Chapter 11

Emerging Insect-Borne Diseases of Agricultural, Medical and Veterinary Importance

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Additional information is available at the end of the chapter

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Abstract

Current migrations, due to several causes, but mainly consequence of climate changes, are causing several problems in Southern Europe. Some migrations are evident and attract attention immediately; others are silent, but still important, like several ones involving agriculture and livestock. In the future, a number of products employed in pest control may lose their efficacy. Pesticide resistance should be considered an increasing problem, and more environmental-friendly control approaches against arthropod pests are urgently needed. Two examples from the South of Italy clearly explain the kind of arising alerts and the complex network involving abiotic and biotic causes. The first case is the growing number of blue-tongue disease outbreaks, vectored by *Culicoides* sp. The second case is the alarm concerning the olive trees epidemic disease in Apulia, due to the bacterium *Xylella fastidiosa*. The development of new pest control methods is required in order to minimize negative effects of currently marketed synthetic pesticides. In this scenario, natural product research can afford solutions as part of an integrated pest control system. Preliminary results concerning the use of neem, *Azadirachta indica*, in control of insect vectors are discussed.

Keywords: Arthropods, Asian tiger mosquito, mosquito-borne diseases, blue-tongue, *Xylella fastidiosa*, neem

1. Introduction

In 1962, *Silent Spring*, the book written by Rachel Carson, documented the detrimental effects on the environment of the indiscriminate use of synthetic pesticides [1]. The book claimed that DDT and other pesticides had been shown to cause cancer and that their agricultural use was a threat to wildlife, particularly birds. She explicitly accused chemical industry of spreading misleading information and public officials of accepting industry claims unquestioning about consequences. Despite the fierce opposition by chemical companies, the book's impact on the American public was a seminal event for the environmental movement, spurring a reversal in national pesticide policy. Still the issue has great actuality, considering the recent debate [2–4] about effects of neonicotinoids on honey bees and birds [5].

In 1972, DDT was banned on the agricultural uses in the US, and soon after in EU. Before DDT was banned, more than 600,000 tonnes were applied in the US. The environmental movement led to the creation of the US EPA (Environmental Protection Agency). The first resistance episode concerned DDT in 1914 [6]. In May 2015, US President Obama, considering the final report of the Commission, stated the primary importance of antibiotic multiresistance, leading to the ban of the use of antibiotics in agriculture and farm practices. However, deep divide exists between American and European regulation of pesticides and other chemicals, many chemicals that are banned or strictly regulated in EU are permitted in the US.

Accordingly to the current definitions, resistance can be defined as "a heritable change in the sensitivity of a pest population that is reflected in the repeated failure of a product to achieve the expected level of control when used according to the label recommendation for that pest species" [7]. In this chapter, we will consider resistance as "the inherited ability of an organism to become tolerant to a dosage of the chemical that would be lethal to a definite species." Evidence for pesticide resistance in arthropods of agricultural and medical importance is an emerging threat. It is possible that in the next 20–30 years, all the synthetic pesticides now employed for pest control will lose their efficacy. Research on newer and safer control tools may be helpful in future scenarios for mankind, dramatically involving feed and food.

Synthetic insecticides are usually utilized to reduce damages caused by insects that destroy crops or transmit diseases. To be effective, an insecticide should be lethal to the majority of the individuals in a normal wild population. However, the insecticide can lose its efficacy, thus many pest populations developed resistance to the toxic effects [8]. This key point is only a further example of the consequences of human tendency to amplify the natural resources beyond any limit in order to obtain the maximum of the effects and not considering the consequences. The problem is inherent: the resistance is related to a massive and persistent use of pesticides, exactly like for antibiotics in microorganisms. Many species have resistant populations, which can resist to one or many treatments [9]. In the moment of the insecticide use, some individuals result resistant. Sensitive insects exposed to the insecticide will die, except the few resistant ones, which can continue to develop and proliferate. Continuing the use, they will be favored. More use of pesticide fuels the dominance of resistant populations. The consequence of the mechanism is that, in the right time, insecticides, once effective, are not sufficient in controlling insects [10]. Nowadays, the above definitions risk to be insufficient to describe the complex system that generates the resistance phenomenon to insecticides or the absence of any real efficacy in control of insect pests. The solution can be considered simply as "find the best weapon to kill the bacterium and solve the problem," as so far mainly considered. As in the general medicine, where the "chemical magic bullet" was considered the central solution for any disease, this approach is nowadays in crisis because the physiologic aspects are more complex and complicated by interactions at several levels. Resistance is the consequence of a series of events.

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This chapter attempts to overcome the paradigm substance → replay of organism → resistance, in favor of a more complex approach, leading to integrated pest management. A focus on two current vector-borne emergencies was provided.

2. A new situation

So far, much attention of research and public concern was focused on vector-borne human diseases in order to eradicate their presence and to save as many lives as possible [11]. However, if the resistance will affect our life supports, the surviving struggle will be in balance due to two key factors: resistance to many pesticides and/or the impossibility of using current effective substances because of the consequences of the effects on environment. Even pyrethroids are now considered dangerous for nontarget animals since they can impair memory and movement [12]. Currently, we have to face new epidemic emergencies due to several factors (including climate change) concerning crops and livestock. Insects are vectors of important diseases involving nonhuman targets, causing important effects on plants and animals of strategic economic relevance. Recently, some of such diseases are of increasing concern to the general population, attracting a level of attention never experimented before and generating great alarms for the consequences of their rapid diffusion. The economic negative effects are enormous, and the damages on the local economic system are dramatic. Agricultural production must increases in relation to the needs of world population. However, losses due to arthropod pests account now for around 20–30% of the production [13]. Insect damages are important in the field and in the stored products. Agricultural production resorts to the use of a large quantity of insecticides to raise production and preservation of foodstuff. According to the increasing needs, the use of insecticides has increased even more than necessary, although it was demonstrated that the excessive and inappropriate use of synthetic pesticides is frequently even counterproductive. Effects are not limited to the treated field, involving undesirable consequences on public health and environment. Current pesticide pollution of Adige valley in Northern Italy, due to the continuous heavy treatments of apple monoculture, is a clear example. The introduction of OGM did not afford solutions, inducing additional problems to farmers.

Most of insecticides are usually employed to reduce the damages caused by insects that destroy crops or transmit diseases. Up to now, agricultural pests account for 59% of the resistant insect species, while veterinary pests account for 41%. Antibiotics in the US and the UK mainly utilized to treat livestock are considered immunostimulants, similarly, most insecticides are used in agricultural practice to improve the production and preservation of foodstuff. In both cases, the use is now widespread, excessive, and inappropriate.

Recently, some of such diseases have rapidly gained media's attention, generating great alarm for the consequences of their diffusion. The economic negative effects are enormous, and the damages on the local living system are dramatic. Several epidemic emergencies are in act, and the emergency is going to be converted into a normal trend, as a consequence of the permanence of several factors, including in first place the climate changes [14].

On the basis of novel knowledge, some new approaches are emerging, changing the aspect of insect control. Integrated pest management is an important approach, developed in the last years to control disease vectors and limit economic agricultural damages, improving crop yield with minimum cost. Main goals are (a) to increase basic knowledge of biology of the insect pest and its relationship with other organisms sharing the same ecological niche [15]; (b) to reduce pesticide application and quantity developing biological controls, farming practices, farmers collaboration, and mechanical and physical controls; and (c) to build new models of integrated managements on the basis of laboratory and field experiments, including the research of new active compounds. Novel pesticides to be suitable must be low cost, eco-friendly, from renewable raw material, nontoxic to nontarget organisms, of rapid degradation, and no accumulation in the environment.

However, resistance is only the last consequence of a series of events. Most of this paper will be dedicated to the deep knowledge of this sequence, being considered the key to struggle resistance. Insect-borne diseases are the result of complex multiorganism interactions. The network of several different collaborating organisms is on the basis of diffusion, effectiveness, and metabolism of insect vectors, including the resistance phenomenon. The integrated network acts like a "superorganism," integrating functions of all the different types of involved organisms. Disease is the result of a brave and useful collaboration between organisms totally different, from bacteria to insect, giving rise to an integrated system that is the key to survive and proliferate. It is the example that we must learn, asking for several levels of eco-friendly interactions. The consequences that we consider as negative are only the collateral effects of the competitive struggle.

In these years, the Mediterranean Sea was an incubation sap of several massive migrations of organisms, mainly due to climate changes and commercial routes, which radically modified previous equilibrium. Migrations start from distant sites but are able to spread in a large area until they find the right conditions to set up and rapidly become dominant.

Therefore, insects, like microorganisms, are particularly able to change their genome. It is a problem of survival. In some cases, the change generates an organism more aggressive and virulent. Previous treatments are usually not useful, in particular when they are the cause of the genetic change, like the exaggerated use of pesticides and/or climate changes.

In Italy, at least three cases are focusing on scientific, social, and policy attention, causing strong alert for the consequences of their anomalous increasing speed of spreading. The first one concerned *Aedes albopictus* (Skuse), commonly known as the Asian tiger mosquito [16–18]. This species is currently retained the most invasive mosquito species in the world since it is able to rapidly adapt to different anthropogenic environments, thanks to its ecological and physiological plasticity [19]. Recently, the Asian tiger mosquito has invaded many countries, spreading rapidly to Europe, North and South America, the Caribbean, Africa, and the Middle East [20,21]. *A. albopictus* is both a nuisance and a disease vector. Its medical importance is mainly due to the aggressive daytime human-biting behavior and to its ability to transmit many diseases. It works as a vector for many viruses, including dengue, yellow fever, West Nile, Japanese encephalitis, St. Louis, encephalitis virus (Flaviridae, genus *Flavivirus*); chikungunya, Eastern equine encephalitis, Venezuelan equine encephalitis, Western equine encephalitis, Ross River, Sindbis, Mayaro, Getah (Togaviridae, genus *Alphavirus*); Potosi, San Angelo, La Crosse, Jamestown Canyon (Bunyaviridae, genus *Bunyavirus*); Rift Valley fever (Bunyaviridae, genus Phlebovirus), and Orungo virus (Reoviridae, genus *Orbivirus*). *A. albopictus* is also the vector of different filariasis, such as *Dirofilaria immitis* Leidy, *Dirofilaria repens* Railliet and Henry, and *Setaria labiatopapillosa* Perroncito [19]. Although the introduction of Tiger mosquito was casual, probably due to the commerce of old tires, the permanence is clearly due to the climate change with the rising of temperature.

Two recent exceptional cases of overflowing insect-borne diseases not directly involving human health are reported to evidence of the difficulties of fighting new insect emergencies. Both emergencies are the results of a complex multiorganism interaction. The network of several different organisms is on the basis of the mechanism of survival and diffusion, conditioning effectiveness and metabolism of insect vectors, including the resistance phenomenon. The network acts like a "superorganism," integrating functions of all the different types of involved organisms, asking for several levels of eco-friendly actions. Integrated methods are urgently needed for control of these pests. On the basis of novel knowledge, some new approaches are emerging, changing the aspect of insect control.

Both cases present several, not casual, similarities and therefore can be considered as paradigms of next future or actual situations. The first case is the expanding relevance of bluetongue disease, vectored by *Culicoides* sp., so far concerning Southern Europe (Sardinia in particular) and going to be present in other countries. The second case is the alarm concerning the olive trees epidemic disease, probably due to *Xylella fastidiosa*, which may lead to the disappearance of extensive areas cultivated with olive trees in Southern Italy. Actually, no useful control tools have been reported.

3. *Xylella fastidiosa:* A threat for olive trees

In 2013, one of the most beautiful part of South Italia, the Salento Peninsula, well known for the production of the olive oil and wines, was alerted by a dramatic phenomenon, never reported since human memory. The centenary olive trees that are the hearth of the monumental natural architecture of the region started to die, as Golias killed by invisible Davids. The sentence was that the responsible bacterium, *X. fastidiosa*, causes so-called Pierce's disease. So far, Pierce's disease (PD) was mainly known as a deadly disease of grapevines [22]. It is caused by the bacterium *X. fastidiosa*, which is spread by xylem feeding leafhoppers, known as sharpshooters. PD is known to be prevalent within the US from Florida to California and outside the US in Central and South America (Table 1).

Figure 1. The effect of *Xylella fastidiosa* on olive trees in Apulia (Photo: Marcello Nicoletti, October 2014).

1870 Reports in California of grape wine "mysterious disease"
1890 The disease practically disappeared
1892 Newton B. Pierce reports on the disease on grapes in California.
1920 New epidemic diseases in California, apparently not linked to the previous episode
1920 Alfa-alfa disease (AD), no other cases reported
1930 Hewitt names the rediscovered grape wine disease as Pierce disaster (PD)
1930 Reports on peach disease
1933 PD spreads in South US
1940 Major epidemic disaster; vectors come from alpha-alpha (AD) through the some "virus"; they are xylem sap-feeders and disease is xylem-limited
1970 Almonds and oaks also affected; symptomless plant host discovered
2011 First cases of dehydrated olive trees near Lecce town in Puglia
2012–2014 Most of the peninsula of Puglia region, named Salento, evidences the presence of *Xylella* in the centenary olive trees.
2014 The disease spreads interesting more than 9000 hectares.
2015 The eradication campaign, in accordance with the EU protocols, starts with the destruction of dozens of trees, against the population concern. The regional court accepts the considerations against the eradication, but the campaign goes on.

Table 1. Chronology of *Xylella fastidiosa* outbreaks

X. fastidiosa works blocking the xylem, which conducts the water around the plant. Symptoms include chlorosis and scorching of leaves, and entire vines will die after 1–5 years. Pierce's disease is less prevalent where winter temperatures are cold, that is, at high altitudes and in inland northern areas [23–26]. The anomalous recent diffusion of the bacterium *X. fastidiosa* is causing a great alert and enormous damage. The disease risks to menace the surviving of olive trees, at least in Southern Italy. Starting from a little area in Gallipoli, near the town Lecce, most of the olive trees of a great part of Apulia region in the South of Italy were totally destroyed during the last 2 years. Therefore, the diffusion was very rapid, epidemic, and devastating. However, the disease is known by long time and so far considered mainly affecting grape wine and sporadically other plants, like oleander, almond, cherry tree, *Polygala myrtiflora*, and *Spartium junceum*. Concerning olive trees, so far it was considered one of the hundred diseases affecting the species, without any report of epidemic virulence.

In October 2013, the bacterium was found to be infecting olive trees in the region of Apulia in southern Italy. The disease was causing a rapid decline in olive plantations, and by April 2015, it was affecting the whole Province of Lecce and other zones of Apulia, focused in the Salento Peninsula. Almond and oleander plants in the region have also tested positive for the pathogen. The disease has been called Olive Quick Decline Syndrome (OQDS). The disease causes withering and desiccation of terminal shoots, distributed randomly at first but which then expands to the rest of the canopy. This results in the collapse and death of the trees (Fig. 1). In the affected groves, all of the plants show symptoms. By the beginning of 2015, it had infected up to a million trees in the southern region of Apulia [25–30]. The epidemic damage affected thousands of centenary olive trees, completely dehydrated by the disease. After the eradication, the treated areas appeared totally desertified. Beside the great economic damage, loss of the olive trees means a tremendous cultural and environmental impact for a territory, where they are the symbol of region's identity. Centenary olive trees are the living monuments of the Apulia. Furthermore, there are convincing hypotheses that the epidemic infection will propagate rapidly to the neighboring regions and later in several parts of the Mediterranean area. Therefore, interested countries, like France and Greece, are asking for a rapid control of the disease before further pandemic diffusion.

In conclusion, there are two main hypotheses about future scenarios: (a) a natural stress-induced dieback: this is consistent with widespread groves of various ages all suffering to different degrees and slowly declining rather than a virulent point infection that can be seen to spread. In other words, the disease is due to a "normal" increasing of virulence coupled with the "stress effect," derived from climate change and agricultural loss, that will affect mainly the old trees, causing a turnover in favor of the new stronger generations; (b) a modified, more virulent pathogen appeared, and plants defenses will be not able to face the new challenge, with devastating consequences.

So far, the only real performed action was the application of the UE protocol consisting in the eradication of any olive tree and creating a defensive line of 2 km in extension in the northern part of Salento Peninsula, where any plant must be eradicated, in order to isolate the disease. This measure should stop the diffusion of *X. fastidiosa*? The bacterium relies to insect vectors. Known vectors of *X. fastidiosa* are xylem-sap feeder insects belonging to the families Cicadellidae, Aphrorophoridae, Cercopidae, and Cicadidae within the Cicadomorpha. Among them, the meadow spittlebug, *Philaenus spumarius*, is one of the most abundant field insect in that region, although other species are probably involved. The spittlebug xylem-sap feeding possesses a piercing-sucking beak, named rostrum. Introducing the rostrum into the tree for feeding, the insect causes the infection and the bacterium propagation close the xylem vessels, causing the dehydration of the plant. It is very difficult that the EU protective approach will give significant effects. The vectors are not good flyers; they usually move by jumping, but they can be efficiently transported by the wind and human of animal occasional transportation, travelling for many kilometers in only one day. The block of plant import from America is in act. Also, in this case, the total control is quite impossible.

The bacterium cannot be controlled by the use of antibiotics, either because they are banned in agriculture in EU or because they are highly costly and complicated. The insect vector could be controlled by adequate insecticide. Insecticides usually select as target the adults, but the larval stage is the best situation to act on the insects, before they are able to move and fly away.

In any case, to obtain any real result, we must learn the Nature's lesson. Olive tree disease evidences three main actors: the bacterium, the vector and the plant, and probably a symbiotic fungus. They work together, acting like a "superorganism." It is a very complex system, but in some way also very efficient. The only way to face the *X. fastidiosa* challenge is an integrated pest management. It is necessary to operate considering together the several involved aspects: a treatment of soil to sustain the plant; an insecticidal agent to control the insect; a natural, low cost, and eco-friendly antibiotic to be inserted inside the plant.

A key step is the reply to the following question: How did *X. fastidiosa* become so dangerous in the last 2 years? We know the presence of bacterium in Italy from at least 30 years, and so far it was considered just one of the several diseases involving olive oil. Something happened during the last years changing completely the equilibrium between the microorganism and the host. There are several hypotheses about the causes of the change and a consequent relevant debate. The local official institutions have accepted the idea that some infected plants of oleander imported from Costa Rica were the epidemic start. Everything could be, but it is strange that only a single little point of Apulia was affected by the only infected imported plant.

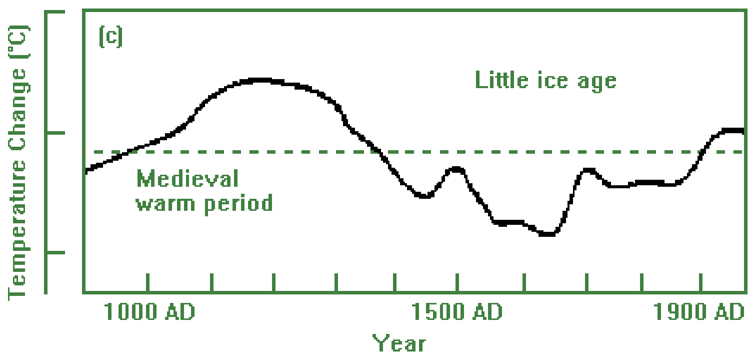

Figure 2. Temperature changes over the last centuries.

The second hypothesis is that a change in the *X. fastidiosa* genoma occurred, giving rise to more aggressive and dangerous strains. In this case, there are two possibilities: the change is derived from some experiment or a biological cause. Due to climate change (Figure 2), some virulent strains from hotter countries could be able to survive and proliferate against local populations. Other causes, like the absence of the traditional treatment of the soil and the trees due the urbanization of the population, could have interfered. The phenomenon is quite well known and possible, as explained in the next part of this chapter.

4. The climate hypothesis

There have been several marked changes in climatic conditions in Europe during the last century. In the 20th century two main periods of warming have occurred in Europe (Fig. 2). The second was the warmest decade (the 1976–2000) on record, and it is still in act. The evidence was an increasing of approximately 1.2°C over the past 100 years, which means twice the average global rate. Turnover of hot and glacial periods is a normal trend for our planet, mainly due to the CO_2 the quantity of this gas in the atmosphere. We are now in a warming period, and the trend is accelerated by the emission due to human activities [30–34]. Warning consequences are higher nighttime temperature, with limited difference between day and night, and few frost days in winter, associated with milder temperature in all winter period, longer dry periods, and peaks of temperature. In particular, temperature increases were most marked in both Central Europe (Italy, Corsica, and Balearic Islands) and Eastern Europe (western Bulgaria, northern Greek, Albania, Macedonia, Bosnia, Montenegro, and Croatia). On the contrary, central Iberia and the region around the border between Morocco and Algeria have cooled. Simplifying the model tendency, Europe is getting warm and North Africa is cooling. This resulted in changes in precipitation dividing Europe in two parts: the number of wet days have increased in the North Europe and decreased on the South, increasing the already presence tendency to desertification of several regions. North Europe, including the UK, northern Iberia, and Scandinavia, is becoming wetter, whereas southern Iberia, France, Germany, and Italy are becoming drier.

Vector-borne pathogens are particularly sensitive to climate, a fact that has led to widespread and continued speculations that anthropogenic climate change will increase the incidence and intensity of their transmission. Other nonclimatic abiotic and biotic factors can also affect disease distribution. Diffusion can be very rapid and effective. Adult insects are usually not strong fliers, but they can be passively dispersed by the wind, possibly up to several kilometers in a single night, especially over the sea. Thus, natural barriers cannot be considered an efficient control of the diffusion. Otherwise, they can travel utilizing ancient transportations, like other animals (street ruminants), or new unexpected ones, i.e., inside old tires as happened for *A. albopictus*.

There is an urgent need for ecologically sound, equitable, and ethical pest management, based on control agents that are pest-specific, nontoxic to humans and other biota, biodegradable, less prone to pest resistance and resurgence, and relatively less expensive. The last aspect is fundamental for a large-scale use in emerging countries.

5. Bluetongue spread in Europe

Bluetongue is a devastating disease of ruminants, mostly restricted to certain breeds of sheep, particularly fine wool and mutton breeds common in Europe [35] (Table 2). Until 1998, bluetongue has made only sporadic incursions in Europe, until six strains of the bluetongue virus (BTV), from the Middle East, were transferred to Europe, through two main pathways. One spread northward involving Greece and Balkans. The second one interested the North Africa, and from Tunisia/Algeria landed to Sardinia, Sicily, Corsica, and Balearic islands. Clearly, these pathways are coincident with the traditional livestock trade routes, such ruminant street. The same routes were used in the last years by human migrants to reach the Italian peninsula, i.e., across the Adriatic Sea from Albania and across the Sicily channel from North Africa [36, 37].

1969	First isolation in Greece of strain BTV-9 and BTV-1
1998	Isolation in Tunisia of the strain BTV-2, endemic of sub-Saharan Africa, and belonging to strains from South Africa, Nigeria, Sudan, US.
2000–2001	Isolation in Greece and Turkey of the European strain BTV-1, similar to viruses that have been isolated in India. First outbreak in Bulgaria.
2003	A new strain BTV-4 type, different from that of Greece and Turkey, is isolated in Corsica, Sardinia and Balearics.
2004	Detection of strain BTV-4 in Sicily and France.
2005–2014	Distinct strains are still entering in Europe, affecting at least 12 countries and more than 800 km further north than before.
2014	In Sardinia, the BT disease caused the deaths of 13,000 sheep and damages for 42 million of euro. 5772 infection sites were detected.

Table 2. Chronology of blue tongue virus (BTV) spread in Europe

Since its arrival, BTV has caused the deaths of more than one million sheep, and the loss of trade in animals and animal products, with an estimated damage of US$125 million in the US alone [38]. Sardinia was in particular affected, being the economy largely based on sheep, producing very appreciated like cheese (pecorino) and fine wool. The widespread use of a vaccine, although effective, caused a series of problems for its distribution and episodes of corruption.

6. Natural products help

Natural products are mainly derived from plants as the result of coevolution between organisms and environment. For this reason, they are used for centuries in popular and traditional medicines, as well as often as spices and insecticides. Unlike modern pharmacology and drug development based on single chemical entity, natural product preparations are multi-ingredient, derived from the historical references and empirical experiences. A single herbal drug contains at least hundred of compounds making a complex matrix, named phytocomplex in which not the single active constituent is considered the only responsible for the overall efficacy. The phytocomplex utilization is not a philosophy because many data afford the validity of this approach and others can be obtained using the modern pharmacological devices.

The study produced in 2010 by MIT and the Broad Institute of Harvard University (US) is a clear step in the direction of a scientific validation of natural products [39]. The argument strictly relates to the past and future role of natural products and the endless debate about their efficacy, often resulting into a fighting contrast between natural products supporters vs. synthetic drugs defenders. The key argument was to understand what is going on between the two main levels of the metabolism, involving the functional connection between genes and genes products and between targets and genes. The MIT researchers decided to commit the argument to the neutral judgment of artificial intelligence. The computational work was based on the comparison of cumulative connectivity distribution of small molecules, natural or synthetic, grouped according to connectivity associated with the target, assuming that proteins form biological networks. The result is simple: natural products target the proteins with a high number of protein–protein functional interactions (higher network connectivity), whereas the synthetic ones act on limited protein network: "We observe that approved drug targets that are not also natural product target exhibit a connection distribution much closer to the case for human disease genes that natural product targets, which remain the most highly connected targets."

Natural products tend to target proteins more essential and general to an organism than other groups of small-molecule targets, like those related to disease genes. They therefore work as a nonspecific basic defense against predators or pathogens acting on more highly connected proteins, interrupting essential protein activity of the environmental competitor or invader. However, natural products are not only defense and toxic substances. On the contrary, the story of plant evolution and the experience evidence the progressive production of positive substances produced in favor of a collaboration with the animals present in the same habitat. They may be tailored for a positive or negative influence in physiologic activities and basic metabolism. These argumentations are in favor of the potential use of natural products as insecticides.

7. The neem opportunity

The tree *Azadirachta indica* A. Juss (sin. *Melia azadirachta*) is commonly known as neem or nimba, margosa or Indian neem, Indian lilac, the last one to distinguish from the similar species *Melia azedaracht* L., named Melia o Persian lilac (Fig. 3). Several exceptional terms were used to describe the importance and the value of neem, i.e., "the marvellous tree, the tree of XXI century, the divine tree, India's tree of life, Nature's drugstore, Panacea for all diseases, a tree for solving global problems" [40–45]. In 1989, WHO/UNEP considered the neem tree as one of the most promising tree of the 21st century. In 1992, the US National Academy of Sciences published a report having the significant title "Neem—A Tree for Solving Global Problems" [45]. The medicinal use of neem is strongly eradicated into the Indian tradition. All its parts are largely used for many illnesses, and in Indian rural areas, the plant is called "the village pharmacy." Neem is considered a natural exemplar insecticide.

Figure 3. The neem tree (*Azadirachta indica* A. Juss)

The neem tree pertains to the Meliaceae (Mahogany family). It is a fast-growing evergreen tree, native of Indian subcontinent and distributed in the Tropical and Subtropical areas. Neem [46–48], owing to its ability of growing so easily and surviving on dry, nutrient-lean soil, is now cultivated in tropical and subtropical countries, including South Asia, West Africa, central (i.e. Cuba) and South America, and Australia. Flowering occurs from January to May. Flowers are fragrant, beautiful, and abundant. Fruits ripening from June through August are green ellipsoidal drupes containing one seed. A single mature tree may produce annually 5–8 kg of seeds.

The main product of neem is the oil obtained by expressing the kernels containing the seeds (Figs. 4 and 5). Neem seed oil (NSO) is obtained by different extraction methods. Most of the NSO is produced in India by familiar little producers using very simple machines, but many other countries are now producing NSOs. Therefore, considering also the possible different geographical origin of the raw material, combined pre- and postharvesting factors can result in great differences in constituents present in marketed NSOs, as already reported [49].

The chemistry of neem is very complicated and still far to be completed, despite the great number of dedicated researches. More than 300 compounds have been characterized from the seeds. One-third of them are nortriterpenoids, which means triterpenoid lacking some carbon atoms. Partial loss of lateral chain is combined by a complicated rearranging of the remaining part, giving rise to different polyclicic molecular skeletons full of oxygenated functional groups, partially acylated.

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Figure 4. The kernels, containing the seeds, that are used as raw material for the production of the oil, mainly produced in India by little and simple producers (figure on the right).

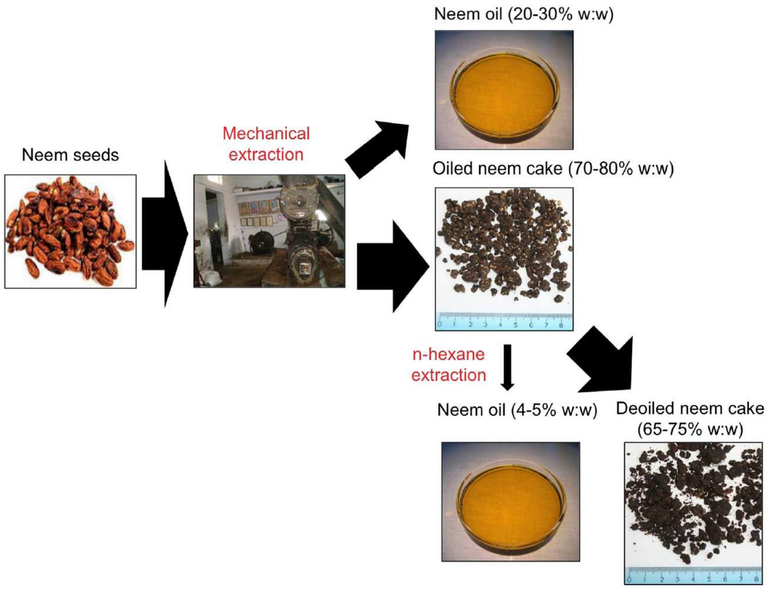

Figure 5. The process of neem oil production and neem cake (modified from Benelli et al., 2015).

Among major nor-triterpenes are limonoids, azadirachtin A and B, nimbin, nimbidin, salannin, and nimbolides (Fig. 6) [50]. Unlike chemical insecticides, neem compounds work on the insect's hormonal system, not on the digestive or nervous system, and therefore do not lead to development of resistance in future generations. The limonoids present in neem make it a harmless and effective insecticides, pesticide, nematicide, fungicide, etc. The most significant limonoids found in neem with proven ability to block insect growth are azadirachtin, salanin, meliantriol, and nimbin. Azadirachtin is currently considered as neem main agent for controlling insects. It appears to cause 90% of the effect on most pests. It does not kill insects – at least not immediately – instead both repels and disrupts their growth and reproduction. Research over the past years has shown that it is the most potent growth regulator and feeding deterrent ever assayed. It can repel or reduce the feeding of many species of pest insects as well as some nematodes. In fact, it is so potent a deterrent that a mere trace of its presence prevents some insects from even touching plants.

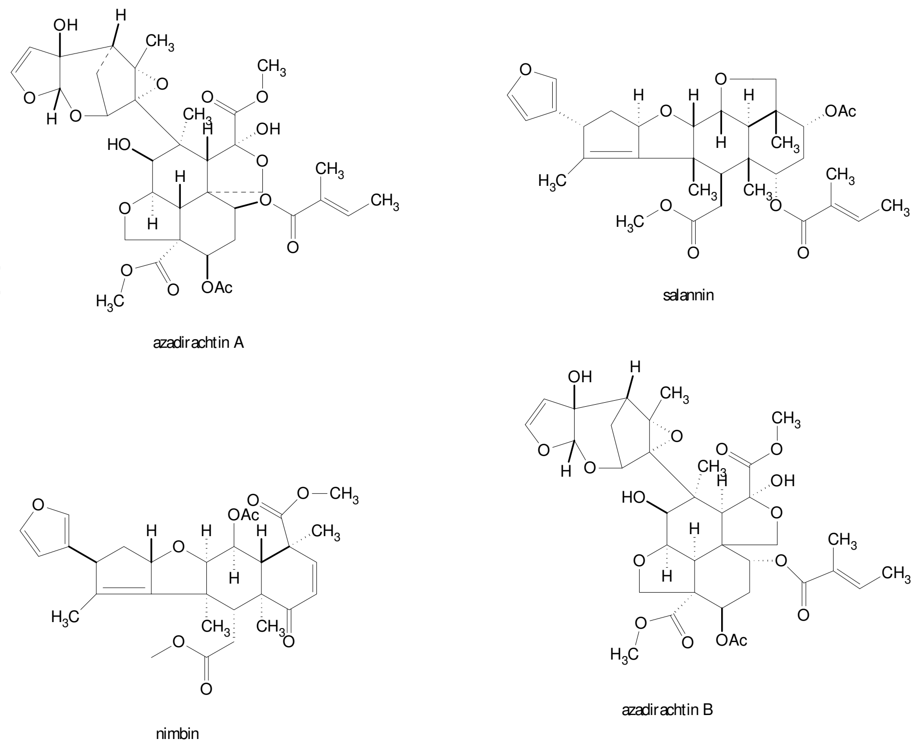

Figure 6. The main limonoids of neem.

Many formulations deriving from neem seeds show antifeedancy, fecundity suppression, ovicidal and larvicidal activity, insect growth regulation, and/or repellence against insect pests, even at low dosages [51–56], including ticks, house dust mites, cockroaches, raptor bugs, cat fleas, bed bugs, *Sarcoptes scabiei* mites infesting dogs, poultry mites, and beetle larvae parasitizing the plumage of poultry. The insecticidal properties, environmental safety, and public acceptability of neem and its products have been certified by the US EPA [57] and have led to its adoption into some control programs against Diptera pests [58]. Noticeably, emulsified formulations of *A. indica* oil showed an excellent larvicidal potential against different mosquito genera, including *Aedes*, *Anopheles*, and *Culex*, also under field conditions.

Action mechanisms include repellence, feeding and oviposition deterrence, but hormonal effects are the key of the inversion of control strategy, changing the target from the adult everywhere dispersed to the locally maintained larvae, through growth inhibition, mating disruption, chemo-sterilization, etc. In fact, hormones are necessary for to complete the process of metamorphosis as the insects pass from larva to pupa to adult. In any case, if the larva manages to enter the pupal stage, the adult emerging from the pupa is 100% malformed, absolutely sterile without any capacity for reproduction. The insect populations decline drastically as they become unable to reproduce. However, also antifeedant and deterrent activities are important to defend crops. The ideal plant-derived product, including insecticide, should be eco-friendly, sustainable, low cost, and target specific, leaving unaffected the beneficial ones. Neem products do not leave any residue on the field, being biodegradable by the action of sunlight. Azadirachtin in open space after dissipation has a half-time of about 20 h. The degradation slowly occurs also when neem products are stored under appropriate

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conditions [59–60]. Neem, at usual concentrations, is harmless to nontarget and beneficial organisms like pollinators, honeybees, mammals, and other vertebrates [61]. The absence of toxicity is largely evidenced by the millenary use in Indian traditional medicine, as well as by the EPA report and the large use during the last 30 years, including products for pet care.

Neem cake is the residue that is left over when the kernel is crushed from neem kernels containing seeds and the remaining is pressed to obtain the oil (Fig. 5). In fact, although the overall marketed name is seed neem oil, not only the seeds are utilized. Neem cake looks more like flour than cake, with differences in color and size of particles. Two products are therefore in the market: neem oil cake obtained by cold pressure, with 6% of the oil still residue, and neem cake deoiled, with a residue up to 1.5% [62].

Actually, it is not approved as pesticide, and mainly it is highly appreciated as organic fertilized. Neem cake acts as a natural fertilizer with pesticide properties, protecting crops from nematodes, soil grubs, and white ants.

India alone has an annual potential of 80,000 tons of oil and 330,000 tons of neem cake from 14 million plants that grow naturally. To this potentiality, the high number of cultivations actually occurring in many parts of the world must be added. This situation evidences the neem's high sustainability and possibility to have an increasing production of low cost products to be utilized in many fields, not only insecticides, from medicine to the cosmetic one. The importance of the neem future is strictly related to this wild range of utilizations, which are strictly linked to the new market of plant natural products.

8. The neem cake alternative

Despite the evidence of efficacy, several factors limit the massive use of neem oil in control of insect vectors. Limits include high cost, photosensibility, and persistence in the soil. A network of several Italian universities and research institutions decided to investigate the larvicidal activity of the neem cake as an alternative. In fact, neem cake is a low cost by-product of neem oil production (Fig. 7).

Figure 7. Nem cake ready for exportation.

The first step of the network was analytical. Several neem cakes from different producers and importers were analyzed by high-performance liquid chromatography (HPLC), evidencing still the presence of neem limonoids, but with different pattern in comparison with neem oil. Percentage was low but very different in each sample and salannin was the prevalent nortriterpene (3750 ppm of azadirachtin A+B, 7980 ppm of salannin, and 1850 ppm of nimbin) [63–65]. The high-performance thin layer chromatography (HPTLC) analyses, performed in the laboratories of Environmental Biology at the Sapienza University of Rome, allowed to evidence a great complexity of the neem cake extract, showing at least more than 30 secondary metabolites spread in all range of polarity (Fig. 8). On the basis of the information obtained in the chromatographic analyses, a neem cake product was selected and used for the activity tests, realized at the ENEA laboratories. Laboratory essays evidenced a significant activity of neem cake n-hexane and ethylacetate extract against *A. albopictus* mosquito larvae [66].

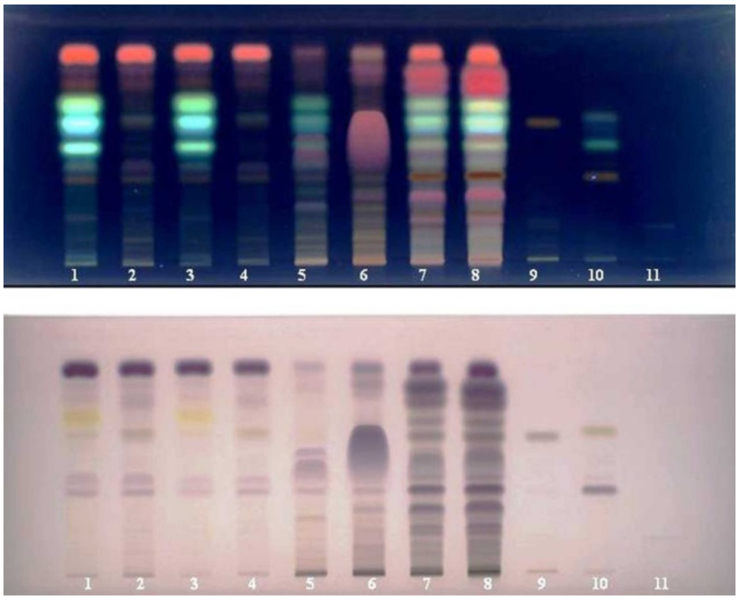

Figure 8. HPTLC analysis of different neem products. Mobile phase: toluene, ethyl acetate (4:6 v/v). Derivatization: Anisaldehyde. Plate on the top, visualization: UV366 nm. Plate on the bottom visualization: white light. Tracks: (1) neem oil marketed in Italy extracted with ethyl acetate, (2) neem oil marketed in India extracted with ethyl acetate, (3) neem oil marketed in Italy, (4) neem oil marketed in India, (5 and 6) neem cakes extracted with ethyl acetate, (7 and 8) neem cakes of tracks, (5) defatted and concentrated (track 8 more concentrated), (9) nimbin, (10) salannin, and (11) azadirachtin A.

In the same time, another group of the network, at the University of Sassari in Sardinia, worked on Blue Tongue disease. *Culicoides* species are vectors of BTV [67]. These insects breed in mist microhabitats, like small pools, irrigation channels, beverage sites, and drainage pipes. *Culicoides imicola* is the main vector, representing about 10% of all emerged *Culicoides* adults. In the laboratory essays, larvae of *C. imicola* resulted highly sensitive to the commercial neem cake. The larval mortality in water after 7 days gave a lethal concentration value (LC50) of 0.37 g/l. In order to define the chemical nature of active constituents, a neem cake methanol extract was separated by different solvents. Fractions of increasing polarity were assayed on *Culicoides* larvae. The most active resulted the ethyl acetate fraction, containing 1 ppm of azadirachtin, 1.5 ppm salannin, and 0.3 ppm of nimbin. The fraction was more toxic than a commercial formulation at the same azadirachtin concentration.

Strategy in field trials was based on the deposit of neem cake in the typical larval sites of *Culicoides*, and again the product was found to be very effective. A treatment with neem cake at dose of 100 g/m was applied in a larval breeding site of *Culicoides* located in a riverside of a pond margin of a livestock farm in Sardinia, Italy. The treatment with the neem cake resulted in a significant reduction in *Culicoides* emergence until 28 days.

Finally, activity tests are now in progress at the ENEA laboratories to measure the larvicidal toxicity against *P. spumarius*, as main vector of *X. fastidiosa*. First experiments were positive but limited to the laboratory conditions. Field experiments are urgently needed.

Although the mentioned results need confirmation and utilization in larger scale, neem cake is a promising material for the development of newer products useful in the control of vectors of insect-borne diseases at the larval stage.

9. Conclusions

The previous samples are related to Southern Italy, but situations in the other parts of the world are probably very similar. Chun-Xiao et al. reported the relationship between insecticide resistance and genome mutations of *Aedes aegypti* in Southern China [68]. The relationship has been detected for several insecticides, but the mechanisms of resistance are not totally understood. The kind of resistance to both pyrethroids and DDT, known as knockdown resistance, has been related to amino acid substitution in the sodium channel. In the paper, the causes of the resistance are not only attributed to the extensive recent use of pyrethroids and related to a series of different factors, first of all the climate changes, but also associated to the rapid development of tourism, transportations, and increasing urbanization that could increase *A. aegypti* breeding sites. Therefore, the development of resistance must be considered a complex multifactor phenomenon. The complex solution should consist into a multitreatment in at least three steps.

The soil must be considered not only to sustain the plant with adequate fertilizer able to give the necessary elements but also to positively change the biome living underground. Roots must be considered not only as the corm part necessary for collection of water and minerals but also as a part of the plant integrated to the underground habitant, including the living system. Insect vectors must be controlled possibly at the larval stage, when the insects are not able to move and need mist conditions to survive. Insecticide must be eco-friendly and low cost, targeted to preserve pollinators and other useful insects. Natural substances could be the starting point to develop antibiotics of new generation, based on different mechanism and useful to be used in large scale, without relevant damages to the environment. This multidisciplinary approach highlights the need of stronger cooperation among pharmacologists, chemists, parasitologists, entomologists, and behavioral ecologists.

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References

[1] Carlson R. Silent Spring. Farwcett World Library. USA. (1962).

[2] Caspar A. Hallmann, Ruud P. B. Foppen, Chris A. M. van Turnhout, Hans de Kroon, Eelke Jongejans (9 July 2014). Declines in insectivorous birds are associated with high neonicotinoid concentrations. Nature. Retrieved 14 July 2014. doi: 10.1038/nature13531.

[3] J. P. van der Sluijs, V. Amaral-Rogers, L. P. Belzunces, M. F. I. J. Bijleveld van Lexmond, J-M. Bonmatin, M. Chagnon, C. A. Downs, L. Furlan, D. W. Gibbons, C. Giorio, V. Girolami, D. Goulson, D. P. Kreutzweiser, C. Krupke, M. Liess, E. Long, M. McField, P. Mineau, E. A. D. Mitchell, C. A. Morrissey, D. A. Noome, L. Pisa1, J. Settele, N. Simon-Delso, J. D. Stark, A. Tapparo, H. Van Dyck, J. van Praagh, P. R. Whitehorn, M. Wiemers (10 October 2014). Conclusions of the worldwide integrated assessment on the risks of neonicotinoids and fipronil to biodiversity and ecosystem functioning. Environmental Science and Pollution Research 22: 3229. doi: 10.1007/s11356-014-3229-5.

[4] Neonicotinoid Pesticides and Honey Bees. Washington State University Extension Fact Sheet FS122E.

[5] Gill, R. J., O. Ramos-Rodriguez, N. E. Raine (2012). Combined pesticide exposure severely affects individual and colony-level traits in bees. Nature 491(7422): 105–108. doi: 10.1038/nature11585.

[6] Tadeusz Banaskiewicz (2010). Evolution of pesticide use. In: Contemporary Problems of Managements and Environmental Protection. Vol. 5. Influence of the Pesticide Dump on the Environment, p. 7–18.

[7] Insecticide Resistance Action Committee. Resistance Management for Sustainable Agriculture and Improved Public Health. 2007. Retrieved December 2014.

[8] J. Hemingway, H. Ranson (2000). Insecticide resistance in insect vectors of human disease. Annual Review of Entomology 45: 371–391.

[9] D. W. Onstad (2008). Insect Resistance Management. Elsevier: Amsterdam.

[10] S. U. Karaagac (2011). Insecticide Resistance. In: Advances in Integrated Pest Management, Farzana Perveen (ed.). InTech, Rijeka, Croatia, p.469–478.

[11] G. Benelli (2015). Research in mosquito control: current challenges for a brighter future. Parasitology Research. doi: 10.1007/s00436-015-4586-9.

[12] W. G. Ware, D. M. Whitacre (2004). The Pesticide Book. Meister Media Worldwide, Willoughby, Ohio.

[13] Bayo F. Sanchez (2011). Ecological impact of insecticides. In: Advances in Integrated Pest Management, Farzana Perveen (ed.). InTech. Rijeka, Croatia, p. 61–90.

[14] C. Gordon (2003). Role of environment stress in the physiological response to chemical intoxicants. Environmental Research 92: 1–7.

[15] G. Benelli, K. M. Daane, A. Canale, C. Y. Niu, R. H. Messing, R. Vargas (2014). Sexual communication and related behaviours in Tephritidae—current knowledge and potential applications for integrated pest management. Journal of Pest Science 87, 385–405.

[16] R. G. Estrada-Franco, G. B. Craig (1995). Biology, Disease Relationship and Control of *Aedes albopictus*. Pan American Health Organization, Washington, DC. Technical Paper No. 42.

[17] C. J. Mitchell (1995). Geographic spread of *Aedes albopictus* and potential for involvement in arbovirus cycles in the Mediterranean Basin. Journal of Vector Ecology 20: 44–58.

[18] R. Romi (1995). History and updating of the spread of Aedes albopictus in Italy. Parassitologia 37: 99–103.

[19] M. Q. Benedict, R. S. Levine, W. A. Hawley, L. P. Lounibos (2007). Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. Vector Borne and Zoonotic Disease 7: 76–85.

[20] C. Caminade, J. M. Medlock, E. Ducheyne, K. M. McIntryre, S. Leach, M. Baylis, A. Morse (2012). Suitability of European climate for the Asian tiger mosquito *Aedes albopictus*: recent trends and future scenarios. Journal of the Royal Society Interface 9: 2708–2717.

[21] C. Paupy, H. Delatte, L. Bagny, V. Corbel (2009). Fontenille. *Aedes albopictus*, an arbovirus vector: from the darkness to light. Microbes and Infection 11:1177–1185.

[22] J. M. Wells, B. C. Raju, H. Y. Hung, W. G. Weisburg, L. M. Parl, D. Beemer (1987). *Xylella fastidiosa* gen. nov., sp. nov.: Gram-negative, xylem-limited, fastidious plant

bacteria related to *Xanthomonas* spp. International Journal of Systematic Bacteriology 37(2): 136–143. doi:10.1099/00207713-37-2-136.

[23] Minimizing the Spread of Disease in Italy's Famous Olive Trees. Our Environment at Berkeley (in English) (9 February 2015). University of California at Berkeley, Department of Environmental Science, Policy, and Management (ESPM). Retrieved 5 May 2015.

[24] Public Intellectual Property Resource for Agriculture. PIPRA. Pierce's disease. Pierce's Disease website. "Major consequences" of olive disease spreads across EU. BBC News. 9 January 2015.

[25] First report of *Xylella fastidiosa* in the EPPO region. European and Mediterranean Plant Protection Organization (EPPO).

[26] Expert Says Eradication of New Olive Tree Disease in Europe Unlikely (29 March 2014). Olive Oil Times. Retrieved 1 March 2015.

[27] Italy warns deadly olive tree bacteria could spread across Europe (27 February 2015). The Telegraph. Retrieved 1 March 2015.

[28] S.E. Randolph (2004). Evidence that climate change has caused "emergence" of tick-borne disease in Europe. International Journal of Medical Microbiology 37, 5–15.

[29] P. Reiter (1998). Global warning and vector disease in temperate regions and at high latitudes. Lancet 351, 839–849.

[30] P. Reiter (2001). Climate change and mosquito-borne disease. Environmental Health Perspectives 109 (Suppl. 1): 141–161.

[31] V. P. Pursue, P. S. Mellor, D. J. Rogers, A. R. Samuel, P. C. Mertens, M. Baylis (2005). Climate change and the recent emergence of bluetongue in Europe. Nature Review/ Microbiology 3: 171–181.

[32] T. M. L. Wigkey, D. Schimel, eds. (2000). The Carbon Cycle. Cambridge, Cambridge University Press.

[33] G. Cook (1992). Effect of global warning on the distribution of parasitic and other infectious diseases: a review. Journal of the Royal Society of Medicine 85: 688–691.

[34] P. S. Melior, E. J. Wittiman (2002). Bluetongue virus in the Mediterranean basin, 1998–2001. Veterinary Journal 164: 20–37.

[35] P. Calistri, V. Caporale (2003). Bluetongue in Italy: a brief description of the epidemiological situation and the control measures applied. Bulletin—Office International des Épizooties 15–17.

[36] J. H. W. Slingenbergh, G. Hendrickx, G. R. W. Wingt (2002). Will the new livestock revolution succeed? Agriworld Vision 2: 31–331.

http://dx.doi.org/10.5772/61467

[37] W. J. Tabachnick (1996). *Culicoides variipennis* and bluetongue virus epidemiology in the United States. Annual Review of Entomology 41: 23–43.

[38] V. Dancik, K. P. Seile, D. W. Young, S. L. Schreiber, P. A. Clemons (2010). Distinct biological network properties between the targets on natural products and diseases genes. JACS Communications.

[39] National Research Council (1992). Neem: a tree for solving global problems. Academic, Washington.

[40] R. P. Singh, R. C. Saxena (1999). *Azadirachta indica* A. Juss. Oxford 7 IBH Publishing Co. Pvt. Ltd.

[41] H. Schmutterer (1995). The Neem Tree, VCH. Verlagsgesellschaft mnH, Germany.

[42] D. N. Tewari (1992). Monograph of Neem. International Book Distributors, Dehradun, India.

[43] N. S. Randhawa, B. S. Parmar (eds.) Neem Research and Development, Society of Pesticide Science, India.

[44] H. S. Puri (1999). Neem the Divine Tree, Harwod Academic Publishers.

[45] M. Nicoletti, K. Murugan (2013). Neem the tree of 21st century. Pharmacologyonline 3, 115–121.

[46] K. N. Kabra (2000). Development & Ecological Role of Neem in India, Neem Foundation, India.

[47] R. T. Gahukar (1995). Neem in Plant Protection. Agri-Horticultural Publishing House.

[48] R. P. Singh et al. (1996). Neem & Environment. Oxford & IBH Publishing Co. Pvt. Ltd.

[49] . F. R. Gallo, G. Multari, E. Federici, G. Palazzino, M. Nicoletti, V. Petitto (2012). The modern analytical determination of botanicals and similar novel natural products by the HPTLC fingerprint approach in studies in natural products chemistry. Atta-ur-Rahman (Ed.). Elsevier 37: 217–258.

[50] M. Nicoletti, C. Toniolo (2013). Fingerprint HPTLC analysis of marketed products. Pharmacologyonline 3(6): 30.

[51] A. J. Mordue, A. Blackwell (2003). Azadirachtin: an update. Journal of Insect Physiology 39: 903–924.

[52] M. Rossi Forim, M. F. da Silva, F. das Gracas, J. B. Fernandes. Secondary metabolism as a measurement of efficacy of botanical extracts: the use of *Azadirachta indica* (Neem) as a model in insecticides—advances in integrated pest management. Farzana Perveen (Ed.). InTech. Rijeka, Croatia. 2011, p. 367–390.

[53] C. B. Wandscheer, J. E. Duque, M. A. N. da Silva, Y. Fukuyama, J. L. Wohlke, J. Adelmann, J. D. Fontana (2004). Larvicidal action of ethanolic extracts from fruit endocarps of *Melia azedarach* and *Azadirachta indica* against the dengue mosquito *Aedes aegypti*. Toxicon 8:829–835.

[54] R. P. Prakash Srivastava (2001). Neem and Pest Management. International Book distributing Co.

[55] T. Amorose (1995). Larvicidal efficacy of neem (*Azadirachta indica*) oil and defatted cake on Culex quinquefasciatus Say. Geobios 22: 169–173.[55] M. S. Mulla, T. Su (1999). Activity and biological effects of neem products against arthropods of medicinal and veterinary importance. Journal of the American Mosquito Control Association 15: 133–152.

[56] F. O. Okumu, B. G. J. Knols, U. Fillinger (2007). Larvicidal effects of a neem (*Azadirachta indica*) oil formulation on the malaria vector *Anopheles gambiae*. Malaria Journal 6: 63–67

[57] Cold Pressed Neem Oil (025006) Fact Sheet. EPA. 2012. Cold Pressed Neem Oil (025006).

[58] V. P. Sharma, R. C. Dhiman (1993). Neem oil as a sand fly (Diptera: Psychodidae) repellent. Journal of the American Mosquito Control Association 9:364–366.

[59] T. Wei-Hong, S. Zhan-Qian (2006). Research on effect of four natural ultraviolet light absorbers on photostabilization of azadirachtin-A. Agricultural Sciences in China 5: 885–894.

[60] S. J. Boeke, M. G. Boersma, G. M. Alink, J. J. van Loon, A. van Hnis, M. Dicke, I. M. Rietjens (2004). Safety evaluation of neem (*Azadirachta indica*) derived pesticides. Journal of Ethnopharmacology 94:25–41

[61] M. Gandhi, R. Lal, A. Sarkaranarayaman, C. K. Baneyel, P. L. Sharma (1988). Acute toxicity of the oil from *Azadirachta indica* seed (neem oil). Journal of Ethnopharmacology 23:39–51 518.

[62] G. Benelli, M. Nicoletti (2013). Shedding light on bioactivity of botanical by-products: neem cake compounds deter oviposition of the arbovirus vector *Aedes albopictus* (Diptera: Culicidae) in the field. Parasitology Research 110: 2013–202.

[63] M. Nicoletti, M. Serafini, S. Mariani, T. Ciocchetti, K. Murugan (2012). Neem cake: chemical composition and larvicidal activity on Asian tiger mosquito. Parasitology Research 111 (1), 205–213.

[64] S. Mariani, M. Nicoletti (2013). Antilarval activity of neem cake extracts against *Aedes aldopictus*. Pharmacologyonline 3:128–132.

[65] M. Nicoletti, M. Serafini, A. Aliboni, A. D'Andrea, S. Mariani (2010). Toxic effects of neem cake extracts on *Aedes albopictus* larvae. Parasitology Research 107:89–84.

[66] G. Benelli, K. Murugan, C. Panneerselvam, P. Madhiyazhagan, B. Conti, M. Nicoletti (2015). Old ingredients for a new recipe? Neem cake, a low-cost botanical by-product in the fight against mosquito-borne diseases. Parasitology Research 114:391–397.

[67] C. Foxi, G. Delrio (2013). Efficacy of a neem cake for the control of Culicoides. Pharmacology Online 3: 110–114.

[68] C.-X. Li, P. E. Kaufman, R.-D. Xue, M. H. Zhai, G. Wang, T. Yan (2015). Relationship between insecticide resistance and Kdr mutations in dengue vector *Aedes aegypti* in Southern China. Parasites Vectors 8(1): 325. doi: 10.1186/s13071-015-0933-7.