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Development of RNAi in Insects and RNAi-Based Pest Control

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1. Introduction

In agricultural systems, insect pests can cause crop damage mainly through loss in yield or quality resulting in a loss in profits for farmers. Worldwide pests cost billions of dollars due to damage and use of pesticides. Chemical pesticides are still the major approach for controlling insect pests, but they are associated with significant hazards to the environment and human health. The alternative commercial biotechnological system relies mostly on the expression of *Bacillus thuringiensis* insecticidal proteins (Cry toxins). Its effectiveness however is threatened by the development of resistance in some species such as *Ostrinia nubilalis* (Lepidoptera, Pyralidae) and *Heliothis virescens* (Lepidoptera: Noctuidae) (Ferre and Van Rie, 2002; Baum *et al*., 2007). As a result, there is an urgent need to develop economically and ecologically sound alternatives for pest control.

Gene silencing has been suggested as one of the new alternatives to reduce damage from insect pests. RNA interference (RNAi) is first described by Fire *et al*. (1998), and its mechanism lies in that a double-stranded RNA (dsRNA) introduced in an organism has the capacity to silence post-transcriptional genes (Hannon, 2002; Geley and Muller, 2004). RNAi is highly conserved in eukaryotic organisms (Fire, 2007). It is considered as a specific type of defence mechanism (Terenius *et al*., 2011). Four different types of RNAi have been described including short interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), endogenous siRNAs (endo-siRNAs or esiRNAs), and microRNAs (miRNAs) (Terenius *et al*., 2011). To date, RNAi has been proven promising for research on gene function determination and gene knockdown in eukaryotes and medical control of cancers and viral disease (Huvenne and Smagghe, 2010).

In insects, studies have mainly targeted the understanding of the RNAi mechanism, and the function, regulation and expression of genes. Introduction of dsRNA into an organism has been tested by using different techniques such as microinjection (Bettencourt *et al*., 2002;

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Tomoyasu and Denell, 2004; Ghanima *et al*, 2007), soaking, or, most preferably, oral feeding of artificial diet (Eaton *et al*., 2002; Turner *et al*., 2006; Baum *et al*., 2007; Mao *et al*., 2007; Chen *et al*., 2008; Tian *et al*., 2009). Transgenic plants producing dsRNAs directed against genes function in Lepidoptera, Coleoptera, and Hemiptera pests are becoming more common (Gordon and Waterhouse, 2007; Baum *et al*., 2007; Mao *et al*., 2007, Chen *et al*., 2010). For example, Chen *et al*. (2010) report the successful feeding of TPS (trehalose-6-phosphate synthase for the synthesis of trehalose, main sugar reserve in haemolymph) dsRNA solutions to silence this gene thus proposing it as a useful pest control agent. These results suggest that over time, new generations of insect-resistant crops will be created to manage agriculturally important insect pests.

In this review chapter, we summarize the current knowledge on the recent RNAi research on insects, including the application of RNAi techniques in research involving functional insect genes and functional genomics, the methods of dsRNA uptake RNAi in insects, the systemic diffusion of RNAi silencing molecules in the insect body and the mechanism underlying this diffusion, and the potential application of RNAi in integrated pest management (IPM). The main purpose of this review is to help entomologists become familiar with RNAi research, a rapidly growing field where new avenues and techniques are being used to investigate insect RNAi mechanisms for the development of pest control.

2. Study on the function of insect genes using RNAi methods

RNAi is a powerful tool for the study on the function of insect genes. It was first used in the study of a model insect, the fruit fly *Drosophila melanogaster* (Lipardi *et al*., 2001). RNAi studies in *D. melanogaster* have laid a solid foundation for the development of insect RNAi technology and the elucidation of the RNAi mechanisms in insects. Recently, Huvenne and Smagghe (2010) have reviewed the definitions of RNAi in insects while Terenius *et al.* (2011) have analyzed the variability and the implications of over 150 published and unpublished studies, mainly focusing the analysis on lepidopteran insects, on the need for further studies on RNAi mechanisms.

In this section, we discuss a selected group of published studies and the main orientations used by researchers in exploring these techniques. Table 1 summarizes the studied functions, methods used for RNAi introduction and the main responses of insects. Besides *D. melanogaster*, 20 other insect species are reported here, including 7 species of Lepidoptera, 3 species of Coleoptera, 3 species of Orthoptera, 2 species of Hymenoptera, 2 species of Homoptera, 1 species of Diptera, 1 species of Isoptera, and 1 species of Hemyptera. These selected papers have all in common the successful use of RNAi mechanisms as potential pest control agent. It is important to note that, except for a few exceptions (e.g. circadian clock gene), most studies have targeted different genes. Responses also greatly vary from minor effects such as disruption in functional rhythm to reduction in fitness and increased mortality. As reported by Terinius *et al*. (2011) for lepidopteran species, most of the studies have helped better understand developmental processes and the immune system.

3. Internal diffusion of RNAi molecules within insect body

The effects of RNAi inside the body of insects are determined by an important factor, the spread of silencing RNA molecules inside the insect body (so-called systemic RNAi). In

Insects	Genes and References	Methods	Effects
Silkworm *Bombyx mori*	Circadian clock gene *per* (Sandrelli *et al*., 2007)	Transgenics	Disruption of egg-hatching rhythm
	Ecdysis-triggering hormone gene *ETH* (Dai *et al*., 2008)	Transgenics	Lethal at pharate second-instar larval stage
Egyptian cotton leafworm *Spodoptera littoralis*	*β-actin* gene (Gvakharia *et al*., 2003)	Injection	Disruption of sperm release
	Circadian clock gene *per* (Kotwica *et al*., 2009)	Injection	Delayed sperm release
Light brown apple moth *Epiphyas postvittana*	Carboxylesterase gene *EposCXE1* and pheromone binding protein gene *EposPBP1* (Turner *et al*., 2006)	Feeding	Inhibition of gene expression
Cotton bollworm *Helicoverpa armigera*	Cytochrome P450 gene *CYP6AE14* (Mao *et al*., 2007)	Feeding	Inhibition of larval growth
	Glutathione-S-transferase gene *GST1* (Mao *et al*., 2007)	Feeding	Successful inhibition of gene expression
Beet armyworm *Spodoptera exigua*	Chitin synthase gene (Chen *et al*., 2008)	Injection	Disorder in the insect cuticle, no expansion of the larval trachea epithelial wall, and other larval abnormalities
Japanese pine sawyer *Monochamus alternatus*	Laccase gene *MaLac2* (Niu *et al*., 2008)	Injection	Pupal and adult cuticle sclerotisation, death at a high dose
Red flour beetle *Tribolium castaneum*	Chitin synthase genes *TcCHS1* and *TcCHS2* (Arakane *et al*., 2005)	Injection	Disruption in all types of moulting(larva-larva, larva-pupa, and pupa-adult), cessation of ingestion, decrease in larval size, and reduction of chitin content in the midgut
	Chitinase-like proteins *TcCHT5*, *TcCHT10*, *TcCHT7*, and *TcIDGF4* (Zhu *et al*., 2008)	Injection	Effects on pupal-adult moulting Effects on egg hatching, larval moulting, pupation, and adult metamorphosis. Effects on abdominal contraction and wing/elytra extension. Effects on adult eclosion

Insects	Genes and References	Methods	Effects
Western corn rootworm *Diabrotica virgifera virgifera* LeConte	Vacuolar ATPase (*v-ATP*) (Baum *et al.*, 2007)	Feeding	Delayed larval development and increased mortality
Striped flea beetle *Phyllotreta striolata*	Arginine kinase gene *AK* (Zhao *et al.*, 2008)	Feeding	Delayed development, increased mortality, and reduced fertility
Mediterranean field cricket *Gryllus bimaculatus*	Circadian clock gene *per* (Moriyama *et al.*, 2008)	Injection	Complete loss of circadian control of locomotor activity and electrical activity in the optic lobe
	Nitric oxide synthase gene *NOS* (Takahashi *et al.*, 2009)	Injection	Destruction of long-term memory
German cockroach *Blattella germanica*	*BgRXR* gene (Martin *et al.*, 2006)	Injection	Inhibition of pupal eclosion
	Pigment-dispersing factor gene *pdf* (Lee *et al.*, 2009)	Injection	Effects on insect night activity
American grasshopper *Schistocerca americana*	Eye colour gene *vermilion* (Dong and Friedrich, 2005)	Injection	Suppression of ommochrome formation and systematic expression
Brown planthopper *Nilaparvata lugens*	Trehalose phosphate synthase (*TPS*) (*NlTPS* mRNA) (Chen *et al.*, 2010)	Feeding	Disturbed development through disruption in the TPS enzymatic activity, reduction of insect survival rate
Turnip sawfly *Athalia rosae*	*Ar white* gene (Sumitani *et al.*, 2005)	Injection	White phenocopy in embryonic eye pigmentation
European honey bee *Apis mellifera*	Transcription factor gene *Relish* (Schlüns and Crozier, 2007)	Injection	Inhibition of *Relish* gene expression and reduction in the expression of two other immune genes, *abaecin* and *hymenoptaecin*
Triatomid bug *Rhodnius prolixus*	Salivary nitrophorin 2 gene *NP2* (Araujo *et al.*, 2006)	Injection and feeding	Shortened plasma coagulation time
Savannah tsetse fly *Glossina morsitans morsitans*	*TsetseEP* gene and transferrin gene *2A192* (Walshe *et al.*, 2009)	Feeding	Inhibition of *TsetseEP* gene expression, but no inhibition of *2A192* gene expression

Insects	Genes and References	Methods	Effects
Eastern subterranean termite *Reticulitermes flavipes*	Cellulase enzyme gene *Cell-1* and caste-regulatory hexamerin storage protein gene *Hex-2* (Zhou *et al.*, 2008)	Feeding	Reduction in group fitness and increased mortality

Table 1. RNAi research on functional genes in insects

plants, the nematode *Caenorhabditis elegans*, and the planarian *Schmidtea mediterranea*, RNAi is systemic as the RNAi signal spreads throughout the entire biological system by travelling between cells (Fire *et al*., 1998; Newmark *et al*., 2003). In insects, RNAi is not always to be systemic. For example, fly cells take up dsRNA, which cannot spread throughout the entire body (Saleh *et al*., 2006). Whangbo and Hunter (2008) have defined different mechanisms for dsRNA uptake: cell-autonomous and non-cell autonomous. Huvenne and Smagghe (2010) described these two types of RNAi and their level of spread which would be greater in systemic non-cell autonomous RNAi than in the cell autonomous RNAi. While most research in insects has been conducted with cell-autonomous RNAi, it is suggested that studies should focus towards non-cell autonomous RNAi as a better potential for defining agent of insect control.

In summary, studies have shown that the ability to distribute an RNAi signal is different in different insects. The intake of dsRNA by *Drosophila* cells leads to localised gene silencing, without systemic distribution of the RNAi signal (Van Roessel *et al*., 2002; Roignant *et al*., 2003; Dietzl *et al*., 2007). On the other end, *Tribolium* (Tomoyasu *et al*., 2008) and *Schistocerca americana* (Dong and Friedrich, 2005) have strong systemic RNAi reactions. The gene responsible for nematode systemic RNAi is *sid-1* (Winston *et al*., 2002). Correspondingly, the *sid-1* gene is not found in the *Drosophila* genome, whereas the grasshopper has a *sid-1* ortholog (Dong and Friedrich, 2005), and *Tribolium* also has a *sid-1*-like gene (Tomoyasu *et al*., 2008). Further BLAST searches at the NCBI website have identified one species in Coleoptera, one in Lepidoptera, two in Hymenoptera, and three in Hemiptera containing *sid-1* homologs, whereas no homologous gene has been found in Homoptera (Walshe *et al*., 2009).

Further studies have found that the RNAi mechanisms in *Tribolium* and the nematode *C. elegans* are different. *Tribolium* does not have some of the key elements that are required for RNAi in *C. elegans*, such as RNA-dependent RNA polymerase (RdRP) and the RNA channel transporter (SID) (Fire *et al*., 1998; Winston *et al*., 2002). Furthermore, the function of the *sid-1*-like gene of *Tribolium* is not to absorb RNAi but, instead, is similar to the function of the *tag-130* gene of *C. elegans* (Tomoyasu *et al*., 2008). Therefore, further verification is needed to define the function of the *sid-1* gene in insect RNAi. Recent studies have shown that the anti-viral RNAi reaction in *Drosophila* depends on a virus-specific immune signal and systemic spreading (Saleh *et al*., 2009). Further studies need to be conducted to understand the spread of silencing RNA within the insect body and the genes involved in this process. Understanding and revealing the molecular mechanisms of determining how RNA spreads systemically inside the insect body will facilitate the application of RNAi technology for pest control.

4. Methodology of dsRNA uptake in insects

Methods of dsRNA uptake in insects can greatly vary and strongly influence the efficiency of gene silencing, thus their potential as insect pest control agent. It is important to note that

since gene silencing is only limited to cells that are infected, the main challenge is the selection of the delivery system (Terenius *et al*., 2011). In both types, methods of delivery must be first defined, being effectively easier and better understood for cell-autonomous RNAi machinery (Siomi and Siomi, 2009). The main uptake (or delivery) methods include injection, soaking, feeding, transgenic technique, and viral infection. This section examines these various mechanisms and their effectiveness in delivering RNAi and gene silencing in various species.

4.1 Microinjection

Microinjection, i.e. the direct injection of dsRNA into the body of insects, has been one of the most effective delivery methods for systemic RNAi types. Short dsRNA have had the most success with this mechanism (Siomi and Siomi, 2009). In addition, the 5′ end of the dsRNA can affect the effectiveness of RNAi; a phosphorylated 5′ end exhibits better gene silencing rate than does a hydroxylated 5′ end (Boutla *et al*., 2001).

The major advantage of injecting dsRNA into the insect body is the high efficiency of inhibiting gene expression. There are however some limitations with micro-injection. First, the cost for in vitro synthesis and storage of dsRNA is relatively high, and the steps are complicated. In addition, injection pressure and the wound generated inevitably affect the insects. It has been shown that skin damage stimulates the immune response. In practice, this delivery method would have very limited application as pest control agent.

4.2 Soaking

Soaking *D. melanogaster* embryos in a dsRNA solution can inhibit gene expression, and its effectiveness is comparable to the injection method in that it requires a higher concentration of dsRNA (Eaton *et al*., 2002). Soaking *D. melanogaster* S2 cells in *CycE* and *ago* dsRNA solutions has been shown to effectively inhibit the expression of these two genes for cell cycle, thereby elevating levels of protein synthesis (March and Bentley, 2007). The soaking method is suitable only for certain insect cells and tissues as well as for specific insects of developmental stages that readily absorb dsRNA from the solution, and therefore, it is rarely used.

4.3 Feeding of artificial diet

Compared to other methods, dsRNA feeding is the most attractive primarily because it is convenient and easy to manipulate. Since it is a more natural method of introducing dsRNA into insect body, it causes less damage to the insect than microinjection (Chen *et al*., 2010). It is especially popular in very small insects that are more difficult to manipulate using microinjection. Early insect RNAi feeding studies were frustrating; for example, the injection of dsRNA effectively silenced the aminopeptidase gene *slapn*, which is expressed in the midgut of *Spodoptera littoralis*, but feeding with dsRNA did not achieve RNAi (Rajagopal *et al*., 2002).

Fortunately, there are other studies showing that dsRNA feeding can be successful for RNAi studies in insects. Feeding dsRNA to *E. postvittana* larvae has been shown to inhibit the expression of the carboxylesterase gene *EposCXE1* in the larval midgut and also to inhibit the expression of the pheromone-binding protein *EposPBP1* in adult antennae (Turner *et al*., 2006). dsRNA feeding also inhibite the expression of the nitrophorin 2 (*NP2*) gene in the salivary gland of *Rhodnius prolixus*, leading to a shortened coagulation time of plasma

(Araujo *et al*., 2006). dsRNA feeding has also been successful in many other insects, including insects of the orders Hemiptera, Coleoptera, and Lepidoptera (Baum *et al*., 2007; Mao *et al*., 2007).

The main challenge remains that there needs to be a greater amount of material for delivery as silencing has been shown to be incomplete (Chen *et al*., 2010). This phenomenon has been observed after ingestion of *CELL-1* dsRNA by the termite *Reticulitermes flavipes* (Zhou *et al*., 2008), *TPS* dsRNA in *N. lugens* nymphae (Chen *et al*. 2010), *Nitrophorin 2* dsRNA by *Rhodnius prolixus* (Araujoa *et al*., 2006). In addition, different species of insects have different sensitivities to RNAi molecules when delivered orally. For example, *Glossina morsitans* fed with dsRNA may effectively inhibit the expression of *TsetseEP* in the midgut, but cannot inhibit the expression of the transferrin gene *2A192* in fat bodies due to lack of transfer capacity between tissues (Walshe *et al*., 2009). The mechanisms associated with the transfer of gene expression through feeding delivery method still need further study.

In addition, one method that may be better than direct feeding with dsRNA is the use of transgenic plants to produce dsRNA (Baum *et al*., 2007; Mao *et al*., 2007). The advantage of this method is the generation of continuous and stable dsRNA material. Genetically engineered dsRNA-producing yeast strains have also been developed to feed *D. melanogaster*, but gene silencing was not successful (Gura, 2000). However, dsRNA produced in bacteria is effective in *C. elegans* (Timmons and Fire, 1998). Therefore, the use of bacteria, especially insecticidal microorganisms, to produce dsRNA for insect RNAi merits further study.

4.4 Developing transgenic insects

The advantage of using transgenic insects that carry the dsRNA is that as it is inheritable, the expression can be stable and continuous. The technique has been proposed to help either reduce population through introduction of sterile insects or for population replacement. In this case, dsRNA must be first injected in the host insect. Tests are being conducted on several species with promising results but as stated by Scolari *et al*. (2011), there is a need to understand environmental and genetic influences when assessing the potential use of such transgenics. The transgenic method has been first used in *D. melanogaster* with the *GAL4/UAS* transgenic system that leads to the expression of hairpin RNA (Kennerdell and Carthew, 2000; Tavernarakis *et al*., 2000). Subsequently, transgenic technology has generated transgenic *Aedes aegypti* that produces dsRNA (Travanty *et al*., 2004). Through the use of a U6 promoter in *D. Melanogaster*, S2 cells can generate short hairpin RNA (shRNA) to inhibit gene expression (Wakiyama *et al*., 2005). RNAi molecules targeting the circadian clock gene *per* have also introduced into *Bombyx mori* embryos by a piggyback plasmid to obtain gene-silenced transgenic individuals (Sandrelli *et al*., 2007). The transfection technique has been used to silence the *D. melanogaster* mitochondrial frataxin gene *dfh*, generating large-sized, long-lived larvae and short-lived adults (Sandrelli *et al*., 2007). The *GAL4/UAS* transgenic system has also been used in *B. mori* (Sandrelli *et al*., 2007) to allow for induction of the transgenic construct. Therefore, gene function can be studied within a certain time period, and the study of gene functions in development, physiology, and the nervous system is possible.

4.5 Virus-mediated uptake

Virus-mediated RNAi methods involve the infection of the host with viruses carrying dsRNA formed during viral replication and targeting the gene of interest in the host. For

example, recombinant Sindbis virus introduced into *B. mori* cells through electroporation can produce dsRNA to inhibit *BR-C* gene expression, causing the larvae not to pupate or leading to adult defects (Uhlirova *et al*., 2003). Virus-mediated RNAi studies are still rare. However, this method takes advantage of the infection and ability of the virus to spread rapidly in a host population. Virus-mediated RNAi does not require screening for transgenic insects or tissues, and thus, it has unique advantages.

5. RNAi-based pest control

In our struggle to minimizing the damage caused by insect pests, we have to acknowledge that pests cannot be efficiently managed by utilizing a single pest control agent. Several studies have shown that pest resistance to chemical pesticide and more recently to Bt has increased requiring new techniques to be applied to reduce the impacts of pest on crop production. While commonalities regarding the development of resistance to chemical and biological control agents remain to be determined, research suggests that both biochemical and genetic factors can contribute to this resistance. It is therefore crucial to continue examining the potential of integrated pest control or management (IPM) to reduce the threat of pests on agroecosystems. IPM has been suggested as a strategy to control incidence of pests since the 1950's and is based on six components: controlled pest populations, healthy crops, monitoring, mechanical and biological controls, and responsible use of pesticides (Kogan, 1998). The basis of for IPM is to balance ecological gain with economic loss (Southwood and Way, 1970). Over the past decade, the number of studies examining these issues has been increasing with, RNAi, as a novel pesticide-free way, to be integrated into IPM.

Research is still in its infancy to examine the application of RNAi machinery for pest control, as most of the focus has been on functional studies of insect genes. Huvenne and Smagghe (2010) however describe in some detail the potential application of RNAi in insect control through cell-line and feeding-in-plant experiments. Based on the currently available literature, they suggested five important factors largely influencing the silencing effect and the efficiency of RNAi as insect pest control technique: concentration of dsRNA, nucleotide sequence, length of dsRNA fragment, persistence of the silencing effect, and life stage of the target pest.

Through insect gene function studies involving injecting or feeding with dsRNA, we have found that some gene silencing can dramatically affect insect growth and development. Theoretically it would be possible to use RNAi to inhibit insect gene leading to insect control. Already, pest control using transgenic plants expressing dsRNA have been published (Baum *et al*., 2007; Mao *et al*., 2007). Transgenic corn expressing dsRNA against the vacuolar ATPase gene (*v-ATP*) significantly decreases the damage caused by *D. virgifera virgifera* LeConte and, notably, protects corn crops (Baum *et al*., 2007). Introduction of RNAi elements targeting the *CYP6AE14* gene, which is directly related to gossypol detoxification in *Helicoverpa armigera*, into *Arabidopsis* or tobacco inhibits *CYP6AE14* gene expression in *H. armigera* feeding on the transgenic plants and, therefore, increases the toxicity of gossypol (Mao *et al*., 2007). Although there needs to be more testing in the field and at large scale, transgenic insects have also been tested as a mechanism for pest control (Scolari et al., 2011). For both transgenic plants and insects, very few species have been investigated and it is clear that further research is essential to explore the potential use of transgenics as an effective means for pest control. Like any other control mechanisms, risk assessment will be

required to determine whether RNAi technology as a form of pest control will be safe and likely create a new era in pest control.

6. Conclusion

Widespread increase in the application of RNAi technology in insect research has facilitated the identification of insect gene function. Research has shown that while dsRNA is particularly conservative, there are various functions and development factors among insect species. Such variations are yet to be fully understood but certainly can serve as a basis for determining their capacity to control insect genes. The main challenge for moving towards larger scale projects remains the development of effective delivery mechanisms. Feeding is very popular in insect RNAi research and may have the most promising future in pest control, especially with the creation of transgenic plants producing dsRNA. Overtime, the use of transgenic insects will also lead to more efficient pest control.

Our understanding of the types of dsRNA and their spreading mechanisms within an organism can limit our ability to move further. Indeed, existing studies have not provided enough evidence that systemic RNAi, with silencing RNA molecules spreading throughout the entire body, can be achieved in all insects. Which insects have characteristics promoting systemic RNAi? Are the mechanisms underlying systemic RNAi the same in different insect species? Such questions need to be answered before moving further in developing large scale pest control systems. Undoubtedly, there is broad potential for the application of RNAi technology in pest control, mainly if combined into IPM strategies.

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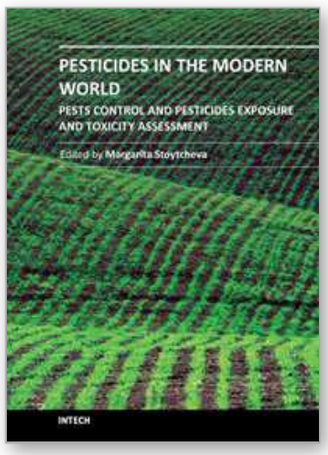

Pesticides in the Modern World - Pests Control and Pesticides Exposure and Toxicity Assessment

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The present book is a collection of selected original research articles and reviews providing adequate and up-to-date information related to pesticides control, assessment, and toxicity. The first section covers a large spectrum of issues associated with the ecological, molecular, and biotechnological approaches to the understanding of the biological control, the mechanism of the biocontrol agents action, and the related effects. Second section provides recent information on biomarkers currently used to evaluate pesticide exposure, effects, and genetic susceptibility of a number of organisms. Some antioxidant enzymes and vitamins as biochemical markers for pesticide toxicity are examined. The inhibition of the cholinesterases as a specific biomarker for organophosphate and carbamate pesticides is commented, too. The third book section addresses to a variety of pesticides toxic effects and related issues including: the molecular mechanisms involved in pesticides-induced toxicity, fish histopathological, physiological, and DNA changes provoked by pesticides exposure, anticoagulant rodenticides mode of action, the potential of the cholinesterase inhibiting organophosphorus and carbamate pesticides, the effects of pesticides on bumblebee, spiders and scorpions, the metabolic fate of the pesticide-derived aromatic amines, etc.

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