



Azadirachtin-Based Insecticide: Overview, Risk Assessments, and Future Directions

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In the context of the major crop losses, pesticides will continue to play a key role in pest management practice in absence of practical and efficient alternatives; however, increasing awareness regarding environmental and human health impacts of conventional pesticides as well as the development of resistance and cross-resistance reduced their availability and promoted the search for alternative control strategies and reduced-risk pesticides. Among the various alternatives, a drastic re-emergence of interest in the use of plant-derived compounds, called allelochemicals, was noted and demand for an organic product is rising. Currently, azadirachtin, a tetranortriterpenoid derived from the neem seed of the Indian neem tree [*Azadirachta indica* A. Juss (Meliaceae)], is one of the prominent biopesticides commercialized and remains the most successful botanical pesticide in agricultural use worldwide. Azadirachtin is a powerful antifeedant and insect growth disruptor with exceptional low residual power and low toxicity to biocontrol agents, predators, and parasitoids. This review summarizes the state of the art on key azadirachtin insecticidal activities and risk assessment, identifies knowledge gaps that could serve as the basis for future research direction and highlights limitation in agricultural use and the development of novel strategies by the use of nanotechnology to control its release rate and improve its stability and sustainability.

Keywords: azadirachtin, *Azadirachta indica*, nanotechnology, alternative pest control, agroecosystems

INTRODUCTION

The United Nations predicts that the global population will increase from 7.7 billion in 2019 to 9.7 billion in 2050 (United Nations, 2017), this evolution is the main factor that will increase the demand for food production which is expected to continue to grow and is projected to increase by 25–70% in 2050 to meet the increasing human demand (Hunter et al., 2017; Silva, 2018). Annual crop losses caused by insects, weeds, and diseases are estimated between 20 and 40 percent, similar to those of 50 years ago due to the intensification of agricultural production together with the effects of climate change (FAO, 2017). To safeguard and improve food security, crop protection from pests is required and aimed to avoid or prevent crop losses or to reduce them to an economically acceptable level (Karuppuchamy and Venugopal, 2016).

Over the years and since the 1950s, conventional synthetic insecticides have played a crucial role in increasing agricultural productivity (Aktar et al., 2009; Popp et al., 2013). In the context of the major crop losses, pesticides will continue to play a key role in pest management practice in absence of practical and efficient alternatives. Indeed, the beneficial outcome from the use of

pesticides remains vital for avoiding hunger and food insecurity and meeting the demand of today and future generations especially in the developing countries (Deravel et al., 2014); however, the extensive use of pesticides generates human and environmental health risk and hazards (Carson, 1962; Aktar et al., 2009; Nicolopoulou-Stamati et al., 2016; Jars et al., 2018) and a growing resistance to targeted pests by exerting selection pressure on insect pests (Harrop et al., 2014; Helps et al., 2017).

After the publication of the *Silent Spring* by Rachel Carson (Carson, 1962), and to attenuate the negative impacts of pesticides in the environment and public health, search for alternative control strategies and reduced risk pesticides became a real challenge (Pimentel, 1997; Khater, 2012). Consequently, a drastic re-emergence of interest in the use of natural pesticides known as biopesticides was noted (Cantrell et al., 2012; Kumar, 2015; Mishra et al., 2018; Haddi et al., 2020). Although there is no formally agreed definition, biopesticides are eco-friendly pest management agents based on living organisms or natural products (Chandler et al., 2011). They may be derived from animals (ex: nematodes), microorganisms (ex: *Bacillus thuringiensis*), plants (ex: *Azadirachta*) as well as certain minerals (Damalas and Koutroubas, 2018). If biopesticides are gaining popularity as reduced environmental impact alternatives to conventional synthetic pesticides, the biopesticides market remains small (5%) to the worldwide pesticide market (Olson, 2015). However, this segment of the industry is experiencing rapid growth in recent years with a compound annual growth rate of 8.64% and is projected to outpace that of chemical pesticides (Olson, 2015; Damalas and Koutroubas, 2018).

The main advantages of biopesticides are that they are inherently less toxic than conventional pesticides by offering more targeted action against specific pests (Damalas and Koutroubas, 2018). Indeed, conventional pesticides which exert their effects on the nervous system of insects often affect a broad spectrum of pests along with bird and mammalian species (Thakora, 2006). Furthermore, biopesticides often are effective in very small quantities and decompose quickly, resulting in lower exposures and largely avoiding the pollution problems caused by conventional pesticides (FAO). When using as a component of integrated pest management (IPM) programs, biopesticides can supplement the conventional pesticides and greatly reduce their use and offer potentially higher crop yields (Thakora, 2006; Damalas and Koutroubas, 2018).

Recently, among the biopesticides, plants with pesticidal properties have been the subject of an increasing number of academic researches as a potential option for environment friendly pest management tools for developing sustainable agricultural practices and promote human and environmental safety (Isman, 2006; Cantrell et al., 2012; Hikal et al., 2017). Plants, the most common source of biopesticides, produce a great variety of secondary metabolites potentially applicable in IPM programs (Céspedes et al., 2014).

Growing attention has been given to the neem tree, *Azadirachta indica* A. Juss. (Meliaceae), as the most prominent biopesticide (Isman and Grieneisen, 2014; Aribi et al., 2020). In Asia, the neem tree is regarded as a wonder tree and has been used for centuries in Ayurvedic medicine as one of oldest

medical systems in humanity (Biswas et al., 2002; Pasquoto-Stigliani et al., 2017). Among its many attributed properties, it acts as an antidiabetic, immunostimulant, antimicrobial, antiviral, cholesterol-lowering agents, contraceptive and anticancer remedy, and it has long been revered by ancient Indian people and is entitled “village drugstore” (Tinghui et al., 2001; Hummel et al., 2016; Moga et al., 2018; Blum et al., 2019). Additionally, aqueous extracts of powdered neem kernels have been used as an insecticide in India for about 2,000 years for the control of insect pests (Schmutterer, 1995). In recent time, and following the isolation of azadirachtin, the major active compound, that is mainly responsible for the insecticidal activity of neem, the use of neem-based insecticide has increased in the last 30 years (Chaudhary et al., 2017; Pasquoto-Stigliani et al., 2017). Currently, azadirachtin is one of the prominent biopesticides commercialized and remains the most successful botanical pesticide in agricultural use worldwide (Isman and Grieneisen, 2014; Chaudhary et al., 2017; Aribi et al., 2020); however, its mechanisms of action still unclear and remain to be clarified especially in relation to the neurophysiological and the possible long-term activities.

THE NEEM TREE

Neem is an evergreen fast-growing tree native to India and Burma, it grows in arid, semiarid, and tropical regions (Schmutterer, 2002). Today, the neem tree is widely distributed throughout tropical and subtropical Asia, Africa, Australia, and South America (Kumar et al., 2016). Neem products have been obtained from several species of neem trees belonging to the Meliaceae family. *A. indica* Juss, is the most important species of this group considered a renewable resource of various useful domestic, medicinal, and agricultural products (Kumar et al., 2016). All parts of the tree (leaf, flower, seed kernel, wood, bark, and twig), are a source of biologically active ingredients, and the maximum of activity is associated with the seed kernel (Kumar et al., 2016). More than 300 different phytochemicals have been reported from different parts of the neem tree (Gupta et al., 2017) and over 130 of these compounds belongs to limonoid-type triterpenoids that are endowed with potent medicinal and insecticidal properties (Chen et al., 2018); However, the chemical composition of neem is far to be completely elucidated, as evidenced by the novel compounds reported each year (Nicoletti et al., 2016; Chen et al., 2018). The most important neem limonoids include azadirachtin, nimbolide, salannin, nimbin, deacetylnimbin, mahmoodin, epoxy-azadiradione, deacetylgedunin, and gedunin (Nagini, 2014; Gupta et al., 2017). These compounds have been shown to possess many useful properties of which, antifeedancy, insecticidal, and insect growth disruption are used in the management of pest (Schmutterer, 1995). Most of the triterpenoids of neem were found in very small quantities in various parts of the tree and account for the total bioactivity of the neem seed extract (Mordue et al., 2010). Azadirachtin A is the major active component and is responsible for 72 to 90% of the biological activity (Schmutterer, 1990; Mordue et al., 2010).

AZADIRACHTIN: PROPERTIES AND INSECTICIDAL ACTIVITIES

Azadirachtin is a complex tetranortriterpenoid with 16 chiral carbon centers, derived from the mevalonic acid pathway in the neem tree (Hansen et al., 1993; Aarthy et al., 2018). It is a highly oxidized tetranortriterpenoid natural product related to limonin, the bitter principle of citrus fruits and known as limonoids (Benuzzi and Ladurner, 2018). Azadirachtin A is considered as the main constituent and azadirachtin commercial formulations, available on the world market for insect control in organic farming, contain a stated amount of azadirachtin A (Table 1) (Benuzzi and Ladurner, 2018). It has a complex molecular structure and following the determination of its correct structure in 1985 (Kraus et al., 1985), the first total synthesis of this molecule was published two decades after the discovery of the compound (Jauch, 2008). Azadirachtin is a broad-spectrum insecticide (Figure 1), its acts as a feeding deterrent, insect growth disruptor (IGD), and sterilant and is used to control various agricultural pest species, including Coleoptera, Heminoptera, Diptera, Orthoptera, and Isoptera (Morgan, 2009). The toxicity of azadirachtin varies among insect orders and is influenced by the different penetration rates and activities of detoxifying enzymes (Table 2).

The chemical complexity of azadirachtin minimizes the potential risk of insect resistance (Mordue et al., 2010). Feng and Isman (1995) reported development of resistance to pure azadirachtin over 40 generations in the peach potato aphid *Myzus persicae* but no resistance was reported with neem seed extract. Bomford and Isman (1996) also showed habituation to pure azadirachtin in the tobacco cutworms with less sensitivity to the antifeedant properties of azadirachtin, but not to neem with the same absolute amount of azadirachtin. This might account for avoiding desensitization to commercial neem-based insecticides containing additional non- AZA-compounds (Bomford and

Isman, 1996). Azadirachtin A is very well-received by the root system, and, subsequently, it is systematically distributed through the xylem into the green parts of plant tissues and stored in leaves in an unchanged form. In addition, a very low content of azadirachtin A in plant tissues may protect significantly plant damage against phytophagous pest larvae (Pavela, 2016).

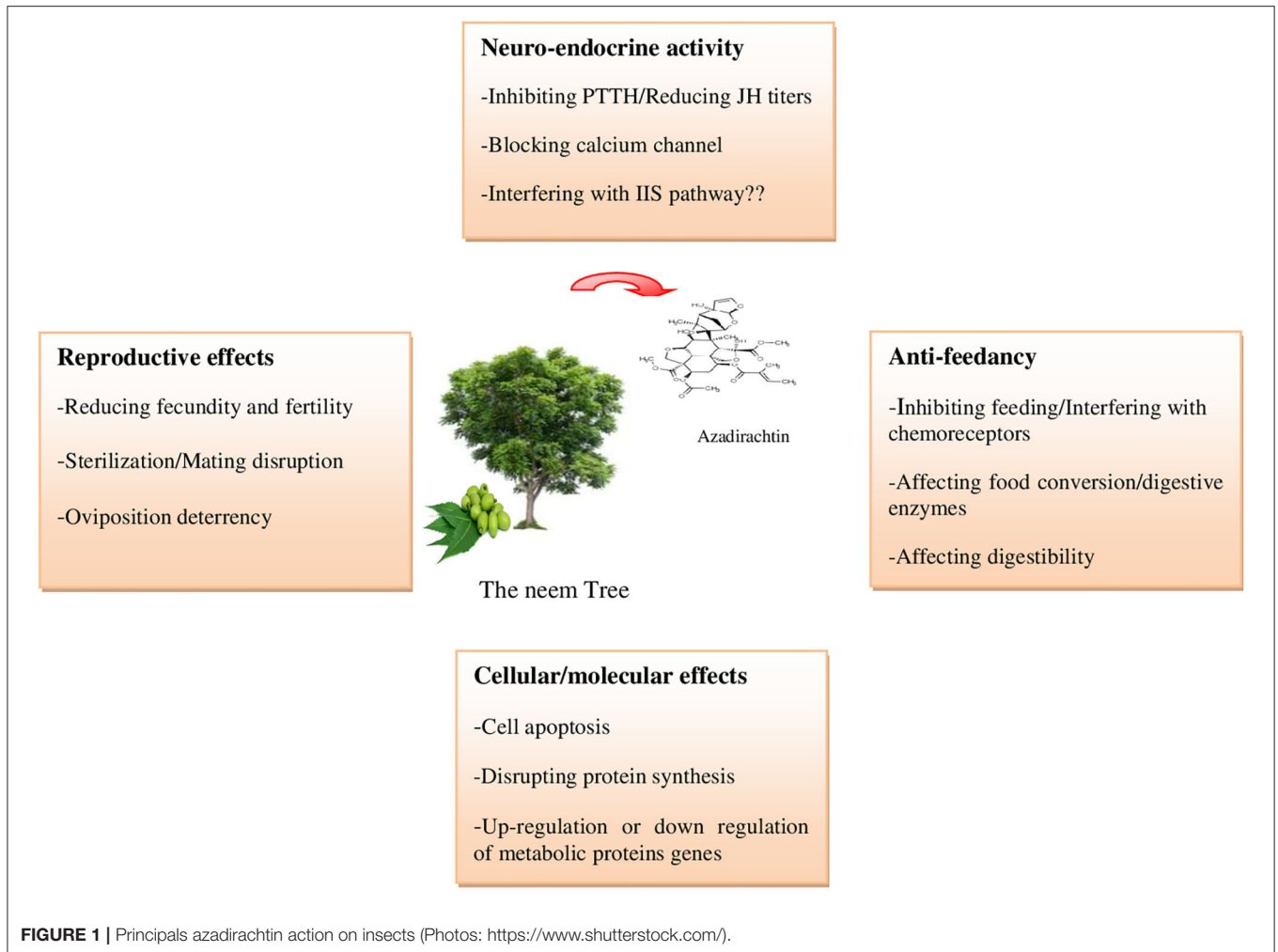
In addition, azadirachtin has displayed remarkable selectivity with low mammalian toxicity (Mordue et al., 2010). According to Raizada et al. (2001), azadirachtin has shown an LD₅₀ value of more than 5,000 mg/kg which falls into class U (Unlikely to present an acute hazard) of the WHO (2009) toxicity rating. Azadirachtin is registered in the United States as a general-use pesticide with a toxicological class Environmental Protection Agency (EPA) of IV (relatively non-toxic). Azadirachtin seems to be selective, non-mutagenic, and readily degradable and has also been reported as safer for non-target organisms and beneficial organisms (Medina et al., 2004; Cordeiro et al., 2010; Mordue et al., 2010; Celestino et al., 2014; Dai et al., 2019); however, the presumed safety of azadirachtin has been questioned, especially, in relation to natural enemies and pollinators (Barbosa et al., 2015; Lima et al., 2015; Xavier et al., 2015; Bernardes et al., 2017, 2018; Francesena and Schneider, 2018). Nevertheless, semi-field and field studies may enable to reliably predict potential side effects of azadirachtin on non-target insects. However, azadirachtin is still considered as one of the best alternatives to conventional insecticides in IPM programs and considered as one of the most promising plant compounds for pest control organic agriculture (Tomé et al., 2013; Bezzar-Bendjazia et al., 2017). Despite the progress on the physiological and biological activities and agricultural application of azadirachtin, its exact mechanism of action, especially, at the molecular level is not yet fully understood (Lai et al., 2014; Dawkar et al., 2019).

Effects on Neuro-Endocrine Activity

In insects, 20-hydroxyecdysone (20E) and juvenile hormone (JH) play a central role in the regulation of growth and development (Bensebaa et al., 2015), and the hormonal balance determines the outcome of each developmental transition (Dubrovsky, 2005). Therefore, any interference with hormonal homeostasis leads to interrupted development and is considered as a potential specific target for pest control (Pener and Dhadialla, 2012). Azadirachtin is known as an antagonist of these two principles hormones; its major action was its ability to modify or suppress hemolymph ecdysteroid and JH titers through inhibition of the secretion of morphogenetic peptide hormone (PTTH) and allatotropins from the *corpus cardiacum* complex and this account for its well-documented IGD effects defined mostly as reduced pupation, malformation or a failure of adult emergence (Mordue and Blackwell, 1993; Bezzar-Bendjazia et al., 2017). Furthermore, this compound is known to cause degenerative structural changes of the nuclei in all endocrine glands (prothoracic gland, *corpus allatum*, and *corpus cardiacum*) responsible for controlling molting and ecdysis in insect which would contribute to a generalized disruption of neuroendocrine function (Mordue et al., 2010). Azadirachtin applied on the diet at 74 ppm affects the growth, suppresses ecdysis, and inhibits ecdysteroids synthesis

TABLE 1 | Commercial azadirachtin-based products available worldwide.

Products	Manufacturer	Azadirachtin Percent %
Agroneem plus	Agro logistic systems Inc	0.15
Azagro	India MART	1
AzaGuard	BioSafe systems	3
Azamax	E.I.D parry Ltd	1.2
AzaPRO	CANN-CARE company	1.2
Azasol	ARBORJET Inc	6
Debug TRES	AGROLogistic systems	3
EcoZin plus	AMVAC chemical Corp.	1.2
Fortune aza	Fortune biotech Ltd	3
MOLT-X	BIOWORKS Inc	3
Neem Azal TS	Trifolio-M GmbH	1
Neemarin	AZA-Direct Gowan company LLC	0.15
Neemfol	Gassin pierre	5
Neemix	Certis	0.25
Ornazin	SEPRO corporation	3

**TABLE 2** | Azadirachtin LC₅₀ in some species of pest.

Species	Mode of application	Developmental stage	LC ₅₀ (ppm)	References
<i>Plutella xylostella</i>	Orally	3rd instar larvae	0.63	Zada et al., 2018
<i>Tuta absoluta</i>	Orally	2nd instar larvae	5.62	Tomé et al., 2013
<i>Tirathaba rufivena</i>	Orally	Larval	28.79	Zhong et al., 2017
<i>Helicoverpa armigera</i>	Orally	3rd instar larvae	12.95	Abedi et al., 2014
<i>Coridius viduatus</i>	Orally	Adults	0.003	Aljedani, 2018
<i>Megaselia scalaris</i>	Dipping	1st instar larvae	13.79	Abdel-Gawad, 2018
<i>Agonosceca pistaciae</i>	Dipping	5th instar larvae	0.22	Izadi et al., 2012
<i>Heteracris littoralis</i>	Topically	4th instar larvae	101.20	Ghazawi et al., 2007
<i>Lobesia botrana</i>	Orally	1th instar larvae	2.1	Irigaray et al., 2010
<i>Galleria mellonella</i>	Topically	Immature stage	16.56	Er et al., 2017

in the larvae of *Ostrinia furnacalis* Guenée (Min-Li and Shin-Foon, 1987). In *Tenebrio molitor*, the injection of 1 µg of azadirachtin into freshly ecdysed pupae induced a significant depletion of levels of immunoreactive ecdysteroids affecting 20-hydroxyecdysone levels and suppressing the ecdysteroid peak that normally appears at the middle of the instar (Marco et al.,

1990). A drastic reduction of hemolymph ecdysteroid titers was also reported in *Rhodnius prolixus* after a unique dose of azadirachtin (Garcia et al., 1990). In addition to its effects on morphogenetic PTTH, azadirachtin affects ecdysone 20-monooxygenase activity, the insect cytochrome P450-dependant hydroxylase responsible for the conversion of the steroid

hormone ecdysone to its more active metabolite, and 20E (Smith and Mitchell, 1988). Indeed, *in vitro* analysis of three insect species, homogenates of wandering third instar larvae of *Drosophila melanogaster*, fat body or midgut from last instar larvae of *Manduca sexta* and abdomens from adult female *Aedes aegypti*, incubated with radiolabelled ecdysone and azadirachtin revealed inhibition of the ecdysone 20-monooxygenase with a dose-dependent relationship (Smith and Mitchell, 1988); however, ingested or injected azadirachtin had no effect on ecdysone 20-monooxygenase activity in *Spodoptera frugiperda* (Yu, 2000). Besides its negative effects on molting hormone, azadirachtin induced a delay or a reduction in JH titters, primarily by hindering the release of the allatotropins and thereby blocking the synthetic and release processes of the JH (Mordue et al., 2010; Dhra et al., 2018).

Azadirachtin is reported to impair the growth and molting process of insects and induced robust developmental delays in the larva-to-pupa and the pupae-to-adult transition compromising their survival (Hasan and Ansari, 2011; Tomé et al., 2013; Lai et al., 2014; Bezzar-Bendjazia et al., 2016). In addition, growth and nutrient intake are functionally linked processes in development and growth and body mass are directly affected by nutrient uptake principally governed by the insulin/insulin-like growth factor signaling (IIS) pathway (Tennesen and Thummel, 2011). Lai et al. (2014) reported that the inhibition of growth and development in *D. melanogaster* after azadirachtin treatment was similar to those caused by disruption of the IIS pathway. In addition, azadirachtin can inhibit the excitatory cholinergic transmission and block partly the calcium channel (Qiao et al., 2014), and this might interfere with different endocrinological and physiological actions in insects.

Effects on Reproduction

The negative effects of azadirachtin on reproduction were reported in several insect orders (Pineda et al., 2009; Tine et al., 2011; Tomé et al., 2013; Boulahbel et al., 2015; Er et al., 2017; Oulhaci et al., 2018). Reduced fecundity and fertility has been recorded in many insects including *Spodoptera littoralis*, *D. melanogaster*, *Galleria mellonella*, *Dysdercus cingulatus*, *Tuta absoluta*, and *Helicoverpa armigera* (Pineda et al., 2009; Pandey and Tiwari, 2011; Tomé et al., 2013; Ahmad et al., 2015; Er et al., 2017; Oulhaci et al., 2018) and could be due to the interference of azadirachtin with yolk protein synthesis and or its uptake into oocytes (Boulahbel et al., 2015). In leaf-cutting ant queens *Atta sexdens*, azadirachtin affects oviposition, decreases, and inhibits vitellogenin reserve, which impact negatively the egg development (Amaral et al., 2018).

Sterility effects in females due to interference with vitellogenin synthesis and uptake into oocytes were also reported. A single injection of 10 µg of azadirachtin resulted in sterilizing effect on *Locusta migratoria migratorioides* with an arrest of terminal oocytes maturation and oviposition (Rembold and Sieber, 1981). In *Heteracris littoralis*, ovaries in azadirachtin-treated females showed complete shrinkage with oocyte growth arrest with disintegration and destruction in follicular cells and mitochondria (Ghazawi et al., 2007). In males, azadirachtin decreases significantly the number of cysts and the apical nuclei

within the cysts in *D. melanogaster* (Oulhaci et al., 2018). The inhibition of spermiogenesis was also reported in *Mylabris indica* (Vivekananthan and Selvisabhanayakam, 2014) and *Heteracris littoralis* (Ghazawi et al., 2007).

For the normal progress of oogenesis and spermatogenesis, a proper balance between JH and 20E is needed, antagonist action of azadirachtin on these two principal hormones account for the deleterious effects on reproductive parameters. Indeed, the application of exogenous 20E after azadirachtin treatment can compensate for its depressive effects on *D. melanogaster* and restored normal values of yolk protein content in the fat body and ovaries (Boulahbel et al., 2015).

In addition, azadirachtin was finding to alter reproductive behavior in *D. melanogaster* by reducing mating success (Aribi et al., 2017; Oulhaci et al., 2018). The impact of azadirachtin on sex behavior and mating response to sexual pheromones was also reported in *Oncopeltus fasciatus* (Dorn et al., 1987) and the predator *Neoseiulus baraki* (Lima et al., 2015). Oviposition sites treated with azadirachtin or other neem-based compounds induce an oviposition repellence, deterrence, or inhibition in several species of insects after a probable detection of the bioinsecticide on the treated surface (Schmutterer, 1990; Dhar et al., 1996; Cordeiro et al., 2010; Tomé et al., 2013). Pure azadirachtin was also reported to deter the oviposition in *Nezara viridula* (Riba et al., 2003). A single larval exposure to a commercial formulation of azadirachtin, the Neem Azal, was found to reduce fecundity in *D. melanogaster* and enhance avoidances to this compound (Bezzar-Bendjazia et al., 2016). These effects were observed in the next non-exposed generations which can be used as repellent strategies in pest management programs (Ferdenache et al., 2019).

Anti-feedancy

Azadirachtin is usually associated with a marked antifeedant activity and even behavioral avoidance in a large number of insect species including hemipterans (Kumar and Poehling, 2007), lepidopterans (Charleston et al., 2006; Shannag et al., 2015), orthopterans (Capinera and Froeba, 2007), coleopterans (Baumlner and Potter, 2007), and dipterans (Kilani-Morakchi et al., 2017).

Insects use an olfaction system to search and locate potential food and thereafter contacting chemoreception, called primary antifeedancy, which could confirm its quality and provide a basis for food selection and discrimination (Lee et al., 2010). A signal to the brain provokes avoidance from further approach or feeding.

The primary antifeeding effect of azadirachtin seems to be mediated by gustatory chemosensillas and linked to inhibition on the rate of firing of sugar-sensitive cells of the gustatory chemoreceptors by activating bitter sensitive gustatory cells (Lee et al., 2010; Weiss et al., 2011; Delventhal and Carlson, 2016). Indeed, the sensitivity to primary antifeedancy of azadirachtin was reported in different species, which starve to death rather than ingest the biopesticide (Mordue and Nisbet, 2000). An internal feedback mechanism called secondary antifeedancy, including a long-term reduction in food intake, and deleterious effects on different insect tissues (muscles, fat body, gut epithelial cells), is also reported (Mordue et al., 2010; Khosravi and Sendi,

2013; Shannag et al., 2015). Third-instar larvae of *S. littoralis* orally treated with sublethal concentrations of azadirachtin display a reduction in food intake, conversion efficiency, and feeding behavior (Martinez and van Emden, 1999). In second instar larvae of *Spodoptera eridania*, short-term consumption (2 days) of food treated with Azatrol, a commercial formulation of azadirachtin, reduced relative consumption rate, the efficiency of conversion of ingested food, relative growth rate, approximate digestibility, and assimilation rate of food during the entire larval developmental period (Shannag et al., 2015). In *D. melanogaster*, a single topical application of azadirachtin on early third instars larvae decreased significantly the amount of larval food intake and disrupted the ability of the insect to digest food by interfering with digestive enzymes activities (Bezzar-Bendjazia et al., 2017). This effect is also observed in adults surviving the pre-imaginal treatment, which suggests a long-term antifeedancy and delayed effects through the developmental stage with a possible reinforcement of the insecticidal activity of azadirachtin (Kilani-Morakchi et al., 2017).

In addition, azadirachtin showed an agonistic effect on dopaminergic neurons and can induce aversive taste memory in *D. melanogaster*, and such memory is regulated by dopaminergic signals in the brain resulting in inhibition of proboscis extension response (PER) (Yan et al., 2017).

Cellular and Molecular Effects

Besides the above mentioned effects, accounting for its broad-spectrum activities, azadirachtin was also shown to cause upregulation of p53, resulting in cell cycle mediated cells apoptosis induction and cell proliferation inhibition in *S. Spodoptera litura* S1-1 cell line (Huang et al., 2011). In the same species, Shu et al. (2018) demonstrated that azadirachtin induced structural alteration in the larval midgut by apoptosis activation including increased expression of caspase family members and apoptosis-binding motif 1 and the release of cytochrome c from mitochondria to cytoplasm, which may affect the digestion and absorption of nutrients. The induction of apoptosis through caspase-dependent pathways by azadirachtin was also reported in *S. frugiperda* cultures cell line Sf9 (Shu et al., 2015). Based on proteomic studies, Sun et al. (2018) reported that the molecular response mechanism of male infertility induced by azadirachtin in *S. litura* may be linked to regulation of many proteins in the pathway of focal adhesion exerting influences in detachment of cell attachment, the loss of cell-cell interactions, and inducing apoptosis at the pupal stage. Furthermore, many proteins in the adenosine monophosphate-activated protein kinase (AMPK) pathway were also changed at the adult stage after azadirachtin treatment as larvae (Sun et al., 2018). In *D. melanogaster*, a depolymerization of actin causing a cell arrest and apoptosis caspase-independent was reported after azadirachtin treatment (Anuradha et al., 2007; Anuradha and Annadurai, 2008).

At the cellular level, azadirachtin disrupts protein synthesis and secretion. In *Schistocerca gregaria*, injections of 3 µg azadirachtin/g body weight induce an inhibitory effect of the incorporation of radiolabelled glycine into the protein of the whole locust (Paranagama et al., 2004). Roberston et al. (2007) reported that the heat-shock protein, hsp 60, in cultured

Drosophila Kc 167 cells could bind to azadirachtin A which might be associated with a failure of protein synthesis and release.

At the molecular level, azadirachtin alters or prevents the transcription and/or expression of several proteins. Ingestion of 10 ppm of azadirachtin in third instars larvae of *Ostrinia furnacalis* significantly affected the fat body by interfering with protein expression related to hemolymph lipid (Huang et al., 2007). Lai et al. (2014) reported that azadirachtin downregulated expression of genes of cuticular protein and amylase and upregulated gene odorant-binding protein 99b (Obp99b) in *D. melanogaster*, which may be related to the development, molting defects, and antifeedancy action of the biopesticide. Azadirachtin treatment was shown to increase superoxide dismutase activity (SOD) and malondialdehyde contents (MDA) in *D. melanogaster* and induce antioxidant enzymes, such as SOD, catalase (CAT), and glutathion S-transferase (GST), by an upregulation of gene expression to protect against oxidative damage caused by elevated and accumulation of reactive oxygen species (ROS) triggered by a stress response to azadirachtin (Zhang et al., 2018). Azadirachtin also inhibits the expression of ferritin and thioredoxin peroxidase genes, in the sweet potato of whitefly *Bemisia tabaci*, related to protective roles against oxidative stress (Asaduzzaman et al., 2016).

Recently, azadirachtin was found to regulate the growth of *S. frugiperda* by affecting the insect chitin synthesis pathway by a downregulation of 31 cuticle proteins and several other genes encoding important enzymes involved in insect chitin and hormone biosynthesis, such as, trehalase, chitin-synthase, chitin deacetylase, chitinase (Shu et al., 2020). The suppressed expression of chitin biosynthesis and cuticle genes by azadirachtin might represent the molecular basis for the retardation of molting and growth.

Genes encoding enzymes responsible for key steps in hormone biosynthesis were also affected by azadirachtin. Azadirachtin also affected genes encoding key enzymes in hormone biosynthesis, such as genes encoding farnesol dehydrogenase, responsible for oxidization of farnesol, a precursor of JH named farnesal (Mayoral et al., 2009); the gene encoding an aldehyde dehydrogenase, which is responsible for converting farnesol into farnesoic acid and CYP15A1_C1, which converts the farnesoic acid to JH-III acid (Qu et al., 2015); the gene encoding JH epoxide hydrolase, responsible for JH degradation by hydrolyzing the epoxide of JH (Zhao et al., 2017); the gene encoding cytochrome oxidase-related proteins CYP307A1 and CYP314A1, which catalyze the 20-Hydroxyecdysone (Liu et al., 2019). All these changes in the expression levels of these key genes account for the disruption of the synthesis of JH and ecdysone, and therefore, interfere with the balance of these hormones, contributed to the growth inhibition.

RISK ASSESSMENTS

Azadirachtin-based pesticides act on a wide range of pestiferous insects from different orders as well as some ectoparasites which present high sensitivity to these compounds. The major property of azadirachtin is the blockage of neurosecretory peptides,

which regulate the synthesis and release of ecdysteroids and JH leading to disruption of endocrine events. The important roles of these hormones in arthropods physiology for normal development leave open the possibility that azadirachtin may pose a hazard to non-target species. Indeed, Barbosa et al. (2015) reported that long-term chronic exposure with azadirachtin may affect reproduction and behaviors of the bumblebee *Bombus terrestris* under laboratory conditions. Similarly, *in vitro* chronic exposure of azadirachtin affects stingless bee, *Partamona helleri*, by reducing the survival, development time, growth, and affecting reproductive organs but did not affect the larval food intake, the rate of emergence of queen and walking activity (Bernardes et al., 2018); however, the instability of azadirachtin and its low residual potential persistence makes these chronic conditions unexpected under semi-field and field situations. Azadirachtin was also found to be selective to the honeybee, *Apis cerana*, based on three essential risk assessment criteria [selectivity ratio, probit substitution method (%), and hazard ratio/risk quotient (Challa et al., 2019)].

In the case of predatory insects and parasitoids, azadirachtin, and neem-based insecticides show slight to moderate toxic effects and are considered to be harmless and with a certain degree of selectivity, especially for the adult insects (Raguraman and Kannan, 2014); however, pre-imaginal instars of beneficial organisms (nymphal/larval instars) are more susceptible to neem insecticides under laboratory conditions (Raguraman and Kannan, 2014). Hence, it is important to control the stage of parasitoids/predators used and the timing of application to avoid any toxicity in semi-field and field applications.

According to European Food Safety Authority (European Food Safety Authority, 2011), azadirachtin has moderate to high toxicity to aquatic organisms (acute $LC_{50} = 0.048$ mg azadirachtin A/L, chronic $NOEC = 0.0047$ mg azadirachtin A/L) and aquatic insects (chronic $NOEC = 0.0016$ mg azadirachtin A/L), with an aquatic half-life of around 30 days. The risk assessment for this compound focused on freshwater organisms as there are no marine or estuarine data. However, the risk values did not exceed the criteria and were predicted to be low when azadirachtin was used following the label instruction of the product (Goktepe et al., 2004; European Food Safety Authority, 2011).

Azadirachtin is not highly mobile in soil due to its oily composition. Its half-lives in soil are about few hours to 1 or 2 days reducing the risk to earthworms and soil macro-organisms. The hazard index of heavy metal contamination in vegetables after soil treatment with azadirachtin was <1 and does not exceed the WHO/FAO permissible limit in vegetables, suggesting it is safer for consumption (Egwu et al., 2019).

However, information regarding the fate, behavior, and toxicity of individual compounds, and the degradation of products are needed to complement its relatively favorable ecotoxicological profile (European Food Safety Authority, 2011). In general, European Food Safety Authority (2018) reported that the margin safety of the risk assessment performed for azadirachtin A is considered sufficient to estimate the risk from the whole azadirachtin. In addition, semi-field and field studies

should be performed considering situations that may include acute and chronic exposure in the risk assessment setup.

FUTURE DIRECTIONS

Azadirachtin has a variety of physiological effects on many insect pests, such as antifeedancy (Qin et al., 2020), growth and development inhibition (Zhao et al., 2019), impairment of oocyte structure, inhibition of fecundity, and egg viability (Bezzar-Bendjazia et al., 2016; Amaral et al., 2018; Oulhaci et al., 2018; Ferdenache et al., 2019). Despite extensive studies of the mechanisms that highlight the physiological effects of azadirachtin, the behavioral effects remain more controversial (Charleston et al., 2006; Hasan and Ansari, 2011; Tomé et al., 2013).

The fitness and survival of insects strongly depends on successful localization of host plants, food source, mating partners, and oviposition sites. Many insect behaviors are heavily dependent on chemosensation, especially on the perception of olfactory and gustatory cues (Herrero, 2012; Depetris-Chauvin et al., 2015; Walker et al., 2016). In addition to these olfactory and gustatory cues, locomotion represents an integral part of insect behaviors as is essential for food-seeking, mating, and escape response (Zhu et al., 2020). The ability of insects to modify their behavior based on prior experience is essential for their survival (Chia and Scott, 2020). Increasing evidence has highlighted the critical role of early life experience in adult behavior in insects (Caubet et al., 1992; Bezzar-Bendjazia et al., 2016; Ferdenache et al., 2019). In addition, exposure to a stressor, such as pesticides, has been shown to prompt a range of behavioral effects which can be inherited to the next generation (Ferdenache et al., 2019; Lu et al., 2020). Recent work demonstrated for the first time that *D. melanogaster* can modulate its behavior based on previous experiences of early life (third instars larvae) with azadirachtin affecting oviposition site preference and food selection and enhancing avoidances of this compound in adults of parent generation as well as the non-exposed F1 generation (Bezzar-Bendjazia et al., 2016; Kilani-Morakchi et al., 2017; Ferdenache et al., 2019). These changes in insect behavioral responses are influenced by individual sensory experience and may leave an “imprinted” trace into adult life in accordance to experience-induced learning by changes in the neurophysiology of insects (Dukas, 2008; Little et al., 2019). Indeed, biogenic amines, octopamine (OA), serotonin (5-HT), and dopamine (DA) are known to convey the reinforcing cues for many different types of associative memory in *Drosophila* (Masek and Keene, 2016). Azadirachtin treatment was found to reduce OA, 5-HT, and DA levels in both the brain and the hemolymph of *Acherontia styx* (Awad et al., 1997). Furthermore, azadirachtin interferes with the amount of 5-HT in the endocrine organs and, mainly, in the brain of locusts (Banerjee and Rembold, 1992).

Moulin et al. (2020) reported that transient dysregulation of the dopaminergic signaling can produce behavioral alterations in *D. melanogaster* adults, which can then be carried to descendants. In addition, azadirachtin can excite different clusters of dopaminergic neurons, such as PPL1, and increase

dopamine release inducing aversive taste memory in *Drosophila* (Yan et al., 2017); however, the neurophysiological actions of azadirachtin remain to be clarified. In addition, insecticides are known to be able to provoke epigenetic alterations, which can be inherited in the next generations (Vandegheuchte and Janssen, 2011); this possible epigenetic alteration induced by azadirachtin treatment was never investigated. The comprehension of the mechanisms that induce the transgenerational conservation of the aversive effects of azadirachtin may contribute to better use of this compound in IPM programs.

In addition, azadirachtin had the potential to be used in synergy with other botanical compounds. Indeed, azadirachtin and clarified neem oil can significantly synergize the pyrethrum activity while reducing or eliminating the need for pipronyl butoxide as an agent to augment pyrethrum activity, which represents a significant cost advantage when compared with existing pyrethrum/pipronyl butoxide formulations (Chang et al., 1996). On the other hand, phenol compounds in neem were suspected to synergize with the main component (azadirachtin) in increasing the antifeedant activity on *S. litura* (Prianto et al., 2019). The use of azadirachtin in synergy with *B. thuringiensis* (Bandyopadhyay et al., 2014) and karanj (*Pongamia pinnata* Pierre) was also reported (Kumar et al., 2007). Azadirachtin was found to enhance the efficacy of *B. thuringiensis* in *Cydia pomonella*, *S. exigua*, and *Dendrolimus pini* (Konecka et al., 2019). This synergistic effect was observed between azadirachtin and multicausid nucleopolyhedrovirus (SfMNPV) on the mortality of *S. frugiperda* (Pineda et al., 2014).

More studies are needed for synergism between azadirachtin and other insecticides to find combinations that can effectively control pests. Essential oil or their main compounds, especially compounds (linalool, borneol) with antifeeding activities, might represent a good candidate.

PRACTICAL PROBLEMS OF AZADIRACHTIN APPLICATION

If rapid degradation by sunlight and low persistence in the environment are considered as advantages of the use of azadirachtin and neem derived products, it also represents a problem for their use on a large scale and is disadvantageous from an agribusiness perspective, since they result in lower efficiency and necessitates a greater number applications (Pasquoto-Stigliani et al., 2017).

The chemical nature of the media containing azadirachtin formulation is important and influences its stability. Indeed, studies on the effect of various solvents on the stability of azadirachtin in extracts and formulations reported higher stability of azadirachtin in alcoholic and other aprotic solvents, which are neutral, as compared with protic solvents (Pereira et al., 2019). Furthermore, azadirachtin was most stable in mildly acidic solutions between pH 4 and 6 (Pereira et al., 2019).

The neem-based oil in water emulsion formulation by high shear mixing also improves stability and bio-efficacy of the biopesticide by a decrease of particle size of the emulsion with the increase of stirring time leading to excellent emulsion

stability (Iqbal et al., 2020). In addition, the stability of neem oil-based microemulsion can be enhanced by the use of botanical synergists, such as aqueous extract of *Prosopis Juliflora* (Sharma et al., 2019).

The use of nanotechnology also represents a way to overcome such limitations, and the development of controlled-release formulations of botanical insecticides by polymeric encapsulation has been studied in recent years (Das et al., 2014; Pasquoto-Stigliani et al., 2017). Flores-Céspedes et al. (2015) reported that natural polymers, such as kraft lignin and alginate, protect azadirachtin against photodegradation and could be used to improve its stability and delivery to its site of action. These new procedures to encapsulate botanical pesticides provide several benefits including slow-release, enhanced stability of compounds, use of small dose, and masking of odor (Chaudhary et al., 2017). Poly(ϵ -caprolactone) nanocapsules loaded with neem oil are safe to soil microbiota during 300 days of exposure and did not affect the net photosynthesis and stomatal conductance of maize plants, and present lower toxicity against non-target organisms (Pasquoto-Stigliani et al., 2017); however, the same nanocapsule containing a mixture of neem oil and oleic acid presented higher toxicity and led to negative effects. Recently, Shanmugapriya et al. (2019) demonstrated that azadirachtin loaded in silica nanoparticles at 500 ppm showed high mortality of adult *Bemisia tabaci* and can be used as an alternative to chemical pesticides.

Although nanotechnology is still at an early stage in the agricultural sector, it is clear that there is growing interest in its use; however, studying the toxicity of nano pesticides and understanding their mechanism of action in target organisms is a key factor in the selection of the best formulations for use in agricultural applications (Feng and Peng, 2012; Seugling et al., 2019; Jesser et al., 2020).

In addition, a sublethal dose of azadirachtin was reported to induce hormesis in the Mexican Bean Weevil, *Zabrotes subfasciatus*, with increased fecundity daily to compensate for azadirachtin-induced decreased longevity (Vilca Malqui et al., 2014). In addition, the population of *Z. subfasciatus* engendered from females exposed to azadirachtin present a higher rate of population increase and a higher net reproductive rate (Vilca Malqui et al., 2014). Similar results were reported in *Myzus persicae* exposed to sublethal concentrations of azadirachtin with a modest hormetic response under laboratory conditions (Cutler et al., 2007). Evidence-based toxicology under field conditions must be used to solidify the importance of hormesis to understand the risk of exposure to azadirachtin and neem-based compounds. In addition, new research tools, such as toxicogenomics and statistical modeling processes, must be designed to evaluate possible hormetic responses when devising pest management strategies.

CONCLUSION

Health and environmental concerns have influenced the use of safe and non-hazardous pest control measures. Azadirachtin-based insecticides have recently been promoted as an alternative

pest control method, especially in agroecological farming and organic agricultural systems. Azadirachtin has broad-spectrum activity for combating numerous pests in different crops, and it has not yet reached most of its potential utilization. Currently, information is sparse on the possible long-term and transgenerational effects of azadirachtin on insects; a better comprehension of this phenomenon could improve its use in IPM programs by reducing the concentrations used, frequency of application and targeting the best time of application, which might enhance its ecotoxicological profile.

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- In addition, the nanoencapsulation of this biopesticide provides a novel way to enhance its stability and sustainability, since they protect it against degradation and modulate its release.

AUTHOR CONTRIBUTIONS

SK-M wrote the manuscript. SK-M, HM-G, and KS contributed to the collection of the information and the discussion and revised the manuscript. All authors approved the final version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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