure, but it affected the expression of the antimicrobial peptide hymenopteran (Al Naggar *et al.*, 2015). In the class of neonicotinoids, compounds such as thiacloprid and imidacloprid can affect immune parameters at concentrations similar to those used on the field, whereas the clothianidin can influence total hemocyte count only at non-realistic concentrations but it does affect encapsulation at all the concentrations assessed in *A. mellifera* (Brandt *et al.*, 2016). These discrepancies are probably to be related to the chosen compound, the doses applied and the selected immune parameters.

The information collected on *A. mellifera* is consistent with data collected on other bees. In *Apis bursata* five commercial formulations of the SI, endosulfan, bifenthrin, diafenthiuron, imidacloprid and ethofenprox affect bee hemocyte number, shape and integrity (Perveen and Ahmad, 2017). In the bumblebee *Bombus impatiens* Cresson (Hymenoptera: Apidae) field-realistic 6-day pulses of imidacloprid revealed that the immune response to a non-lethal immune challenge can be maintained only in control bumblebees and in those exposed to the lowest concentration. The animals exposed to the highest concentration did not sustain the immune response and had a higher chance to die after the immune challenge (Czerwinski and Sadd, 2017). In this way, it is confirmed that both the insecticide and the immune challenge act as a stressor and they could present additive or synergistic effects, highly deleterious for the pollinators (Czerwinski and Sadd, 2017). Walderdorff *et al.* (2018) designed a study to determine the effects of separated and simultaneous exposure of *A. mellifera* to the SI imidacloprid and the Gram-negative *Escherichia coli*. Results demonstrated that imidacloprid alone decreased phagocytosis and production of hydrogen peroxide and nitric oxide, while *E. coli* slightly increased these immune reactions compared to sole insecticide exposure. Finally, the authors reported that imidacloprid had more severe effects on the unspecific oxidants hydrogen peroxide and nitric oxide than on phagocytosis. Hernández López *et al.* (2018) reported that sub-lethal dose of dimethoate, an organophosphate SI, led to the increase of total- and differentiated hemocyte (granulocytes and oenocytoids, but not plasmatocytes) counts in *A. mellifera* larvae while LD₅₀ concentration decreased total hemocyte count compared to control although no statistical

differences were observed in case of differentiated hemocyte count. The SI clothianidin, a neonicotinoid, increased total hemocyte count and activated cellular responses but it had no effect on differentiated hemocyte count. The pyrethroid fluvalinate showed no statistical effects on hemocyte counts and cellular immunity compared to control. The authors also demonstrated synergistic and negative effects of demethoate and clothianidin on bees simultaneously exposed to the bacterium *Paenibacillus larvae* and concluded that fluvalinate might be the less aggressive insecticide for *A. mellifera* larvae.

Recently, Santos *et al.* (2018) designed the series of experiments to determine the effects of *Cymbopogon martinii* essential oil and its major constituent, geraniol (a BI), along with the SI imidacloprid on *A. mellifera*. At first, the authors investigated the oral and contact toxicities of the two compounds against adult bees. Second, the LD₂₀ concentration of the compounds separately was exposed to evaluate the encapsulation and foraging behavior of *A. mellifera*. The oral exposure was more toxic than contact exposure, but *C. martinii* essential oil and geraniol toxicity were lower than that of imidacloprid. No significant difference was observed in encapsulation and foraging behavior of the individuals treated the BIs and the SI, while negative effects were observed in the average distance traveled, light orientation and movement in the same direction among the orally treated honeybees, especially for bees treated with imidacloprid. The authors attributed the lower toxicity of the essential oil and geraniol to their efficient metabolic degradation thanks to the activity of glutathione S-transferase, esterase and cytochrome P₄₅₀-dependent monooxygenase. The higher toxicity of the nicotinoid imidacloprid is correlated to its neurotoxic action which is not counteracted by specific metabolic activities.

Conclusion

This overview of the action of different class insecticides, SI, IGRs and BI highlight the relevance of the effects that all these compounds have on the IIS. The effects are in some cases controversial because there is not a unique effect in terms of immune activation or inhibition, but in general, the immune functions are altered in a way that makes the IIS less efficient and responsive. While these can be useful for controlling insect pests, it may also represent a double-edged sword, because immune impairment extends also to beneficial pollinators. The development of new generation and eco-friendly pesticides, especially those of natural origin or based on pathogens, specifically targeting the immune system of the insect pest under treatment, may represent a valid alternative. However, long-term effects, persistence in the environment and side effects on beneficial insects must be carefully evaluated for all the products that will be developed in the future. This represents a further obstacle because for several users the cost, availability and environmental persistence are considered advantageous points in favor of present-day chemical/synthetic pesticides.

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