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REVIEW ARTICLE

Neonicotinoids and their Metabolites in Human Biomonitoring: A Review

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Summary Neonicotinoids (NNDs) constitute a major class of insecticides with a broad and versatile spectrum of applications in agriculture. Hence, their residues are found in several environmental compartments and can be transferred *via* several pathways to numerous organisms. Despite their profound impact on honeybees and wild bees (impairment of memory, impact on immune system), their presence in humans is far less reported, possibly due to the low to moderate toxicological effects that they elicit. The aim of the present review is to emphasize on developments in the biomonitoring of NNDs. It focuses mainly on chromatographic analysis of NNDs and their metabolites in human biological fluids, discussing key features, such as sample preparation and analytical method validation. Nonetheless, case reports regarding intoxication incidents are presented, highlighting the significance of such cases especially in the developing world.

Additional Keywords: LC-MS, urine, Imidacloprid

Introduction

Insecticides are substances of chemical or biological origin that are used to control insects. Amongst the plethora of insecticides, neonicotinoids (NNDs) comprise a significant class of insecticides with numerous applications in agriculture. NNDs family includes, imidacloprid (IMI), thiamethoxam (THIAM), clothianidin (CLOTH), thiacloprid (THIAC), acetamiprid (ACET), dinotefuran (DINOT), nitenpyram (NITEN), nithiazine (NITH), imidaclothiz (IMCL), flonicamid (FLON), the fourth generation member sulfoxaflor (SULF) and cycloxaprid (CCLX). Exemplary compound of NNDs is IMI, whose sales in 2008 were estimated to ca. 5,450 tones in 2010 (Pollack, 2011) and its production was estimated at ca. 20,000 tones [see (Simon-Delso *et al*., 2015) and references therein]. The above data indicate the significance of NNDs for plant protection; nevertheless they imply their ubiquitous presence in the environment.

NNDs are systemic insecticides with chemical structures based on nicotine moiety (Figure 1). Consequently, their mode of action is similar to that of nicotine. Research studies have shown that NNDs bind in several and sometimes different domains in the insect nicotinic acetylcholine receptors [for the description of these receptors see the chapter by Jones and Sattelle, 2010] that results in differentiation of their bioactivity (Matsuda *et al*., 2005; Tomizawa *et al*., 2007a; Tomizawa *et al*., 2007b). Briefly, NNDs target the nicotinic receptors and provoke excitation of the nerve cells, causing trembling and shaking and eventually paralysis. The latter can lead to the death of the insects, depending on dose and exposure duration.

Active substances of NNDs have been alleged as one of the factors that lead to the development of the honeybee colony collapse disorder (CCD) syndrome (Vanengelsdorp *et al*., 2009). NNDs in particular seem to interplay in CCD (Lu *et al*., 2014), however further research is needed to elucidate CCD causality, since combined stress that

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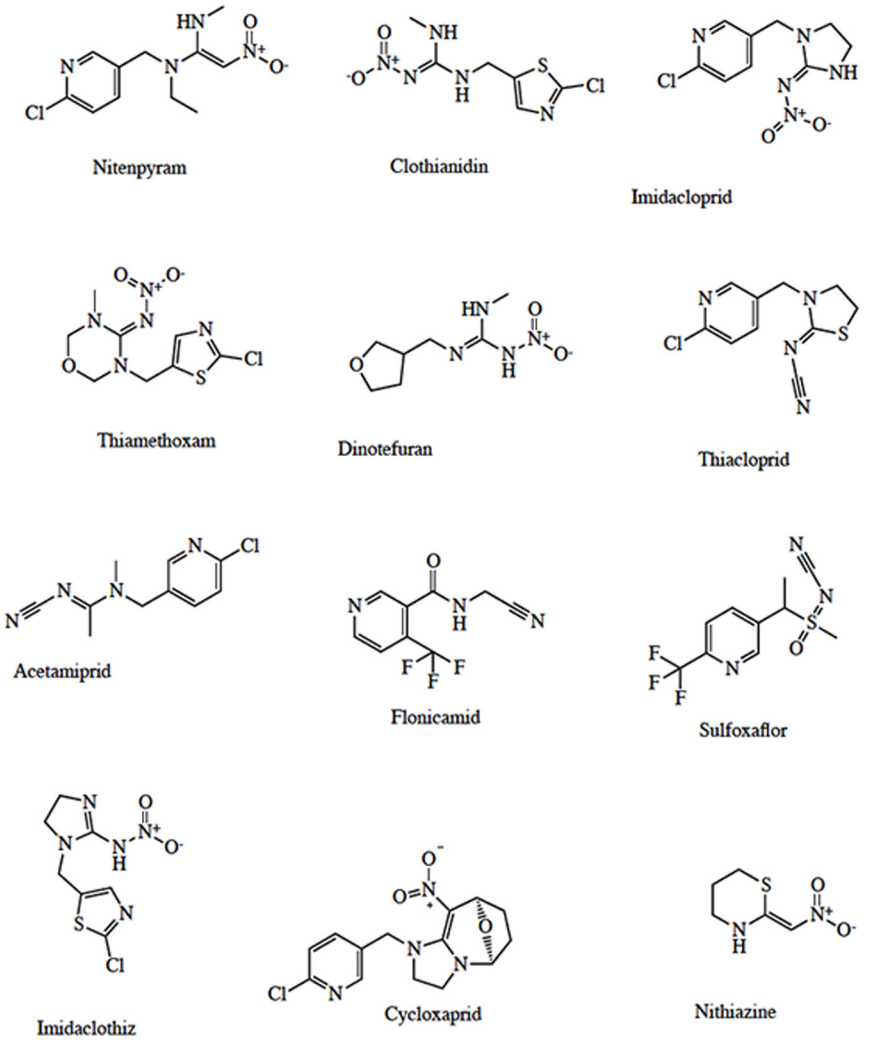

Figure 1. Chemical structures of Neonicotinoids

stems from parasites, pesticides and the poor flower diversity seems to be related to bee declines (Goulson *et al.,* 2015). Indicative symptoms provoked by sublethal doses of IMI in honeybees are the decrease in the hypopharyngeal gland size, and respiratory rhythm (Hatjina *et al*., 2013). Nonetheless, impairment of memory and brain metabolism has also been reported (Decourtye *et al*., 2003). In this context, NNDs' presence in human organisms is overshadowed by their impact especially on bees, and the relatively moderate to low toxicity that they exhibit with respect to other more hazardous for human health pesticides, such as organophosphates, carbamates and pyrethroids (Dawson *et al*., 2010).

Humans are exposed to numerous pollutants *via* their diet (Domingo *et al*., 2008; Lu *et al*., 2008; Marti-Cid *et al*., 2008b, a, c), the drinking water (Benotti *et al*., 2009), and the pollution of several environmental compart-

ments such as air (Dockery and Pope, 1994; Chen and Liao, 2006). Amongst the organic pollutants that impact the environment, pesticides possess a predominant role. Increasing number of works and modern applications are published in the domain of presence of pesticides in human biological fluids [see (Hernandez *et al*., 2005; Inoue *et al*., 2007; Jia *et al*., 2008)]. The latter are often encompassed in prospective cohort studies that try to elucidate diseases' causes and associate them with chemicals' exposure. Such studies have proved their efficacy to unveil important aspects of prenatal exposure (Bouchard *et al*., 2011; Engel *et al*., 2007).

Human biomonitoring is a leading field in bioanalysis, which covers all parts of the analysis of contaminants in biological fluids such as urine, blood, serum, saliva and body tissues. Amongst all matrices (invasive and non-invasive), blood and urine are the most frequently investigated. The collection of biomonitoring data regarding pesticides is of great interest since human exposure is portrayed, and association of concentration levels with potential toxicological effects is plausible.

Gagliardi and Pettigrove (2013) reported the improvement of aquatic ecosystem health after removal of intensive agriculture from an Australian region. Similarly, minimization of pesticides' use should be sought projecting in lowered residue levels found in human biological fluids and tissues and subsequently less impact on human health. Given this aspect, NNDs should be encompassed in monitoring schemes, and collection of pertinent works is of primary importance.

The present review summarizes all developments in the field of determination of NNDs and metabolites in human biological fluids. To our knowledge, all available works are included, and highlights of each one are discussed. In addition, case reports are presented that in some cases contain analytical approaches. In the same context, future prospects are provided with emphasis on the directions towards pertinent research endeavors should be focused.

Bioanalytical Methods

A biomonitoring study comprises a study population, data and biospecimen collection, sample preparation and purification, and finally chemical analysis. A fundamental prerequisite for a human biomonitoring study is to obtain information from the target population group regarding possible exposure to particular pollutants. By this way, the analytical methods become focused, and results more easily interpreted and related to possible health problems that might emerge. However, biological fluids are complex materials that contain macromolecules such as proteins, and other organic compounds that share common physicochemical parameters with the analytes of interest. Thus, the sample preparation step is also critical in providing pure samples, enriched in analytes considering that compounds of interest are usually found at low concentrations. Exceptions are the intoxication incidents in which levels are usually higher. One of the most common sample preparation techniques is liquid-liquid extraction (LLE) (Kataoka, 2003). It works through the extraction of analytes from the matrix using an organic solvent. Its traditional form has certain downsides that are observed in some occasions, such as the non-miscibility of solvents with the samples, and their difficulty in extracting polar and ionic compounds from aqueous media. Advances on LLE and several of its modified protocols have gained ground the last two decades and are extensively used in analysis of contaminants in various commodities (Bosch-Ojeda and Sanchez-Rojas, 2009; de Pinho *et al*., 2010). Another routine approach in the sample preparation is solid phase extraction (SPE). SPE has been broadly used in preparing the analysis of pesticides in biological fluids (Kataoka, 2003). It possesses certain advantages, such as high recovery, enrichment of analytes through pre-concentration, relatively short preparation time, and automation compatibility (Li, 2013; Li *et al*., 2013; Togola *et al*., 2014). Last but not least, protein precipitation is of the oldest ways of processing samples in bioanalysis. It

entails a denaturation stage that is accomplished by heating or the use of an organic solvent. After the solvent addition (usually acetonitrile (ACN) or methanol), the organic phase is separated from the protein by cyclomixing and centrifugation (Kole *et al*., 2011). Kole *et al*. (2011) have also reviewed recent advances in sample preparation in the bioanalysis domain including all available preparation steps prior to analysis.

A last but imperative parameter is the validation of the analytical methods. In the bioanalysis field, several validation guidelines and protocols (Bioanalytical-Validation; ICH, 2005) are adopted, which deal with particular validation parameters such as the limit of detection and quantification (LOD and LOQ), accuracy and precision. Matrix effects are also critical, considering the complexity of matrices, from which the analyst has to selectively extract the compounds of interest. A Belgian group reviewed successively these effects in bioanalytical methods, and proposed solutions to reduce or eliminate matrix interferences (Van Eeckhaut *et al*., 2009).

A critical feature that deserves attention is the *in vivo* metabolism of NNDs. It is acknowledged that the insertion of chemicals into humans' body is accompanied by several reactions that occur and usually breakdown the parent compounds to smaller molecules. Breakdown products should not be neglected, since it is well reported that some of these molecules exhibit significant toxicity in several organisms that can surpass those of parent molecules (Nauen *et al*., 2001; la Farre *et al*., 2008). Metabolism of NNDs is extensive, including several metabolic products produced by reactions such as reduction, demethylation, hydroxylation, and olefin formation. Hence, it is practically impossible to incorporate all metabolites in a targeted analytical method since many of them are not commercially available. In the group of NNDs, 6-chloronicotinic acid (6-CNA) is a common metabolite for IMI, NITEN, THIAC and ACET, considering that the latter share the chloropyridinyl moiety in their structure, and therefore it is widely included in analytical methods. CLOTH and THIAM contain the chlothiazole core and one of their key metabolites is 2-chloro-1,3-thiazole-5-carboxylic acid (2-CTCA). Finally, DINOT that contains the furanyl moiety is converted to 3-furoic acid (3-FA). Fundamental *in vivo* metabolites of NNDs are depicted in Figure 2.

Biomonitoring Studies

Of the first works published on NNDs and their metabolites was that of Uroz *et al*. (2001). The authors developed an analytical method for the monitoring of 6-CNA in human urine by gas chromatography tandem mass spectrometry (GC-MS/MS). Sample preparation consisted of acidification of urine and heating (for deconjugation to take place) and then the passage of the resulting mixture through an Amberlite XAD-4 cartridge. Amberlite XAD-4 resin, which is a polymeric sorbent with adsorbing potency for hydrophobic molecules, was selected after comparison with octadecyl carbon chain material (C18). Clean up was achieved with water of low pH and hexane. Then, 6-CNA was eluted with diethyl ether. Recoveries were optimum with acidic pH, although very low pH would decrease the resin's adsorption capacity. Though, 6-CNA is a molecule that is more compatible with liquid chromatography (LC), the authors choose gas chromatography. Hence, the dry sample was reconstituted in hexane and subjected to derivatization with a hexafluoroisopropanol (HFIP) using a carbodiimide as a coupling agent. After neutralization of the extract, the latter was injected into the GC-MS/MS system. Analysis time was short (6 min), monitoring the precursor ion of derivatized 6-CNA and several daughter ions. LOD of the method was determined to 16 pg/mL, which is the lowest reported in the literature for 6-CNA (LODs of NNDs and metabolites for selected works incorporated in this review are presented in Table 1). Finally, the application of the method to five urine samples of agricultural workers did not disclose the presence of 6-CNA.

Taira *et al*. (2011) reported 6-CNA pres-

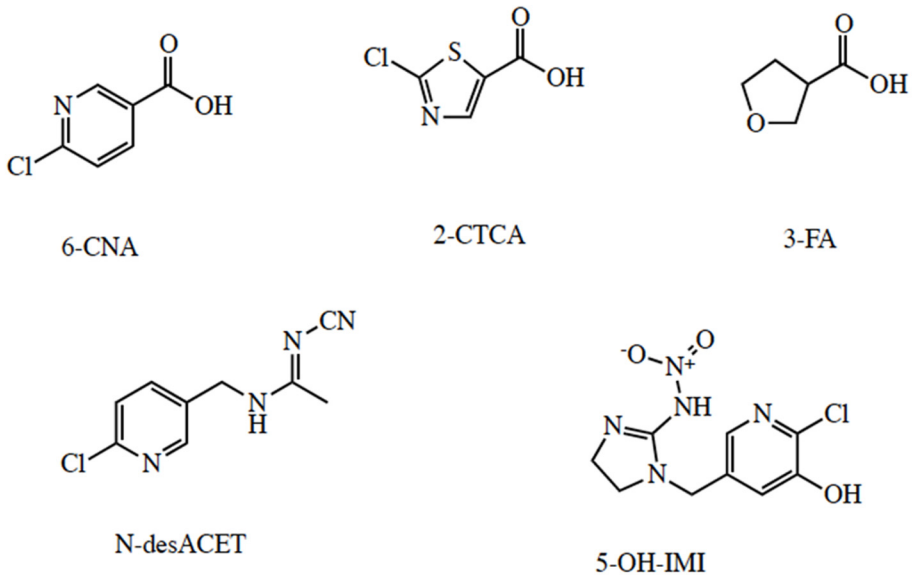

Figure 2. *In vivo* metabolites of Neonicotinoids.

Table 1. NNDs' and metabolites' LODs from selected works in human urine.

Analytes	LOD	Matrix	Detection	Reference
6-CNA	16 pg/mL	Urine	GC-MS/MS	(Uroz *et al.*, 2001)
6-CNA	400 ng/mL 2 ng/mL	Urine Urine	IC LC-MS (SIM)	(Taira *et al.*, 2011)
ACET N-desACET	0.068 ng/mL (LOQ) 0.55 ng/mL (LOQ)	Urine	LC-MS/MS	(Taira *et al.*, 2013)
6-CNA 2-CTCA 3-FA	0.1 ng/mL	Urine	GC-MS	(Nomura *et al.*, 2013)
ACET N-desACET IMI CLOTH DINOT FLON NITEN THIAC THIAM	0.2 ng/mL 0.2 ng/mL 0.2 ng/mL 0.5 ng/mL 0.2 ng/mL 1 ng/mL 1 ng/mL 0.1 ng/mL 0.2 ng/mL	Urine	LC-MS/MS	(Yamamuro *et al.*, 2014)

ence in the urine of six individuals that developed subacute nicotinic symptoms. Spot urine samples (spontaneous voided by the individuals) on the first visit and after were collected and subjected to ion chromatography (IC) analysis. IC was selected as a screen-

ing method since it exhibited acceptable recovery for 6-CNA. Confirmatory LC-MS was applied only to positive samples. Samples prior to chromatography were ultra-filtrated to remove proteins. LOD for the IC method was 0.4 mg/L_{urine}.

Regarding the LC-MS methodology, standard reversed phase separation was performed. Selected ion monitoring (SIM) was used to quantify 6-CNA, using the $[M+1]^+$ as quantitation ion. The LOD of the method was 2 μg/L_{urine}. Maximum 6-CNA concentrations ranged from 7.5 to 84.8 μg/L_{urine}. The origin of 6-CNA was attributed to the excessive intake of tea beverages and conventionally grown fruits. Nevertheless, the authors did not present an analytical study on NNDs' and metabolites' detection in food commodities, so as to strengthen the mentioned statement.

The same group two years later investigated NNDs metabolites in human urine of 3 patients suspected of subacute exposure to NNDs (Taira *et al*., 2013). This work came to fill gaps of the previous work, such as the non-inclusion of other metabolites of key members of NNDs family (showing selected metabolites of IMI, ACET, and CLOTH). To proceed to this study, a qualitative step was adapted aiming to unveil possible metabolic products. The latter was accomplished by liquid chromatography time-of-flight mass spectrometry (LC-TOF/MS). TOF/MS is known for its inherent advantages, which are: sensitivity, high mass range, and high-speed analysis. TOF/MS functioned using a database of nominal molecular weights of 57 known metabolites of the 3 NNDs of the study. For ACET, the dominant metabolite was *N*-desmethyl-acetamiprid (N-desACET) that until this work was reported only in rats' biological fluids. Subsequently, quantitation of TOF/MS identified compounds was performed with LC-MS/MS. Human urine samples were solid-phase extracted, and 10-folds concentrated. After loading urine samples in preconditioned cartridges, a wash step with H_2O was applied, and analytes were extracted with ACN. Acidic and basic extraction was also used utilizing 1.25 μL of formic acid and 10 μL of an ammonium hydroxide solution, respectively. Data analysis was based on data mining approach, which is a computerized procedure used to unveil patterns in large data sets. An almost 50% of nominated compounds were detected in positive controls by this screening methodology. Acidic SPE conditions exhibited the highest retention with only two undetectable substances. The qualitative TOF/MS analysis of human urine confirmed the presence of 8 metabolites, including N-desACET and 5-OH-IMI. The highlight of this work was the first report on N-desACET detection and quantitation in human urine at 3.2 ng/mL (one sample) that indicated human exposure to ACET. Taira has also reviewed the suspected health effects as a consequence of NNDs exposure in Japan, focusing on inhalation and oral exposure (Taira, 2014).

Nomura *et al.* (2013) published work on the detection and quantitation of NNDs' metabolites in human urine using GC-MS. Metabolites studied were 6-CNA, 2-CTCA and 3-FA. The method was validated after optimizing particular parameters. More specifically, a hydrolysis step was applied in order deconjugation to take place. Deconjugation was tested under acidic and basic conditions. It was found that addition of 50 μL of sulfuric acid was the optimum condition for deconjugation of the 3 NNDs. Then SPE was applied, by eluting compounds from preconditioned cartridge with methanol after column washing with 0.5 mL of 2% formic acid. SPE was performed on polymeric strong cation exchange column, also characterized by its non-polar retention mechanism. Methanol was chosen instead of ACN due to the observed minimization of interferences in the chromatogram. Division of the eluate was performed in order to proceed in separate analysis for 2-CTCA, and 3-FA and 6-CNA respectively. Derivatization of the analytes with trimethylsilyl group (a typical group used for such reactions) using BSTFA-TMCS proceeded smoothly for all target compounds. For the assessment of recovery, spiking was conducted at two different stages: initially at the beginning of

the extraction and then before derivatization. The calibration curve was constructed in the range of 0.6 to 10 μg/L, using pooled urine, exhibiting correlation coefficients for all analytes above 0.99. In the same context, within-run precision was determined at five concentration levels, with acceptable % relative standard deviation (RSD) values. Between-run precision was assessed similarly at 0.6 and 5 μg/L for five consecutive days, with RSD% below 13%. LOD and LOQ were calculated using the signal to noise (S/N) ratio of 3 and 10 in respect and were 0.1 and 0.3 μg/L, correspondingly. Application of the method to real samples unveiled a high frequency of detection for 3-FA, which is attributed to the frequent use of DINOT in Japan. Even though the presence of CLOTH is unambiguous in agricultural commodities in Japan, its metabolite 2-CTCA displayed low detection rate. The latter, as the authors state is unclear. Thus, it can be a challenge for future endeavors. Overall mean concentrations were 1.8 and 2.6 μg/L for 6-CNA and 3-FA, respectively. 2-CTCA was detected only in one farmer at 0.1 μg/L.

One year later, and as a compendium of their previous work, Ueyama *et al.* (2014) dealt with urinary NNDs metabolites. The main principal of this work was to focus solely on the urinary metabolites, overcoming overestimation of concentrations resulting from dietary intake. Consequently, they developed a straightforward method for simultaneous determination of urinary NNDs using LC-MS/MS. Sample preparation began with acidification of urine sample and addition of internal standard (IS). Then, the urine sample was incubated and applied to SPE. After conditioning, and a washing step, the majority of analytes were eluted with MeOH. NITEN and the IS were finally eluted with MeOH:ACN that contained 5% of ammonia (NH_3) solution. The use of NH_3 aided the elution of NITEN that exhibits high ionic binding to the SPE material. LODs varied from 0.01 to 0.12 μg/L. The concentration results showed that the Japanese population was exposed to NNDs. In particular, the detection frequencies were higher than 50% for all analytes, excepting NITEN. Overall, the authors pointed out two limitations. The first regarded the use of only one IS, and the second the difficulty to identify NNDs peak near LOD.

Yamamuro *et al.* (2014) developed a novel analytical method, for detecting NNDs in serum and urine. Until then most works on NNDs and metabolites were concentrated either in environmental or food samples that might contain NNDs or in biological fluids but with limited number of analytes. Therefore, this work came to fill this gap since it dealt with almost all NNDs. In addition, the authors included three ACET metabolites. The sample preparation step was simple. A low volume of sample was diluted with water and then purified, through a cartridge containing diatomaceous earth. This step although it seems as an SPE step, it works via LLE that occurs among the eluate (chloroform: isopropanol, 3:1), and a gel formed on the diatomaceous earth surface. Acceptable analytical performance was obtained only when elution was repeated with ten portions of low volumes (3 mL each) of the mentioned solvent mixture. The optimum mobile phase was a pH 3-buffered methanol, which provided substantial sensitivity, except FLON. Linearity of the calibration curve for each analyte was studied over a range of concentrations, starting from the LOQ up to 1 μg/mL. All correlation coefficient values were above 0.99, thus acceptable. Extensive validation of the method proved its efficacy and robustness. Sensitivity was substantial as depicted by the respective LOD values (serum 0.1-0.2 ng/mL, urine 0.1-1 ng/mL). It is foreseen that this approach can become a useful vehicle in forensic laboratories, which investigate human poisoning incidents with NNDs.

Jamin *et al.* (2014) published a cutting-edge work, in an untargeted profiling of pesticide metabolites in urine from pregnant women from a French epidemiological cohort. To carry out such profiling, the authors generated a pesticide metabolite list based on the likelihood of pesticide use in the study area. Analysis was accomplished

by liquid chromatography high-resolution mass spectrometry (LC-HRMS) using an Obritrap system. HRMS has already proved its effectiveness in the drug discovery domain (Ramanathan *et al*., 2011), in metabolomics studies (Xiao *et al*., 2012) and recently was reported in human exposure evaluation. This approach made effective the investigation of molecules on the basis of the theoretical mass of their quasi-molecular ions. Nineteen metabolites of IMI were screened. Nevertheless, no residues were detected.

Case Reports

Case reports usually refer to intentional (suicide attempts) ingestion of an amount of a pesticide formulation, a severe problem, especially in developing countries (Gunnell and Eddleston, 2003). Increased risk of suicide with exposure to pesticides has been reported particularly in intensive agricultural regions (Parron *et al*., 1996). The latter is becoming critical considering the risk projected to young people in these areas that have relative easy access to pesticides formulations (Kong and Zhang, 2010). Several case reports are published where NNDs are implicated. Typical symptoms-manifestations that develop in humans after such exposure to NNDs are disorientation, drowsiness, dizziness, cough, vomiting and abdominal pain. In the case of non-fatal incidents, after an initial treatment in the hospital (nasogastric lavage, instillation of activated charcoal), the patients are treated symptomatically and supportive, and finally discharged.

Wu *et al.* (2001) have presented a case report of acute poisoning with IMI formulation. More specifically a 64-year-old farmer was attempted to suicide using a bottle of insecticide containing IMI in *N*-methyl pyrrolidone (NMP), and a low percent of surfactant. The researchers concluded that it was rather difficult to determine whether the symptoms of drowsiness and dizziness, were provoked by IMI. However, the relative high concentration of the solvent (NMP) seemed to play a decisive role in intoxication. Proenca *et al.* (2005) published work on the fatal poisoning with IMI. To assess exposure in post-mortem samples the authors developed an LC-DAD-ESI/MS method that was capable to detect IMI and two of its metabolites (6-CNA and 5-OH-IMI). Samples of blood, urine and tissues were collected for toxicological analysis. Sample preparation was based on LLE with dichloromethane as organic solvent. Analysis was conducted by concomitant use of diode array (DAD) and mass spectrometer detector. IMI was detected in all post-mortem samples, but none of its two metabolites was detected. All specimens prior to targeted IMI and metabolites analysis were subjected to screening of other substances as well. None drug or pesticide was found in the samples. From analytical standpoint, the method was sensitive, exhibiting an LOD of 0.002 μg/mL$_{blood}$ and LOQ of 0.01 μg/mL$_{blood}$. Respective limits in urine were not reported. IMI levels varied from 0.29 μg/mL (in urine) to 2.05 μg/mL in blood. Conclusively, this study demonstrated how analytical methodologies could assist the resolving of cases that under routine examinations is difficult to understand the causative agents.

David *et al.* (2007) reported an incident regarding IMI poisoning. More specifically, a 22-year-old male with clinical toxicity was hospitalized after ingestion of 30 mL of IMI (17.8% concentration). Symptoms were as those above mentioned, and the patient was released on the 5th hospital day. Mohamed *et al.* (2009) published incidents from Sri Lanka, where IMI was involved after intentional self-poisoning (Mohamed *et al*., 2009). The latter was an outcome of a prospective observational cohort study of all poisoning presentations that was established during 2002 and lasted until 2007. More precisely, blood samples were taken, whenever possible so as to determine IMI levels (none metabolite were included). Plasma was isolated; SPE extracted, and the extract was subjected to LC-MS/MS analysis. Over this period, 68 patients were presented with a history of IMI exposure. Seven cases were occupational dermal exposure and not worrying, five involved co-ingestion of IMI with another

active substance and 56 were acute IMI self-poisoning. IMI residues were detected in 28 patients with a median admission plasma concentration of 10.6 ng/L.

Kumar *et al.* (2013) reported an accidental human poisoning with IMI, from rural India. The case report regarded a 60-year-old farmer that was exposed through inhalation. After hospitalization, the man was released. Data regarding IMI concentration in fluids was not provided, possibly due to the route of exposure. The same year, Lin *et al.* (2013) published a paper regarding a case report from Taiwan. The latter considered a suicide attempt by ingesting an IMI formulation. The authors, however, did not refer to IMI concentrations in biological fluids. An overview of cases reported until 2013 was also presented, including clinical details that are useful in incidents that end up to hospitalization and subsequent treatment of patients.

Fuke *et al.* (2014) reported the detection of IMI in biological fluids in a case of fatal intoxication. The authors developed an HPLC-DAD method monitoring IMI and 6-CNA. Specimens studied were whole blood, cerebrospinal fluid, humor, and urine. Sample preparation consisted of initial vortex mixing of a low volume of the liquid sample and concomitant extraction with ACN. Evaporation and two centrifugation steps provided the organic phase that was injected to the HPLC system. Prior to HPLC, a screening with GC-MS verified the presence of IMI, however, the poor chromatographic performance, favored HPLC analysis. Validation was performed after spiking drug-free blank blood. All validation characteristics were acceptable, with recoveries for IMI ranging from 86 to 105% for all fluids. 6-CNA was not detected in the samples analyzed. IMI in the femoral blood reached a maximum concentration of 105 μg/mL. Regarding cerebrospinal fluid its concentration was approximately half the one determined in the femoral blood. In this fatal case, since no other cause of death was evidenced, IMI intoxication was the cause of death. Same year Yeter and Aydn reported on the determination of ACET and one of its metabolites after fatal intoxications (Yeter and Aydin, 2014). Both biological fluids (postmortem blood and urine) and tissues were processed. Chromatographic analysis revealed ACET at 2.7 μg/mL in blood while its metabolite was not evidenced. None of the compounds was detected in urine samples.

Yeh *et al.* (2010) reported the acute multiple organ failure with IMI and alcohol ingestion. Specifically a 67-year-old man was transferred to the emergency in Taiwan hospital, after ingestion of an unknown amount of an insecticide containing IMI mixed with liquor. The incident led to arrhythmia and multiple organ failure within hours of intake. This incident argued the belief of the low mammalian toxicity of IMI and added a point to the increasing evidence that IMI can provoke kidney damage and other organ damages. Same year Iyyadurai *et al.* (2010) reported a fatal incident regarding IMI, after a suicidal attempt. The authors although stated (an often shortcoming also observed in other studies) that no data regarding serum IMI level were available.

Forrester (2014) provided an overall picture of NNDs exposure that occurred in Texas, USA, from 2000 to 2012. Of 1,142 exposures the 77% contained IMI and in less extent DINOT (17%). Both substances were detected along with other active substances as well. A seasonal trend favored mid-spring to mid-summer exposure reaching 50%. Almost all NNDs were detected including NITEN, ACET, THIAM, and CLOTH. The most common routes of exposure were ingestion, accounting for a 51%, dermal (44%) and ocular (11%).

Greek IMI biomonitoring

Our group the last decade is involved in biomonitoring studies in which pesticides are the target analytes (Kasiotis *et al.*, 2008; Kasiotis *et al.* 2011; Kasiotis *et al.*, 2012). In the frames of the ECOPEST project (ECOPEST) a biomonitoring study was conducted (blood and urine) that included 27 farmers. These farmers among several field applications, they applied seed treatment with IMI in cot-

ton crop fields. IMI and 6-CNA were incorporated in the developed analytical method that included a total of ten analytes (publication under preparation). The analyses showed the presence of IMI at levels ranging from 9.7-20.1 ng/mL$_{urine}$, and 6-CNA from 5.1 to 9.4 ng/mL$_{urine}$. Only one serum sample was positive with 6-CNA.

Conclusions

Human biomonitoring constitutes an indispensable tool in public health surveillance since it entails the prevalence of diverse pollutants in human organisms. It serves also as a valuable source of information in integrated health impact assessment. NNDs comprise a major pesticide category that deserves attention mostly due to their frequent use and broad spectrum of applications. Although, their human toxicity is moderate to low, monitoring schemes should encompass them. The latter are important in multiple exposure assessment that considers both cumulative toxicity and synergistic effects. Several reported analytical studies, verified the presence of NNDs and their metabolites. In this context, modern analytical tools, such as HRMS, is the trend that biomonitoring laboratories should pursue and encompass in their analytical schemes. Last but not least, the elucidation of undiscovered metabolic products should be a constant goal that is firmly connected to the diversity of pathways and reactions that organic substances undergo after their insertion in humans' body.

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ΑΡΘΡΟ ΑΝΑΣΚΟΠΗΣΗΣ

Ανασκόπηση της βιοπαρακολούθησης στον άνθρωπο, των νεονικοτινοειδών και των μεταβολιτών τους

Κ.Μ. Κασιώτης και Κ. Μαχαίρα

Περίληψη Τα νεονικοτινοειδή αποτελούν μια σημαντική κατηγορία εντομοκτόνων με ένα ευρύ φάσμα εφαρμογών στον τομέα της γεωργίας. Ως εκ τούτου, τα υπολείμματά τους βρίσκονται στο περιβάλλον και μπορούν να μεταφερθούν μέσω διαφόρων οδών σε πολλούς οργανισμούς. Παρά τις σοβαρές επιπτώσεις τους στις μέλισσες και τις άγριες μέλισσες (απώλεια μνήμης, επιπτώσεις στο ανοσοποιητικό σύστημα), η παρουσία τους στον άνθρωπο είναι λιγότερο περιγεγραμμένη, πιθανώς λόγω των χαμηλών έως μετρίων τοξικολογικών επιδράσεων που προκαλούν. Σκοπός της παρούσας ανασκόπησης είναι να δοθεί έμφαση στις εξελίξεις στη βιοπαρακολούθηση των νεονικοτινοειδών στον άνθρωπο. Επικεντρώνεται κυρίως στη χρωματογραφική ανάλυση των μητρικών μορίων και των μεταβολιτών τους σε ανθρώπινα βιολογικά υγρά, σχολιάζοντας βασικά χαρακτηριστικά των μεθόδων, όπως η προετοιμασία του δείγματος και η επικύρωση της αναλυτικής μεθόδου. Επιπλέον, παρουσιάζονται οι αναφορές σε περιστατικά δηλητηρίασης από νεονικοτινοειδή, τονίζοντας τη σημασία τους ιδίως στον αναπτυσσόμενο κόσμο.

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Pathogenicity of indigenous strains of three entomopathogenic fungi to the sisal weevil, *Scyphophorus acupunctatus* (Gyllenhal) (Coleoptera: Curculionidae)

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Summary The pathogenicity of indigenous isolates of *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* was evaluated in the laboratory against larvae and adults of the sisal weevil, *Scyphophorus acupunctatus*. Inoculation was achieved via immersion of individuals into conidia suspensions of different concentrations. All three fungal species proved high pathogenicity against larvae of the weevil, causing 100% mortality in most of the treatments. *Beauveria bassiana* caused the highest mortality of the adults (86.67±12%), followed by *M. anisopliae* (46.67±17.8%) and *I. fumosorosea* (40±17.5%). Mean survival time also differed significantly among treatments and life stages of the weevil. In total, larvae survived significantly fewer days than adults post infection. Results of the present study indicate the potential of indigenous strains of entomopathogenic fungi as biological control agents against the invasive weevil.

Additional keywords: agave, *Beauveria bassiana*, Curculionidae, *Isaria fumosorosea, Metarhizium anisopliae*

Introduction

The sisal weevil, *Scyphophorus acupunctatus* (Gyllenhal), is amongst the most severe pests of both cultivated and ornamental agave plants (Pott, 1975; Valenzuela-Zapata, 1994; Camino Lavin *et al.,* 2002). In its place of origin, the Nearctic region, it has been found to cause severe economic losses by strongly damaging *Agave tequilana* (Weber) the tequila producing agave (Solis *et al.,* 2001). The sisal weevil was introduced in Europe around 1980, when it was first recorded on imported *Yucca* sp. in the Netherlands (van Rossem *et al.,* 1981). Since then it has been found in many European countries (i.e. Italy, France, Greece) on ornamental plants (Colombo, 2000; EPPO 2008; Kontodimas and Kallinikou, 2010).

The females use the basal parts of the agave leaves for feeding and as oviposition sites. Furthermore, once the neonate larvae hatch they start to bore galleries within the plant's head and form a cocoon made by plant's fibers to complete their development (Lock, 1965). In addition to the destructive activities of the larvae, the adults are often natural vectors of plant pathogenic bacteria, such as *Erwinia carotovora* (Dye) (Aquino Bolanos *et al.,* 2011). In Central and South American agave cultivations chemical insecticides are commonly applied to control the sisal weevil, however there are many problems and controversies connected to their use (Solis *et al.,* 2001; Terán-Vargas *et al,* 2012). Firstly, as the weevil completes its development within a protected habitat, inside the agave leaves, insecticides have a low effectiveness (Figueroa-Castro *et al.*, 2013). Secondly, their extended application harbors the risk of resistance development in the pest species (Terán -Vargas *et al.,* 2012). Finally, there are certain restrictions in the use of synthetic insecticides in urban landscape areas, where the weevil is mainly found, due to their effects on the environment and health risks. Therefore, the development of biological and biotechnical control methods is of great environmental and economic importance.

Although a few arthropod natural enemies of the sisal weevil could potentially be regarded as biological control agents in its

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site of origin (Velazquez *et al.,* 2008), these arthropods are not found in the invaded regions. Entomopathogenic fungi and nematodes have been reported as potential biological control agents of the weevil (Hueso *et al*., 2005; Velazquez *et al.,* 2008). Both the fungi and the nematodes are of great importance for the biological control of *S. acupunctatus*, as they are able to overcome the barrier of the concealed environment in which the weevil develops. While the nematodes are able to move within the agave leaves and are easily able to reach the concealed pest (Dembilio *et al.,* 2010a), the entomopathogenic fungi infect their hosts by direct contact and subsequent penetration of their cuticle (Dembilio *et al.,* 2010b). This makes insects susceptible both to direct contact with the pathogen, but also to passive, horizontal transmission, between healthy and infected individuals vectoring spores (Klein and Lacey, 1999; Quesada-Moraga *et al*., 2004).

It has been widely accepted that entomopathogenic fungi show high intra-specific diversity, which results in different levels of pathogenicity among different strains of the same species (Ferron, 1978). Furthermore, the virulence of a specific strain to specific insect genera has been proved to depend upon the host insect identity from which it was isolated (Fargues and Robert, 1983; Maurer *et al*., 1997). This is attributed to the strong selective pressure of the host insect environment on the pathogen (Roy *et al.,* 2006) through which the origin of the entomopathogens could affect their pathogenicity against pests (Cherry *et al.,* 2005).

Despite the importance of alternative methods for the control of the sisal weevil, research on the evaluation of potential biological control agents of the pest is very limited (Molina-Ochoa *et al.,* 2004; Valdes Estrada *et al.,* 2014). In the present study we aim to evaluate the virulence of locally isolated strains of *Beauveria bassiana* (Balsamo), *Metarhizium anisopliae* (Metchnikoff) Sorokin and *Isaria fumosorosea* (Wize) to adults and larvae of the introduced weevil *S. acupunctatus*. It is expected that the use of indigenous isolates of fungal entomopathogens is advantageous, as they are already adapted in specific environmental conditions and could be part of a conservation biological control strategy.

Material and Methods

Entomopathogenic fungi

All entomopathogenic fungi strains used in the laboratory assays were obtained from the entomopathogenic fungi collection of the Benaki Phytopathological Institute. The strain of *B. bassiana* IBB 010 was initially isolated from a *Rhynchophorus ferrugineus* (Olivier) cadaver, found in Ellinikon region (Athens, Greece). Both strains IBB020 of *I. fumosorosea* and TMB04 of *M. anisopliae* were isolated from soil samples with the *Galleria* bait method (Zimmerman, 1986) from the Agios Stefanos and Marathon regions (Attica, Greece), respectively. The molecular identification of the strains of *I. fumosorosea* and *M. anisopliae* was previously conducted by Beris *et al.* (2013). The fungal isolations were cultured on Sabouraud dextrose agar (SDA) at 25°C, under dark regulated conditions. Spore (conidia) suspensions were prepared by scraping the surface of 2 week old cultures into an aqueous solution of 0.2% Tween 80, after which the suspensions were vortexed for 60 s and filtered twice using a sterile nylon membrane to remove mycelium. Subsequently, the concentration of the spore suspensions was determined by the use of a standard haemocytometer. The concentrations of spore suspensions used in bioassays were 2.12×10^7 and 2.12×10^6 spores/ml for *B. bassiana*, 1.27×10^7 and 1.27×10^6 spores/ml for *M. anisopliae* and 7.45×10^6 spores/ml for *I. fumosorosea*. Spore concentrations are not equal due to the different developmental rate of each fungus (Kontodimas and Gkotsi, 2009). Spore viability measured over 96% for all selected strains.

Insects

Both adult and larvae of the sisal weevil were collected from ornamental aga-

ve plants, *Agave americana* L., in the urban landscape area of Ardittos hill (Athens, Greece). The infestation of the agave plants was spotted in the summer of 2012 and no action for controlling the pest was taken prior to the collection of the weevils. The adults were collected by hand at site, while whole plants were cut from the basis and taken to the laboratory for the isolation of 4th instar larvae. The adults were kept in polyester cages (30 x 30 x 30cm) and the larvae in plastic boxes (20 x 20 x 20cm), with an opening covered by mesh for ventilation. All insects were kept at controlled laboratory conditions (25±1°C, 65±5% R.H. and 14:10 L:D) and fed on a natural diet (agave slices; twice per week) until they were used in the bioassays.

Bioassays

The application of the spore suspensions was achieved through direct cuticle contact. Groups of five, 4th instar larvae and adults were immersed in the prepared aqueous suspensions for 30 sec after which each group of insects was transferred in 1L rearing boxes and provided with fresh natural diet. The treatment was repeated 15 times (replicates) for each fungus strain and concentration per life stage. Furthermore, a control treatment was included for both adults and larvae, where insects were immersed into an aqueous solution of 0.2% Tween 80. All bioassays were carried out in controlled environment conditions (25±1°C, 65±5% R.H. and 14:10 L:D). The mortality was recorded daily for up to 11 and 21 days for larvae and adults, respectively. When a weevil died, the cadaver was transferred individually to a Petri dish lined with a moistened sterile filter paper to ascertain the involvement of the entomopathogens to the death of weevils. The Petri dishes were sealed with parafilm and kept at room temperature, in darkness, to monitor for external signs of fungal infection.

Statistical analyses

General Linear Model (GLM) with a binomial distribution was used to obtain Analysis of Deviance for a level of significance α=0.05. The analysis included life stage (larval and adult), species of fungi (*B. bassiana*, *I. fumosorosea* and *M. anisopliae*), and concentration of spore suspensions (10^6 and 10^7 spores/ml) as variables. Additionally, the average survival times were calculated and compared by the Kaplan-Meier survival analysis (Kaplan and Meier, 1958). All the GLM procedures and survival analysis were carried out in R (v. 2.1; R Foundation for Statistical Computing, Vienna, AT).

Results

The examined strains of entomopathogenic fungi caused different levels of mortality to *S. acupunctatus*. The mean mortality of weevils differed significantly at different life stages, and fungus species, but it did not differ between different concentrations of spore suspensions (X^2=22.939, *df*=1, *P*<0.0001; X^2=9.5758, *df*=2, *P*<0.0001 and X^2=2.5984, *df*=1, *P*=0.1070, respectively). No mortality was recorded for adult weevils or larvae when treated with the aqueous solution of 0.2% Tween 80 (i.e. control treatment). Interactions among the variables did not affect the mortality levels, thus, they were omitted from the initial model.

All treatments with fungi resulted in almost all concentrations at 100% mortality of larvae (Figure 1a). Mortality in adults depended on fungus species but not on the concentration of the suspensions (X^2=11.4223, *df*=2, *P*=0.0033 and X^2=2.9638, *df*=1, *P*=0.0851, respectively). The treatment with *B. bassiana* at the concentration of 2.12 x 10^7 spores/ml caused the highest mortality to adult weevils (86.67±12%) compared to *M. anisopliae* and *I. fumosorosea* treatments (Figure 1b). The lowest mortality levels to adults were caused by *M. anisopliae*, when applied at the low spore concentration (26.67±15.8%).

The examined strains of *B. bassiana, M. anisopliae* and *I. fumosorosea* developed external fungal growth and sporulated on all treated cadavers of both adults (Figure 2) and larvae, regardless the spore concentration.

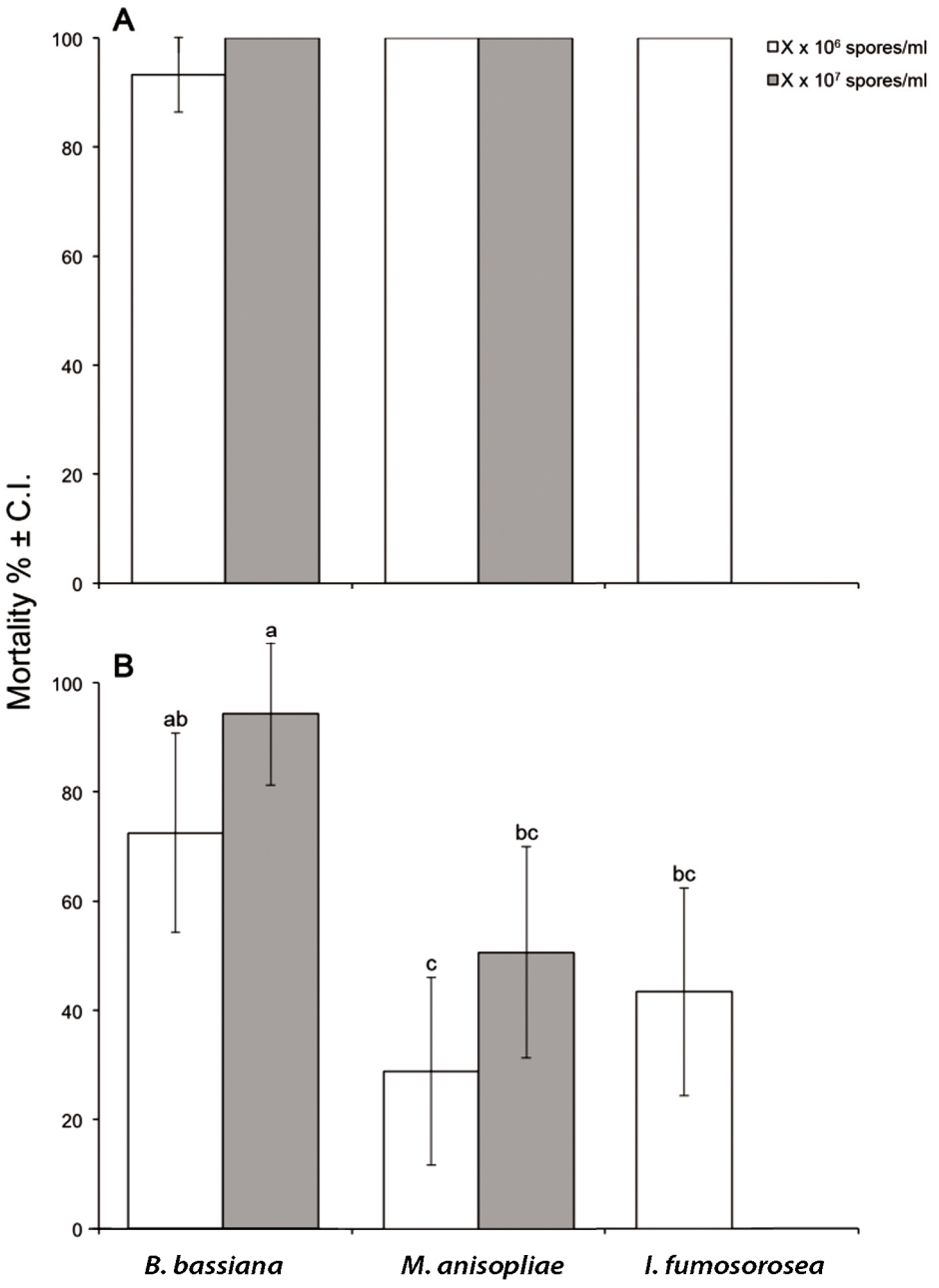

Figure 1. Mean mortality ± SE of (A) larvae and (B) adults of *Scyphophorus acupunctatus* after immersion into spore suspensions of entomopathogenic fungi.
*Different letters within the same chart indicate significant differences (LR test, α=0.05).

Figure 2. Development of external fungal mycelium on cadavers of *Scyphophorus acupunctatus* adults: *Beauveria bassiana* (a), *Metarhizium anisopliae* (b) and *Isaria fumosorosea* (c).

The mean survival time of the treated larvae differed significantly at different fungi species and different concentrations, namely the low concentration of *B. bassiana* (6.80±0.251 days) compared to the other treatments (4.73-5.67 days) (X^2=8.3, *df*=4, *P*=0.0807; Table 1).

The mean survival time differed significantly for adult weevils that received different treatments (X^2=17.5, *df*=4, *P*=0.0016; Table 1). Adult weevils treated with the high concentration of *B. bassiana* exhibited the shortest survival time (10.20±1.055 days) (Table 1). Overall, the survival time of larvae was significantly shorter than that of adults (X^2=105, *df*=1, *P*<0.001).

Discussion

All species of fungi proved to be highly effective on larvae of *S. acupunctatus*. Further wider dose range tests would be necessary to show the dose effect, the differences among strains and the potential of the tested strains in lower concentrations. However, our results showed that the examined strains of entomopathogenic fungi are able to infect larvae and adults of the sisal weevil. Although all strains managed to fully complete their life cycle by sporulating on *S. acupunctatus* cadavers, *B. bassiana* proved to be the most pathogenetic to adult weevils. Several studies have pointed out the relation between strains of entomopathogens isolated from specific hosts and the high virulence towards the host genera (Fargues and Robert, 1983; Maurer *et al.,* 1997). Therefore, the high virulence of the strain IBB010 of *B. bassiana* might be related to its source of isolation, an infected red palm weevil cadaver, as both the red palm weevil and the sisal weevil belong to the same tribe of Rhynchophorini.

Until now, *B. bassiana* is the only entomopathogenic fungus species mentioned as a natural enemy of the weevil in its place of origin (Aquino Bolanos *et al.,* 2011). Our results showed that the concentration of 2.12 x 10^7 spore suspension of *B. bassiana* caused a quicker death of adults compared to spore suspensions of *M. anisopliae* and *I. fumosorosea*. Moreover, a 10-fold increase of the diluted spore suspension of the *B. bassiana* strain from 2.12 x 10^6 to 2.12 x 10^7 decreased the mean survival time for adults by almost 4 days .

The sisal weevil has identical life hab-

Table 1. Mean survival time ± SE (days after treatment) for larvae and adults of *Scyphophorus acupunctatus* treated with *Beauveria bassiana*, *Metarhizium anisopliae* and *Isaria fumosorosea* spore suspensions.

Treatment	Concentration (spores/ml)	Mean survival time ± SE (days)	
		Larvae	Adults
B. bassiana	2.12×10^7	5.47 ± 0.351^a	10.20 ± 1.055^a
	2.12×10^6	6.80 ± 0.251^b	14.74 ± 0.999^b
M. anisopliae	1.27×10^7	5.00 ± 0.359^a	15.65 ± 1.031^{bc}
	1.27×10^6	5.67 ± 0.308^{ab}	17.20 ± 1.210^c
I. fumosorosea	7.45×10^6	4.73 ± 0.314^a	17.50 ± 1.025^c

* Mean survival times within columns followed by the same letter are not significantly different (LR test, α=0.05)
* Mortality was recorded for up to 11 and 21 days for larvae and adults, respectively

its with the red palm weevil and its development is completed within its host plant, adults are the only exposed stage to entomopathogenic fungal treatments (Gindin *et al.,* 2006; Dembilio *et al.,* 2010b). Therefore, the longer survival of an infected host increases the possibility of interacting with other individuals and probably of transmitting the pathogen. In this sense, a lower spore concentration of *B.bassiana* might be more advantageous in a field application, especially because our study showed that the mean mortality did not differ significantly between the two concentrations. However, other aspects of the application of these entomopathogenic fungi are not yet fully studied. For example, potential horizontal transmission between adults or from adults to larvae and vertical transmission from infected adults to their offspring should be elucidated (Glare *et al.,* 2002; Gindin *et al*., 2006; Dembilio *et al.,* 2010b). Furthermore, *M.anisopliae* and *B. bassiana* have been found to reduce the fecundity of *R. ferrugineus* (Gindin *et al*., 2006; Dembilio *et al.,* 2010b). If a similar effect can be proved on *S. acupunctatus*, then even strains that caused lower levels of mortality might be a useful tool in the control of the weevil.

Another important finding of the present study is the high mortality of larvae caused by all the strains tested. In most of the cases, mortality in the larval stage reached 100%. Although our methodology of immersing the larvae differs considerably from the natural infection, such high mortality levels in controlled conditions support these strains as candidate biological control agents. While the larvae of the weevil are the most destructive stage they are also the most concealed life stage (Camino Lavin *et al.,* 2002; Lopez-Martinez *et al*., 2011), challenging the application of the entomopathogenic fungi. It is, however, possible that entomopathogenic fungal endophytes are effective against larval stages of the weevil. Among many others, the entomopathogenic fungi *B. bassiana*, *M. anisopliae* and *I. fumosorosea* have been reported as endophytes of several plants (Gomez-Vidal *et al., 2006;* Quesada-Moraga *et al*., 2006; Vega, 2008) and attempts have been made to introduce entomopathogens into host plants for the control of pests (Gomez-Vidal *et al.,* 2006, Posada *et al.,* 2007; Valdes Estrada *et al*., 2014). If the strains studied here could be introduced into agave plants they could possibly act as successful biocontrol agents due to their high pathogenicity against larvae.

In conclusion, our results prove that the strain IBB 010 of *B. bassiana* is highly pathogenic to both adults and larvae of *S. acupunctatus* and could be considered as a biological control agent of the weevil. Additionally, application of lower concentrations of IBB 010 was shown to prolong the adults' survival time, without reducing the mortality levels. This should be taken into consideration, as a positive effect for strategies

based on attracting and infecting adults. Strains IBB020 and TMB04 of *I. fumosorosea* and *M. anisopliae,* respectively, caused lower mortality levels to adults, but were equally effective on larvae. Hence, they could be considered as potential control agents into an IPM plan against the pest.

Despite the lack of field data on the effectiveness of these strains, to the best of our knowledge, this is the first attempt of screening locally isolated entomopathogenic fungi as biological control agents against the introduced *S. acupuntatus*. Further research in field applications and possibility of transmission is needed in order to strengthen current findings.

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Παθογένεια ιθαγενών στελεχών τριών εντομοπαθογόνων μυκήτων επί του εντομολογικού εχθρού της αγαύης *Scyphophorus acupunctatus* (Gyllenhal) (Coleoptera: Curculionidae)

Β.Τ. Γκουντή, Δ. Μαρκογιαννάκη και Δ.Χ. Κοντοδήμας

Περίληψη Η παθογένεια ιθαγενών απομονώσεων των εντομοπαθογόνων μυκήτων *Beauveria bassiana, Metarhizium anisopliae* και *Isaria fumosorosea* αξιολογήθηκε στο εργαστήριο εναντίον προνυμφών και ακμαίων του εντομολογικού εχθρού της αγαύης *Scyphophorus acupunctatus*. Ο πειραματισμός διεξήχθη με εμβάπτιση ατόμων του εντόμου σε διαφορετικές συγκεντρώσεις εναιωρημάτων κονιδίων των μυκήτων. Και τα τρία είδη μυκήτων έδειξαν υψηλή παθογένεια εναντίον των προνυμφών του σκαθαριού, προκαλώντας 100% θνησιμότητα στις περισσότερες επεμβάσεις. Ο μύκητας *Beauveria bassiana* προκάλεσε την υψηλότερη θνησιμότητα στα ακμαία (86.67±12%), ακολουθούμενος από τον *Metarhizium anisopliae* (46.67±17.8%) και τον *Isaria fumosorosea* (40±17.5%). Παρατηρήθηκαν στατιστικώς σημαντικές διαφορές στο μέσο όρο του χρόνου επιβίωσης μεταξύ των επεμβάσεων και των βιολογικών σταδίων του σκαθαριού. Σε όλες τις επεμβάσεις οι προνύμφες επιβίωσαν σημαντικά λιγότερες ημέρες συγκριτικά με τα ακμαία. Τα αποτελέσματα της παρούσας μελέτης δείχνουν τη δυνατότητα χρήσης αυτών των ιθαγενών στελεχών ως παράγοντα βιολογικής αντιμετώπισης του *S. acupunctatus*.

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Degradation profile and safety evaluation of methomyl residues in tomato and soil

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Summary A high performance liquid chromatography with the photodiode array detector (HPLC-DAD) analytical method was developed to determine the residue levels and investigate the dissipation pattern and safety use of methomyl in tomato and soil. Methomyl residues were extracted from tomato and soil samples with ethyl acetate. The extract was cleaned up with the QuEChERS method. The results showed that the average recoveries were in the range of 87.1–94.5%, with RSD of 6.9-11.2%. Limits of detection (LOD) and quantification (LOQ) were 0.005 and 0.007 mg/kg, respectively. The residue levels of methomyl were best described to first order rate kinetics and half-lives ranged from 1.34 to 1.8 days in tomato and soil, respectively. The theoretical maximum residue contributions for methomyl on tomato were found to be less than the maximum permissible intake values even on zero days, therefore consumer health risks are minimal at the recommended dose on tomato.

Additional keywords: Dissipation, HPLC-DAD, QuEChERS method, Risk assessment

Introduction

Tomato, *Solanum Lycopersicum L.* (Solanaceae), is grown throughout the globe and its berry (fruit) constitutes an important part of human diet (Gupta *et al.,* 2011). In Egypt, tomato is cultivated in about 221 thousand hectares which represent about 34 % of the average area of vegetables, while the fruits are basic component of the daily diet (Malhat *et al*., 2012a). Chemical pesticides are often applied for plant protection of the crop (Singh *et al.,* 1980; 1989; Awasthi, 1986). Nearly all these chemicals are readily soluble in plant oils and waxes (Ripley and Edgington 1983; Malhat and Hassan, 2011), pausing an urgent need for monitoring programs of pesticide residues to properly assess the relevant human exposure and environmental risks.

Pesticide dissipation rate after application is a useful gauge for the assessment of residue levels trend (Malhat *et al.,* 2015). Residue dissipation curves can be used to estimate the time required for residues to reach levels below maximum residue limits (MRLs) (Fong *et al.,* 1999; Malhat *et al*., 2014b). In addition, the MRL regulations require a preharvest interval (PHI) to ensure that dissipation of a pesticide is below the proposed MRL at harvest time. The determination of pesticide residues is usually accomplished by chromatographic techniques and involves many preliminary steps like extraction and clean-up for interference removal.

An adequate description of pesticide degradation in soil is important for the risk assessments within the pesticide registration process. The fate of the pesticides in the soil environment in respect of pest control efficacy, non-target organism exposure and offsite mobility has become a matter of environmental concern (Hafez and Thiemann, 2003) potentially because of the adverse effects of pesticidal chemicals on soil microorganisms (Araújo *et al*., 2003), which in turn may affect soil fertility (Schuster and Schröder, 1990). Several factors influence the final concentration of the pesticide in soil including volatilization, photochemical degradation, chemical and biological transformation, leaching and sorption (Malhat *et*

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al., 2013; Purnama *et al.,* 2015).

Methomyl (5-methyl-N-(methyl carbamoyloxy) thioacetimidate) (Figure 1) has ovicidal, larvicidal and adultcidal action against a variety of insect crop pests as well as an acaricidal effect (Chakraborty and Pahari, 2002; Furness, 2005). Different extraction and quantification methods including HPLC-DAD are used by various scientists for estimation of methomyl residues in several vegetables and fruits (Alawi and Rüssel, 1981; Steven and Lin, 1992). The main criteria for opting any methodology is that analytical method should be fast, easy, inexpensive and applicable to different matrices. Currently, there are no reports in the literature of the analysis of methomyl in tomato and soil using the QuEChERS method coupled with liquid chromatography with a photodiode array detector.

In this study, we set up and validate a modified QuEChERS method followed by HPLC-DAD for quantifying methomyl residues in tomato and soil. Supervised field trials were conducted to determine the dissipation kinetics in tomato and soil. From the generated data, the pre-harvest interval (PHI) was established based upon the dissipation pattern as well as the biological half-life. Furthermore, it is rather imperative to ascertain the food safety hazard by evaluating residues of methomyl in terms of their dietary exposure related to the acceptable daily intake (ADI) and maximum permissible intake (MPI).

Materials and Methods

Chemical and Reagents

The certified reference standard of methomyl was provided from central agricultural pesticide laboratory, Egypt, and was of >99 % purity. All organic solvents were of HPLC grade and were purchased from Merck. Primary secondary amine (PSA, 40 µm Bondesil) and graphite carbon black (GCB) sorbents were purchased from Supelco (Supelco, Bellefonte, USA). Analytical grade anhydrous sodium sulfate was purchased from El Naser pharmaceutical chemical Co. (Cairo, Egypt); it was activated by heating at 250°C for 4 h in the muffle furnace, cooled and kept in desiccators before use.

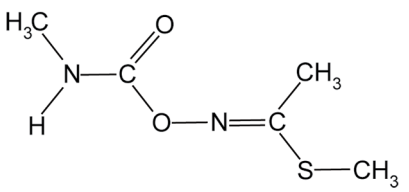

Figure 1. Chemical structure of methomyl

Preparation of standard solution

The stock standard solution (100 mg L^{-1}) of methomyl was prepared in methanol and subsequently stored at -18°C. An intermediate solution (10 mg L^{-1}) was prepared by appropriate dilution with methanol. The calibration standards (five calibration points) ranging from 0.005 to 1.0 mg L^{-1} (0.005, 0.01, 0.05, 0.1 and 1.0 mg L^{-1}) were prepared by successive dilution of the intermediate working standard with pure solvent and matrix extract. All standard solutions were stored at -18°C in amber glass bottles until further analysis.

Instrument and apparatus

The food processor was a Thermomix, Vorwerk. The rotary evaporator was Butch. The HPLC analysis was performed with an Agilent 1260 HPLC system, with quaternary pump, autosampler injector, thermostat compartment for the column and photodiode array detector.

Field experiment

Field experiments were conducted at El-Hakimayia village, Miet-Gamer Province, El-Dkahlyia Governorate, Egypt. The field trail for methomyl was conducted with one commercially available soluble powder formulation (Lannate 90% SP). One treatment was carried out on 2 August 2011 at the maximum recommended dose (675 g.a.i.ha^{-1}). The treatments, including the untreated control, were replicated three times in a complete randomized block design. The average maximum and minimum temperature during the experiment were 25°C and 39°C. There

was no rainfall during the experiment.

Sampling procedures and storage

Samples of pre marked tomato fruits of the same ripening stage and size were harvested at random from each replicate of the treated and control plots separately at regular time intervals on 0 (2 hour after spraying), 1, 2, 4, 7, 10, 13, and 17 days after methomyl application. Soil samples were collected at the same times with the tomato samples, by obtaining a 2 kg of soil for each replicate. Immediately after collecting, samples were transported at 4ºC and in darkness in labelled polyethylene bags to the laboratory, where they were processed. The tomatoes were homogenised in a food processor and the homogenate of each sample was placed in polyethylene containers and frozen at -18 °C until analysis. Soil samples were air-dried at 20 °C, sieved to obtain sub-samples and were placed in polyethylene bags and frozen at -18 °C until analysis.

Extraction and clean up

A sub-sample of 10 g was extracted from each tomato or soil sample with 10 ml ethyl acetate in the presence of 10 g anhydrous sodium sulphate by homogenization followed by centrifugation at 3800 rpm for 10 min. An aliquot of 4 ml of the supernatant was drowning in a 15 ml polypropylene tube containing 100 mg of the clean-up agent PSA and 20 mg GCB. The mixture was shaken vigorously and centrifuged at 3800 rpm for 5 min. 2ml of the supernatant was taken, evaporated to dryness, the residue was re-dissolved in 2 ml of methanol, filtered through a 0.2 µm PTFE syringe filter and then directly measured by HPLC-DAD.

HPLC analysis

Final analysis of methomyl residues was done by HPLC. The chromatographic column was C_{18} Zorbax XDE (250 mm x 4.6 mm, 5 µm film thickness). The column was kept at room temperature. Flow rate of mobile phase (methanol/water = 90/10 v/v) was 1 ml/min., and injection volume was 20 µl. Detection wavelength for detection of methomyl was set at 254 nm. The retention time of methomyl was 5.7 min. The residues in the field incurred samples were tentatively identified by comparing the retention times (RTs) of the sample peaks with that of the injected standard. The chromatographic apparatus was controlled by Chemstation software.

Method validation

The recovery experiment were carried out on fresh untreated tomato and soil by fortifying the samples (10 g) with methomyl at three concentration levels (i.e. LOQ, 5 x LOQ and 25 x LOQ). The fortified samples were processed as previously described and analyzed by HPLC to evaluate the accuracy and the precision of the analytical procedure. Recovery test was replicated five times for each fortification level. The calibration curve for methomyl was obtained by plotting the peak area against the concentration of the corresponding calibration standard. The limit of detection (LOD) of methomyl was determined as the lowest concentration giving response of three times the standard deviation of the base line noise defined from the analysis of three control samples. The limit of quantification (LOQ) was determined as the lowest concentration of a given response that could be quantified with relative standard deviation lower than 20%.

Statistical Analysis

The dissipation kinetics of methomyl in tomato and soil were determined by plotting the residue concentration against time and the maximum squares of correlation coefficient found were used to determine the equations of best fit curves. For all the samples studied, exponential relationships were found to apply, corresponding to the first order rate equation. Confirmation of the first order kinetics was further made graphically from the equation of $C_t=C_0e^{-kt,}$ where *Ct* represents the concentration of the pesticide residue at the time of *t*, C_0 represents the initial deposits after application and k is the degradation rate constant in days $^{-1}$. The half-life ($t_{1/2}$) was calculated from the k value for each experiment, being $t_{1/2}=ln\ 2/k$.

Results and Discussion

Matrix effects and linearity

The matrix effect of the present method was investigated by comparing the relative responses of standards in solvent with matrix-matched standards for 5 replicates at 0.5 mg L^{-1}. The relative responses (response matrix/response solvent) were 1.01 and 1.04 for tomatoes and soil, respectively. It can be concluded that the matrix doesn't significantly suppress or enhance the response of the instrument. The results showed that no interfering endogenous peak appeared, and the retention times of the tested analyte at the spiked sample completely matched those of the standard samples. Good linearity was obtained over the concentration ranges (0.005, -1.0 µg mL^{-1}) with $R^2 > 0.999$, under this condition.

Method performance

The analytical method was developed as to provide a rapid accurate and efficient means of determining methomyl residues in tomatoes and soil. Table 1 shows the fortified results of methomyl in tomato and soil samples. The mean recoveries in tomato samples for methomyl were 87.1–94.5%, with RSD of 6.9-11.2%. Recovery rates and their relative standard deviation were acceptable. The LOQs and LODs were found to be 0.007 mg kg^{-1} and 0.005 mg kg^{-1}, respectively, ensuring LOQ values significantly lower than the MRLs (0.02 mg/kg) established by the European Union. These results demonstrate the good performance of the method according to document SANCO/12571/2013 (SANCO, 2013).

Table 1. Fortification level and recovery percentage (±RSD) of methomyl in tomato and soil samples.

Fortification levels (mg/kg) (n* = 5)	Methomyl	
	Tomato	Soil
0.006	87.10 ± 8.4	92.5 ± 5.2
0.03	94.5 ± 11.2	90.9 ± 12.6
0.15	92.1 ± 6.9	88.3 ± 13

* number of replicates

Dissipation behaviour in tomato

Mean residue levels of methomyl during the sampling period for the recommended application dose derived from the extraction and analysis of three tomato sub samples are shown in Figure 2 and Table 2. The results showed that methomyl residues were easy to be decomposed. The original deposits of methomyl at the recommended dosage were 1.272 mg/kg. These deposits dissipated to 0.007 mg/kg 10 days after methomyl application, thereby, showing a loss of 99.44 %. The residues of methomyl in tomato reached below the LOQ of 0.006 mg/kg in 13 days after application at the recommended dosage. The dissipation curve of methomyl in tomatoes is presented in Figure 1. Degradation equation of methomyl in tomato is as follows: $C_t=1.412^{-0.517t}$ (methomyl). Estimated half-life of methomyl was 1.34 days. A similar $t_{1/2}$ to that found in our results was observed in a study of the dissipation rate of methomyl in cabbage and tomato (Othman *et al.,* 1987), although studies made in grape showed a higher $t_{1/2}$ than ours (Kaushik *et al.,* 2006). Different species, weather conditions, the physical and chemical properties of pesticides, method and rate of application may be responsible for the different dissipation rates of this compound (Romeh and Mekky, 2009; Malhat *et al.,* 2012b, 2014c). In the field, besides the effect of some physical and chemical factors such as light, heat, pH and moisture (Agnihothrudu and Muraleedharan, 1990; Chen *et al.*, 1987; Cosby *et al.,* 1972; Miller and Donaldson, 1994; Malhat, 2012) on the degradation of pesticides, growth dilution factor might play a significant role in the degradation of methomyl residues (Agnihothrudu and Muraleedharan, 1990; Bisen and Ghosh Hajara, 2000; Chen and Wan, 1988; Khay *et al.,* 2008; Cabras *et al.,* 1990, Malhat, 2013).

Dissipation behaviour in soil

Figure 2 shows the decline curve of methomyl in soil. The dissipation dynamics of methomyl can be described by the following first-order kinetics equation $C_t=1.404^{-0.386t}$ (R^2=0.992). The $t_{1/2}$ for methomyl in soil was

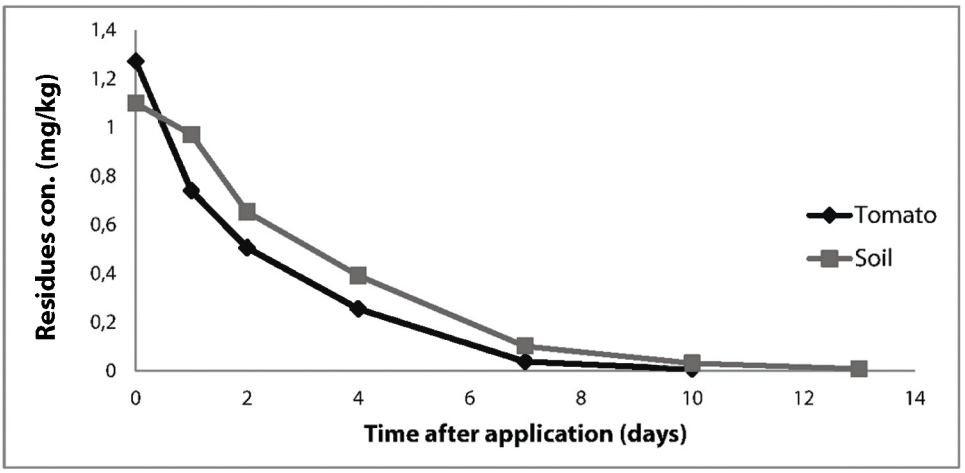

Figure 2. Dissipation pattern of methomyl in tomato and soil at the recommended dosage of application.

Table 2. Residue levels (mg/kg ± SD) of methomyl in tomatoes and soil after application.

Time (days)	Residues (mg/kg) ± SD*	
	Tomatoes	Soil
0	1.272 ± 0.10	1.100 ± 0.30
1	0.740 ± 0.11	0.971 ± 0.10
2	0.506 ± 0.08	0.653 ± 0.09
4	0.254 ± 0.07	0.391 ± 0.05
7	0.038 ± 0.01	0.101 ± 0.03
10	0.007 ± 0.01	0.031 ± 0.01
13	-	0.008 ± 0.005
17	-	-

* Three replicates

1.8 days. The results showed that the dissipation was also fast in the soil. A decline in soil residues may be attributed primarily to growth dilution between application and sampling, as well as to volatilization which occurs during the first few days following application. Other parameters involve sorpation-desorpation, chemical and biological degradation, uptake by plants, run-off and leaching (Fang and Qiu 2002; Spunu, 1989; Malhat and Hassan, 2011).

The results showed that the tested pesticides had a higher degradation in tomato fruits compared with soil, which could be attributed to the high growth rate of fruits which causes a dilution of pesticides. In addition, tomatoes were exposed to various factors, including direct sunlight and daily temperature that affected degradation rates of pesticides.

Risk assessment of methomyl

The risk to the consumer from methomyl on tomatoes has been evaluated by comparing Theoretical Maximum Residue Contribution (TMRC) of the pesticide with its Maximum permissible Intake (MPI). The acceptable daily intake (ADI) for methomyl has been observed to be 0.02 mg/kg body weight per day (Tomlin, 2009). The maximum permissible intake (MPI) was obtained by multiplying the ADI with the average body weight of an adult taken as 60 kg (Malhat *et al.*, 2014-a, Loutfy *et al.*, 2015). MPI was calculated to be 1.2 mg/person/day without any appreciable life risk. The TMRC has been calculated by multiplying the maximum residue levels with average per capita daily consumption of 77 g of total vegetables in Egyptian context (WHO, 2003). The TMRC values on 0 day are found to be 0.098 mg/person/day (Table 3). As the TMRC for methomyl on tomatoes are found to be less than the toxicological estimated MPI value of 1.2 mg/person/day, even on 0 day, the consum-

Table 3. Theoretical maximum residue contribution (TMRC) and maximum permissible intake (MPI) of methomyl in tomatoes.

Pesticide name	Dose (kg a.i. ha^{-1})	ADI (mg kg b.w)	MPI (mg/person/day)	TMRC (mg/person/day)						
				0 day	1 day	2 days	4 days	7 days	10 days	13 days
Methomyl	0.675	0.020	1.2	0.098	0.057	0.039	0.020	0.003	0.0006	-

er health risks are minimal at recommended dose on tomatoes.

In this study, the dissipation dynamic and risk assessment of methomyl were investigated in tomatoes under field conditions to determine consumer safety. A relative simple and fast method was developed to analyze the residue of methomyl in tomatoes fruit and soil. The residue of methomyl dissipated following first order kinetics. The results of this study are expected to help establish the safe and proper use of methomyl in tomatoes crop grown under field conditions in Egypt.

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Αξιολόγηση της ασφάλειας των υπολειμμάτων του methomyl και μελέτη αποδόμησής του σε καλλιέργεια τομάτας και έδαφος

F. Malhat, H. Watanabe and A. Youssef

Περίληψη Στην παρούσα εργασία αναπτύχθηκε και επικυρώθηκε αναλυτική μέθοδος υγρής χρωματογραφίας υψηλής απόδοσης με ανιχνευτή συστοιχίας φωτοδιόδων (HPLC-DAD) για τον προσδιορισμό των υπολειμμάτων της δραστικής ουσίας methomyl σε καρπούς τομάτας και καλλιεργούμενο έδαφος. Η μέθοδος αυτή χρησιμοποιήθηκε σε μελέτες αποδόμησης, για την εκτίμηση της περιβαλλοντικής τύχης του methomyl στην τομάτα και το έδαφος, ύστερα από διενέργεια υπό επίβλεψη εφαρμογών του σε υπαίθρια καλλιέργεια στην Αίγυπτο. Η κατεργασία των δειγμάτων τομάτας και εδάφους βασίστηκε στη μέθοδο QuEChERS χρησιμοποιώντας ως οργανικό διαλύτη οξικό αιθυλεστέρα. Τα αποτελέσματα επικύρωσης της μεθόδου έδειξαν ότι οι μέσες ανακτήσεις κυμαίνονταν από 87,1 έως 94,5%, με τιμές σχετικής τυπικής απόκλισης (RSD%) από 6,9 έως 11,2%. Τα όρια ανίχνευσης (LOD) και ποσοτικοποίησης (LOQ) ήταν 0,005 και 0,007 mg/kg, αντιστοίχως. Η αποδόμηση του methomyl ακολουθεί κινητική πρώτης τάξης, ενώ οι χρόνοι ενώ οι χρόνοι ημιζωής προσδιορίστηκαν σε 1,34 και 1,8 ημέρες στην τομάτα και το έδαφος, αντίστοιχα. Η θεωρητική μέγιστη συνεισφορά καταλοίπων για το methomyl στην τομάτα βρέθηκε να είναι χαμηλότερη από τις μέγιστες επιτρεπόμενες τιμές πρόσληψης, ακόμη και στην ημέρα εκκίνησης (ημέρα 0). Ως εκ τούτου, οι κίνδυνοι για την υγεία των καταναλωτών είναι ελάχιστοι στη συνιστώμενη δόση για την τομάτα.

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SHORT COMMUNICATION

The scale insect *Dynaspidiotus abieticola* (Koroneos) (Hemiptera: Diaspididae) on the Taygetus mountain in Greece

G.J. Stathas

Summary In this study, the presence of the scale insect *Dynaspidiotus abieticola* (Koroneos) (Hemiptera: Diaspididae) has been recorded in fir trees, *Abies cephalonica* Loudon (Pinaceae), on the Taygetus mountain (Peloponnese, South Greece). The biology of the scale insect was observed during a twelve month period, from February 2013 to January 2014. It was determined that the insect completed one generation. It overwintered as a reproductive adult female. The first ovipositions and hatching of crawlers were observed in early May, 2nd instar nymphs appeared at the beginning of August, male nymphs were seen in September and finally the first adult females at the beginning of October.

The species of the genus *Dynaspidiotus* Thiem & Gerneck (Hemiptera: Diaspididae), which have been recorded in Greece to-date, are namely *D. abieticola* (Koroneos) (Koroneos, 1934), *D. abietis* (Schrank) (Koroneos, 1934; Stathas, 2007), *D. britannicus* (Newstead) (Koroneos, 1934; Pellizzari *et al.,* 2011), *D. ephedrarum* (Lindinger) (Koroneos, 1934) and *D. greeni* (Balachowsky) (Kozár *et al.,* 1991).

The presence of *D. abieticola* was first recorded as *Aspidiotus abieticola* in Greece by Koroneos (1934) on *Abies cephalonica,* in the Ano Lekhonia area and in the surrounding region of mount Pelion (Thessaly). Since then, no further records concerning the presence of *D. abieticola* in Greece, data on its biology or ecology, have been reported.

According to ScaleNet (Ben-Dov *et al.*, 2015), *D. abieticola* is a Paleartctic species. It has been recorded in Greece, Iran, Lebanon and Turkey on *Abies cephalonica* and *Cedrus libanotica* subsp. *libani* (A. Rich.) Holmboe (Ben-Dov *et al.*, 2015). Information concerning *D. abieticola* is very scarce and mainly refers to the morphological characters of the adult female scale insect (Koroneos, 1934; Balachowsky, 1948).

In February 2013, a population of *D. abieticola* was observed in fir trees, *Abies cephalonica,* on the Taygetus mountain (Peloponnese, Southern Greece), specifically in a forest area of North Taygetus (37°18′N, 22°20′E) at an altitude of 760m. The species (adult females) was identified by Dr Ferenc Kozár (Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary). The slides of these specimens are deposited in the scale collection of the Hungarian Academy of Sciences.

To obtain data on the scale insect life cycle, samples of fir tree branches infested by the scale insect were regularly collected from February 2013 to January 2014 and the branches were examined under a stereoscope. According to the observations, *D. abieticola* is an oviparous, bisexual scale insect, which develops one generation per year. The scale insect settles on the adaxial needle surface (Figure 1). It overwinters as mated adult female from the beginning of October to the end of April. Egg-laying and hatching of crawlers occur from early May to the end of June. Settled first instar nymphs are present from early June until early September. Second instar nymphs occur from the first days of August until late September, the male nymphs in September and the first adult females at the beginning of October.

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Figure 1. *Dynaspidiotus abieticola* on *Abies cephalonica*: scale covers of male (a) and female (b) nymphs (Photo by G.J. Stathas).

Chlorosis on the needles on infested fir trees by *D. abieticola* was recorded on Taygetus (Figure 1). On heavily infested fir trees, needles get dry and fall and fir trees become weak.

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Το κοκκοειδές έντομο *Dynaspidiotus abieticola* (Koroneos) (Hemiptera: Diaspididae) στο όρος Ταΰγετος στην Ελλάδα

Γ.Ι. Σταθάς

Περίληψη Καταγράφεται η παρουσία του κοκκοειδούς εντόμου *Dynaspidiotus abieticola* (Koroneos) (Hemiptera: Diaspididae) σε έλατα *Abies cephalonica* στο Όρος Ταΰγετος (Πελοπόννησος, Νότιος Ελλάδα). Κατά το διάστημα Φεβρουαρίου 2013 – Ιανουαρίου 2014 το κοκκοειδές συμπλήρωσε μία γενεά. Διαχείμασε στο στάδιο του ενήλικου θήλεος ακμαίου προ-ωοτοκίας. Οι πρώτες ωοτοκίες και ακολούθως οι εκκολάψεις ερπουσών παρατηρήθηκαν κατά τις αρχές Μαΐου, οι νύμφες 2ης ηλικίας αρχές Αυγούστου, οι νύμφες των αρρένων κατά το μήνα Σεπτέμβριο και τα ενήλικα θήλεα αρχές Οκτωβρίου.

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SHORT COMMUNICATION

First record of *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae) in Greece

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Summary The study concerns the first records for the presence of the box tree moth *Cydalima perspectalis* in Greece and subsequent infestations on ornamental box trees in urban environment. Adults of the pest were first spotted in six locations around the country from October 2013 until April 2015, when infestation was also detected (mid April). The pest was found infesting plants of *Buxus sempervirens* in several private and public gardens and parks in the urban environment of Kifissia, Attica. Possible introduction scenarios, as well as preventive and control measures are discussed.

Additional keywords: alien species, box tree moth, *Buxus*, invasive

The box tree moth *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae) (synonyms: *Diaphania perspectalis, Glyphodes perspectalis)* is an invasive species on box tree *Buxus* spp., in Europe, which has been spreading and establishing across the continent during the last decade. The pest was included in the alert list of the European Plant Protection Organisation (EPPO) in 2007 but was removed in 2011 because no particular action was requested by the EPPO member countries (EPPO, 2011). However, the box tree moth could be a serious threat for natural habitats of wild *Buxus* in Europe (Bella, 2013) and a major pest of ornamental *Buxus* in urban landscape, at historical and decorative gardens and parks where they are highly used as design plants (EPPO, 2012; Seljak, 2012) as well as in nursery production (Leuthardt and Baur, 2013).

Herein we provide the first records of *C. perspectalis* in Greece. The presence of the box tree moth was recorded for the first time in Thermi, Thessaloniki, northern Greece, in October 2013 (Theodosis Mamais, personal communication). On 18 May 2014, two adults *C. perspectalis* were found and collected by the first author (IS) in the city of Thessaloniki (40.608°, 22.971°) [voucher numbers: NHMC.85.01.16129.01 and NHMC.85.01.16129.02, Natural History Museum of Crete] and a photograph of a specimen from Thessaloniki taken on May 20, 2014 was published http://www.lepidoptera.eu/ContributorPics.php?ID=1688 (photograph by Theodosis Mamais). On 17 July 2014, the second author (CK) found another specimen at Ano Lechonia village, Pelion mountain (Figure 1) (39.328°, 23.058°). Four more observations made by different citizens followed: on 22 July 2014 an adult specimen was photographed by Dimitris T. Kaloutsikos in Drama city (41.153°, 24.117°); on 22 August 2014 Savvas Vassiliadis photographed another specimen in Katerini city (40.303°, 22.501°); on 27 August 2014 Ersi Augustidou observed and photographed the species in Kalamaria, Thessaloniki (40.586°, 22.941°); in September 2014 Lia Naki photographed an adult specimen in Kifissia, Attica (38.066°, 23.818°). Locations of the first records of *C. perspectalis* are indicated in the

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map of Figure 2. All specimens recorded had the white colour form. The aforementioned citizens contacted the first two authors requesting species identification and provided us their data, thus we include their observations in this short communication, having their written permission.

Heavy infestation by larvae of *C. perspectalis* was observed on plants of *Buxus sempervirens* grown as a tree and in border shrubs at Benaki Phytopathological Institute and several private and public gardens and parks in Kifissia, Athens, in mid April 2015 (Figures 3, 4). The plants had a dry appearance and were covered by dense webs (Figure 3). This is the first report of *C. perspectalis* as a pest in Greece with evidence of its consequent infestation. Samples of infested shoots were transferred to Benaki Phytopathological Institute and kept in cages (30 x 30 cm) at 25 °C, 16:8 L:D h until pupation of the larvae. Pupae were collected and placed in plastic containers until adult emergence (Figure 5). Forty one adults emerged; thirty five presented the white colour form and six of them the brown colour form (Figures 1, 6).

The box tree moth is native to subtropical regions of eastern Asia (India, China, Korea, Japan and the Russian Far East) (Walker, 1859; Hampson, 1896; Inoue, 1982; Kirpichnikova, 2005; Park, 2008; Leraut, 2012). It was introduced in Europe and was recorded for the first time in south-western Germany in 2006 (Krüger, 2008). It spread rapidly across Europe and it is now present in the Netherlands (Muus *et al.,* 2009), Switzerland (Käppeli, 2008; Sigg, 2009), France (Feldtrauer *et al.,* 2009), Austria and Liechtenstein (Rodeland, 2009), United Kingdom (Mitchell, 2009), Belgium (Casteels *et al.,* 2011), Hungary (Sáfián and Horváth, 2011), Czech Republic (umpich, 2011), Romania (Székely *et al*., 2011), Italy (Griffo *et al*., 2012; Tantardini *et al*., 2012), Slovenia (Seljak, 2012), Turkey (Hizal *et al*., 2012), Croatia (Koren and Črne, 2012), Slovakia (Pastorális *et al*., 2013), Denmark (Hobern, 2013), Chechen Republic (Russia) (Proklov and Karayeva, 2013), Spain (Pérez-Otero *et al*., 2014; Pino Perez and Pino Perez, 2014) and Bulgaria (Beshkov *et al*., 2015). This is the first record of the pest in Greece.

The main host plants of *C. perspectalis* are *Buxus* species (common names box tree, box, boxwood), including *B. sempervirens* L., *B. microphylla* Siebold & Zucc., *B. sinica* (Rehder and Wils.) M. Cheng and *B. colchica* Pojark (Buxaceae). In its origin countries, the pest has also been reported on *Euonymus japonicus* Thunb., *E. alatus* (Thunb.) Siebold (Celastraceae), *Ilex purpurea* Hassk. (Aquifoliaceae), *Pachysandra terminalis* Siebold & Zucc. and *Murraya paniculata* (L.) Jack (Rutaceae), but there are no reports of these plant species being attacked in Europe (Wang, 2008; Hizal *et al*., 2012; Bella, 2013; Plantwise Knowledge Bank, 2015). Box trees are evergreen shrubs and small trees. *Buxus sempervirens* lives in

Figure 1. Specimen of *Cydalima perspectalis* from Ano Lechonia, Pelion mountain: adult of white colour form (Photograph by C. Kazilas).

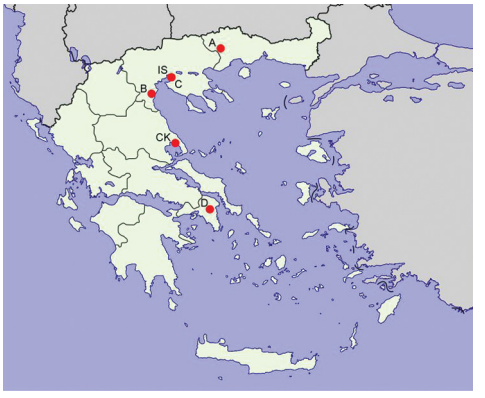

Figure 2. Locations of the first records of *Cydalima perspectalis* in Greece: A) Drama, B) Katerini, C) Kalamaria, Thessaloniki, D) Kifissia, Attica, IS) Thessaloniki and CK) Ano Lechonia, Pelion mountain.

Figure 3. Infestation of *Buxus sempervirens* by *Cydalima perspectalis* in Kifissia, Attica, Greece.

Figure 4. Mature larvae of *Cydalima perspectalis* on infestated twigs of *Buxus sempervirens*.

the wild in different habitats, in open phrygana and forest areas in a large part of Europe (Di Domenico *et al.*, 2011) whereas boxes are also economically important ornamental species grown in nurseries, parks and public and private gardens. The ornamental *Buxus* species and varieties met in Greece are *B. sempervirens*, *B. sempervirens 'Rotundifolia'*, *B. microphylla* and *B. microphylla 'Faulkner'*.

The adult of *C. perspectalis* has a wingspan of 3.5-4 cm, which makes it a large spe-

cies among European Crambidae (Székely *et al.,* 2011). Two colour forms of adults have been described, the white one, which is the most common, and the melanic one, being less common. In the white form, adults have white, slightly iridescent wings with a large dark brown band at the margin and a characteristic white spot in the discoidal cell only in the forewings (Mally and Nuss, 2010). In the melanic form, the wings are completely brown with the exception of a white discoidal spot on the forewings (Figure 6). Eggs are laid in clusters of 5-20 on the underside of the leaves (Leuthardt and Baur, 2013); they are pale yellow when laid and black heads of the larvae are visible before hatching. The late instar larvae have a shiny black head and they are light green with two longitudinal black thick stripes and white dots in between at the lateral part of the body; they also have black dots outlined in white on the dorsal side of the body; in the last larval stage they can reach a length of up to 4 cm (Székely *et al.,* 2011; Bella, 2013). The pupae are 1.5-2.0 cm long and they are concealed in a cocoon of white silk spun among the leaves and twigs. They are initially green with dark stripes on the dorsal surface and towards the end of pupation they turn brown with a dark pattern corresponding to the brown wing borders of the adult (Korycinska and Eyre, 2009).

The pest is reported to have 2-3 generations in Central Europe (Korycinska and Eyre, 2009; Leuthardt *et al*., 2010; Sage and Karl, 2010) and is capable of hibernating and spreading naturally across the continent (Krüger, 2008; Feldtrauer *et al*., 2009; Muus *et al.,* 2009; Sigg, 2009).

Figure 5. Pupae of *Cydalima perspectalis*.

Infestation symptoms include feeding damage on the leaves of the shoot edges by the larvae, which can leave only leaf skeletons and the epidermis behind them. Larvae can also attack the bark (Leuthardt and Baur, 2013). Other associated symptoms are webbing of the branches, frass and residues of moulting such as black capsules of different sizes. Heavy infestation leads to dry plants and their defoliation, which combined with the subsequent attack of the bark results in the death of the plant. Box trees with a low level of damage are often able to recover if they do not suffer from renewed attacks. However, severely damaged boxes in an area where *C. perspectalis* has established are less likely to survive. This also applies to naturally occurring boxes in the understories of forests in the invaded range of *C. perspectalis* (Plantwise Knowledge Bank, 2015).

The species either has actively dispersed in Greece from neighbour countries (e.g. Turkey), where its presence is already confirmed, or it has been passively introduced *via* one or more relatively recent commercial importations of plants of *Buxus* sp. infested with the moth's eggs or larvae. It has been assumed that the species is capable of spreading across Europe in both ways (Käppeli, 2008; Krüger, 2008; Feldtrauer *et al.,* 2009; Muus *et al*. 2009; Sigg, 2009). Introduction seems more likely to have taken place *via* plant importations. However, one cannot yet exclude an active dispersal or a combination of both ways mentioned. Further investigation is needed to determine the origin of each different Greek population.

Figure 6. Adult of *Cydalima perspectalis*: melanic (brown) colour form.

A climate model applied by Nacambo *et al.* (2014) suggests that *C. perspectalis* is likely to continue its spread across Europe, except for Northern Fenno-Scandinavia, Northern Scotland and high mountain regions and become a pest more likely in Southern and Central Europe where the moth is able to complete at least two generations per year; restriction of distribution of the species in the northern range is expected due to the limitation in degree-days above the temperature threshold to complete a generation whereas in the southern zone due to the absence of a cold period necessary to resume diapause.

Investigation of effective preventive and management methods is necessary. *Buxus* plants importation in European countries, such as the Netherlands, Germany and Italy, has largely increased in the recent years, mainly from China (EPPO, 2012). The trade of infested box trees may still be the most important dissemination pathway as detection of early larval stages or eggs is difficult (Leuthardt *et al*., 2010). Campaigns to communicate the risk of displacing eggs, larvae and pupae when moving infested box trees will contribute in public awareness and slow down the dispersal of *C. perspectalis*.

Control of the pest in East Asia, where it was primarily studied until its invasion in Europe, embraces mainly biological control by nematodes (Choo *et al.,* 1991; Lee *et al*., 1996), mating disruption (Kawazu *et al.,* 2007) and chemical control (Zhou *et al.,* 2005). Natural enemies of the pest include polyphagous parasitoids (Nacambo *et al*., 2014) and birds exhibiting low predation, probably due to the high levels of toxic alkaloids sequestered by its larvae (Leuthardt and Baur, 2013). Investigation on specific parasitoids of the moth in the places of its origin in Asia should be envisaged in the perspective of their use in a classical biological control programme which would offer a long-term control option in natural habitats of boxes.

Use of pheromones for monitoring did not give satisfactory results in field trials in Europe (Van den Straten and Muus, 2010; pers. comm. F. Griepink). Chemical control with contact insecticides has been proved very effective but may harm beneficial arthropods using the box trees for shelters, such as arachnids. Insecticides working by ingestions are also very effective, although the lag until death of all larvae is usually longer. Biopesticides based on *Bacillus thuringiensis* are usually the preferred option on ornamental box trees because of their limited impact on the environment (Plantwise Knowledge Bank, 2015). Recent research indicated the susceptibility of *C. perspectalis* larvae to baculovirus *Anagrapha falcifera* nucleopolyhedrovirus (AnfaNPV) as a potential control agent for the pest (Rose *et al.,* 2013). Physical control by cutting the infested material, if applicable, may also be effective (Korycinska and Eyre, 2011). Nevertheless, since introduction of alien arthropods in Europe mainly occurs *via* ornamental plant trade (Rabitsch, 2010), a more efficient inspection of the condition of traded goods is necessary in order to prevent the pest's further spreading (Bella, 2013).

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Πρώτη καταγραφή του *Cydalima perspectalis* (Walker, 1859) (Lepidoptera: Crambidae) στην Ελλάδα

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Περίληψη Η μελέτη αφορά στις πρώτες καταγραφές της παρουσίας του νυκτόβιου λεπιδόπτερου *Cydalima perspectalis* στην Ελλάδα και των επακόλουθων προσβολών από το έντομο σε πυξάρι στο αστικό πράσινο. Ενήλικα άτομα του εντόμου παρατηρήθηκαν αρχικά σε έξι περιοχές της χώρας από τον Οκτώβριο του 2013 έως τον Απρίλιο του 2015, οπότε εντοπίστηκε και η πρώτη προσβολή (μέσα Απριλίου). Η προσβολή αφορούσε φυτά του *Buxus sempervirens* σε πολλούς ιδιωτικούς και δημόσιους κήπους και πάρκα στο αστικό πράσινο της Κηφισιάς. Συζητάμε τους πιθανούς τρόπους εισαγωγής του εντόμου και μέτρα πρόληψης της εξάπλωσης και αντιμετώπισής του.

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