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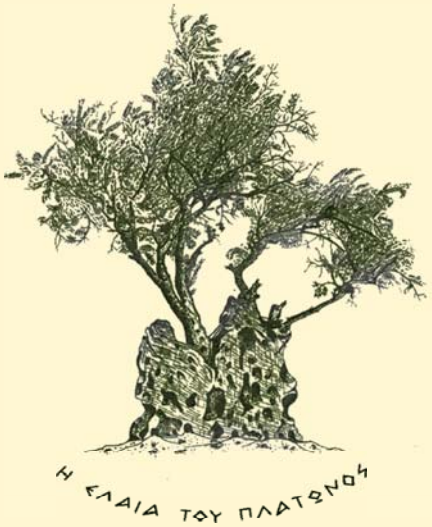

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REVIEW ARTICLE

Pepino mosaic virus

R.A.A. van der Vlugt

Summary *Pepino mosaic virus* (PepMV) is a relatively new plant virus that has become a significant agronomical problem in a relatively short period of time. It is a member of the genus *Potexvirus* within the family *Flexiviridae* and is readily mechanically transmissible. It is capable of infecting tomato (*Solanum lycopersicum*) and other Solaneceous host plants. Since its description in 1980 from pepino plants (*Solanum muricatum*) collected in 1974 in Peru, the virus remained unknown for a long time until it manifested itself in commercial tomato crops in Europe in 1999. Since then the virus has been reported worldwide and the disease it causes has become important in commercial tomato production. Since 1999, new strains of the virus have been described which differ from the original pepino isolate. The fast spread of the virus and the appearance of mixed infections with the new strains may play an important role in the increase of the agricultural importance of this viral disease.

Additional keywords: epidemiology, potexvirus, virus strains, virus symptoms

Introduction

The first finding of *Pepino mosaic virus* (PepMV) dates back to 1974 in field samples of pepino plants (*Solanum muricatum*) collected in the Canete valley in coastal Peru and showing yellow mosaic in young leaves (Jones *et al.*, 1980). Typical filamentous potexvirus particles of approximately 500 nm were observed in the electron microscope (EM). Serologically these particles did not react with antisera against *Potato virus* X (PVX) but appeared most closely related to *Narcissus mosaic virus* (NaMV). Host ranges, however, of the two viruses differed considerably. The authors concluded that *Pepino mosaic virus* was a new and distinct potexvirus.

Since its first description, the virus has not been reported again and was not considered to be of any agricultural significance. In 1999, however, it was detected in protected commercial tomato crops (*Solanum lycopersicum*) in the UK (Mumford and Metcalfe, 2001) and The Netherlands (Van der Vlugt *et al.*, 2000). Since 2005, new strains of the virus have appeared and PepMV has spread very rapidly throughout tomato-producing areas. It is now reported nearly worldwide and can cause significant damage in commercial tomato crops.

Host range and symptomatology

PepMV was originally found on pepino (*S. muricatum)* in Peru (Jones *et al.*, 1980). Later surveys showed several related wild *Lycopersicon* spp. being infected with PepMV (Soler *et al.,* 2002). These included *L. peruvianum*, *L. parviflorum*, *L. chilense*, *L. chmielewskii* and *L. pimpinellifolium*. Infection of these wild species by PepMV appears to be generally symptomless.

PepMV natural host range seems to be mainly restricted to the family Solanaceae of which many species become infected systemically. The virus main hosts appear to be *Lycopersicon* spp. and *Solanum* spp. The originally described pepino strain was found on wild and commercial tuber-

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bearing *S. tuberosum* spp., causing mostly a symptomless systemic infection or mild mosaic symptoms. However, in two local Peruvian *S. tuberosum* cultivars and *S. stoloniferum* PI 230557 the virus caused a severe systemic necrosis (Jones *et al.,* 1980). The virus was shown to be transmitted through tubers.

Several weed hosts were also found to be infected with PepMV. Jones *et al.* (1980) reported *Datura stramonium*, *Nicandra physaloides* and *Physalis peruvianum* as weed hosts in Peru. Studies on material sampled from the mainland of Spain and the Canary Islands (Jordá *et al.,* 2001) showed a large number of weed species being latently infected (i.e. showing no symptoms) with PepMV: *Amaranthus* sp., *Malva parviflora*, *Nicotiana glauca*, *Solanum nigrum*, *Sonchus oleraceus*, *Bassia scoparia*, *Calystegia sepium*, *Chenopodium murale*, *Convolvulus althaeoides*, *C. arvensis*, *Conyza albida*, *Coronopus* sp., *Diplotaxis erucoides*, *Echium creticum*, *E. humile*, *Heliotropium europaeum*, *Moricandia arvensis*, *Onopordum* sp., *Piptatherum multiflorum*, *Plantago afra*, *Rumex* sp., *Sisymbrium irio*, *Sonchus tenerrimus*, and *Taraxacum vulgare*. However, the following species were found not to be infected with the virus: *Capsicum annuum*, *Cucumis sativus*, *Chenopodium amaranticolor*, *C. quinoa*, *Petunia x hybrida*, *Phaseolus vulgaris*, *Physalis floridana*, *Nicotiana rustica*, *N. tabacum*, *Polypogon monspeliensis* and *Senecio vulgaris*.

In the initial PepMV report, the typical symptom caused by the virus was a distinct yellow mosaic on young leaves of pepino. Most of the infected plants also showed dark green enations on the lower surface of some leaves. In *Lycopersicon* spp. the virus caused a symptomless systemic infection, which became apparent only by back-inoculation on susceptible indicator plants, such as *Nicotiana glutinosa*. Only *Datura metel*, *D. stramonium* and some *Nicotiana* spp. showed distinct symptoms upon systemic infection. The virus failed to infect 13 plant species belonging to six other families.

The virus was found in 1999 in commercial tomato crops in the UK and The Netherlands causing more pronounced symptoms. However, symptoms may depend on the PepMV strain, the tomato cultivar, the age of the plant when first infected and the environmental conditions. Generally symptoms include yellow leaf spots, yellow-green mosaic, mottle on the older leaves and/or slight curling of the top leaves (nettle head symptom) or a grayish appearance of the top of the plant (Figure 1a, b & c). Fruit symptoms range from alteration in colour, in some cases sever yellow/orange mottling (Figure 1d) and uneven ripening to netting and cracking and shape distortion (Spence *et al.,* 2006; Hanssen *et al.,* 2008). Particular isolates cause leaf and stem necrosis. Fruit symptoms, such as uneven ripening, marbling or even cracking, may affect fruit quality resulting in different levels of economic damage. Environmental conditions are reported to affect symptom expression with low temperatures and low light conditions favouring the appearance of more pronounced symptoms. Symptoms are also reported to change during the growing season when environmental or growing conditions change. Especially plant stress appears to promote symptom expression. Initially it was thought that the different virus strains induced different symptoms, but isolates belonging to the same strain (i.e. EU-tomato or Chile-2) may also differ greatly in the severity of symptoms they cause in a tomato crop ranging from only a few yellow spots to severe necroses on stems and leaves or very severe symptoms on fruit. Inoculation studies on sets of indicator plants confirmed these differences between different isolates.

Virion properties

PepMV is a potexvirus and as such, has typical filamentous particles with a normal length of 510 nm. Particles are comprised of a single capsid protein (CP) of approximately 26 kDa. In ultrathin sections of infected leaf material inclusions consisting of arrays of filamentous virus-like particles may be observed.

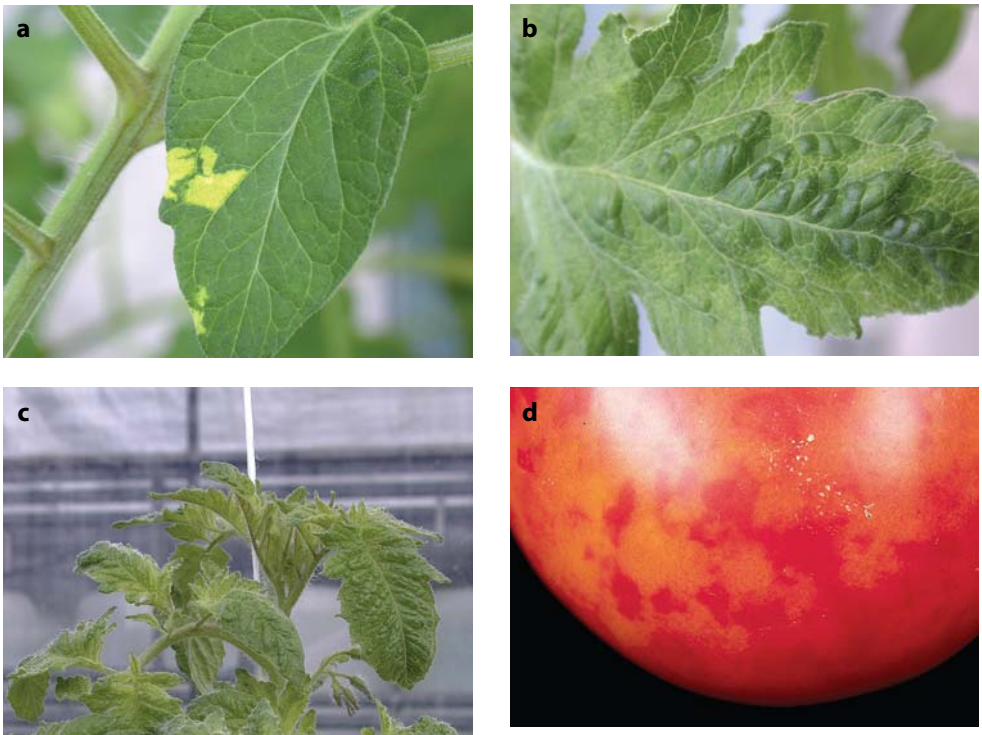

Figure 1. PepMV symptoms on tomato plants: (a) yellow leaf spots, (b) leaf bubbling, (c) nettle head, and (d) fruit marbling.
Courtesy: Dr R.A.A. van der Vlugt, Plant Research International, Wageningen, The Netherlands.

PepMV is fairly stable. In endpoint dilution studies, sap from infected *N. glutinosa* was still infectious at dilutions of 10^{-4} and occasionally at 10^{-5}. Sap lost most of its infectivity after 10 minutes at 65°C and was no longer infectious at 70°C (Jones *et al.,* 1980). Sap stored at 20°C still shows some infectivity after three months, while leaves of *N. glutinosa* desiccated over silica gel were still infectious after six months. Under greenhouse and field conditions, the virus may easily survive for several weeks in plant debris and on surfaces or tools that have come in contact with virus-infected leaves or fruit (C.C.M.M. Stijger, personal comunication). There are indications that virion infectivity is retained longer at lower temperatures (Van der Vlugt, unpublished).

A polyclonal antiserum raised against the original pepino isolate did not react with *Potato virus* X (PVX) and *Potato Acuba mosaic* virus (PAMV), the only two other potexviruses known to infect tomato. Both in immuno-sorbent electron microscopy (IEM) and in ELISA the antiserum clearly reacted with different tomato isolates although at a lower titre (Jones *et al.*, 1980). Comparisons of isolates from the different strains (EU-tomato, Chile-2 and US1) with an antiserum raised against the EU-tomato type isolate (Van der Vlugt *et al.*, 2000) showed differences in heterologous titres between them (Van der Vlugt *et al.*, unpublished). These differences, however, only became apparent at very

low virus concentrations. At virus concentrations more in line with "real-world" situations, no differences in reactivity between the strains could be observed.

Genome organization and expression

The genome organization of PepMV (Aguilar *et al*., 2002, Cotillon *et al*., 2002) is typical for potexviruses (Verchot-Lubicz *et al.,* 2007). Its positive single-stranded RNA is capped at the 5'-end, polyadenylated at the 3'-end and contains 5'- and 3'-non-translated regions. Its 6410 nucleotides encode five putative partly overlapping open reading frames (ORFs 1 to 5, Figure 2). The 5'-nontranlated region (5'-NTR) of the virus is 85 nts long and starts with the pentanucleotide GAAAA, which is typical for potexviruses.

ORF1 (nt 86-4406) encodes a 164 kDa of 1439 amino acids (aa). A putative methyltransferase domain (aa 59-224) specific for the supergroup of 'Sindbis-like' viruses (Rozanov *et al.,* 1992), a NTPase/helicase domain (aa 708-934) with the NTP-binding motifs GCGGSGKS and VVIFDD (Kadaré and Haenni, 1997) and a RNA-dependent RNA polymerase (RdRp) domain (aa1217-1374) characterised by the SGEGPTFDANT-X22-GDD motif (Kamer and Argos, 1984), can be distinguished. The stop codon of the first ORF is followed by a short intergenic region (IR1) of 25 nts and a set of three partially overlapping ORFs typically known as the triple gene block (TGB).

ORF2 (nt 4432-5136) encodes the first TGB protein (TGBp1), a 234 aa protein of 26 kDa. TIt contains a typical NTPase/helicase motif (AA26-233) characterised by seven conserved motifs. Two of these motifs may be involved in NTP binding (Kadaré and Haenni, 1997). TGBp1 belongs to the superfamily I of RNA helicases.

ORF3 (nt 5117-5488) overlaps 19 nucleotides with the 3'-end of ORF2 and extends 148 nucleotides past the start codon of ORF4. It encodes a small 14 kDa protein of (TGBp2), which contains a potexvirus specific consensus motif: PxxGDxxHxL/FPxG-GxYxDGTKxxxY (Wong *et al.,* 1997).

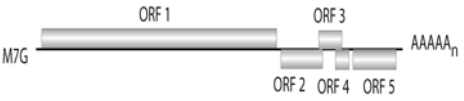

Figure 2. Schematic organization of the RNA genome of *Pepino mosaic virus* with its five open reading frames (ORFs). M7G = 5' cap, AAAAAn = poly(A) tail.

ORF4 (nt 5340-5594) encodes the third TGB protein (TGBp3), a 85 aa protein of 9 kDa. This protein is the most variable among potexviruses. It contains a CxV/IxxxG consensus motif among potexvirus TGBp3 proteins.

Following ORF4 is the second intergenic region (IR2) of 38 nts (nt 5595-5632). This IR2 is highly variable in sequence between the different strains. It precedes ORF5 (nt 5633-6346), which encodes the 238 aa coat protein (CP) of 25 kDa. This CP contains the amphipathic core sequence KFAAFDFFDGVT. A similar sequence is also found in the CP of other potexviruses (Wong *et al.,* 1997) and it might be responsible for binding of virus RNA to the CP through hydrophobic interactions.

The 64 nts long 3'-non-translated region (3'-NTR: nt 6347-6410) precedes the poly(A) tail and contains the hexameter 5'-ACUUAA sequence, which is also present in the 3'-NTR of all potexviruses sequenced so far. This motif is proposed to be a cis-acting element involved in the positive and negative viral RNA synthesis (Bancroft *et al.,* 1991; White *et al.,* 1992). The 5'-AAUAAA polyadenylation signal terminates the RNA genome, whereby the AAA portion forms the first A residues of the poly(A) tail.

Virus isolates and strains

The first described PepMV isolate (BBA1137; Jones *et al.,* 1980) was found on pepino (*S. muricatum*) in Peru. This isolate is now considered the type isolate of the Peruvian (PE) strain of PepMV. Its full sequence has been determined and was published as SM74 (acc

no. AM109896). Full length sequence data were also obtained from an isolate collected from a wild *L. peruvianum* during a survey in Peru (LP2001; Soler *et al.*, 2002).

The EU-tomato isolate of PepMV, first described in 1999, clearly differs in a number of characteristics from the original pepino isolate from Peru. The differences are most pronounced in the reactions of both isolates on tomato (*S. lycopersicum*) in which the pepino isolate does not cause symptoms, whereas the tomato isolate causes more distinct symptoms. The two isolates could be also distinguished on a number of test plants, most clearly on *N. glutinosa* and *D. stramonium* (Verhoeven *et al.,* 2003).

Since 1999, a large number of different PepMV isolates were described in commercial tomato crops in different countries. Many partial sequences and a number of full length sequences of tomato isolates have been obtained. Comparisons show that isolates of the Peruvian and the EU-tomato strains are closely related and share approximately 95% overall nucleotide sequence homology between their genomes. Comparison of full length nucleotide sequences and deduced amino acid sequences of a number of tomato isolates from different countries (Aguilar *et al*., 2002; Cotillon *et al*., 2002; López *et al.,* 2005*)* confirmed the very high levels of sequence identity (over 99%) within the tomato isolates. Table 1 lists the currently available full length sequences of PepMV.

In 2005, Maroon-Lango *et al.* (2005) published the full sequences of two isolates (US1 and US2) collected in 2000 in the USA. These sequences were obtained directly from total RNA isolated from a mix of dried symptomatic tomato leaves collected in Arizona. Direct nucleotide sequence comparisons showed only 79-82% overall identity between European tomato isolates from France and Spain and US1 and US2 and 86% overall nucleotide identity between the RNA genomes of both US isolates. Pairwise comparisons of each of the ORFs of US1 and US2 with European tomato isolates showed similar levels of identity. Based on the overall levels of homology between them, US1 and US2 were considered as new PepMV strains distinct from the Peruvian and EU-tomato strains.

Table 1. Full genome sequences of *Pepino mosaic virus* isolates.

Acc no	Host	Isolate	Strain
FJ212288	Tomato		?
EF408821	Tomato	PK	Ch2
DQ000984	Tomato seed		US1
DQ000985	Tomato seed		Ch2
AY509926	Tomato		US1
AY559927	Tomato		US2
AM491606	Tomato	Hungary	EU-tomato
AM109896	Pepino	BBA1137 (SM74)[1]	Peruvian
AJ606359	Tomato	LE2000	EU-tomato
AJ606360	Tomato	LE2002	EU-tomato
AJ606361	Tomato	LP2001	Peruvian
AJ438767	Tomato	Fr	EU-tomato
AF484251	Tomato	Sp13	EU-tomato
FJ940223	Tomato	PD99901066	EU-tomato
FJ940224	Tomato	DB1	EU-tomato
FJ940225	Tomato	US1	US1
FJ612601	Tomato	PA	

[1] Original PepMV isolate, as described by Jones *et al.* (1980).

Two additional full length sequences, namely Chile-1 (Ch1) and Chile-2 (Ch2), were obtained from tomato seed batches imported from Chile into the US (Ling, 2007). Both showed the highest nucleotide similarity with the previously described US1 and US2 isolates; 98.7% between Ch1 and US1 and 90.7% between Ch2 and US2. Ch1 and Ch2 shared only 78% nucleotide identity and between 78 and 86% identity to five isolates of the EU-tomato strain.

At that point the Ch1 isolate was considered to belong to the US1 group and the Ch2 isolate to the US2 group. However, detailed sequence alignments of the various ORFs between Ch2 and US2 (Van der Vlugt, unpublished) showed a possible recombination point in the ORF1 nt sequence of US2. Up to nt 2305 the US2 sequence clearly resembles the Ch2 sequence. However, between nt 2306 and 4742 the US2 sequence is nearly identical to the US1 sequence. From

nt 4742 until the 3' poly(A) tail the US2 sequence again resembles the Ch2 sequence. Whether this represents a true recombination event in the US2 isolate or is an artefact from the sequence assembly process (both US1 and US2 sequences were obtained from one RNA sample) remains unclear. Unfortunately, the original infectious material is no longer available.

Currently four different strains of PepMV isolates are recognized:

1. The Peruvian (PE) strain, originally found on pepino (*S. muricatum*) and wild *Lycopersicon* spp.
2. The EU-tomato (*S. lycopersicum*) strain.
3. The US1/Ch1 strain.
4. The Ch2 strain.

Several studies have shown that isolates from the EU-tomato and Ch2 strains can readily occur simultaneously in a mixed infection in plants (Pagán *et al.,* 2006; Hanssen *et al.,* 2008; Van der Vlugt, unpublished). Detailed sequence analysis showed different recombination events between the EU-tomato and Ch2 genotypes. The findings of these recombinants in different geographical locations with different recombination points, suggest that genetic recombination between PepMV strains may not be a rare event.

The differences in symptom expression between isolates of one strain as well as between isolates of different strains, do not allow biological differentiation between strains. Serological differentiation is also not possible with the currently available (polyclonal) antisera. Given the clear genetic differences between strains, a number of molecular tests have been developed that can differentiate between strains. These are based on strain-specific RFLP patterns (Martínez-Culebras *et al.,* 2002) or strain-specific RT-PCR primer sets (Ling *et al.*, 2007; Hanssen *et al.,* 2008; Mumford, unpublished; Van der Vlugt, unpublished) or a combination of both (Alfaro-Fernández *et al.,* 2009).

Virus epidemiology

The origin of PepMV clearly lies in South America. Studies showed that the virus is likely to be widespread in wild *Lycopersicon* spp. in Peru, even in isolated wild populations (Soler *et al.,* 2002), suggesting that Peru might be its centre of origin. Interestingly most infected wild *Lycopersicon* spp. showed no distinct symptoms, which might suggest a long term relationship with the virus. Several studies have confirmed that the original pepino isolate is characterised by the absence of, or only very mild symptoms on commercial tomato crops (Van der Vlugt *et al.*, 2002). The strain now known as the EU-tomato strain of PepMV caused more distinct symptoms on tomato although these were still relatively mild.

Detailed population studies from Spain on material collected between 1998 and 2004 confirmed the prevalence and homogeneity of the tomato strain isolates as well as their presence since 1998 (Pagán *et al.,* 2006). However, there were clear indications for several independent introductions of this virus strain both in the Canary Islands and the Spanish mainland. Interestingly, in addition to the tomato strain, the original pepino strain and the US2/Ch2 strain were also found, but always in mixed infections with the tomato strain. Both strains were shown to be already present in Spain since 2000. This is surprising since the Ch2 strain was only recognised for the first time in 2006. Recently the US1 strain was also found on the Canary Islands (Alfaro-Fernández *et al.,* 2008), and this is the first official report on the occurrence of this strain outside the US.

The above data suggest that different strains of PepMV, at different occasions, have spread from South America, most likely from different host plants, such as pepino, wild *Lycopersicon* spp. or other as yet unidentified hosts, resulting in different introductions of the virus in different parts of the world. A number of virus characteristics are likely to have contributed to this:

- The original pepino strain is (nearly) symptomless in commercial tomato.
- Proper diagnostics for the virus only became available after the recognition of

the tomato strain in commercial tomato crops.

- As a potexvirus, PepMV is easily mechanically transmissible and remains infectious for several weeks in plant debris or on contaminated surfaces.
- Tomato fruit harvested from infected plants contain high concentrations of the virus and as such, they can easily spread the virus over long distances.

Due to the absence of explicit symptoms, the initial introduction of PepMV into Europe in commercial tomato crops may, thus, have remained largely unnoticed. Nothing is known about the possible occurrence of the EU-tomato, Ch2 or US1 strains in pepino or in wild and cultivated *Solanum* spp. in South America and the precise origin of these strains remains unknown. It has been suggested that the EU-tomato and Ch2 isolates show an increased fitness and virulence in commercial tomato crops. This might explain the fast worldwide spread of the virus problem.

Although PepMV was already present in Spain since 2000, the Ch2 strain has spread quickly over Europe after 2005. No obvious reasons for this have been identified. It is also not clear if this strain is more competitive than the EU-tomato strain. Both strains often occur in mixed infections and genetic recombinants between the EU-tomato and Ch2 strain have been identified both in Spain (Pagán *et al.,* 2006) and Belgium (Hanssen *et al.,* 2008). It is not known to what extent these mixed infections and recombination events have contributed or will contribute to the severity of the virus symptoms or to the spread of the virus problem.

Seed transmission has been implicated in the spread of the virus. The introduction through infected seed of such a highly contagious virus in only a few plants in a nursery can obviously result in the infection of many thousands of plants in a very short time. For this reason, PepMV has a quarantine status on tomato seeds in the European Union. Directive 2004/200/EC prohibits the trade of seeds collected from PepMV infected plants or from plants not officially tested for the absence of PepMV. Recently, a study on the possibility of seed transmission of PepMV was conducted within the framework of the EC FP6 funded research project PEPEIRA (*'Pepino mosaic virus: epidemiology, economic impact and pest risk analysis'*; www.pepeira.wur.nl). In this study, 100,000 seeds were harvested from an infected crop. Following only marginal cleaning without any disinfection, the seeds were sown and the seedlings were tested for PepMV infection by DAS-ELISA. Of nearly 9,000 tested batches of 10 seedlings each, only 23 were positive (Hanssen *et al*., in press). This corresponds to a seed transmission rate of 0.026%, which is in good agreement with earlier tests (Krinkels, 2001; Córdoba-Sellés *et al*., 2007).

Virus damage and control

Initially PepMV infections in commercial tomato crops were reported as relatively insignificant with no apparent plant or fruit symptoms and no or very limited yield reduction. Studies from the UK, however, reported significant effects on fruit quality (Spence *et al.,* 2006). Smaller sized fruit with different grades of uneven ripening and discoloration and occasionally misshaped fruit due to PepMV infection led to production of fruit unsuited for the fresh UK market and hence to economic damage.

Studies indicate that symptom induction by PepMV is highly dependent on environmental conditions. In Spain, PepMV-infected tomato plants showed symptoms only from autumn through spring and symptoms disappeared in late spring when higher temperatures occurred. Similar effects of high temperatures and high light conditions have been reported from other countries. However, symptoms on PepMV-infected plants can be highly variable, ranging from very mild leaf symptoms to severe leaf and stem necrosis and fruit symptoms. Mild, chlorotic or necrotic PepMV isolates from both the EU-tomato and the Ch2 strains have been reported. This suggests that the capacity of the virus to induce dif-

ferent symptoms is a property of the isolate rather than of the strain. Mixed infections with different strains of PepMV are relatively common, especially with the EU-tomato and Ch2 strains. Synergism resulting from mixed infections of the EU-tomato and Ch2 strains has been reported (Hanssen *et al.,* 2009), but this is not very common. The precise effects of the mixed infections on the symptomatology of the disease caused by PepMV are unknown. In addition it should be noted that it is not always clear what the possible contribution of other plant pathogens in the expression of symptoms may be.

PepMV is a mechanically transmitted virus. The most important transmission routes are through contaminated tools, clothes and surfaces. The virus is relatively stable at room temperature and can survive and stay infectious for several weeks in plant debris and on contaminated surfaces. Fruit from infected plants may contain high concentrations of the virus even if they do not show symptoms. Long distance transmission of the virus with fruit is highly likely and much more likely than transmission through infected seeds. Insects may also play a role in spreading the virus within and between crops. Bumble bees, often used as pollinators in glasshouses, were shown to transmit the virus (Shipp *et al*., 2008). Implementation of strict hygiene protocols during the growing season and thorough cleaning of greenhouses at the end of the growing season have shown to effectively control the introduction and spread of the virus.

Effective control of the virus in commercial tomato crops should be based on the use of virus-free seeds and planting material, strict hygiene measures and a constant monitoring of possible infections.

Literature cited

Aguilar, J.M., Hernandez-Gallardo, M.D., Cenis, J.L., Lacasa, A. and Aranda, M.A. 2002. Complete sequence of the *Pepino mosaic virus* RNA genome. *Archives of Virology*, 147: 2009-2015.

Alfaro-Fernández, A., Cebrián, M.C., Córdoba-Sellés, M.C., Herrera-Vásquez, J.A. and Jordá, C. 2008. First report of the US1 strain of *Pepino mosaic virus* in tomato in the Canary Islands, Spain. *Plant Disease*, 92: 1590.

Alfaro-Fernández, A., Sánchez-Navarro, J.A., Cebrián, M.C., Córdoba-Sellés, M.C., Pallas, V. and Jordá, C. 2009. Simultaneous detection and identification of *Pepino mosaic virus* (PepMV) isolates by multiplex one-step RT-PCR. *European Journal of Plant Pathology*. DOI 10.1007/s10658-009-9466-7.

Bancroft, J.B., Rouleau, M., Johnston, R., Prins, L. and Mackie, G.A. 1991. The entire nucleotide sequence of foxtail mosaic virus. *Journal of General Virology*, 72: 2173-2181.

Córdoba-Sellés, M.C., García-Rández, A., Alfaro-Fernández, A. and Jordá-Gutiérrez, C. 2007. Seed transmission of *Pepino mosaic virus* and efficacy of tomato seed disinfection treatments. *Plant Disease*, 91: 1250-1254.

Cotillon, A.C., Girard, M. and Ducouret, S. 2002. Complete sequence of genomic RNA of a French isolate of *Pepino mosaic virus*. *Archives of Virology*, 147: 2231-2238.

Hanssen, I.M., Paeleman, A., Wittemans, L., Goen, K., Lievens, B., Bragard, C., Vanachter A.C.R.C. and Thomma, B.P.H.J. 2008. Genetic characterization of *Pepino mosaic virus* isolates in Belgian greenhouses reveals genetic recombination. *European Journal of Plant Pathology*, 121: 131-146.

Hanssen, I.M., Paeleman, A., Vandewoestijne, E., Van Bergen, L., Bragard C., Lievens, B., Vanachter A.C.R.C. and Thomma, B.P.H.J. 2009. *Pepino mosaic virus* isolates and differential symptomatology in tomato. *Plant Pathology*, 58: 450-460.

Hanssen, I.M., Mumford, R Blystad, D-R, Cortez, I., Hasiów-Jaroszewska, B., Hristova, D., Pagán, I., Pereira, A.M., Peters, J., Pospieszny, H., Ravnikar, M., Stijger, I., Tomassoli, L., Varveri, C., Van der Vlugt, R. and Nielsen, S.L. Seed transmission of *Pepino mosaic virus* in tomato. *European Journal of Plant Pathology* (in Press).

Jones, R.A.C., Koenig, R. and Lesemann, D.E. 1980. *Pepino mosaic virus*, a new potexvirus from pepino (*Solanum muricatum*). *Annals of Applied Biology*, 94: 61-68.

Jordá, C., Lázaro-Pérez, A. and Martínez-Culebras, P.V. 2001. First report of *Pepino mosaic virus* on natural hosts. *Plant Disease*, 85: 1292.

Kadaré, G. and Haenni, A-L. 1997. Virus-encoded RNA helicases. *Journal of Virology*, 71: 2583-2590.

Kamer, G. and Argos, P. 1984. Primary structural comparisons of RNA-dependent RNA-polymerases from plant, animal and bacterial viruses. *Nucleic Acids Research*, 12: 7269-7282.

Krinkels, M. 2001. PepMV causes sticky problems. *Prophyta*, May 2001: 30-33.

Ling, K.S. 2007. Molecular characterization of two *Pepino mosaic virus* variants from imported tomato seed reveals high levels of sequence identity

between Chilean and US isolates. *Virus Genes*, 34: 1- 8.

Ling, K.S., Wechter, W.P. and Jordan, R. 2007. Development of a one-step immunocapture real-time TaqMan RT-PCR assay for the broad-spectrum detection of *Pepino mosaic virus*. *Journal of Virological Methods*, 144:65-72.

López, C., Soler, S. and Nuez, F. 2005. Comparison of the complete sequences of three different isolates of *Pepino mosaic virus*: size variability of the TGBp3 between tomato and *Lycopersicon peruvianum* isolates. *Archives of Virology*, 150: 619-627.

Maroon-Lango, C.J., Guaragna, M.A., Jordan, R.L., Hammond, J., Bandla, M. and Manquardt, S.K. 2005. Two unique US isolates of *Pepino mosaic virus* from a limited source of pooled tomato tissue are distinct from a third (European-like) US isolate. *Archives of Virology*, 150: 1187-1201.

Martínez-Culebras, P.V., Lazaro, A., Abad-Campos, P. and Jordá, C. 2002. A RT-PCR assay combined with RFLP analysis for detection and differentiation of isolates of *Pepino mosaic virus* from tomato. *European Journal of Plant Pathology*, 108: 887-892.

Mumford, R.A. and Metcalfe, E.J. 2001. The partial sequencing of the genomic RNA of a UK isolate of *Pepino mosaic virus* and the comparison of the coat protein sequence with other isolates from Europe and Peru. *Archives of Virology*, 146: 2455-2460.

Pagán, I., Córdoba-Sellés, M., Martinez-Priego, L., Fraile, A., Malpica, J.M., Jordá, C. and García-Arenal, F. 2006. Genetic structure of the population of *Pepino mosaic virus* infecting tomato crops in Spain. *Phytopathology*, 96: 274.

Rozanov, M.N., Koonin, E.V. and Gorbalenya, A.E. 1992. Conservation of the putative methyltransferase domain: a hallmark of the 'Sindbis-like' supergroup of positive-strand RNA viruses. *Journal of General Virology*, 73: 2129-2134.

Shipp, J.L., Buitenhuis, R., Stobbs, L., Wang, K., Kim, W. S. and Ferguson, G. 2008. Vectoring of *Pepino mosaic virus* by bumble-bees in tomato greenhouses. *Annals of Applied Biology*, 153: 149-155.

Soler, S., Prohens, J., Díez, M.J. and Nuez, F. 2002. Natural occurrence of *Pepino mosaic virus* in *Lycopersicon* species in Central and Southern Peru. *Journal of Phytopathology*, 150: 49-53.

Spence, N.J., Basham, J., Mumford, R.A., Hayman, G., Edmondson R., Jones, D.R. 2006. Effect of *Pepino mosaic virus* on the yield and quality of glasshouse-grown tomatoes in the UK. *Plant Pathology*, 55:595-606.

Van der Vlugt, R.A.A., Stijger, C.C.M.M., Verhoeven, J.Th.J. and Lesemann, D.E. 2000. First report of *Pepino mosaic virus* in tomato. *Plant Disease*, 84: 103.

Van der Vlugt, R.A.A., Cuperus, C., Vink, J., Stijger, C.C.M.M., Lesemann, D.E, Verhoeven, J.Th.J. and Roenhorst, J.W. 2002. Identification and characterization of *Pepino mosaic potexvirus* in tomato. *EPPO Bulletin*, 32: 503.

Verchot-Lubicz, J., Ye, C-M and Bamunusinghe, D. 2007. Molecular biology of potexviruses: recent advantages. *Journal of General Virology*, 88: 1643-1665.

Verhoeven, J.Th.J., Van der Vlugt and Roenhorst, J.W. 2003. High similarity between tomato isolates of *Pepino mosaic virus* suggests a common origin. *European Journal of Plant Pathology*, 109: 419-425.

White, K.A., Bancroft, J.B. and Mackie, G.A. 1992. Mutagenesis of a hexanucleotide sequence conserved in potexvirus RNAs. *Virology*, 189: 817-820.

Wong, S.M., Mahtani, P.H., Lee, K.C., Yu, H.H., Tan, Y., Neo, K.K., Chan, Y., Wu, M. and Chang, C.G. 1997. *Cymbidium mosaic potexvirus* RNA: complete nucleotide sequence and phylogenetic analysis. *Archives of Virology*, 142: 383-391.

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ΑΡΘΡΟ ΑΝΑΣΚΟΠΗΣΗΣ

Ιός του μωσαϊκού του πεπίνο (*Pepino mosaic virus*)

R.A.A. van der Vlugt

Περίληψη Ο ιός του μωσαϊκού του πεπίνο (*Pepino mosaic virus*, PepMV) είναι ένας νέος σχετικά ιός που αναδείχθηκε σε σημαντικό αγρονομικό πρόβλημα μέσα σε σύντομο χρονικό διάστημα. Ο ιός είναι μέλος του γένους *Potexvirus* της οικογένειας *Flexiviridae* και μεταδίδεται εύκολα με μηχανικό τρόπο. Προσβάλλει την τομάτα (*Solanum lycopersicum*) και άλλα σολανώδη. Από την αρχική του περιγραφή το 1980 σε φυτά πεπίνο (*Solanum muricatum*), που συλλέχθησαν στο Περού το 1974, ο ιός παρέμει-

νε άγνωστος για μεγάλο χρονικό διάστημα μέχρι την εμφάνισή του σε καλλιέργειες τομάτας στην Ευρώπη το 1999. Από τότε ο ιός έχει εξαπλωθεί παγκόσμια και προκαλεί σημαντικά προβλήματα σε εμπορικές καλλιέργειες τομάτας. Από το 1999 έχουν εμφανιστεί νέα στελέχη του ιού που διαφέρουν από την αρχική απομόνωση από φυτά πεπίνο. Η ταχεία μετάδοση του ιού καθώς και η εμφάνιση μικτών μολύνσεων με τα νέα στελέχη παίζουν πιθανόν σημαντικό ρόλο στην αύξηση της σοβαρότητας της ασθένειας που προκαλεί ο ιός.

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Effectiveness of *Apis mellifera* and *Bombus impatiens* as dispersers of the Rootshield® biofungicide (*Trichoderma harzianum*, strain T-22) in a strawberry crop

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Summary Two experiments were performed in Québec (Canada), in the field and in a greenhouse, to assess the effectiveness of *Apis mellifera* L. and *Bombus impatiens* Cresson (Hymenoptera: Apidae) in transmitting *Trichoderma harzianum* Rifai, strain T-22, to strawberry flowers from hives-mounted dispensers containing the biofungicide Rootshield®. The number of Colony Forming Units (CFU) of *T. harzianum* was determined: (i) on bees exiting from hives-mounted dispensers containing the biofungicide; (ii) on foragers visiting the strawberry flowers; (iii) on open flowers exposed to pollinator visits; (iv) on flowers that had only one visit, and (v) on flowers without visits. The results showed that both honey bees and bumble bees have the potential to get dusted with *T. harzianum* from the Houle-dispensers and disperse the biofungicide to the flowers. The assay with bumble bees allowed to obtain positive correlations between the CFU per bumble bee and the CFU per flower and also, between the length of visit and the CFU per flower. The density of inoculum that was deposited after one single visit of honey bee or bumble bee was also determined. Advantages and limitations of the used hive-dispensers were identified for future improvements.

Additional keywords: bumble bees, disseminators, honey bees, inoculum dispenser

Introduction

The use of pollinator insects to disperse biological control agents is a relatively recent technique, which has been tested in several scientific works since 1990. The technique has already been tested for the control of several pests affecting diverse agricultural crops (2, 7, 11, 14, 16, 23, 30, 31). The pollinators mainly used were honey bees and bumble bees as they have hives easier to manipulate although, more recently, these types of studies have been using other species such as *Osmia cornuta* Latreille (Hymenoptera: Megachilidae) (22). This new technique makes pollinators useful by two means which influence productivity of crops: pollination and crop protection. This method can be a practical option for growers working under integrated and biological pest management requirements (20).

Historically, however, this biocontrol method has mostly been used to fight grey mould (*Botrytis cinerea* Persoon: Fries), which is the main fungal pathogen affecting berry fruit crops like strawberry (17, 18, 19, 25, 27, 29). Since in this pathosystem the flower serves as an infection site (6), the dispersal of a control agent directly to the flowers against this pathogen thus explains the success of this technique on this crop. Furthermore, the use of bee colonies to assure a better pollination of strawberry is a common practice among farmers since several stud-

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ies have shown the importance of pollinating insects for the improvement of strawberry crop production, in terms of fruit quality (size, weight and shape) (1, 4, 8, 9, 24).

In Québec, the CRAAQ (Centre de Référence en Agriculture et Agroalimentaire du Québec) recommends strawberry growers to apply the fungicide chlorothalonil at the vegetation and two other sprays of other registered fungicides for grey mould during flowering. As a consequence, only those flowers that are open during that moment become protected. After flowering, the fungicide labels recommend several applications at 7- to 10-day intervals or at 5-day intervals if conditions are favourable for the spread of the disease. Although some studies point out the negative consequences of some fungicides on strawberry pollen germination (13), these products are most often used without the knowledge of this fact. Furthermore, the repulsive behaviour of those fungicides to pollinators has apparently never been examined. Another identified problem is the development of resistant forms of *B. cinerea* to some fungicides used in strawberry plantations (12).

The present study is part of a wider framework that aims at reducing the amount of fungicides used to fight the grey mould fungus, bringing down production costs and increasing the treatment efficacy by dispersing selected fungicides that are non-toxic for pollen and non-repulsive for pollinators, such as honey bees and bumble bees. This project is pioneering in the sense that it included the testing of the dispersion of a biofungicide and synthetic fungicides in its global scope, using dispensers that are adapted either to the hives of honey bees or those of bumble bees. Previous tests were performed in order to select the non-toxic and non-repulsive fungicides both for strawberry pollen and pollinators, respectively. Among these fungicides the biofungicide Rootshield®, a commercial formulation of the antagonist *Trichoderma harzianum* Rifai, and several synthetic fungicides were selected (10).

T. harzianum is an antagonistic fungus of a wide range of pathogens including *B. cinerea* that is responsible for grey mould on strawberry (3, 15). There is a diversity of mechanisms available to *Trichoderma* spp. for pathogen suppression, including (i) the production of antifungal metabolites, (ii) mycoparasitism, and (iii) competition with the pathogen for nutrients and space. These features make this fungus attractive as a biocontrol agent (26).

The scope of the present study included the need to demonstrate the efficiency of the dispersion of fungicides by pollinators that pass through the dispenser to the flowers that they visited. Because this task is easier and cheaper to achieve using conidia of *T. harzianum* that can be grown on agar medium, than synthetic fungicides which require costly analysis, this validation was performed only with the Rootshield® biofungicide.

The main purpose of this study - which intends to satisfy one of the tasks of the wider project that was mentioned above – was therefore to determine the effectiveness of honey bees and bumble bees in transmitting the powder formulation of *T. harzianum* from hives-mounted dispensers containing the biofungicide Rootshield® to flowers in open field and greenhouse assays.

Materials and Methods

1. Dispensers

The construction of the first dispenser model for honey bees was based on works by Gross *et al.* (16) and Kovach *et al.* (17). Modifications were made to this model by Emile Houle. After several tests, the original model was gradually improved before a final model, now called the Houle-dispenser, was attained (Figure 1). This later model is entirely made of folded sheets, providing the potential for its fast and easy construction. Furthermore, several holes were incorporated on the sides in order to avoid internal water condensation. The new design allows the dispenser to be adjusted to the beehive entry without difficulty. The dispenser has a system that makes bee entries and exits in-

dependent from each other. The fungicide container (drawer) can be removed and replaced in order to be emptied and cleaned without effort.

A small dispenser that is adjustable to commercial bumble bee hives was also developed by Emile Houle, based on work by Kovach *et al.* (17). Following preliminary tests, it was also modified in order to allow bees to exit and enter the hive by separate paths (Figure 2) therefore avoiding fungicide to be carried inside the hive. Additional procedures were later carried out in order to assure a better adaptation of bumble bees to the dispensers: (i) the area surrounding the entrance holes were coloured in yellow (A') (Figure 2) in order to help the insects to detect and remember their location, and (ii) the dipensers were installed on the very first day the hives were open. Koppert hives, used in our experiments, have an one-way door for entries (A) and a two-way door for entries and exits (B) (Figure 2). Following the application of the above mentioned procedures, bumble bees were observed always entering door A' and exiting door B' (Figure 2).

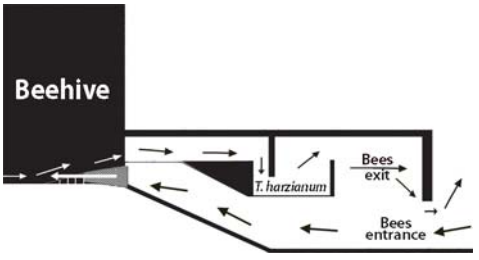

Figure. 1. Houle-dispenser adapted to hives for honey bees [drawing by Jocelyn Boulianne in Chagnon *et al.* (10)].

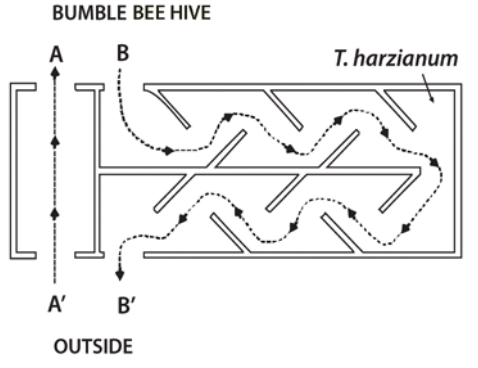

Figure 2. Houle-dispenser adapted to commercial hives for bumble bees. A - colony box entrance, B - colony box entrance/exit, A'- dispenser entrance, B'- dispenser exit.

2. Experimental procedure

The assays for both honey bees and bumble bees took place in Québec, Canada, during June and July 2005. The honey bee assay was done at Île d'Orléans, and at the Saint-Lambert Experimental Station (IRDA). Four honey bee [*Apis mellifera* L. (Hymenoptera: Apidae)] hives with 20 frames each were placed at about 50 meters from the 1 ha experimental strawberry [*Fragaria* × *ananassa* Duch. (Rosaceae)] fields with the cultivar Seascape. The beehives at the Saint-Lambert Experimental Station were used only for the capture of honey bees exiting from hives-mounted dispensers containing *T. harzianum*. The bumble bee assay took place in July 2005 in a greenhouse (80 m^2) at the IRDA (Institut de Recherche et Développement en Agroenvironnement), in which a Koppert commercial hive for bumble bees [*Bombus impatiens* Cresson (Hymenoptera: Apidae)] was installed. The cultivar used was also Seascape.

The methodological approaches were similar for both the field and greenhouse activities as well as for the laboratory procedures. In order to allow a better adaptation of the bees, empty dispensers were fastened to the hives a few days before the experiments begun.

2.1. Fieldwork

All the dispensers used in the experiments were filled on a daily basis with the biocontrol agent *T. harzianum*, strain T-22 (commercial label: Rootshield®, BioWorks, Inc). Every morning, when the weather conditions were favourable for bee activity, the dispensers in the honey bee and bumble bee hives were filled with 6g and 2g of the biofungicide, respectively. The biofun-

gicide was kept in the freezer (-16º C) until it was placed in the dispensers. The preparation of *T. harzianum* used in the experiments contained a 9.8 x 10^6 Colony Forming Units (CFU)/g formulation.

2.1.1. Inoculum on pollinators

For the honey bee assay, a sample of 30 honey bees exiting from hives-mounted dispensers containing *T. harzianum* was collected. These captures were performed about one hour after the filling. In the bumble bee assay a sample of 10 bumble bees exiting from the hives-mounted dispensers with *T. harzianum* was collected in the following three hours after the filling.

In both experiments, a sample of 30 pollinator foragers visiting the strawberry flowers was also collected. During the honey bee assay, there were no other beehives installed at a radius of 3 to 4 km. The foraging bee samples collected in the experimental field thus most probably belonged to the hives-mounted dispensers.

2.1.2. Inoculum deposited on flowers

During each assay (honey bee and bumble bee), a total of 120 flower buds were randomly marked in two groups: 60 flower buds were isolated with a small tulle bag to exclude all insect visits during the experiment (control flowers); the other 60 flower buds were kept unscreened to bee visits. For this second group, every morning the flower buds were checked, and the newly open flowers were left exposed to the bees for that day and collected at the end of it.

Additionaly to these two groups of flowers, a third group was defined in each of the assays: flowers exposed to a single visit. For that, in a given location of the field and of the greenhouse, a large area of the strawberry plants was kept screened with a mesh cloth until experimental observations were performed. During the observation periods, the screens were removed and the open virgin flowers were exposed to a single visit. In the honey bee and bumble bee assays, single bee visits were recorded for 20 and 30 flowers, respectively.

For the bumble bee assay, when an open virgin flower was visited, the arrival and departure of the visiting bee was vocally registered with a tape recorder for a later determination of the length of each visit. After a sample of 30 flowers (mentioned above), the following 12 observed flowers were allowed to be visited two or three times each, performing a total of 42 observed flowers. Whenever possible, the bumble bee that made the corresponding visit was captured, in a total of 30 insects.

2.2. At the laboratory

The insect and flower samples collected during each assay were individually stored in a glass container and carried to the laboratory. All samples were kept preserved in a freezer (-16º C) before their analysis.

To each sample collected during fieldwork (flowers and insects) 20 ml of distilled water with Tween (0.01%) were added and the resulting suspension was shaken for one hour. Subsequently, 400µl of the original suspension were plated on a *Trichoderma* selective medium. This growth medium, modified from Smith *et al.* (28), contained the following chemical compounds (g/L): $Ca(NO_3)_2$, 1.0; KNO_3, 0.26; $MgSO_4 . 7H_2O$, 0.26; KH_2PO_4, 0.12; $CaCl_2.2H_2O$, 1.0; citric acid, 0.05; sucrose, 2.0; agar, 20.0. After adjusting the pH to 4.5 and when temperature decreased to 45ºC, the following antibiotics and fungicides were added (g/L): tetracycline, 0.05; streptomycine, 0.10; Senator® (70%) (oxychloride + maneb + sulphur), 0.0143 and quintozene (75%), 0.02666. Three Petri dishes (replicates) were used for each sample. No dilutions were performed with the exception of the samples of bumble bees that were captured while exiting the hives.

The Petri dishes were kept in the laboratory at 22ºC and the number of colonies that developed was counted about eight days after plating took place.

2.3. Data analysis

For all the samples (flowers and insects) that resulted from the two experiments, the mean number of CFU was estimated from

the three replicates mentioned above. For each sample group, the means were averaged and the respective standard deviations were computed. The percentage of flowers or insects in which the presence of *T. harzianum* was detected was also calculated for each analysed sample group. In order to compare the *T. harzianum* CFU density per flower between the flowers freely exposed to pollinator's visits and those exposed solely to a single visit, non-parametric Mann-Whitney *U* tests were performed. For the bumble bee assay Spearman correlations between the number of CFU per visiting bumble bee and the number of CFU per flower, and also between the total length of visits and the number of CFU per flower, were computed.

The significance level used for all the tests was 5%. Statistical tests were performed with STATISTICA version 7.0 [StatSoft, Inc. (2004)].

Results

1. Inoculum on pollinators

In both the honey bee and bumble bee assays, all the individuals that were captured while exiting the hives-mounted dispensers carried the inoculum. The mean density of inoculum transported per insect was 3.92 x 10^3 ± 1.73 x 10^3 CFU for honey bees and 7.19 x 10^4 ± 2.17 x 10^4 CFU for bumble bees.

Approximately 40% of the honey bees that were captured during their foraging activity carried remaining traces of *T. harzianum,* with a density of 53.33 ± 95.43 CFU per bee. On all bumble bees that were caught while visiting flowers the presence of the biofungicide was detected with a mean density of 6.59 x 10^3 ± 1.92 x 10^3 CFU per bumble bee.

2. Inoculum deposited on flowers

In both the assays, the presence of *T. harzianum* was not detected in the control flowers. However, the fungus was detected in other samples of plant material taken from locations where the experiments took place.

In the honey bee assay, there was no significant difference between the mean density of inoculum found on freely exposed flowers and on flowers that were exposed to a single visit (Z= 0.75; P=0.45), although the latter showed a higher density of CFU (Table 1). The percentage of flowers having detectable biofungicide was almost the same for both flower samples (Table 1).

In the bumble bee assay, the flowers exposed to a single bumble bee visit showed an average number of CFU per flower significantly lower than flowers that were freely exposed to bee visits (Z= -7.28; P < 0.0001) (Table 2). While 100% of the freely exposed flowers contained *T. harzianum*, only 75% of the flowers exposed to single visits had ves-

Table 1. Percentage of flowers in which the presence of *T. harzianum* has been detected, and means of the number of CFU per freely exposed flowers and per flower exposed to a single honey bee visit.

	% of flowers with *T. harzianum*	CFU/flower (mean ± s.d.)
Freely exposed flowers to honey bee visits	33.33%	26.27 ± 87.99 a
Flowers exposed to a single honey bee visit	34.78%	118.84 ± 222.44 a

Means within a column followed by the same letter do not differ significantly.

Table 2. Percentage of flowers in which the presence of *T. harzianum* has been detected, and means of the number of CFU per freely exposed flowers and per flower exposed to a single bumble bee visit.

	% of flowers with *T. harzianum*	CFU/flower (mean ± s.d.)
Freely exposed flowers to bumble bee visits	100%	1.25 x 10^3 ± 8.97 x 10^2 b
Flowers exposed to a single bumble bee visit	75%	123.44 ± 196.01 a

Means within a column followed by the same letter do not differ significantly.

tiges of the biofungicide presence (Table 2).

Spearman correlation analyses performed on data obtained from the bumble bee assay showed a positive correlation between the number of CFU per bumble bee forager and the number of CFU per correspondent visited flower (r_s = 0.45; n= 28; P= 0.01). A positive correlation was also found between the total length of visits and the number of CFU per flower (r_s = 0.33; n= 42; P= 0.03).

Discussion and conclusions

Inoculum picked up by pollinators exiting the hive-mounted dispenser

The average load of inoculum carried by each honey bee exiting the hives-mounted dispensers was lower than that mentioned in other works for *T. harzianum* (17, 19, 27). Although the sample size was not very large, the amount of inoculum on bumble bees exiting the hive was comparable to that obtained in other work with *Bombus terrestris* using another dispenser model [OP-dispenser- 4.3 x 10^4 CFU per bumble bee (21)].

However, the results of the studies mentioned above cannot be directly compared to ours, since (i) most of the studies used dispenser models different from ours (ii) some of them do not mention the quantity of inoculum that is placed in the dispenser, and (iii) some do not indicate the length of time between the filling of the dispenser with biofungicide and insects capture. Moreover, in other studies (17) small nuclear honey bee hives were used while in the present study we used standard hives. As mentioned by Bilu *et al.* (5), some of these factors may influence the dispenser performance, and, therefore, in case these factors are not considered and measured, correct comparison of the effectiveness between distinct dispenser types is not possible.

Bee vectoring dynamics in open field and greenhouse conditions

According to the results, the load of inoculum on the bees while exiting from hives dispensers is apparently more rapidly lost to the environment when the vector is a honey bee than when it is a bumble bee. In the honey bee assay the inoculum could have been lost in several ways: (i) losses during flight activity and grooming behaviour, (ii) transfer of inoculum to the flowers of the target crop, or (iii) transfer of inoculum to competitor flowers. Since the honey bee assay was carried out in open field conditions and within a more extensive intervention area, honey bees probably lost the inoculum deposited on their bodies more rapidly as they were successively transferring it to the flowers throughout their foraging trip. As a result, only 40% of the honey bees captured during their foraging activity had vestiges of the inoculum on their body, and quantities were much smaller than those found on the bumble bee foragers. Probably for the same reason, only 33.33% of flowers freely exposed to honey bee visits were shown to carry the inoculum. The high percentage of non-colonised flowers in the honey bee assay could be due to the lack of honey bee visits or because visits were depleted of inoculum. Yu and Sutton (31) point out the same explanation for the absence of propagules of *Gliocladium roseum* that was recorded in 5-20% of the flowers exposed to pollinator visits.

In both assays the inoculum density that was deposited on flowers exposed to pollinators was very variable. The large variability in the number of deposited CFU per flower, reflected in high standard deviations, imposes some caution in this data interpretation. Other studies also detected this variability and several factors were pointed out for explaining that: heterogeneity of the inoculum loads in bees, bee vectoring dynamics, orientation of flowers, redistribution of inocula by the bees, other organisms, air currents, rain and other factors (2, 31) and, finally, microclimatic conditions that could influence the attractiveness of flowers (27).

The mean density of inoculum detected on freely exposed flowers, in the assay with honey bees, was considerably lower than

that observed in other studies focused on the dispersion of several biocontrol agents in open field conditions (Table 1) (11, 30, 31). Shafir *et al.* (27) have detected 2.2 x 10^4 CFU of *T. harzianum* per flower on strawberry.

As mentioned earlier, these comparisons should be made with caution since most of these studies differ in some methodological details, namely in the features - and hence in their performance - of the dispensers used, in the visiting rates and in the respective length of visits to which flowers have been exposed. For example, while in most experimental studies the flowers are exposed to foragers for two (11) or three days (27), in our honey bee assay, flowers were exposed for only one day. Although attention was paid to collecting flower samples during favourable sunny days, we observed that foraging activity in the experimental field was relatively low. The low foraging activity was attributed to the existence of competitor flora in the vicinities of the experimental field or the relatively low attractivity of the chosen cultivar. Nevertheless, this result shows that this biological control method may be compromised by factors that have an effect on pollinator activity, such as climatic conditions, attractiveness of the chosen cultivar and presence of competitor flora in the vicinity of the target crop.

Factors mentioned earlier to explain the high variability of *T. harzianum* densities on flowers exposed to pollinators, together with low foraging activity, may explain the inexistence of significant differences between the densities of *T. harzianum* deposited on freely exposed flowers and on those that had a single honey bee visit. As the foraging activity was so low, the *T. harzianum* that is brushed off from freely exposed flowers by different factors, such as air currents and redistribution of inocula by the honey bees and other organisms, is probably not resettled by successive visits of honey bees. As a consequence, the resulting density of inoculum would then be statistically comparable to that obtained with a single visit.

In the experiment using bumble bees, undertaken in a closed greenhouse and with a limiting number of available flowers, inoculum losses during flight and grooming could only occur in a restricted space and through the repeated transfer of biofungicide within the same set of flowers. As a result, the bumble bees in foraging activity retained a high amount of inoculum and consequently all the flowers that were freely exposed to their visits showed inoculum densities comparable to those mentioned in other works (Table 2). More specifically, Yu and Sutton (31) detected densities of about 0.45 x 10^3 – 2.5 x 10^3 of *G. roseum* on raspberry flowers and Maccagnani *et al.* (21) observed densities of about 0.14 x 10^3 of *T. harzianum* on tomato flowers, using the OP-dispenser. Under greenhouse conditions, not only the probability of *T. harzianum* being brushed off from flowers was lower, since the factors early mentioned were controlled (for example air currents, rain and the presence of other organisms that could act as inoculum dispersers), but also bumble bee activity was greater allowing the accumulation of biofungicide throughout several visits.

This study also suggests the importance of some parameters like the number of CFU per bumble bee and the length of visit in determining the amount of inoculum that is deposited on flowers. We also determined the density of inoculum that was deposited after one single visit of honey bee or bumble bee. Even though the present work does not allow a direct comparison between honey bees and bumble bees in their effectiveness as dispersers, since the assays were undertaken at different experimental conditions, the results demonstrated that bumble bees ensured, in greenhouse conditions, a higher percentage of flowers dusted with *T. harzianum* – even after one single visit - than honey bees, in open-field conditions. Previous works (31) showed that bumble bee colonies, although possessing fewer individuals, were at least as efficient vectors of *G. roseum* as honey bee colonies. The same authors demonstrated that *B. impatiens* would be the preferred vector when the weather is cool and wet, and honey bees would be preferable under warm conditions.

Advantages and limitations of dispensers

The Houle-dispensers for honey bees, when loaded with *T. harzianum* and used as in this study, had some of the requisites that distinguish efficient dispensers: (i) by avoiding the transfer of inoculum to the hive's interior by having separate entrances and exits, (ii) by assuring that insects get dusted while exiting the hives-mounted dispensers, and (iii) by not having negative impacts on the health of honey bee colonies. This latter point was shown by an extensive study on honey bee colony development and honey production in relation to the use of the inoculum loaded dispensers placed on hives during the three week strawberry flowering period (10). Nevertheless, in an open field situation some technical problems were detected after a long-term use of the dispenser for honey bees. The main limitation found was related to the maintenance of the biocontrol agent in good condition for its dispersal. According to our observations, some water condensation in the interior of the dispensers was observed, which resulted in alterations in the properties of the biocontrol agent (cluster formation) that obviously affected the efficacy of its dispersion by pollinators. What happens is that the humidity formed inside the hive during the night turns out to be more difficult to evaporate due to the presence of the dispenser in the hive entrance. This problem was partially solved by drilling holes in the sidewalls of the dispensers. Other practical recommendations to minimize this problem are to place the hives in early morning sun locations and to replace the biofungicide on a daily basis after cleaning the dispensers.

Regarding Houle-dispensers for bumble bees, the most important advantage was to have entries and exits independent from each other. This was an important improvement when compared with previous dispensers (17) allowing less impact on hive tunnel traffic and avoiding the transfer of inoculum into the hive. As in the Houle-dispenser for honey bees, the major limitation detected was related to the maintenance of the biocontrol agent in good condition for its dispersal. In this case, the main problem was bumble bees letting their liquid excrements over the powder formulation, which causes it to be no longer available for dispersion. Maccagnani *et al.* (21) identified the same problem with their SSP-dispenser.

Further investigation should be directed towards increasing the dispensers' efficiency, which necessarily implies the optimisation of the existing models and eventually the creation of new models. As such, we agree with Bilu *et al.* (5) that stressed the need for comparing different kinds of dispensers, using standard methodologies in order to establish an adequate dataset for correctly assessing the technique for the dispersion of biocontrol agents by bees.

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Literature Cited

1. Albano, S., de Oliveira, D., Salvado, E., Quaresma, M., Borges, P. and Mexia, A. 2005. Avaliação da importância dos insectos polinizadores na melhoria da produção na cultura do morango. Actas da Associação Portuguesa de Horticultura, II Colóquio Nacional da Produção de Morango e Pequenos Frutos, São Teotónio: 121-129.
2. Al-mazra'awi, M.S., Shipp, L., Broadbent, B. and Kevan, P. 2006. Biological control of *Lygus lineolaris* (Hemiptera: Miridae) and *Frankliniella occidentalis* (Thysanoptera: Thripidae) by *Bombus impatiens* (Hymenoptera: Apidae) vectored

Beauveria bassiana in greenhouse sweet pepper. *Biological Control*, 37: 89-97.

3. Batta, Y.A. 1999. Biological effect of two strains of microorganisms on *Botrytis cinerea*: causal organism of gray mold on strawberry. *An-Najah University Journal for Research* – A (Natural Sciences), 13: 67-83.
4. Bigey, J., Vaissière, B.E., Morison N., Longuesserre, J. and Capy, A. 2002. Fraise/cultures précoces - Amélioration de la pollinisation. *Réussir Fruits & Légumes*, 203: 76-78.
5. Bilu, A., Arnon, D., Yigal, E. and Shafir, S. 2004. Honeybee dispersal of biocontrol agents: an evaluation of dispensing devices. *Biocontrol Science and Technology*, 14: 607-617.
6. Bristow, P.R., Mcnicol, J.R. and Williamson, B. 1986. Infection of strawberry flowers by *Botrytis cinerea* and its relevance to grey mould. *Annals of Applied Biology*, 109: 545-554.
7. Butt, T.M., Carreck, N.L., Ibrahim, L. and Williams, I.H. 1998. Honey-bee-mediated infection of pollen beetle (*Meligethes aeneus* Fab.) by the insect pathogenic fungus, *Metarhizium anisopliae*. *Biocontrol Science and Technology*, 8: 533-538.
8. Chagnon, M., Gingras, J. and de Oliveira, D. 1989. Effect of honeybee (Hymenoptera: Apidae) visits on the pollination rate of strawberries. *Journal of Economic Entomology*, 82: 1350-1353.
9. Chagnon, M., Gingras, J. and de Oliveira, D. 1993. Complementary aspects of strawberry pollination by honey and indigenous bees (Hymenoptera). *Journal of Economic Entomology*, 86: 416-420.
10. Chagnon, M., Thibodeau, P-O., de Oliveira, D., Albano, S., Boulianne, J. and Cadorette, E. 2006. Amélioration des rendements et de la qualité des fraises et framboises par une gestion écologique et rentable des fongicides et des pollinisateurs. Project report no.2070. Conseil pour le développement de l'agriculture du Québec. 81 pp.
11. Dedej, S., Delaplane, K.S. and Scherm, H. 2004. Effectiveness of honeybees in delivering the biocontrol agent *Bacillus subtilis* to blueberry flowers to suppress mummy berry disease. *Biological Control*, 31: 422-427.
12. Dianez, F., Santos, M., Blanco, R. and Tello J.C. 2002. Fungicide resistance in *Botrytis cinerea* isolates from strawberry crops in Huelva (southwestern Spain). *Phytoparasitica*, 30: 529-534.
13. Eaton, G.W. and Chen, L.J. 1969. Effect of the captan on strawberry pollen germination. *Journal of the American Society of Horticultural Sciences*, 94: 558-560.
14. Escande, A.R., Laich, F.S. and Pedraza, M.V. 2002. Field testing of honeybee-dispersed *Trichoderma* spp. to manage sunflower head rot (*Sclerotinia sclerotiorium*). *Plant Pathology*, 51: 346-351.
15. Freeman, S., Minz, D., Kolesnik, I., Barbul, O., Zveibil, A. and Maymon, M. 2004. *Trichoderma* biocontrol of *Colletotrichum acutatum* and *Botrytis cinerea* and survival in strawberry. *European Journal of Plant Pathology*, 110: 361-370.
16. Gross, H.R., Hamm, J.J. and Carpenter, J.E. 1994. Design and application of a hive-mounted device that uses honey-bees (Hymenoptera, Apidae) to disseminate *Heliothis* nuclear polyhedrosis-virus. *Environmental Entomology*, 23: 492-501.
17. Kovach, J., Petzoldt, R. and Harman, G.E. 2000. Use of honeybees and bumblebees to disseminate *T. harzianum* 295-22 to strawberries for *Botrytis* control. *Biological Control*, 18: 235-242.
18. McCandless, L. 1999. Honeybees deliver beneficial fungi to strawberries and increase yield. New York State Agricultural Experimental Station. Cornell University, Geneva, New York [on line]. Available from http://www.nysaes.cornell.edu/pubs/press/1999/bees.html [accessed 5 March 2008].
19. Maccagnani, B., Mocioni, M., Gullino, M.L. and Ladurner, E. 1999. Application of *Trichoderma harzianum* by using *Apis mellifera* as a vector for the control of grey mould of strawberry, first results. *IOBC Bulletin*, 22: 161-164.
20. Maccagnani, B., Ladurner, E., Tesoriero, D., Sgolastra, F. and Burgio, G. 2003. The use of *Osmia cornuta* (Latreille) (Hymenoptera, Megachilidae) for pear pollination: a reason to adopt low impact farming system in a landscape management perspective. *IOBC Bulletin*, 26: 101-106.
21. Maccagnani, B., Mocioni, M., Ladurner, E., Gullino, M.L. and Maini, S. 2005. Investigation of hive-mounted devices for the dissemination of microbiological preparations by *Bombus terrestris*. *Bulletin of Insectology*, 58: 3-8.
22. Maccagnani, B., Bazzi, C., Biondi, E., Tesoriero, D. and Maini, S. 2006. Potential of *Osmia cornuta* as a carrier of antagonistic bacteria in biological control of the fire blight: A comparison with *Apis mellifera*. *Acta Horticulturae*, 704: 379-386.
23. Ngugi, H.K., Dedej, S., Delaplane, K.S., Savelle, A.T. and Scherm, H. 2005. Effect of flower-applied Serenade biofungicide (*Bacillus subtilis*) on pollination-related variables in rabbiteye blueberry. *Biological Control*, 33: 32-38.
24. Paydas, S., Eti, S., Kaftanoglu, O., Yasa, E. and Derin, K. 2000. Effects of pollination of strawberries grown in plastic greenhouses by honeybees and bumblebees on the yield and quality of the fruits. *Acta Horticulturae*, 513: 443-451.
25. Peng, G., Sutton, J.C. and Kevan, P.G. 1992. Effectiveness of honey-bees for applying the biocontrol agent *Gliocladium roseum* to strawberry flowers to suppress *Botrytis cinerea*. *Canadian Journal of Plant Pathology*, 14: 117-129.
26. Perelló, A., Mónaco, C., Simón, M.R., Sisterna, S. and Dal Bello, G. 2003. Biocontrol efficacy of

Trichoderma isolates for tan spot of wheat in Argentina. *Crop Protection*, 22: 1099-1106.

27. Shafir, S., Dag, A., Bilu, A., Abu-Toamy, M., and Elad, Y. 2006. Honeybee dispersal of the biocontrol agent *Trichoderma harzianum* T39: effectiveness in suppressing *Botrytis cinerea* on strawberry under field conditions. *European Journal of Plant Pathology*, 116: 119-128.
28. Smith, V.L., Wilcox, W.F. and Harman, G.E. 1990. Potential for biological control of *Phytophthora* root and crown rots of apple by *Trichoderma* and *Gliocladium* spp. *Phytopathology*, 80: 880-885.
29. Sutton, J.C. 1995. Evaluation of micro-organisms for biocontrol: *Botrytis cinerea* and strawberry, a case study. *Advances in Plant Pathology*, 11: 173-190.
30. Thomson, S.V., Hansen, D.R., and Flint, K.M. 1992. Dissemination of bacteria antagonistic to *Erwinia amylovora* by honeybees. *Plant Disease*, 76: 1052-1056.
31. Yu, H. and Sutton, J.C. 1997. Effectiveness of bumblebees and honeybees for delivering inoculum of *Gliocladium roseum* to raspberry flowers to control *Botrytis cinerea*. *Biological Control*, 10: 113-122.

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Αποτελεσματικότητα των εντόμων *Apis mellifera* και *Bombus impatiens* ως παραγόντων διασποράς του βιομυκητοκτόνου Rootshield® (*Trichoderma harzianum*, στέλεχος T-22) στην καλλιέργεια της φράουλας

S. Albano, M. Chagnon, D.de Oliveira, E. Houle, P.O. Thibodeau and A. Mexia

Περίληψη Στην παρούσα μελέτη εκτιμήθηκε η αποτελεσματικότητα των ειδών *Apis mellifera* L. και *Bombus impatiens* Cresson (Hymenoptera: Apidae) ως παραγόντων μεταφοράς του βιομυκητοκτόνου Rootshield® (*Trichoderma harzianum*, στέλεχος T-22) στα άνθη φράουλας από διασπορείς προσαρμοσμένους σε κυψέλες (hives-mounted dispensers). Πραγματοποιήθηκαν δύο βιοδοκιμές, μία σε αγρό και μία σε θερμοκήπιο, στο Κεμπέκ του Καναδά. Ο αριθμός των πολλαπλασιαστικών μονάδων (Colony Forming Units, CFU) του *T. harzianum* προσδιορίστηκε: α) πάνω στις μέλισσες, κατά την έξοδό τους από τους διασπορείς, β) σε συλλέκτριες μέλισσες που επισκέπτονταν τα άνθη της φράουλας, γ) σε ανοικτά άνθη που δέχθηκαν την επίσκεψη επικονιαστών, δ) σε άνθη που δέχθηκαν μια επίσκεψη επικονιαστή, και ε) σε άνθη που δεν δέχθηκαν επίσκεψη επικονιαστή. Τα αποτελέσματα έδειξαν ότι τόσο οι μέλισσες όσο και οι βομβίνοι μπορούν να προσλάβουν το βιομυκητοκτόνο παράγοντα από τους διασπορείς και να τον μεταφέρουν στα άνθη. Η βιοδοκιμή με τους βομβίνους έδειξε ότι υπήρχε θετική συσχέτιση μεταξύ του CFU ανά άτομο βομβίνου, του CFU ανά άνθος φράουλας, καθώς και μεταξύ της διάρκειας της επίσκεψης του επικονιαστή και του CFU ανά άνθος. Επιπλέον, προσδιορίστηκε η πυκνότητα του μολύσματος που εναποτίθετο στα άνθη μετά από κάθε επίσκεψη μελισσών ή βομβίνων. Τέλος προσδιορίστηκαν τα πλεονεκτήματα αλλά και οι περιορισμοί των χρησιμοποιηθέντων κυψελών-διασπορέων με σκοπό τη μελλοντική τους βελτίωση.

Hellenic Plant Protection Journal **2:** 57-66, 2009

Hellenic Plant Protection Journal **2:** 67-69, 2009

SHORT COMMUNICATION

Occurrence of *Phomopsis* sp. on kiwi plantations in Northern Greece

K. Elena

Summary Symptoms of severe shoot cankers with internal wood discoloration were observed in commercial kiwi plantations, cv. Hayward, in the region of Skydra, Northern Greece. Additional symptoms included browning of the abaxial surface of leaves, necrotic, slightly depressed lesions with internal rot on fruit and peduncle necrosis resulting in formation of smaller fruit. The fungus *Phomopsis* sp. was consistently isolated from the diseased tissues. In artificial inoculations of shoots and detached fruit, symptoms of the disease similar to those occurring in nature (internal wood discoloration and fruit rot) were reproduced.

In September 2008, severe shoot necroses of kiwi plants (*Actinidia deliciosa* C.F. Liang. & A.R. Ferguson), cv. Hayward, with internal wood discoloration (Figure 1) were observed in commercial plantations in the region of Skydra, Northern Greece. Fruit rots commencing from necrotic, slightly depressed lesions (Figure 2), peduncle necrosis bearing fruit of reduced size and brown discoloration of the abaxial surface of the leaves, were also observed. Isolations were made from diseased tissues on PDA medium in order to identify the causal agent. Black pycnidia were produced in culture with two types of hyaline conidia: α-conidia, ellipsoid, one-celled, 5-8.5 × 2-3.5 μm and β-conidia, filiform, curved or bent, one-celled, 17.5-27.5 x 1.3 μm (Figure 3). The cultural and morphological characteristics of the isolates fitted the description of *Phomopsis* sp.

Pathogenicity was determined by wound-inoculating shoots of 2-year-old potted kiwi (cv. Hayward) plants and detached fruit with either a conidial suspension (10^6 conidia/ml) or a mycelial disk (8 mm in diameter), both deriving from 3-week-old cultures grown on PDA dishes at 22°C. One droplet (20 μl) of the conidial suspension was placed on top of each shoot or fruit wound (8 mm-diameter). In addition, an 8 mm in diameter disc of bark was removed from each shoot with a cork borer and a mycelial disk (8 mm in diameter) was inserted with the mycelium facing the shoot. Fruit were inoculated by inserting the mycelial disk 3-4 mm under the skin. Wounded shoots were wrapped with Parafilm one hour after inoculation, whereas fruit were placed in polyethylene bags immediately after inoculation. In another experiment, unwounded fruit were similarly inoculated with a conidial suspension or a mycelial disk. Control shoots and fruit were treated with sterile distilled water or sterile PDA disks. For each treatment, seven shoots and/or fruit with two wounds each were used as replicates. Inoculated and control shoots and fruit were incubated at 20-25°C. Ten and 15 days after inoculation, disease symptoms were observed on wounded shoots and fruit by means of wood discoloration and fruit rot, respectively and *Phomopsis* sp. was re-isolated from the artificially inoculated shoots and fruit. No symptoms were observed on unwounded fruit or control shoots and fruit.

The fungus has been reported to cause

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Figure 1. Kiwi shoot showing internal wood discoloration due to *Phomopsis* sp. infection.

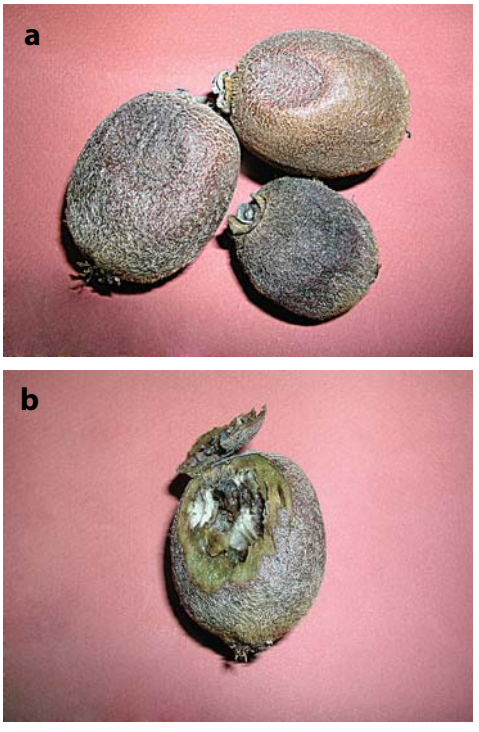

Figure 2. Necrotic lesions (a) and internal rot (b) of kiwi fruit infected by *Phomopsis* sp.

problems in other kiwi-growing countries too, and *Diaporthe actinidiae* is considered to be its perfect stage (1, 2, 3). *Phomopsis* sp. has been previously reported to cause leaf spots and shoot necroses of kiwi plantations in Western and Northern Greece, respectively (4). Kiwi is an economically important crop in Greece grown in areas characterised by humid climatic conditions, which presumably favour the infection and the spread of the disease during the growing season.

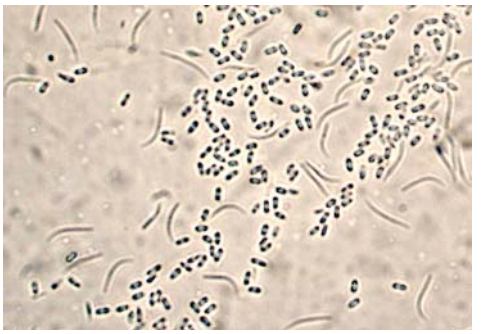

Figure 3. Conidia of a- and β-type of *Phomopsis* sp.

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Literature Cited

1. Jeong, I.H., Lim, M.T., Kim, G.H., Han, T.W., Kim, H.C., Kim, M.J., Park, H.S., Shin, S.H., Hur, I.S., Shin, J.S. and Koh, Y.J. 2008. Incidence of leaf spots and blights of kiwifruit in Korea. *Plant Pathology Journal,* 24: 125-130.
2. Lee, J.G., Lee, D.H., Park, S.Y., Hur, I.S. and Koh, Y.S. 2001. First report of *Diaporthe actinidiae*, the causal organism of stem-end rot of kiwifruit in Korea. *Plant Pathology Journal*, 17: 110-113.
3. Pintos Varela, C., García-Jiménez, J., Mansilla, J.P., Ciurana, N., Sales, R. and Armengol, J. 2000. Presencia de *Diaporthe actinidiae* afectando al Kiwi (*Actinidia deliciosa*) en el noroeste de la península ibérica. *Boletín Sanidad Vegetal-Plagas*, 26: 389-399.
4. Tjempelikou, K. and Elena, K. 1993. *Phomopsis* sp. on kiwi in Greece. In *Summaries of the 16th National Congress of the Hellenic Society of Horticultural Science*, Volos, Greece, 19-21 October 1993, p. 59 (in Greek).

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Παρουσία του μύκητα *Phomopsis* sp. σε οπωρώνες ακτινιδιάς στη Βόρεια Ελλάδα

Κ. Ελένα

Περίληψη Συμπτώματα έντονων ελκών με εσωτερικό μεταχρωματισμό του ξύλου παρατηρήθηκαν σε ετήσιους βλαστούς ακτινιδιάς ποικιλίας Hayward σε εμπορικούς οπωρώνες στην περιοχή της Σκύδρας, Β. Ελλάδα. Επιπλέον παρατηρήθηκε καστανός μεταχρωματισμός της κάτω επιφάνειας των φύλλων, νεκρωτικές, ελαφρώς βυθισμένες κηλίδες με εσωτερική σήψη στους καρπούς και νέκρωση του ποδίσκου με αποτέλεσμα τη μείωση του μεγέθους των καρπών. Από τους προσβεβλημένους ιστούς απομονώθηκε ο μύκητας *Phomopsis* sp. Σε δοκιμές παθογένειας που έγιναν, αναπαράχθηκαν τα συμπτώματα του μεταχρωματισμού του ξύλου των βλαστών και της σήψης των καρπών.

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SHORT COMMUNICATION

First record of *Echinothrips americanus* in Serbia

L. Andjus[1], M. Jović[1] and S. Trdan[2]

Summary In 2005, *Echinothrips americanus* Morgan (Thysanoptera: Thripidae) was first recorded in Serbia. Adults and larvae were found on leaves of *Syngonium* sp. in one of the largest florist shops in Belgrade. *E. americanus* exhibits several advantageous properties, which made its introduction into Serbia easier. The species causes direct damage to the leaves of its host plants. Compared to the western flower thrips, *Frankliniella occidentalis* (Pergande), *E. americanus* is less resistant to insecticides and less able to inhabit new areas. For this reason we suggest the use of environmentally friendly control methods for the pest species in question.

E. americanus Morgan (Thysanoptera: Thripidae) is the only representative of the genus recorded outside the Nearctic region. It was first recorded in southeastern USA (6). Today in that region the insect is primarily a pest of greenhouse ornamentals, though under warmer climatic conditions it can also overwinter in the open, mostly on weeds in the forests (10). There are references to its occurrence in California, Mexico, Hawaii and the Bermuda islands (1). The first significant occurrence of *E. americanus* in Europe was reported in 1993 in The Netherlands, where the species was found on plants of the genera *Syngonium* and *Homalomena* (10). The pest was later recorded in England (1) and in at least nine other European countries (8).

The thrips has a wide host plant range and is extremely polyphagous. It prefers plants of the Araceae and Balsaminaceae families, though it has been found on plants belonging to 24 families (10). In the USA, the most common hosts are those belonging to the genera *Dendranthema*, *Euphorbia*, *Impatiens* and *Medicago* as well as some woody ornamentals. The most important hosts of *E. americanus* in England are those of the genera *Syngonium*, *Dieffenbachia*, *Acalypha*, *Dracaena*, *Fatsia*, *Ficus*, *Hibiscus* and *Spathiphyllum* (1). In The Netherlands this thrips represents an ever increasing threat for the sweet pepper grown in greenhouses. Generally, the host plant range of *E. americanus* is more typical of the thrips species of the Panchaetothripinae subfamily than that of the Thripinae subfamily (9).

On December 1, 2005 a large population of thrips was detected on leaves of *Syngonium* sp. in one of the largest florist shops in Belgrade. The origin of the plants was unknown. More than 20 adults and larvae were recorded on each plant. Adults and larvae were collected and stored in 70% ethanol. The specimens, which were deposited in the thysanopterological collection of the Natural History Museum in Belgrade, were identified as *E. americanus,* based on their morphological characteristics. For the purpose of specimen identification, the zur Strassen's dichotomous taxonomic key (11) was used. The thrips were most probably introduced into the country with plant material. This is the first record of this species in Serbia.

The thrips (more frequently present as adults rather than larvae) were more numerous on the lower leaf surfaces and only some individuals were found on flowers.

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These data correspond well with what is known on the bionomics of *E. americanus* (1, 6). The species possesses several characteristics that could facilitate its establishment in the European greenhouses; it is polyphagous, it reproduces with facultative arrhenotoky, has a short developmental time and is polyvoltine. The introduction in an area of the so-called 'opportunistic thrips' becomes easier if they exhibit any of the above mentioned properties (4).

Damage results from the sucking of adults and larvae. The symptoms of infestation on leaves appear as small chlorotic areas and silvering. The aesthetic appearance of the plants is also affected due to the black fecal material present on leaves. However, compared to the western flower thrips, *Frankliniella occidentalis* (Pergande), the direct damage caused by *E. americanus* is considerably smaller. There is no evidence of plant viruses being transmitted by this species (1).

E. americanus is known to be more susceptible to insecticides compared to the western flower thrips and thus, it can be relatively easily controlled with most of the insecticides commonly used against these insects (3). Some experts claim that, in the absence of effective predators and parasitoids, this Thysanoptera species could become a considerably important pest in European greenhouses within a rather short time (10). Among the significant potential predators of *E. americanus*, *Franklinothrips vespiformis* Crawford (5) and *Macrolophus caliginosus* Wagner (7) are often mentioned.

It can be assumed that this first detection of *E. americanus* in Serbia is a rather early one and the pest has probably not yet spread through greenhouses to a greater extent. This provides an opportunity to take the measures needed in order to prevent its further spread. In addition and given the lack of serious threat posed by *E. americanus*, this species may be an ideal target for research on biological control methods. More specifically, it is well known that control methods employing predators of thrips give satisfactory results only when the population of the pest is low (2). Should this work for species such as *E. americanus*, then perhaps these new control methods could then be extrapolated to more aggressive, resistant and damaging thrips.

Literature Cited

1. Collins, D.W. 1998. Recent interceptions of *Echinothrips americanus* Morgan (Thysanoptera, Thripidae) imported into England. *Entomologist's Monthly Magazine*, 134: 1-4.
2. Jarosik, V. and Pliva, J. 1995. Assessment of *Amblyseius barkeri* (Acarina: Phytoseiidae) as a control agent for thrips on greenhouse cucumbers. *Acta Societatis Zoologicae Bohemicae*, 59: 177-186.
3. Łabanowski, G. 2007. Poinsettia thrips (*Echinothrips americanus* Morgan) - occurrence in Poland and possibilities of control. *Progress in Plant Protection*, 47: 289-302 (in Polish).
4. Mound, L.A. and Teulon, D.A.J. 1995. Thysanoptera as Phytophagous Opportunists. In PARKER, BL. *et al.* (eds) *Thrips Biology and Management*. New York: Plenum Press, p. 3-19.
5. Nedstam, B. 2001. Tripsarter i gron innemiljo. *Växtskyddsnotiser*, 65: 6-9.
6. Oetting, R.D. and Beshear, R.J. 1993. Biology of the greenhouse pest *Echinothrips americanus* Morgan (Thysanoptera: Thripidae). *Zoology* (*Journal of Pure and Applied Zoology*), 4: 307-315.
7. van Schelt, J., Hoogerbrugge, H., van Houten, Y. and van Bolckmans, K. 2002. Biological control and survival of *Echinothrips americanus* in pepper. *Bulletin OILB/SROP*, 25: 285-288.
8. Varga, L. and Fedor, P.J. 2008. First interception of the greenhouse pest *Echinothrips americanus* Morgan, 1913 (Thysanoptera: Thripidae) in Slovak Republic. *Plant Protection Science*, 44: 155–158.
9. Vierbergen, G. 1997. Amerikaanse trips. Nieuwe belager in paprika. *Groenten + Fruit/Glasgroenten*, 7: 12-13.
10. Vierbergen, G. 1998. *Echinothrips americanus* Morgan, a new thrips in Dutch greenhouses (Thysanoptera: Thripidae). *Proceedings of the section Experimental and Applied Entomology of the Netherlands Entomological Society (N.E.V.)*, 9: 155-160.
11. zur Strassen, R. 2003. Die terebranten Thysanopteren Europas und des Mittelmeer-Gebietes. *Die Tierwelt Deutschlands*, 74: 1-277.

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Πρώτη καταγραφή του *Echinothrips americanus* στη Σερβία

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Περίληψη Το έτος 2005 καταγράφηκε για πρώτη φορά στη Σερβία το έντομο *Echinothrips americanus* Morgan (Thysanoptera: Thripidae). Ενήλικα και προνύμφες του εντόμου βρέθηκαν σε φυτά του γένους *Syngonium* σε ένα από τα μεγαλύτερα ανθοπωλεία του Βελιγραδίου. Τα ιδιαίτερα χαρακτηριστικά της βιολογίας του θρίπα *E. americanus* διευκόλυναν την είσοδο και εγκατάστασή του στη Σερβία. Το έντομο προκαλεί άμεσες βλάβες στα φύλλα των ξενιστών του και συγκριτικά με τον θρίπα *Frankliniella occidentalis* (Pergande), ο *E. americanus* είναι λιγότερο ανθεκτικός στα εντομοκτόνα και διαθέτει μικρότερη ικανότητα αποίκισης νέων περιοχών. Για το λόγο αυτόν προτείνονται φιλικές προς το περιβάλλον μέθοδοι αντιμετώπισής του.

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A multiresidue method for analysis of 56 pesticides in peaches using liquid chromatography with tandem mass spectrometry detection

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Summary A liquid chromatography–tandem mass spectrometry (LC–MS/MS) multiresidue method for the simultaneous analysis of 56 multiclass pesticides and their metabolites in peaches is presented. Pesticide residues were extracted from the samples with acetone and a mixture of dichloromethane/light petroleum ether (50:50 v/v) and the determination was performed by liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-MS/MS), in a single chromatographic run. The analytical performance was demonstrated by the analysis of blank peach samples spiked at three concentration levels, 0.01, 0.05 and 0.5 mg/kg, for each pesticide or metabolite. Good sensitivity and selectivity of the method were obtained with limits of quantification of 0.01 mg/kg in most cases. The majority of the pesticides and their metabolites gave recoveries within the range of 65.7–125.3%, with relative standard deviations lower than 20%, for all concentration levels. Pesticide residues were found in five out of the 14 samples. However, in most cases, the concentrations of the analytes found in the samples were lower than the MRLs established at the time of the analysis by the European Union.

Additional keywords: acetone-based extraction method, LC–MS/MS, multiclass pesticides, validation

Introduction

The protection of crops against pests and diseases by various, mostly synthetic pesticides is a common approach in conventional farming. Even when pesticides are applied in accordance with good agricultural practices (GAP), they can leave residues on plants. Screening for residues in matrices, such as cereals, requires analytical methods with effective extraction, followed by a specific final determination step (15). Analytical methodologies employed must be capable of measuring residues at very low levels and must also provide unambiguous evidence to confirm both the identity and the concentration of any residue detected (7).

The choice of the solvent(s) for extraction is one of the most crucial decisions to be made when developing new multiresidue methods. Numerous different organic solvents and mixtures of organic solvents have been used to extract a wide range of pesticides with different physicochemical properties from foods. Nowadays, the solvents most commonly used for multiresidue analysis of pesticides are acetonitrile, acetone and ethylacetate. Each solvent has some advantages and disadvantages in terms of selectivity and convenience. Since the 1980s, we have applied an acetone-based extraction method that is amenable to all our chromatographic separation systems (4).

Acetone is the least toxic, least expensive and the most volatile of the three solvents most commonly used in multiresidue methods (acetone, ethyl acetate and acetonitrile) (1). Acetone is completely miscible with water allowing a good penetration in the aqueous part of the crop. However, to induce a distinct separation from the water phase, acetone essentially needs addition of a non-polar solvent, which leads to dilution and lower recoveries of more polar analytes (11). One of the most popular extraction

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methods, based on acetone, was the modified Luke method [acetone followed by partitioning with a mixture of dichloromethane (DCM) and light petroleum] (16). Luke *et al*. (9) developed a multiresidue method that included not only organochlorine but also organophosphorus compounds. According to this method, samples of fruit and vegetables (200 gr) were extracted with 200 ml of acetone and the extracts were then partitioned into dichloromethane/petroleum ether mixed solvent (50:50 v/v) by a Florisil clean-up step similar to that used by Mills *et al*. (12). Luke *et al.* (10) improved this procedure by eliminating the Florisil clean-up step and adding petroleum ether following the initial concentration to remove traces of DCM. Specht and Tilkes (14) published their multiresidue method for 90 pesticides in samples of both vegetable and animal origins. They also untilled DCM to partition the pesticides from aqueous acetone extracts, but added a gel permeation chromatography (GPC) clean-up step.

Chromatographic techniques, mainly gas chromatography (GC) and high-performance liquid chromatography (LC), have usually been applied for the determination of pesticide residues in food samples (7). Liquid chromatography-mass spectrometry (LC–MS) allows the rapid and efficient determination of many compounds (6). It has become a powerful tool for pesticide residue analysis in a variety of complex matrices, due to its inherent advantages: selectivity and sensitivity are notably improved, the sample pre-treatment steps can be minimized and reliable quantification and confirmation can be easily achieved at the low concentration levels required. Many publications on pesticide residue analysis using this technique were dated from the early 2000s (2, 4, 8).

Analytical methodologies employed must be capable of measuring residues at very low levels and must also provide unambiguous evidence to confirm both the identity and the concentration of any residue detected. Within-laboratory method validation should be performed to provide evidence that a method is fit for the purpose for which it is to be used. Method validation is a requirement of accreditation bodies and must be supported and extended by method performance verification during routine analysis (analytical quality control and ongoing method validation). All procedures (steps) that are undertaken in a method should be validated, if practicable (5).

The purpose of this paper is to present the validation of a rapid multiresidue method by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with electron spray interface (ESI), using an extraction method based on acetone–dichloromethane–petroleum ether for the determination of various pesticides from different chemical classes in fruit and vegetables of high water content. The procedure has been applied for the screening, confirmation and quantification of 56 representative multiclass pesticides in peaches, a representative commodity of fruit and vegetables of the high water content category (5) and was extended to the analysis of 14 samples of stone fruit taken from the market.

Materials and methods

1. Chemicals and reagents

In the present work, 56 analytes were selected with distinct physicochemical characteristics, as shown in Table 1. They included polar and non-polar compounds, as well as compounds of various molecular masses. The selected analytes belong to different chemical classes: anilinopyrimidine, benzimidazole, carbamate, N-methyl carbamate, oxime carbamate, chloroacetamide, cinnamic acid, diacylhydrazine, hydroxyanilide, imidazole, morpholine, neonicotinoid, organophosphorous, oxadiazine, pyrazole, pyridinecarboxamide, quinoline, strobilurin, tetrazine and triazole pesticides.

The following pesticide active ingredients, obtained from Dr Ehrenstorfer Laboratories GmbH (Germany), were used in the present study: acetamiprid, alachlor, aldicarb, aldicarb sulfone, aldicarb sulfoxide,

Table 1. Chemical classes and physicochemical properties of the studied analytes.

Analyte	Chemical group	Water solubility (mg/l)	logPow	Volatility (Pa)
acetamiprid	Neonicotinoid	4250	0.8	1.00E-06
alachlor	Chloroacetamide	170.31	170.31	0.002
aldicarb	Carbamate	4930	1.359	0.013
aldicarb sulfone		10000	-0.57	0.012
aldicarb sulfoxide				
boscalid	Pyridinecarboxamide	4.6	2.96	7.00E-07
bromuconazole	Triazole	50	3.24	4.00E-06
buprofezin		0.9	4.3	0.00125
carbaryl	Carbamate	120	1.85	4.10E-05
carbendazim	Benzimidazole	8	1.51	0.00015
carbofuran	Carbamate	351	1.52	3.10E-05
carbofuran 3-Hydroxy	Carbamate. N-methyl			
clofentezine	Tetrazine	0.003	4.1	1.30E-07
cymoxanil		890	0.67	0.00015
demeton-S-methyl	Organophosphorous	22000	1.32	0.04
demeton-S-methyl sulfoxide	Organophosphorous		-0.74	0.0038
dimethomorph	Cinnamic acid	18	2.7	1.00E-06
fenamidone	Imidazole	7.8	2.8	3.40E-07
fenbuconazole	Triazole	0.2	3.23	5.00E-06
fenhexamid	Hydroxyanilide	20	3.51	4.00E-07
fenoxycarb	Carbamate	7.9	4.07	8.70E-07
fenpyroximate	Pyrazole	0.015	5.01	7.50E-06
fenthion	Organophosphorous	4.2	4.84	0.00074
fosthiazate	Organophosphorous	9850	1.68	0.00056
hexaconazole	Triazole	17	3.9	1.80E-05
imidacloprid	Neonicotinoid	610	0.57	4.00E-10
indoxacarb	Oxadiazine	0.2	4.65	2.50E-08
iprovalicarb	Carbamate	11	3.2	7.70E-08
mepanipyrim	Anilinopyrimidine	3.1	3.28	2.32E-05
methiocarb	Carbamate	27	3.08	3.60E-05
methiocarb sulfone	Carbamate		1.26	
methiocarb sulfoxide	Carbamate		0.618	
methomyl	Carbamate	58000	0.093	0.00072
methoxyfenozide	Diacylhydrazine	3.3	3.7	1.48E-06
monocrotophos	Organophosphorous		-0.22	0.00029
myclobutanil	Triazole	142	2.94	0.000213
oxamyl	Carbamate	280000	-0.44	5.10E-05
prochloraz	DMI:imidazole	34.4	4.12	0.00015
profenofos	Organophosphorous	28	4.44	0.000124
pyraclostrobin	Strobilurin	1.9	3.99	2.60E-08
pyrimethanil	Anilinopyrimidine	121	2.84	0.0022
quinoxyfen	Quinoline	0.116	4.66	1.20E-05
spinosyn A	Spinosad			
spiroxamine	Morpholine	200000	2.8	
tebuconazole	Triazole	36	3.7	1.70E-06
tebufenozid	Diacylhydrazine	0.83	4.25	1.56E-07
tebufenpyrad	Pyrazole	2.8	5.04	1.00E-05
tetraconazole	Triazole	156	3.56	0.00018
thiabendazole	Benzimidazole	30	2.39	4.60E-07
thiacloprid	Neonicotinoid	185	1.26	3.00E-10
thiamethoxam	Neonicotinoid	4100	-0.13	6.60E-09
thiodicarb	Carbamate	35	1.62	0.0057
triadimefon	Triazole	64	3.11	2.00E-05
triadimenol	Triazole			6.00E-07
trifloxystrobin	Strobilurin	0.61	4.5	3.40E-06
vamidothion	Organophosphorous	4000000	0.147	

boscalid, bromuconazole, buprofezin, carbaryl, carbendazim, carbofuran, carbofuran 3-hydroxy, clofentezine, cymoxanil, demeton-S-methyl, demeton-S-methyl sulfoxide, dimethomorph, fenamidone, fenbuconazole, fenhexamid, fenoxycarb, fenpyroximate, fenthion, fosthiazate, hexaconazole, imidacloprid, indoxacarb, iprovalicarb, mepanipyrim, methiocarb, methiocarb sulfone, methiocarb sulfoxide, methomyl, methoxyfenozide, monocrotophos, myclobutanil, oxamyl, prochloraz, profenofos, pyraclostrobin, pyrimethanil, quinoxyfen, spinosad (A), spiroxamine, tebuconazole, tebufenozid, tebufenpyrad, tetraconazole, thiabendazole, thiacloprid, thiamethoxam, thiodicarb, triadimefon, triadimenol, trifloxystrobin and vamidothion.

Acetone, dichloromethane, and petroleum ether of pesticide grade analysis were used for the extraction procedure. Methanol and water, LC-MS grade, were used for the preparation of stock and working standard solutions. All solvents were obtained from Lab Scan (Ireland).

2. Preparation of stock standard solutions

Stock standard solutions at 1000 µg/ml were prepared in acetone for each of the 56 pesticides and stored at -20°C. A single composite standard mixture was prepared by combining aliquots of each stock solution and diluting them with a methanol/water (30:70 v/v) mixture to obtain a final concentration of 1 mg/ml. Blank peach samples were spiked at 0.01, 0.05 and 0.5 mg/kg by adding appropriate volumes of the composite standard mixture. Working standard mixture solutions for measurement were prepared in blank peach extract, previously analysed for the absence of peaks interfering with the peaks of the analytes. Calibration curves were constructed from injections of matrix-matched calibration standards in blank matrix of peach in methanol/water (30:70 v/v) at eight concentrations, within the range of 0.01–0.75 mg/ml (i.e. 0.01–0.025–0.05–0.075-0.1-0.25-0.5-0.75 µg/ml) for all pesticides.

For the preparation of the blank extract, the sample extraction procedure mentioned below under paragraph "Sample extraction" was followed. The only difference was that at the final step the blank extract was taken in 3 ml of MeOH. An aliquot of 1 ml was evaporated to dryness by a stream of N_2 and 1 ml of a standard solution of the desired concentration, prepared in methanol/water (30:70 v/v), was added. Prior to injection in the chromatographic system, the final solution was filtered through a disposable PTFE syringe filter, 0.45 µm in diameter.

3. Liquid chromatography

The liquid chromatographic (LC) system used consisted of two Varian Prostar 210 pumps. Chromatographic separation was achieved using a Polaris C_{18} 5 µm particle size, 2 mm x 50 mm analytical column from Varian, at a flow rate of 250 µl/min with mobile phases consisting of methanol/water (10:90 v/v) – 1mM ammonium formate (solvent A) and methanol/water (90:10 v/v) – 1mM ammonium formate (solvent B). A gradient program was used consisting of 90% of solvent A and 10% of solvent B, ramped linearly over the course of 14 minutes to 100% of solvent B. This composition was held for 6 additional minutes before returning to the initial condition. The column was re-equilibrated for 10 min at the initial mobile phase composition. The total run-time was 30 min. The injection volume was 5 µl and in order to avoid carry-over, the autosampler was purged with a mixture of methanol/water (50:50 v/v) before sample injection.

4. Mass spectrometry

Detection was achieved using a triple quadrupole mass spectrometer (Varian model 1200L) equipped with an electrospray ionization interface operating in the positive mode. Typical source parameters were as follows: capillary voltage and collision cell energy varied depending on the precursor ion, as shown in Table 2, source temperature was set at 250°C and drying gas temperature at 200°C. Nitrogen, generated from high purity generator, was used as drying gas and neb-

ulizing gas, with pressures set at 18 and 55 psi, respectively. For the operation in MS/MS mode, Argon 99.999% (Messer) was used as collision gas at a pressure of 1.5 mTorr. The multiple reaction monitoring experiments were conducted with a dwell time of 100 msec, except for aldicarb, hexaconazole and hexythiazox for which the dwell time was set at 300, 200 and 300 msec, respectively. For instrument control, data acquisition and processing, Varian MS Workstation software version 6.8 was used.

5. Sample extraction

The extraction was based on the acetone–dichloromethane–petroleum ether multiresidue method developed by the Dutch Ministry of Public Health, Welfare and Sport (16). The use of acetone (a water-miscible solvent) provides the extraction of the analytes without extracting the larger lipid volumes that later cause emulsions and interferences. With the addition of immiscible to water solvents, such as dichloromethane and petroleum ether, the analytes under study are extracted in the organic phase without additional co-extractives that were previously in the acetone phase.

The sample processing, according to the applied method, was the following (7, 16): an aliquot of 15±0.15 g of the previously homogenized sample was weighted into a 250 ml PTFE centrifuge bottle (Nalgene, Rochester, NY) and 30 ml of acetone were added and stirred for 1 min in an ultra-turrax homogenizer at 15,000 rpm. Then, 30 ml of dichloromethane and 30 ml of petroleum ether were added and the mixture was stirred again for 1 min. The sample was centrifuged at 4,000 rpm for 5 min, 15 ml of the supernatant liquid were transferred in a flat-bottom long necked flask and evaporated to dryness in a water bath at 65–70°C and 3 ml of methanol/water (30:70 v/v) were added as follows: 0.9 ml of methanol were added in the flask, the extract was placed in an ultrasonic bath for 30 sec and transferred into a 3 ml volumetric flask; the flat-bottom long necked flask was rinsed twice with 1 ml of water, the resulting solution was transferred into the 3 ml volumetric flask and water was added to the mark. The final extract was placed in an ultrasonic bath for 30 sec and was then transferred into a vial with a Teflon stopper. The final extract was filtered through a disposable PTFE syringe filter, 0.45 μm in diameter and 5 μl of the final extract were injected in the chromatographic system. The MS/MS acquisition method included the monitoring of one transition, that of the quantification, for each compound, as shown in Table 2.

6. Confirmation

The confirmation of positive findings was achieved by a second injection under the chromatographic conditions described above. Individual MS/MS acquisition methods, including both transitions of Table 2 for each separate analyte, were developed and used for confirmation purposes. Then, an independent confirmation injection was performed for every positive sample. Confirmation included retention times of standard and sample acceptable tolerances and the ion ratios of qualifier and quantifier ions, as they are referred in the Document No SANCO/2007/3131 (5). The retention time of the analyte in the sample extract must match that of the matrix-matched calibration standard with a tolerance of ±2.5%.

Results and discussion

1. Pesticide and parameter selection

The ionization of the 56 pesticides and metabolites in positive electrospray ion mode was studied. Table 2 shows the precursor ions used for data acquisition, the transitions used for quantification and qualification, the capillary voltage and collision cell energy for each transition, segments (time windows) and the retention times of the analytes in which the transitions were scanned. Pesticides and metabolites were ionized in the form of $[M+H]^+$.

Tandem mass spectrometry (MS/MS) provides a powerful confirmatory tool for pesticide residue analysis because it dis-

Table 2. Mass spectrometry (MS) and chromatography parameters of the studied analytes.

Analyte	Precursor ion	Quantification Transition m/z		Capillary (V)	Collision cell energy (eV)	Qualifier Transition m/z		Capillary (V)	Collision cell energy (eV)	RT (min)	Time segments
aldicarb sulfoxide	[M+H]+	207	89	42	18	207	132	40	15	1.2	1
aldicarb sulfone	[M+H]+	240	148	40	15	240	86	25	25	1.52	1
oxamyl	[M+H]+	237	72	36	15	237	90	36	15	1.55	1
demeton-S-methyl sulfoxide	[M+H]+	247	169	21	14	247	109	20	35	1.81	1
methomyl	[M+H]+	163	88	8	8	163	106	15	10	1.94	1
thiamethoxam	[M+H]+	292	211	51	17	292	181	56	31	2.27	1
monocrotophos	[M+H]+	224	127	40	20	224	98	46	17	2.50	1 & 2
imidacloprid	[M+H]+	256	209	45	20	256	175	45	25	3.35	1 & 2
methiocarb sulfoxide	[M+H]+	242	185	25	15	242	168	25	30	3.75	1 & 2
vamidothion	[M+H]+	288	146	10	30	288	118	10	40	3.98	2
carbofuran 3-hydroxy	[M+H]+	238	163	25	15	238	181	21	15	4.00	2
acetamiprid	[M+H]+	223	126	36	27	223	90	31	45	4.14	2
cymoxanil	[M+H]+	199	128	46	13	199	111	41	25	4.41	2
methiocarb sulfone	[M+H]+	275	122	25	25	258	122	25	45	4.61	2
carbendazim	[M+H]+	192	160	40	14	192	132	40	28	4.65	2
thiacloprid	[M+H]+	253	126	81	29	253	186	76	19	5.03	2
aldicarb	$[M+NH_4]+$	208	89	10	20	208	116	10	20	5.3	2
thiabendazole	[M+H]+	202	175	40	30	202	131	40	30	5.84	2
carbofuran	[M+H]+	222	123	25	40	222	165	25	25	6.51	2 & 3
demeton-S-methyl	[M+H]+	248	89	6	15	248	61	6	47	6.9	2 & 3
carbaryl	[M+H]+	202	145	40	15	202	127	40	50	7.13	3
fosthiazate	[M+H]+	284	104	61	27	284	228	61	15	7.31	3
thiodicarb	[M+H]+	355	108	30	25	355	88	30	25	7.43	3 & 4
pyrimethanil	[M+H]+	200	107	30	30	200	82	30	30	8.93	4
dimethomorph	[M+H]+	388	301	60	17	388	165	60	28	9.07	4
fenamidone	[M+H]+	312	92	41	33	312	236	41	19	9.18	4

methiocarb	[M+H]+	243	169	15	25	226	121	60	25	9.22	4
boscalid	[M+H]+	343	307	71	27	343	140	76	27	9.38	4
triadimefon	[M+H]+	294	197	28	21	294	225	28	21	9.63	4
bromuconazole (1st peak)	[M+H]+	378	159	46	30	378	70	46	15	9.69	4
methoxyfenozide	[M+H]+	369	149	36	23	369	133	41	31	9.77	4
myclobutanil	[M+H]+	289	125	36	41	289	70	36	33	9.83	4
iprovalicarb	[M+H]+	321	119	51	23	321	203	51	20	9.86	4
mepanipyrim	[M+H]+	224	106	40	20	224	77	31	49	10.00	4
triadimenol	[M+H]+	296	70	11	11	296	227	11	9	10.01	4
alachlor	[M+H]+	270	162	31	25	270	238	31	25	10.13	4
fenhexamid	[M+H]+	302	97	35	25	302	55	35	35	10.21	4
tetraconazole	[M+H]+	372	159	40	40	372	70	36	45	10.43	4
fenbuconazole	[M+H]+	337	125	41	37	337	70	41	33	10.63	4
spiroxamine	[M+H]+	298	144	41	27	298	100	41	35	10.75	4
bromuconazole (2nd peak)	[M+H]+	378	159	46	30	378	70	46	15	10.76	4
tebufenozid	[M+H]+	353	133	40	20	353	297	40	16	10.80	4
fenoxycarb	[M+H]+	302	116	21	12	302	88	21	20	10.84	4
tebuconazole	[M+H]+	308	70	30	20	308	125	30	35	11.10	4
fenthion	[M+H]+	279	169	21	23	279	247	41	10	11.18	4
hexaconazole	[M+H]+	314	70	30	20	314	159	26	30	11.47	4
pyraclostrobin	[M+H]+	388	163	11	29	388	194	6	19	11.53	4
prochloraz	[M+H]+	376	308	16	17	376	266	31	23	11.54	4
clofentezine	[M+H]+	303	138	56	15.5	303	102	56	47	11.88	4
trifloxystrobin	[M+H]+	409	186	11	23	409	206	6	21	12.14	4
indoxacarb	[M+H]+	528	203	76	50	528	56	76	21	12.22	4 & 5
tebufenpyrad	[M+H]+	334	145	25	30	334	117	51	47	12.66	5
profenofos	[M+H]+	373	303	40	20	373	97	30	30	12.68	5
buprofezin	[M+H]+	306	116	40	21	306	201	40	17	12.85	5
quinoxyfen	[M+H]+	308	197	50	40	308	162	25	50	13.28	5
spinosad	[M+H]+	733	142	65	20					13.82	5
fenpyroximate	[M+H]+	422	366	15	20	422	135	16	41	13.93	5

criminates efficiently between the analyte and the matrix signal. Existing quality control procedures require qualitative confirmation of positive results for LC–MS/MS, preferably via a second MS/MS transition (5). With the present analytical scope of 56 pesticides, this results in monitoring 112 transitions. Stacking more transitions within a time window drastically reduces the number of data points across a chromatographic peak leading to unsatisfactory peak shapes. Therefore, in the first injection, only one transition is recorded, the quantification one, and a second injection of the sample is thus required with both transitions only for the compounds that give a positive result; in this way the false positive results are minimized. The quantification transition, which in all cases is the one with the highest signal to noise (S/N) ratio, is selected for screening in order to minimize the false negative results.

2. MS optimization

Individual standard solutions at 0.01 mg/ml were prepared in methanol/water (30:70 v/v) and infused into the mass spectrometer in order to obtain the optimum values for the cone and capillary voltage for each analyte. The optimum collision cell energy voltage was found to vary between 6 and 81 kV, depending on the analyte. Product ion mass spectra for the pesticides were obtained in the positive mode electrospray ionization using collision induced dissociation (CID). Variation of the collision energy influences both sensitivity and fragmentation. The collision energy was optimized for two selective product ions of each precursor ion. The optimization values obtained are listed in Table 2.

The time–scheduled data acquisition sequence involved five overlapping segments, with 8 to 29 transitions each. Table 2 shows the distribution of the transitions into 5 time windows based on analyte retention times. With dwell times of 100 msec the average scan cycle time for the segments varied between 0.8 and 2.7 sec. Still, a sufficient number of data points can be acquired over the chromatographic peak in order to have enough sensitivity and allow reproducible peak area integration for good quantitative results.

3. LC optimization

Regarding the LC separation, the gradient was optimized in order to have a separation of the 56 selected analytes. Using a dwell time of 100 msec per transition, we obtained satisfactory peak shape for all analytes under study. By distributing the analytes along five overlapping segments, the chromatographic peak was allowed to be centered in the time window, minimizing the risk of peak loss due to unexpected slight changes in retention time.

4. Validation

The validation study carried out was based on the European SANCO guidelines (5), and the method was evaluated for its sensitivity, accuracy, precision, linearity and specificity. This requires performing recovery experiments with spiked blank peach samples to estimate the accuracy of the method. A minimum of five replicates is required to evaluate precision as well as the reporting limit (to assess sensitivity) and at least another higher level (5). In our study, experiments with fortified peach samples were performed at three fortification levels (0.01, 0.05 and 0.5 mg/kg), with five replicates at each level.

Linearity was studied using matrix-matched calibration standards at six concentration levels within the range of 0.01 to 0.75 µg/ml and three replicates at each level. The approximate value of uncertainty (S_u) in the predicted value *C (mg/kg)* due to the variability in the *Area* resulting from the use of the calibration curve was also estimated by the following equation:

$$S_u = \frac{S_{Area/C}}{b}$$

Where $S_{Area/C} = \sqrt{\frac{\sum_i (y_i - \bar{y})^2}{n-2}}$, n is the number of the data points in the calibration, $(y_i - \bar{y})$ is the residual for the ith point and

b the slope of the regression line.

The precision of the method was estimated by assessing the relative standard deviation (RSD) values of the five replicates at both levels. According to the Document No SANCO/2007/3131, repeatability with RSD ≤ 20% is considered to be acceptable. The limit of quantification (LOQ) was established as the lowest concentration that gave satisfactory recovery (70–110%) and precision (RSD ≤ 20%) data.

Linearity

Good linearity was found for most pesticides with correlation coefficients better than 0.990 in most cases. However, demeton-S-methyl, fenpyroximate and methiocarb sulfone did not exhibit a linear behavior at the concentration range under study, but at a narrower linear range, as shown in Table 3. The dynamic linear range in electrospray ionization can be limited and generally depends on the physicochemical characteristics of the analyte (3). In Table 3, the basic calibration line parameters for the 56 compounds are presented (13).

Accuracy and precision

Recovery and repeatability of the method were established in order to evaluate the method's accuracy and precision, respectively. Recoveries of 70–120% with a repeatability RSD ≤ 20% are considered acceptable (5), while in routine analysis, the acceptable recoveries are 60 - 140%. The recoveries were calculated using matrix-matched single-level calibration standards. Table 4 shows the detailed recovery and repeatability data of the pesticides studied. The majority of the pesticides produced recoveries and RSD values within the accepted ones.

The recoveries of the pesticides ranged from 65.7 to 125.3% with relative standard deviations (RSDs) less than 24.7% at the lowest concentration level and from 70 to 110.9% with RSDs less than 27.1% at the highest level. In the cases of alachlor, aldicarb, aldicarb sulfone, aldicarb sulfoxide, bromuconazole, carbofuran 3-hydroxy, demeton–S-methyl, hexaconazole, mepanipyrim, methiocarb sulfone, methiocarb sulfoxide and pyrimethanil, the recovery and repeatability values at the lowest concentration level were outside the acceptable levels. Therefore, the method is not considered suitable for the determination of these compounds at the lowest level. For clofentezine, fenpyroximate, indoxacarb and prochloraz, the RSDs at the highest concentration level were above 20%, but with satisfactory recoveries.

Limit of quantification/Limit of detection

As limit of quantification (LOQ) of the method, the lowest validated level that produced acceptable accuracy and precision results, which in most cases was 0.01 mg/kg, was selected. In the cases of alachlor, aldicarb, aldicarb sulfone, aldicarb sulfoxide, bromuconazole, carbofuran 3-hydroxy, demeton-S-methyl sulfoxide, hexaconazole, mepanipyrim, methiocarb sulfone, methiocarb sulfoxide and pyrimethanil, the LOQ was set at 0.05 mg/kg.

The limit of detection (LOD) of the method is the minimum concentration or mass of the analyte that can be detected with acceptable certainty, though not quantifiable with acceptable precision. The LOD of the method was calculated as three times lower than the LOQ (LOQ = 3xLOD).

5. Analysis of real samples

To evaluate its application, the proposed methodology was applied to the analysis of real stone fruit samples. All samples were extracted and analysed as described above and the results are presented in Table 5. A total of 14 samples of various stone fruit (apricots, cherries, peaches and plums) were analysed. Pesticide residues were found in five out of the 14 samples. The pesticides found were: carbaryl, carbendazim, indoxacarb, imidacloprid, thiamethoxam and thiacloprid. Three fruit samples contained only one pesticide and two samples contained two pesticides of the pesticides studied. However, the concentrations of the analytes found in the samples, except for one sample, were lower than the MRLs estab-

Table 3. Summary of calibration line parameters for the 56 analytes at 0.01–0.75 μg/ml concentration levels.

Area = f(C)							
Analyte	R	r^2	b	S_b	a	S_a	S_u
acetamiprid	0.994	0.989	2.00E+09	8.60E+07	4.15E+07	2.87E+07	0.0306
alachlor	0.980	0.960	2.27E+08	1.90E+07	9.95E+06	6.35E+06	0.0595
aldicarb	0.995	0.989	6.21E+08	2.65E+07	2.94E+05	8.84E+06	3.03E-02
aldicarb sulfone	0.990	0.980	4.40E+08	2.54E+07	5.96E+06	8.48E+06	0.0411
aldicarb sulfoxide	0.994	0.988	8.92E+08	3.90E+07	4.24E+06	1.30E+07	3.11E-02
boscalid	0.996	0.992	1.32E+08	4.96E+06	3.20E+06	1.66E+06	0.0268
bromuconazole	0.983	0.967	2.20E+08	1.64E+07	1.23E+07	5.48E+06	5.29E-02
buprofezin	0.996	0.992	1.65E+09	5.98E+07	2.98E+07	2.00E+07	0.0257
carbaryl	0.992	0.984	3.56E+09	1.87E+08	-4.23E+07	6.24E+07	0.0373
carbendazim	0.998	0.996	2.92E+09	7.40E+07	9.09E+07	2.47E+07	0.0180
carbofuran	0.989	0.979	3.85E+09	2.31E+08	6.30E+07	7.71E+07	0.0427
carbofuran 3-hydroxy	0.994	0.988	8.31E+08	3.76E+07	-1.88E+06	1.26E+07	0.0322
clofentezine	0.997	0.995	2.51E+08	7.60E+06	-3.99E+06	2.54E+06	0.0215
cymoxanil	0.993	0.985	4.63E+08	2.30E+07	1.19E+06	7.68E+06	0.0353
demeton-S-methyl[1]	0.996	0.992	2.18E+09	7.76E+07	1.06E+07	2.59E+07	2.54E-02
demeton-S-methyl sulfoxide	0.996	0.992	2.18E+09	7.76E+07	1.06E+07	2.59E+07	0.0254
dimethomorph	0.995	0.989	4.54E+08	1.93E+07	1.15E+07	6.47E+06	0.0303
fenamidone	0.984	0.968	3.74E+08	2.77E+07	2.38E+07	9.24E+06	0.0526
fenbuconazole	0.997	0.993	3.13E+08	1.04E+07	6.56E+06	3.48E+06	0.0237
fenhexamide	0.992	0.985	4.16E+08	2.13E+07	1.17E+07	7.12E+06	0.0364
fenoxycarb	0.993	0.985	1.51E+09	7.60E+07	5.78E+07	2.54E+07	0.0357
fenpyroximate[1]	0.991	0.981	4.58E+08	2.88E+07	1.32E+07	1.03E+07	0.0453
fenthion	0.997	0.995	1.33E+08	3.99E+06	1.94E+06	1.33E+06	0.0214
fosthiazate	0.993	0.986	2.44E+09	1.18E+08	5.77E+06	3.94E+07	0.0344
hexaconazole	0.988	0.977	1.33E+08	8.32E+06	6.52E+06	2.78E+06	0.0445
imidacloprid	0.993	0.986	5.34E+08	2.64E+07	1.80E+07	8.82E+06	0.0352
indoxacarb	0.989	0.978	2.24E+08	1.37E+07	2.85E+06	4.60E+06	0.0436
iprovalicarb	0.994	0.988	1.59E+09	7.19E+07	5.09E+07	2.40E+07	0.0321
mepanipyrim	0.992	0.984	6.92E+08	3.61E+07	3.02E+07	1.21E+07	0.0371
methiocarb	0.988	0.977	2.92E+08	1.84E+07	-8.73E+05	6.15E+06	0.0448
methiocarb sulfone[1]	0.892	0.795	4.30E+08	8.90E+07	1.83E+07	2.98E+07	0.1475
methiocarb sulfoxide	0.979	0.959	3.27E+09	3.01E+08	-8.54E+07	1.07E+08	0.0671
methomyl	0.991	0.983	1.19E+09	6.37E+07	4.09E+07	2.13E+07	0.0383
methoxyfenozide	0.992	0.985	9.51E+08	4.79E+07	4.00E+07	1.60E+07	0.0358
monocrotophos	0.996	0.993	1.59E+09	5.51E+07	1.30E+07	1.84E+07	0.0247
myclobutanil	0.992	0.984	1.67E+08	8.68E+06	8.86E+06	2.90E+06	0.0369
oxamyl	0.990	0.980	1.00E+09	5.84E+07	-2.02E+07	1.95E+07	0.0416
prochloraz	0.990	0.979	8.10E+08	4.81E+07	2.03E+07	1.61E+07	0.0423
profenofos	0.996	0.993	9.24E+08	3.22E+07	-3.16E+07	1.08E+07	0.0248
pyraclostrobin	0.995	0.989	2.04E+09	8.73E+07	5.83E+07	2.92E+07	0.0305
pyrimethanil	1.000	0.999	7.63E+08	8.37E+06	-1.37E+06	2.80E+06	0.0078
quinoxyfen	0.989	0.978	1.88E+08	1.16E+07	1.53E+06	3.87E+06	0.0437
spinosad	0.991	0.982	9.77E+08	7.46E+07	3.58E+07	2.49E+07	0.0543

Table 3 (continued)

Area = f(C)							
Analyte	R	r^2	b	S_b	a	S_a	S_u
spiroxamine	0.999	0.997	3.30E+09	7.35E+07	1.91E+07	2.46E+07	0.0158
tebuconazole	0.994	0.988	6.38E+08	2.86E+07	1.65E+07	9.57E+06	0.0320
tebufenozid	0.986	0.973	8.30E+08	5.68E+07	3.97E+07	1.90E+07	0.0487
tebufenpyrad	0.996	0.991	5.70E+08	2.16E+07	6.55E+06	7.23E+06	0.0270
tetraconazole	0.997	0.993	2.69E+08	9.07E+06	5.85E+06	3.03E+06	0.0240
thiabendazole	0.994	0.989	1.83E+09	7.89E+07	4.85E+07	2.64E+07	0.0308
thiacloprid	0.996	0.992	1.97E+09	7.08E+07	5.52E+07	2.37E+07	0.0255
thiamethoxam	0.990	0.980	3.76E+08	2.17E+07	1.21E+07	7.24E+06	0.0410
thiodicarb	0.988	0.975	3.60E+08	2.34E+07	-8.52E+06	7.82E+06	0.0462
triadimefon	0.993	0.986	3.41E+08	1.64E+07	1.44E+07	5.49E+06	0.0343
triadimenol	0.991	0.982	7.80E+08	4.33E+07	3.93E+07	1.45E+07	0.0395
trifloxystrobin	0.996	0.993	3.46E+09	1.22E+08	2.16E+07	4.07E+07	0.0251
vamidothion	0.995	0.989	4.91E+08	2.09E+07	5.17E+06	6.99E+06	0.0303

[1] 0.025–0.75 μg/ml concentration levels
r^2: Correlation coefficient
b: Slope of the regression line
S_b: Mean standard deviation of the slope of the regression line
a: Mean of the population that corresponds to x=0
S_a: Mean standard deviation of the mean of the population that corresponds to x=0
S_u: The uncertainty in the predicted value C (mg/kg) due to the variability in the Area

Table 4. Recovery data and relative standard deviation (RSD) values (n = 6) obtained for the 56 pesticides from the spiking experiments in the peach matrix.

Compound	1st level (0.01 mg/kg)		2nd level (0.05 mg/kg)		3th level (0.5 mg/kg)	
	Mean recovery (%)	RSD	Mean recovery (%)	RSD	Mean recovery (%)	RSD
acetamiprid	113.47	18.31	73.7	20.6	87.2	4.3
alachlor	-	-	91.2	15.3	96.4	5.3
aldicarb	-	-	82.6	9.6	96.18	2.5
aldicarb sulfone	-	-	77.2	6.2	82.6	3.9
aldicarb sulfoxide	-	-	55.5	11.8	48.6	5
boscalid	81.33	16.33	119.8	13.4	91.7	6.3
bromuconazole	-	-	89.7	11.8	93.8	6.8
buprofezin	100.43	6.95	98.6	8.6	93.8	3.9
carbaryl	82.68	19.63	106.2	7.7	100.9	2.3
carbendazim	121.48	17.38	118.0	6.2	90.5	3.5
carbofuran	76.81	14.56	80.4	19.6	110.9	2.3
carbofuran 3-hydroxy	-	-	92.8	6.7	99.4	3.6
clofentezine	105.8	6.79	119.7	22.6	97.5	25.7
cymoxanil	95.35	4.89	98.8	10.7	101.3	4.8
demeton-S-methyl	-	-	66.65	11	88.72	5.5
demeton-S-methyl sulfoxide	92.78	15.83	82.2	17.9	71.2	2.1
dimethomorph	91.74	16.38	110.1	4.9	102.8	3.7

Table 4 (continued)

Compound	1st level (0.01 mg/kg)		2nd level (0.05 mg/kg)		3th level (0.5 mg/kg)	
	Mean recovery (%)	RSD	Mean recovery (%)	RSD	Mean recovery (%)	RSD
fenamidone	95.57	14.06	84.8	17.2	97.9	4.4
fenbuconazole	95.71	10.78	101.3	14.1	78.8	8.7
fenhexamid	96.77	19.44	111.0	6.4	92.0	6.8
fenoxycarb	121.22	5.26	110.4	13.6	77.3	9.6
fenpyroximate	125.29	8.19	119.7	26.9	92.2	26.8
fenthion	116.83	5.94	74.4	16.1	70.0	11.6
fosthiazate	105.32	15.58	98.6	3.6	109.4	1.7
hexaconazole	-	-	112.2	11.5	-	-
imidacloprid	103.94	20.09	96.1	4.8	103.7	3.0
indoxacarb	102.80	3.58	119.3	12.3	98.2	25.0
iprovalicarb	65.71	13.97	103.9	4.5	105.0	3.4
mepanipyrim	-	-	110.3	4.8	70.0	10.8
methiocarb	69.63	17.09	87.7	4.5	90.7	4.1
methiocarb sulfone	-	-	110.8	1.8	108.5	1.7
methiocarb sulfoxide	-	-	91.8	4.3	98.0	4.0
methomyl	119.57	19.78	75.4	18.8	95.3	2.1
methoxyfenozide	104.56	4.27	103.0	5.7	104.0	2.9
monocrotophos	116.64	17.56	70.4	6.3	71.9	3.8
myclobutanil	117.91	12.59	91.5	3.8	100.7	4.4
oxamyl	108.00	4.36	80.5	6.0	80.0	4.7
prochloraz	121.35	7.52	106.6	14.6	104.2	27.1
profenofos	119.92	19.64	105.1	18.3	74.0	12.9
pyraclostrobin	121.03	10.85	101.2	14.1	81.1	18.4
pyrimethanil	-	-	88.4	10.2	71.1	7.1
quinoxyfen	111.99	13.04	115.2	19.0	93.7	21.9
spinosad (A)	80.03	18.84	72.8	21.7	71.1	10.6
spiroxamine	123.87	17.23	79.4	10.6	81.5	7.0
tebuconazole	-	-	109.3	11.6	77.4	10.2
tebufenozid	113.83	12.2	101.7	5.3	98.3	5.2
tebufenpyrad	121.15	18.66	111.0	16.7	98.3	26.8
tetraconazole	108.22	24.68	89.8	13.4	80.3	7.4
thiabendazole	-	-	89.3	8.3	73.3	5.5
thiacloprid	122.85	9.31	70.0	19.8	94.3	2.5
thiamethoxam	123.39	20.66	92.6	6.4	91.2	3.1
thiodicarb	97.01	14.76	100.8	4.9	104.2	3.9
triadimefon	116.00	14.35	95.8	7.1	99.8	4.0
triadimenol	102.75	11.42	100.3	9.3	102.9	3.2
trifloxystrobin	101.77	8.62	76.8	20.7	72.3	13.7
vamidothion	94.71	11.44	75.3	6.9	87.9	1.3

lished at the time of analysis by the European Union or FAO. The quantification of the analytes was conducted by single-level calibration. Single-level calibration may provide

Table 5. Results from the 14 stone fruit samples analysed, the multiresidue methods (MRMs) used for confirmation/quantification, the retention times and relative MRM intensities of the samples and the matrix matched standards, the reported results with the measurement uncertainty and the MRLs.

Sample No	Commodity	Analyte	Retention time (min)		Relative intensity (m/z)		C [1] (mg/kg)	Uncertainty [4]	MRL
			sample	standard	sample	standard			
1	apricots	-							
2	apricots	carbaryl	7.65	7.63	10.0%	12.6%	LOQ[5]< C[1] <LOD[6]		0.05[2]
3	apricots	carbendazim	4.50	4.60	7.0%	7.4%	0.1458	± 0.073	0.2[3]
4	apricots	indoxacarb	13.63	13.48	23.4%	18.6%	0.0756	± 0.038	no MRLs
5	cherries	-							
6	cherries	-							
7	cherries	-							
8	cherries	-							
9	cherries	imidacloprid	3.40	3.34	75.4%	67.5%	LOQ[5]< C[1] <LOD[6]		0.5[3]
	cherries	thiamethoxam	2.22	2.17	29.2%	31.1%	0.0223	± 0.011	no MRLs
10	cherries	thiacloprid	5.19	5.19	9.8%	7.3%	0.0345	± 0.017	0.3[3]
	cherries	carbendazim	4.56	4.53	9.6%	7.1%	0.1910	± 0.096	0.5[3]
11	cherries	-							
12	peaches	-							
13	peaches	-							
14	plums	-							

[1] Concentration
[2] According to Commission Directive 2006/59/EC
[3] According to Commission Directive 2008/17/EC
[4] Measurement of uncertainty is a quantitative indicator of the confidence in the analytical data and describes the range around a reported or experimental result within which the true value can be expected to lie within a defined probability. Considering the results obtained to date from EU proficiency tests, a default expanded uncertainty figure of 50% (corresponding to a 95% confidence level), in general covers the inter-laboratory variability between the European laboratories and is recommended to be used by regulatory authorities in cases of enforcement decisions (MRL-exceedances) (5).
[5] Limit of quantification
[6] Limit of detection

more accurate results than multi-level calibration if the detector response is variable with time (5). When single-level calibration is employed, the sample response should be within ±50% of the calibration standard response. Matrix-matched calibration standards in blank extract from previously analysed organically produced stone fruit were used (5).

In the cases of positive samples, a blank extract spiked at a concentration level near the concentration found in the samples was analysed in order to assure the correct execution of the whole procedure. Recovery rates between 60 and 140% were expected for the spiked sample for the analysis to be considered accurate. Figure 1 shows the chromatogram of a commercial cherry sample that contained carbendazim at 0.191 mg/kg.

Conclusions

The 56 selected pesticides were of different physicochemical characteristics and chem-

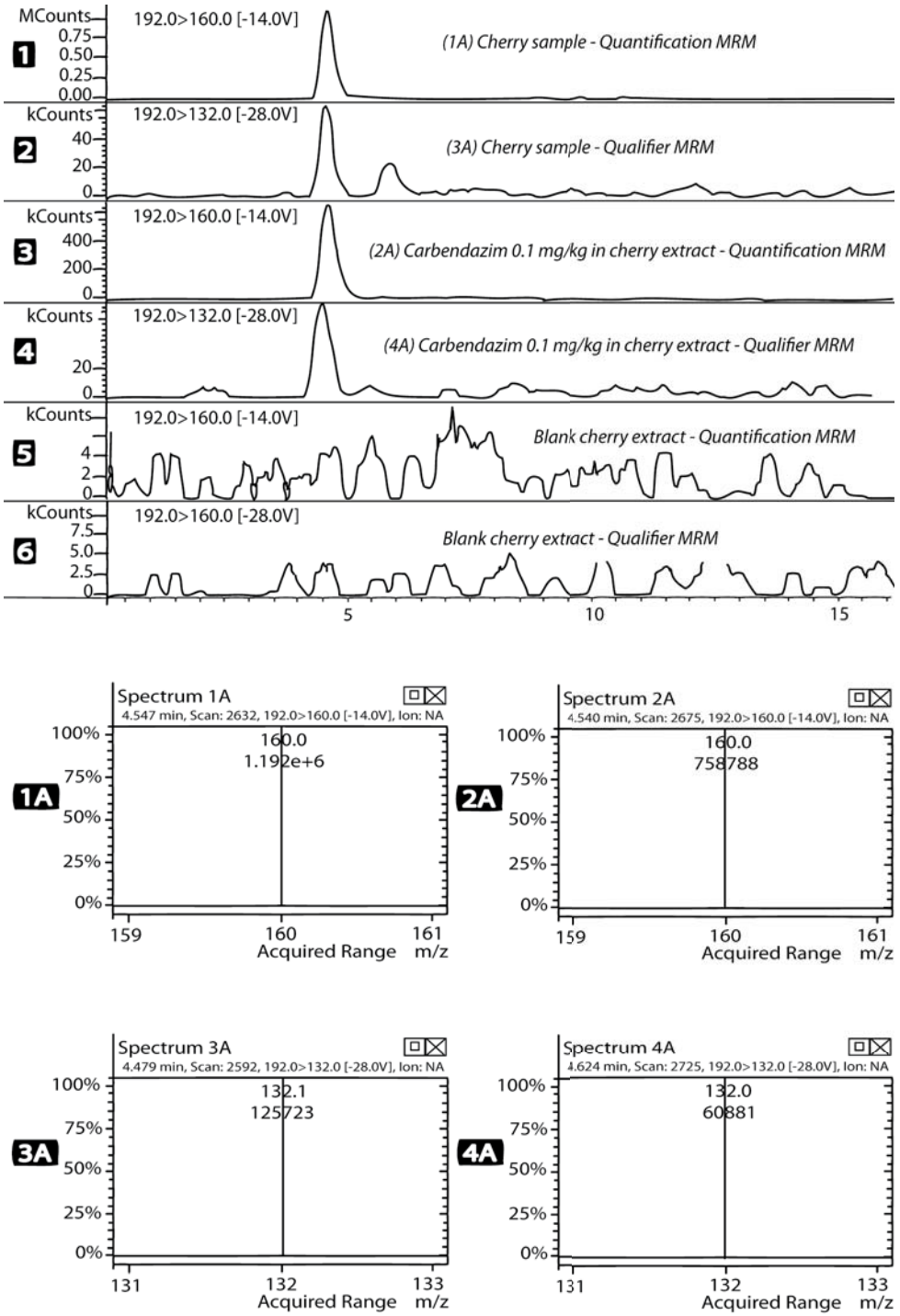

Figure 1. LC-MS/MS chromatogram of a real cherry sample that contained carbendazim at 0.191 mg/kg.

ical classes in order to be representative. The extraction procedure with acetone, followed by dichloromethane and petroleum ether, is easy and the determination performed with liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) at positive ionization mode, is a sensitive technique which provides confirmation of identity. The main advantage of using a LC-MS/MS system is its great chromatographic separation efficiency, which enables analysis to be performed in short chromatographic run. The method was successfully validated at the 10 μg/kg, which was the LOQ objective in this work for 45 compounds and at the 0.05 mg/kg for nine compounds. The method is simple, fast and suitable for routine analysis for the determination of pesticides in fruit and vegetables of high water content meeting the EU guidelines method performance criteria. Based on the good validation data resulting from this study, the suitability of the extraction and determination method on other pesticides of various chemical classes and other matrices of fruit and vegetables of high water content will be further investigated.

Literature Cited

1. Barceló, D. and Fernández-Alba, A. 2005. Chromatographic-mass spectrometric food analysis for trace determination of pesticide residues. Volume XLIII of BARCELÓ, D. (ed). Wilson & Wilson's Comprehensive Analytical Chemistry. Amsterdam: Elsevier, p. 83-84.
2. Bester, K., Bordin, G., Rodriguez, A., Schimmel, H., Pauwels, J. and Van Vyncht, G. 2001. How to overcome matrix effects in the determination of pesticides in fruit by HPLC-ESI-MS-MS. *Fresenius' Journal of Analytical Chemistry,* 371: 550.
3. Cole, R.B. 1997. Electrospray Ionization Mass Spectrometry: Fundamentals, Instrumentation, and Applications. New York: John Wiley & Sons, Ltd., 571 p.
4. Di Muccio, A., Fidente, P., Barbini, D.A., Dommarco, R., Seccia, S. and Morrica, P. 2006. Application of solid-phase extraction and liquid chromatography-mass spectrometry to the determination of neonicotinoid pesticide residues in fruit and vegetables. *Journal of Chromatography A*, 1108: 1–6.
5. European Commission. 2007. Method validation and Quality control procedures for pesticide residues analysis in food and feed, Document No. SANCO/2007/3131. Available from: www.ec.europa.eu/food/plant/protection/resources/qualcontrol_en.pdf
6. Hernández, F., Pozo, O.J., Sancho, J.V., Bijlsma, L., Barreda, M. and Pitarch, E. 2006. Multiresidue liquid chromatography tandem mass spectrometry determination of 52 non gas chromatography-amenable pesticides and metabolites in different food commodities. *Journal of Chromatography A*, 1109: 242–252.
7. Hiemstra, M. and de Kok, A. 2007. Comprehensive multi-residue method for the target analysis of pesticides in crops using liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, 1154: 3–25.
8. Hogenboom, C., Hofman, M.P., Kok, S.J., Niessen, W.M.A. and Brinkman, U.A.T. 2000. Determination of pesticides in vegetables using large-volume injection column liquid chromatography–electrospray tandem mass spectrometry. *Journal of Chromatography A,* 892: 379.
9. Luke, M.A., Froberg, J.E., Doose, G.M. and Masumoto, H.T. 1981. Improved multiresidue gas chromatographic determination of organophosphorus, organonitrogen and organohalogen pesticides in produce, using flame photometric and electrolyc conductivity detectors. *Journal of Association of Official Analytical Chemists,* 64: 1187-1195.
10. Luke, M.A., Froberg, J.E. and Masumoto, H.T. 1975. Extraction and clean-up of organochlorine, organophosphate, organonitrogen, and hydrocarbon pesticides in produce for determination by gas-liquid chromatography. *Journal of Association of Official Analytical Chemists,* 58: 1020-1026.
11. Maštovská, K. and Lehotay, S.J. 2006. Evaluation of common organic solvents for gas chromatographic analysis and stability of multiclass pesticide residues. *Journal of Chromatography A,* 1040: 259.
12. Mills, P.A., Onley, J.H. and Gaither, R.A. 1963. Rapid method for chlorinated pesticide residues in nonfatty foods. *Journal of Association of Official Analytical Chemists*, 46: 186-191.
13. Snedecor, G.W. and Cochran, W.G. 1967. *Statistical methods*. 6th Edition. Ames, Iowa: Iowa State University Press, 593 p.
14. Specht, W. and Tillkes, M. 1980. Gas-chromatographische Bestimmung von Rückständen an Pflanzenbehandlungsmitteln nach Clean-up über Gel-Chromatographie und Mini-Kieselgel-Säulen-Chromatographie. *Fresenius' Journal of Analytical Chemistry*, 301(4): 300-307.
15. Štěpàn, R., Tichà, J., Hajšslovà, J., Kovalczuk, T.

and Kocourek, V. 2005. Fast LC/MS/MS Quantitation of N-Methyl Carbamate Pesticides in Food Using Bond Elut™ Plexa™ and Pursuit™ XRs C18. *Food Additives & Contaminants,* 22: 1231.

16. Working group "Development and improvement of Residue-analytical methods". 1996. Analytical Methods for Pesticide Residues in Foodstuffs. Part I: Multiresidue methods, 6th Edition, Ministry of Public Health, Welfare and Sport, The Netherlands, p. 1-21.

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Μέθοδος προσδιορισμού υπολειμμάτων 56 φυτοπροστατευτικών προϊόντων σε ροδάκινα με την τεχνική της υγρής χρωματογραφίας–συζευγμένης φασματομετρίας μαζών

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Περίληψη Μια γρήγορη πολυ-υπολειμματική μέθοδος αναπτύχθηκε και επικυρώθηκε σε πυρηνόκαρπα για τον προσδιορισμό 56 φυτοπροστατευτικών ουσιών και μεταβολιτών τους διαφορετικών φυσικοχημικών ιδιοτήτων. Η εκχύλιση των ουσιών πραγματοποιήθηκε με ακετόνη και μίγμα πετρελαϊκού αιθέρα:διχλωρομεθάνιου (50:50 κ.ο.). Ο ποιοτικός και ποσοτικός προσδιορισμός των ουσιών πραγματοποιήθηκε με την τεχνική της υγρής χρωματογραφίας–συζευγμένης φασματομετρίας μάζας. Η μέθοδος επικυρώθηκε σε τρία διαφορετικά επίπεδα (0.01, 0.05 και 0.5 mg/kg). Από τα στοιχεία επικύρωσης προκύπτει ότι η μέθοδος παρουσιάζει αποδεκτή ορθότητα με ποσοστά ανάκτησης 65.7–125.3%, καθώς και πιστότητα με σχετικές τυπικές αποκλίσεις μικρότερες από 20%. Το όριο ποσοτικοποίησης της μεθόδου καθορίστηκε για τις περισσότερες περιπτώσεις το 0.01 mg/kg. Η μέθοδος χαρακτηρίζεται από αξιοπιστία και ευαισθησία και κρίνεται κατάλληλη για αναλύσεις ρουτίνας υπολειμμάτων φυτοπροστατευτικών προϊόντων σε πυρηνόκαρπα. Στα πλαίσια ελέγχου της Ελληνικής αγοράς αναλύθηκαν 14 δείγματα πυρηνόκαρπων. Τα πέντε από τα 14 δείγματα βρέθηκαν θετικά, αλλά σε επίπεδα χαμηλότερα από τα Ανώτατα όρια Υπολειμμάτων (MRLs) που θεσπίζονται από την Ευρωπαϊκή Ένωση.

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Sublethal effects of three essential oils on the development, longevity and fecundity of *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae)

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Summary *Acanthoscelides obtectus* larvae and pupae were exposed to three levels (LC_{10}, LC_{40} and LC_{70}) of lavender, rosemary and eucalyptus essential oil vapours. The exposure of larvae and pupae to sublethal doses of essential oil vapours resulted in increased larval and pupal developmental time and reduced longevity and fecundity of the emerged female adults. The observed effects depended on the insect stage and sex as well as the essential oil tested. In all cases no delayed mortality was observed.

Additional keywords: *Eucalyptus globulus, Lavandula hybrida, Rosmarinus officinalis,* volatiles

Introduction

In recent years, the trend towards reducing reliance on synthetic insecticides for the control of stored products pests has oriented worldwide research in the insecticidal properties of different essential oils that often behave like conventional fumigants exhibiting strong adverse effects on both immature and adult stages. In fact, these compounds may act as fumigants, repellents, antifeedants and may also affect some biological parameters such as growth rate, life span and reproduction (11, 12, 16, 18, 19, 20, 21, 23, 26).

Previous studies (18, 19) have established the strong toxic activity of the essential oil vapours of *Lavandula hybrida* Rev. (Lamiacae), *Rosmarinus officinalis* L. (Lamiacae) and *Eucalyptus globulus* Labill. (Myrtacae) against the immature stages of *Acanthoscelides obtectus* (Say), the most destructive pest of *Phaseolus vulgaris* L. (kidney bean), one of the most important food pulses in South America and the Mediterranean region.

Although the insecticide properties of the aforementioned essential oil vapours have been well documented and their LC_{50} values were established, their possible sublethal effects have not been thoroughly explored even though it is well known that many target insects do not receive the appropriate lethal dose during fumigations. In fact, insects exposed to sublethal doses of insecticides may display a variety of symptoms including reduction in growth rate, life span, pupal weight and adult fecundity and fertility (3, 4, 5, 9, 10, 15). In contrast, advantageous effects have also been recorded; these include increased immature stage weight, developmental rate and adult fertility (27). Therefore, the consequences of sublethal exposure have important implications on insect management and from this point of view it is worthy to elucidate such type of effects exhibited by the essential oil vapours (14). Moreover, as Karr and Coats (13) pointed out, when monoterpenoids are investigated as potential insecticides, direct toxicity as well as appraisal of negative effects on biotic potential must be considered in the assessment of overall efficacy.

The aim of this study was to investigate the effects of sublethal doses of lavender, rosemary and eucalyptus essential oil va-

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pours on *A. obtectus* development, longevity and fecundity.

Materials and methods

Insects and essential oils

Second instar larvae and 3-day-old pupae were obtained from laboratory cultures of *A. obtectus* according to the procedure described by Papachristos and Stamopoulos (18). The essential oils tested were extracted from the following plants: *L. hybrida* (flowers), *R. officinalis* (leaves) and *E. globulus* (unripe fruit). Plant samples were collected in mid July 1999 from an experimental farm in the region of Thessaloniki, Northern Greece, where they were cultivated at adjacent sites. Bulk samples of about 1 kg were collected randomly from 20–30 lavender or rosemary plants and three eucalyptus trees. Samples were transferred to the laboratory and 100 g subsamples were subjected to hydro distillation using a Clevenger apparatus (Winzer®) within 24 h. The distilled essential oils were dried over anhydrous sodium sulphate and stored in a refrigerator at 5°C. The chemical composition of the essential oils used in the present study has been reported by Papachristos *et al.* (17).

Exposure to essential oil vapours

Gastight glass jars of 710 ml volume with screwed metallic caps were used as exposure chambers. A small piece of woven dental cotton (diameter = 8 mm, height = 3.5 mm) was attached to the undersurface of the cap to serve as an oil diffuser following the application of the appropriate amount of pure essential oil. Dose levels were selected to cause 10, 40 and 70% mortality according to our findings in a previous study (19). The exact doses of each essential oil applied were: lavender: 0.2, 1.4, 2 µl/l air for larvae and 21, 36.8, 57.9 µl/l for pupae; rosemary: 1.4, 2.1, 2.8 µl/l air for larvae and 26.3, 52.6, 73.7 µl/l for pupae; eucalyptus: 1.4, 4.2, 7.1 µl/l air for larvae and 57.9, 73.7, 89.5 µl/l for pupae. Forty bean seeds containing about 30-40 second instar larvae or 3-day-old pupae of *A. obtectus* were placed in each jar. The same methodology was followed for the control without the application of essential oils. Six replicates were used for each dose and insect stage. After 48 h of exposure to essential oil vapours, the beans were transferred to clean vials for four days and subsequently the beans in half of the replicates were dissected in order to count the dead larvae and pupae. The beans from the other three replicates remained intact and were observed daily until the adult survivors emerged. The developmental time from the end of exposure to adult emergence was also recorded. After emergence the beans were dissected and dead larvae and pupae were counted.

Sublethal effects on adults

The effect of the essential oil vapours on adult insects derived from the survived treated larvae and pupae was also studied. For this purpose male or female survivors from each dose tested were weighted just after their emergence and subsequently, 20-30 of them were placed individually in plastic Petri dishes (9 cm in diameter) in order to record their longevity.

To study the fecundity and fertility of female survivors, about 15 females, emerged from each dose tested, were placed individually with two untreated one-day-old males taken from the mass culture in plastic Petri dishes (9 cm in diameter) containing 10 beans and allowed to mate and oviposit until their death. The number of eggs laid was counted daily. The eggs were kept in the Petri dishes until the end of hatching and the number of hatched and unhatched eggs was recorded.

Experimental conditions

All trials were conducted at 65 ± 5 r.h, 25 ± 1 °C and a 12 h photoperiod.

Statistical analyses

All mortality data were corrected by using Abbott's formula (1). T-test was used in order to compare the average mortality calculated four days after exposure to essential oil vapours with that recorded after the emergence of the adults.

Data concerning the adult weight, adult longevity, fecundity, egg hatchability and pupae developmental time, were analysed by using one way analysis of variance (ANOVA) followed by the least significant difference test (LSD) for means separation (24, 25). Means of the larvae developmental time were compared with the Mann-Whitney U test, as the obtained data did not satisfy the assumptions required for a parametric test (24).

Results

Delayed mortality

The recorded mortality four days after the exposure of larvae or pupae to essential oil vapours did not differ significantly from that recorded after the emergence of adults for all essential oils and doses tested (t test, all P>0.05) (Figure 1) indicating that the majority of larvae and pupae were killed during or immediately after the treatment phase.

Sublethal effects

Lavender essential oil

Exposure of larvae to lavender essential oil vapours did not significantly affect the developmental time from larvae to adult emergence at all doses tested except for that of 2 µl/l air (Mann-Whitney U test, Table 1). Weight and longevity of adults were also not significantly affected (males: $F_{3, 83} = 1.727$; $F_{3, 131} = 0.730$; females: $F_{3, 100} = 2.502$; $F_{3, 115} = 2.618$; all $P > 0.05$, for weight and longevity, respectively). Females derived from larvae exposed to the highest dose of lavender essential oil vapours deposited fewer eggs than control females ($F_{3, 49} = 3.982$, $P < 0.01$), but egg hatchability was not affected ($F_{3, 45} = 2.807$, $P > 0.05$).

Exposure of pupae to lavender essential oil vapours resulted in extension of the developmental time from pupae to adult for both sexes, only at the highest concentration tested (males: $F_{3, 128} = 4.916$, $P < 0.01$; females: $F_{3, 134} = 5.898$, $P < 0.001$) (Table 2). The adult weight and the longevity of male survivors were not affected (weight of males: $F_{3, 53} = 1.001$, $P>0.05$; weight of females: $F_{3, 51} = 0.629$, $P > 0.05$ and longevity of males: $F_{3, 84} = 0.805$, $P > 0.05$). The longevity of the female survivors in all doses tested was significantly lower compared to control ($F_{3, 65} = 11.631$, $P < 0.001$), whereas females laid fewer eggs (32-42%) at the two higher doses ($F_{3, 34} = 3.721$, $P<0.05$). The hatchability of the eggs laid was not significantly affected ($F_{3, 32} = 0.662$, $P > 0.05$).

Rosemary essential oil

After exposure of larvae to rosemary essential oil vapours, the developmental time of larvae was significantly affected only at the highest dose tested (Mann-Whitney U test, Table 3). Exposure to the highest dose also caused a reduction in adult male weight ($F_{3, 78} = 5.841$, $P < 0.001$), whereas the weight of females was not significantly affected ($F_{3, 89} = 1.469$; $P>0.05$). The longevity of the adults of both sexes was not significantly affected (males: $F_{3, 107} = 0.370$; females: $F_{3, 98} = 1.300$, all $P > 0.05$). Females derived from treated larvae laid approximately 23-35% fewer eggs at all doses tested ($F_{3, 49} = 4.486$, $P < 0.01$) compared to control, but egg hatchability was not significantly affected ($F_{3, 49} = 0.496$, $P > 0.05$).

After exposure of pupae to rosemary essential oil vapours, the developmental time from pupae to adult was significantly affected only for the male survivors at the highest dose tested (males: $F_{3, 163} = 4.073$, $P < 0.01$; females: $F_{3, 147} = 2.036$, $P > 0.05$) (Table 4). The weight of the adult survivors did not differ from that of the control insects except for the female survivors derived from the highest dose tested (males: $F_{3, 53} = 1.73$, $P > 0.05$; females: $F_{3, 59} = 4.322$, $P < 0.01$). No negative effects on the longevity of male survivors was recorded ($F_{3, 125} = 2.289$, $P > 0.05$), while the longevity of the adult females was reduced at all doses tested ($F_{3, 89} = 12.036$, $P < 0.001$). Moreover, the female survivors laid fewer eggs at the two higher doses ($F_{3, 36} = 4.253$, $P < 0.01$), but egg hatchability was not significantly affected ($F_{3, 33} = 0.201$; $P > 0.05$).

Eucalyptus essential oil

Exposure of larvae to eucalyptus essential oil vapours prolonged the developmental time from larvae to adult for both sexes at

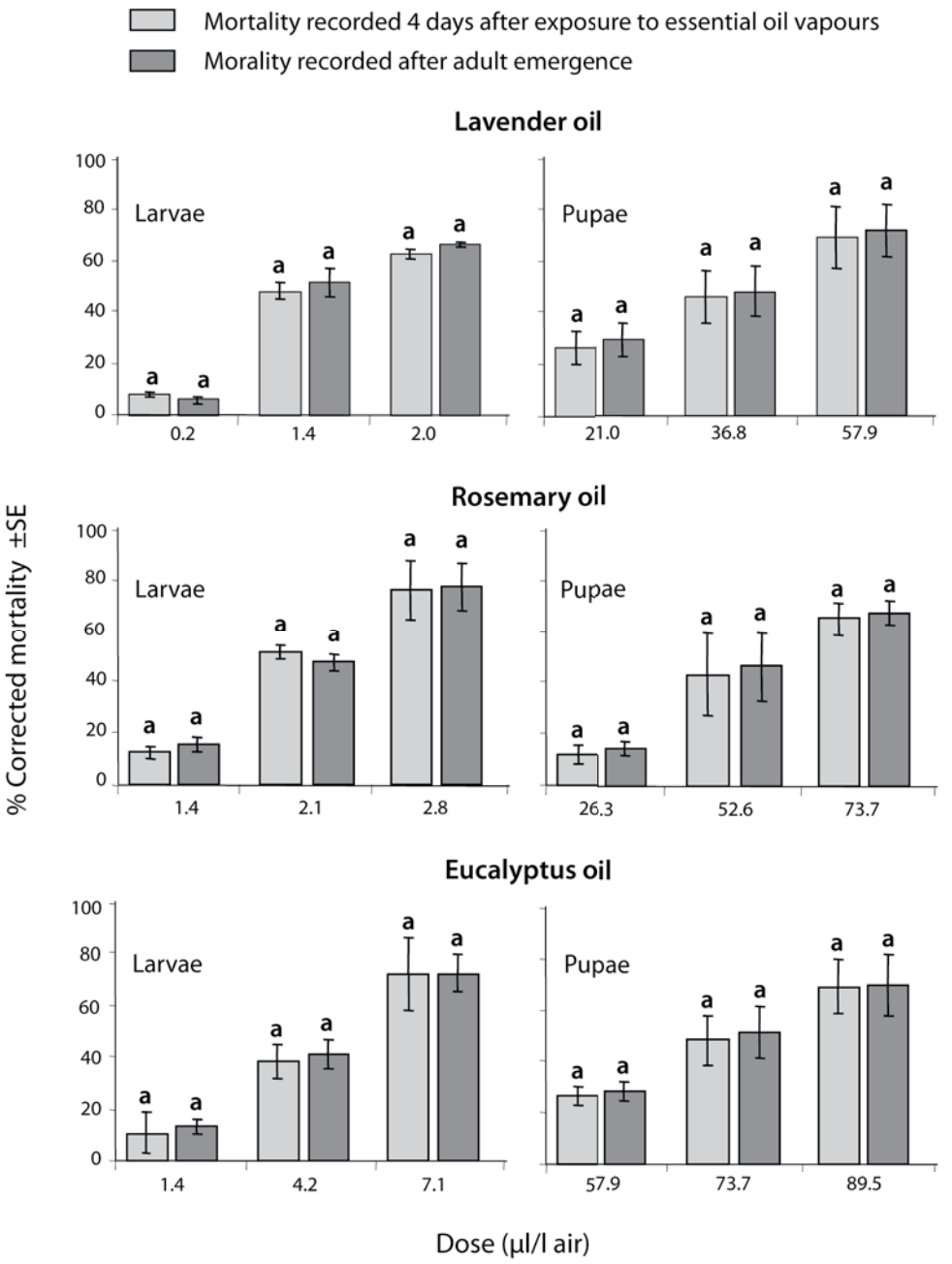

Figure 1. Mortality of *Acanthoscelides obtectus* larvae and pupae after exposure to three essential oil vapours [columns within each dose and essential oil capped by the same letter do not differ significantly (t-test: P>0.05)].

all doses tested (Mann-Whitney U test, Table 5). Males that survived the two higher doses weighted significantly less compared to the control males ($F_{3, 79} = 4.246$, $P < 0.01$), whereas the weight of the female survivors was not significantly affected ($F_{3, 116} = 2.489$, $P > 0.05$).

Table 1. Developmental time (number of days from the end of exposure to adult emergence), weight, longevity, fecundity and fertility of *Acanthoscelides obtectus* adults derived from 2nd instar larvae that survived the exposure to three different doses of lavender essential oil vapours.

Dose (µl/l air)	Mean developmental time ± SE[1] (days)		Mean adult weight ± SE[2] (mg)		Mean adult longevity ± SE[2] (days)		Mean number of eggs laid ± SE[2]	Mean (%) unhutched eggs ± SE[2]
	Males	Females	Males	Females	Males	Females		
0	26.0 ± 0.3a	27.4 ± 0.2a	5.14 ± 0.12a	6.27 ± 0.72a	16.3 ± 0.8a	22.3 ± 0.8a	55.3 ± 2.8a	10.7 ± 1.7a
0.2	26.5 ± 0.3a	27.1 ± 0.3a	4.81 ± 0.11a	6.10 ± 0.10a	16.5 ± 0.8a	21.9 ± 0.8a	43.3 ± 3.4ab	17.5 ± 2.2a
1.4	27.4 ± 0.5ab	28.1 ± 0.6a	4.87 ± 0.13a	6.20 ± 0.14a	16.5 ± 1.0a	19.6 ± 1.0a	45.1 ± 5.2ab	12.8 ± 1.7a
2	28.4 ± 0.6b	28.7 ± 0.6a	4.84 ± 0.09a	5.91 ± 0.09a	14.6 ± 1.4a	17.8 ± 2.4a	33.9 ± 5.8b	11.0 ± 1.2a

Means within a column followed by the same letter do not differ significantly ([1]Mann-Whitney U test, [2]LSD test: P≥0.05).

Table 2. Developmental time (number of days from the end of exposure to adult emergence), weight, longevity, fecundity and fertility of *Acanthoscelides obtectus* adults derived from pupae that survived the exposure to three different doses of lavender essential oil vapours.

Dose (µl/l air)	Mean developmental time ± SE (days)		Mean adult weight ± SE (mg)		Mean adult longevity ± SE (days)		Mean number of eggs laid ± SE	Mean (%) unhatched eggs ± SE
	Males	Females	Males	Females	Males	Females		
0	6.8 ± 0.2a	7.5 ± 0.2a	5.23 ± 0.17a	6.41 ± 0.18a	15.6 ± 0.7a	22.6 ± 0.9a	53.9 ± 3.4a	10.5 ± 2.3a
21	7.4 ± 0.2a	7.5 ± 0.3a	4.94 ± 0.17a	6.11 ± 0.18a	14.5 ± 0.8a	19.5 ± 1.3b	43.3 ± 4.0ab	12.2 ± 1.8a
36.8	7.2 ± 0.2a	7.1 ± 0.2a	4.87 ± 0.12a	6.16 ± 0.22a	14.6 ± 0.6a	18.3 ± 0.9b	36.5 ± 4.8b	8.9 ± 1.3a
57.9	8.5 ± 0.4b	9.1 ± 0.5b	4.9 ± 0.19a	6.08 ± 0.18a	13.9 ± 0.9a	13.8 ± 0.9c	31.0 ± 8.3b	9.4 ± 1.2a

Means within a column followed by the same letter do not differ significantly (LSD test: P≥0.05).

Table 3. Developmental time (number of days from the end of exposure to adult emergence), weight, longevity, fecundity and fertility of *Acanthoscelides obtectus* adults derived from 2nd instar larvae that survived the exposure to three different doses of rosemary essential oil vapours.

Dose (µl/l air)	Mean developmental time ± SE[1] (days)		Mean adult weight ± SE[2] (mg)		Mean adult longevity ± SE[2] (days)		Mean number of eggs laid ± SE[2]	Mean (%) unhatched eggs ± SE[2]
	Males	Females	Males	Females	Males	Females		
0	26.0 ± 0.3a	27.4 ± 0.2a	5.14 ± 0.12a	6.27 ± 0.72a	16.3 ± 0.8a	22.3 ± 0.8a	55.3 ± 2.8a	10.8 ± 1.7a
1.4	25.3 ± 0.7a	26.2 ± 0.6a	4.98 ± 0.14a	6.05 ± 0.09a	16.2 ± 1.1a	19.3 ± 1.2a	35.7 ± 5.3b	17.8 ± 6.2a
2.1	26.2 ± 0.5a	27.3 ± 0.3a	4.87 ± 0.07a	6.00 ± 0.10a	15.2 ± 1.0a	20.5 ± 1.4a	42.1 ± 2.9b	11.6 ± 2.0a
2.8	29.3 ± 0.9b	30.9 ± 0.9b	4.50 ± 0.13b	6.00 ± 0.25a	15.0 ± 1.9a	20.2 ± 2.4a	40.2 ± 5.3b	11.7 ± 1.2a

Means within a column followed by the same letter do not differ significantly ([1]Mann-Whitney U test, [2]LSD test: P≥0.05).

Adult longevity was not significantly affected (males: $F_{3, 144} = 0.408$, $P > 0.05$; females: $F_{3, 101} = 1.647$, $P > 0.05$), but the females surviving the highest dose tested laid fewer eggs compared to the control females ($F_{3, 56} = 4.194$, $P < 0.01$). Egg hatchability was not significantly affected ($F_{3, 54} = 1.644$; $P > 0.05$).

Exposure of pupae to eucalyptus essential oil vapours did not affect the developmental time (males: $F_{3, 207} = 1.31$; females: $F_{3, 195} = 0.389$, all $P > 0.05$) or the weight of the adult survivors (males: $F_{3, 57} = 1.457$; females:

Table 4. Developmental time (number of days from the end of exposure to adult emergence), weight, longevity, fecundity and fertility of *Acanthoscelides obtectus* adults derived from pupae that survived the exposure to three different doses of rosemary essential oil vapours.

Dose (μl/l air)	Mean developmental time ± SE (days)		Mean adult weight ± SE (mg)		Mean adult longevity ± SE (days)		Mean number of eggs laid ± SE	Mean (%) unhatched eggs ± SE
	Males	Females	Males	Females	Males	Females		
0	6.8 ± 0.2a	7.5 ± 0.2a	5.23 ± 1.7a	64.1 ± 0.18a	15.6 ± 0.7a	22.6 ± 0.9a	53.9 ± 3.4a	10.5 ± 2.3a
26.3	6.9 ± 0.2a	7.3 ± 0.2a	5.14 ± 0.22a	6.16 ± 0.26a	15.5 ± 0.5a	19.9 ± 0.7b	53.2 ± 4.1a	11.4 ± 1.2a
52.6	7.3 ± 0.2ab	7.2 ± 0.2a	5.06 ± 0.11a	6.16 ± 0.17a	14.3 ± 1.5a	19.5 ± 0.8b	36.3 ± 5.9b	12.0 ± 3.6a
73.7	8.1 ± 0.3b	8.5 ± 0.6a	5.16 ± 0.12a	5.53 ± 0.18b	13.3 ± 0.6a	14.9 ± 1.0c	32.0 ± 8.4b	8.8 ± 3.5a

Means within a column followed by the same letter do not differ significantly (LSD test: P≥0.05).

Table 5. Developmental time (number of days from the end of exposure to adult emergence), weight, longevity, fecundity and fertility of *Acanthoscelides obtectus* adults derived from 2nd instar larvae that survived the exposure to three different doses of eucalyptus essential oil vapours.

Dose (μl/l air)	Mean developmental time ± SE[1] (days)		Mean adult weight ± SE[2] (mg)		Mean adult longevity ± SE[2] (days)		Mean number of eggs laid ± SE[2]	Mean (%) unhatched eggs ± SE[2]
	Males	Females	Males	Females	Males	Females		
0	26.0 ± 0.3a	27.4 ± 0.2a	5.14 ± 0.12a	6.27 ± 0.07a	16.3 ± 0.8a	22.3 ± 0.8a	55.3 ± 2.8a	10.7 ± 1.7a
1.4	28.9 ± 0.4b	29.8 ± 0.3b	4.89 ± 0.09ab	5.88 ± 0.12a	16.5 ± 0.9a	19.1 ± 1.0a	47.9 ± 2.3a	13.5 ± 2.2a
4.2	31.4 ± 0.5c	32.1 ± 0.4c	4.64 ± 0.08b	5.87 ± 0.24a	16.8 ± 1.3a	19.5 ± 1.3a	51.9 ± 3.3a	21.8 ± 5.3a
7.1	31.3 ± 0.8c	32.8 ± 0.9c	4.74 ± 0.15b	6.02 ± 0.18a	15.1 ± 1.3a	20.1 ± 2.3a	37.8 ± 5.7b	15.9 ± 3.4a

Means within a column followed by the same letter do not differ significantly ([1]Mann-Whitney U test, [2]LSD test: P≥0.05).

Table 6. Developmental time (number of days from the end of exposure to adult emergence), weight, longevity, fecundity and fertility of *Acanthoscelides obtectus* adults derived from pupae that survived the exposure to three different doses of rosemary essential oil vapours.

Dose (μl/l air)	Mean developmental time ± SE (days)		Mean adult weight ± SE (mg)		Mean adult longevity ± SE (days)		Mean number of eggs laid ± SE	Mean (%) unhatched eggs ± SE
	Males	Females	Males	Females	Males	Females		
0	6.8 ± 0.2a	7.5 ± 0.2a	5.23 ± 0.17a	6.41 ± 0.18a	15.6 ± 0.7a	22.6 ± 0.9a	53.9 ± 3.4a	10.5 ± 2.3a
57.9	7.1 ± 0.2a	7.6 ± 0.3a	5.24 ± 0.17a	6.19 ± 0.16a	13.4 ± 0.9b	17.6 ± 0.8b	38.1 ± 4.9b	10.4 ± 2.0a
73.7	7.4 ± 0.2a	7.9 ± 0.3a	5.15 ± 0.18a	6.18 ± 0.16a	13.2 ± 0.6b	16.4 ± 0.7bc	33.7 ± 4.1b	20.7 ± 4.1b
89.5	7.4 ± 0.3a	7.6 ± 0.3a	4.82 ± 0.15a	5.91 ± 0.21a	12.8 ± 0.6b	14.8 ± 1.0c	35.9 ± 5.1b	11.8 ± 2.4a

Means within a column followed by the same letter do not differ significantly (LSD test: P≥0.05).

$F_{3, 53} = 1.294$, all $P > 0.05$) (Table 6). However, adult longevity was significantly decreased (males: $F_{3, 101} = 3.462$, $P < 0.01$; females: $F_{3, 98} = 11.518$, $P < 0.001$) and females laid 29-37% fewer eggs compared to the control females ($F_{3, 37} = 4.071$, $P < 0.01$). Egg hatchability was only affected at the intermediate dose tested ($F_{3, 37} = 3.136$, $P < 0.05$).

Discussion

The study of delayed mortality showed that larvae and pupae of *A. obtectus* within beans died during or soon after their exposure to lavender, rosemary or eucalyptus essential oil vapours. In the case of larvae, this rapid death is a requisite in order to avoid further

damage of the beans. A similar rapid action has been reported for citrus peel essential oil vapours against larvae of *Callosobruchus maculatus* F. (7).

Previous studies have shown that the essential oils and their main components, monoterpenoids, adversely affect the growth and development of larvae of various species via ingestion (8, 13, 15). Our results showed that the essential oils can also affect larval and pupal growth and development as well as fecundity of the survivors via their vapour action.

Exposure of *A. obtectus* larvae and pupae to sublethal doses of lavender and rosemary essential oil vapours did not affect the developmental time to adult emergence except for the doses causing about 70% mortality, where a slight prolongation of the developmental time was observed mainly in the male adults. On the contrary, in the case of the eucalyptus essential oil, a prolonged larval developmental time was observed at all doses tested. Differences in the chemical composition of the three essential oils tested (levanter, rosemary and eucalyptus) (17) may account for their different biological action against *A. obtectus*.

Regarding the effect of essential oil vapours on the weight of adults derived from treated larvae or pupae, a slight decrease was observed which, in most cases, was not statistically significant. In similar experiments, Gunderson *et al.* (8) and Lee *et al.* (15) recorded higher levels of decrease in adult weight (in some cases up to 60%). However, in those experiments the insects were not exposed to oil vapours as the essential oils had been incorporated into their diet.

Adult longevity of *A. obtectus* seems to depend on the stage exposed to the essential oil vapours and the sex of the surviving adult. In fact, the longevity of the adults of both sexes derived from larvae exposed to essential oil vapours was not affected at all doses tested, whereas the exposure of pupae to essential oil vapours resulted in an average 14-34% reduction in the longevity of the female adults at all doses tested. The males were less affected than females, as the longevity of male adults was reduced only in the case of eucalyptus essential oil.

The most severe physiological effect of exposure of larvae and pupae to sublethal doses of the essential oil vapours was the reduction in the female fecundity although the viability of the eggs laid was not decreased significantly. In fact, females emerged from treated larvae or pupae laid 25-40% fewer eggs than control females. The effects of essential oil vapours on the male reproductive system were not investigated in the present study, but similar studies (8) have shown that males were less sensitive than females.

Overall, an adverse impact on the development and especially fecundity of *A. obtectus* individuals surviving the exposure to vapours of three different essential oils should be added to their main rapid mortality action. However, in the interpretation of sublethal effects it is difficult to differentiate whether these effects are due to insecticidal action or to inherent characteristics of the insecticide-selected individuals (6). Subsequently, comparison between treated and control individuals would, thus, be biased (2). In fact, in preliminary experiments with *A. obtectus* adults resistant to lavender essential oil vapours (DP Papachristos, unpubl.), a reduced biological potential (longer developmental time and lower intrinsic rate of increase compared to susceptible population) was observed, but no data are available at the moment for the other insect stages. The practical implications of these findings suggest that, when *A. obtectus* is exposed to lavender, rosemary or eucalyptus essential oil vapours, satisfactory control may be achieved even if some insects survive the treatment.

Literature Cited

1. Abbott, W.W. 1925. A method of computing the effectiveness of an insecticide. *Journal of Economic Entomology*, 18: 265-267.
2. Alford, A.R. and Holmes, J.A. 1986. Sublethal effects of carbaryl, aminocarb, fenitrothion, and *Bacillus thuringiensis* on the development and fecundity of the spruce budworm (Lepidoptera:

Tortricidae). *Journal of Economic Entomology*, 79: 31-34.

3. Ansari, M.A., Vasudevan, P., Tandon, M. and Razdan, P.K. 2000. Larvicidal and mosquito repellent action of peppermint (*Mentha piperita*) oil. *Bioresource Technology,* 71: 267-271.
4. Bazzoni, E., Passino, G.S., Moretti, M.D.L. and Prota, R. 1997. Toxicity of anethole and its effects on egg production of *Ceratitis capitata* Wied. (Dipt., Tephritidae). *Annals of Applied Biology,* 131: 369-374.
5. Collart, M.G. and Hink, W.F. 1986. Sublethal effects of D-limonene on the cat flea, *Ctenocephalides felis*. *Entomologia Experimentalis et Applicata,* 42: 225-229.
6. Croft, B.A., 1990. Arthropod biological control agents and pesticides. New York: John Wiley and Sons, 723 p.
7. Don-Pedro, K.N. 1996. Fumigant toxicity of citruspeel oils against adult and immature stages of storage insect pests. *Pesticide Science,* 47: 213-223.
8. Gunderson, G.A., Samuelian, J.H., Evans, C.K. and Brattsten, L.B. 1985. Effects of the mint monoterpene pulegone on *Spodoptera eridania* (Lepidoptera: Noctuidae). *Environmental Entomology*, 14: 859-863.
9. Gurusubramanian, G. and Krishna, S.S. 1996. The effects of exposing eggs of four cotton insect pests to volatiles of *Allium sativum* (Liliaceae). *Bulletin of Entomological Research*, 86: 29-31.
10. Huang, Y., Lam, S.L. and Ho, S.H. 2000. Bioactivities of essential oil from *Elletaria cardamomum* (L.) Maton. to *Sitophilus zeamais* Motschulsky and *Tribolium castaneum* (Herbst). *Journal of Stored Products Research*, 36: 107-117.
11. Ignatowicz, S. and Gersz, M. 1997. Extracts of medical herbs as repellents and attractants for the dry bean weevil, *Acanthoscelides obtectus* Say (Coleoptera: Bruchidae). *Polskie Pismo Entomologiczne*, 66: 151-159.
12. Kalinović, I., Martinčić, J., Rozman, V. and Guberac, V. 1997. Insecticidal activity of substances of plant origin against stored product insects. *Ochrana Rostlin*, 33: 135-142.
13. Karr, L.L. and Coats, J.R. 1992. Effects of four monoterpenoids on growth and reproduction of the German cockroach (Blattodea: Blattellidae). *Journal of Economic Entomology*, 85: 424-429.
14. Kerns, D.L. and Stewart, S.D. 2000. Sublethal effects of insecticides on the intrinsic rate of increase of cotton aphid. *Entomologia Experimentalis et Applicata*, 94: 41-49.
15. Lee, S., Tsao, R. and Coats, J.R. 1999. Influence of dietary applied monoterpenoids and derivatives on survival and growth of the European corn borer (Lepidoptera: Pyralidae). *Journal of Economic Entomology*, 92: 56-67.
16. Niber, B., Helenius, J. and Varis, A.-L. 1992. Toxicity of plant extracts to three storage beetles (Coleoptera). *Journal of Applied Entomology*, 113: 202-208.
17. Papachristos, D.P., Karamanoli, K.I., Stamopoulos, D.C. and Menkissoglu-Spiroudi, U. 2004. The relationship between the chemical composition of three essential oils and their insecticidal activity against *Acanthoscelides obtectus* (Say). *Pest Management Sciences*, 60: 514-520.
18. Papachristos, D.P. and Stamopoulos, D.C. 2002a. Repellent, toxic and reproduction inhibitory effects of essential oil vapours on *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae). *Journal of Stored Products Research*, 38: 117-128.
19. Papachristos, D.P. and Stamopoulos, D.C. 2002b. Toxicity of three essential oils vapours to the immature stages of *Acanthoscelides obtectus* (Say) (Coleoptera, Bruchidae). *Journal of Stored Products Research*, 38: 365-373.
20. Rąkowski, G. and Ignatowicz, S. 1997. Effects of some plant extracts on fecundity and longevity of the dry bean weevil, *Acanthoscelides obtectus* Say (Coleoptera: Bruchidae). *Polskie Pismo Entomologiczne*, 66: 161-167.
21. Regnault-Roger, C. and Hamraoui, A. 1994. Inhibition of reproduction of *Acanthoscelides obtectus* Say (Coleoptera), a kidney bean (*Phaseolus vulgaris*) bruchid, by aromatic essential oils. *Crop Protection*, 13: 624-628.
22. Regnault-Roger, C. and Hamraoui, A. 1995. Fumigant toxic activity and reproductive inhibition induced by monoterpenes on *Acanthoscelides obtectus* (Say) (Coleoptera), a bruchid of kidney bean (*Phaseolus vulgaris* L.). *Journal of Stored Products Research*, 31: 291-299.
23. Regnault-Roger, C., Hamraoui, A., Holeman, M., Theron, E. and Pinel, R. 1993. Insecticidal effect of essential oils from Mediterranean plants upon *Acanthoscelides obtectus* Say (Coleoptera, Bruchidae), a pest of kidney bean (*Phaseolus vulgaris* L.). *Journal of Chemical Ecology*, 19: 1233-1244.
24. Sokal, R.R. and Rohlf, J.F. 1995. *Biometry*, 3d end. New York: W. H. Freeman and company, 887 p.
25. SPSS. 2004. SPSS 14 for Windows User's Guide. SPSS, Chicago, IL, USA.
26. Stamopoulos, D.C. 1991. Effects of four essential oil vapours on the oviposition and fecundity of *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae): Laboratory evaluation. *Journal of Stored Products Research*, 27: 199-203.
27. Zanuncio, T.V., Serrão, J.E., Zanuncio, J.C. and Guedes, R.N.C. 2003. Permethrin-induced hormesis in the predator *Supputius cincticeps* (Stal, 1860) (Heteroptera: Pentatomidae). *Crop Protection*, 22: 941-947.

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Υποθανατηφόρες επιδράσεις τριών αιθέριων ελαίων στην ανάπτυξη, μακροβιότητα και γονιμότητα του *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae)

Δ.Π. Παπαχρήστος και Δ.Κ. Σταμόπουλος

Περίληψη Προνύμφες και νύμφες του εντόμου *Acanthoscelides obtectus* εκτέθηκαν σε τρεις διαφορετικές συγκεντρώσεις (LC_{10}, LC_{40} και LC_{70}) ατμών των αιθέριων ελαίων λεβάντας, δεντρολίβανου και ευκάλυπτου. Οι προνύμφες και οι νύμφες που δέχθηκαν την επίδραση των αιθέριων ελαίων και επιβίωσαν εμφάνισαν καθυστέρηση στην ανάπτυξή τους. Επίσης, τα θηλυκά που προέκυψαν από προνύμφες και νύμφες που είχαν δεχθεί την επίδραση των ατμών των αιθέριων ελαίων εμφάνισαν μειωμένη διάρκεια ζωής και γονιμότητα. Το είδος και η ένταση των επιδράσεων βρέθηκε να εξαρτάται από το στάδιο ανάπτυξης και το φύλο του εντόμου που δέχθηκε την επίδραση των αιθέριων ελαίων καθώς και από το είδος και τη δόση των αιθέριων ελαίων. Επιπλέον σε καμία περίπτωση δεν παρατηρήθηκε καθυστερημένη θνησιμότητα.

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Toxic effects of insecticide residues on three aphidophagous coccinellid species

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Summary The toxicity of 1- and 8-day residues of three insecticides (imidacloprid, thiacloprid and methomyl) was tested in the laboratory against the larvae (first and third instars) and adults of the predacious coccinellids *Ceratomegilla undecimnotata* (Schneider), *Oenopia conglobata* L. and *Propylaea quatuordecimpunctata* L. Predators were placed for 24 h on broad bean leaves sprayed with the tested insecticides at the recommended doses. Larval mortality was checked 1 day after exposure, upon pupation and adult emergence. Adult mortality was checked 1, 10 and 20 days after exposure to insecticides. Generally, 1- and 8-day residues of all insecticides tested caused high mortality (up to 100%) to larvae and adults.

Additional keywords: adults, imidacloprid, larvae, methomyl, thiacloprid, toxicity

Introduction

Beetles of the Coccinellidae family are among the most common aphid predators (5, 8, 10). However, their conservation in agroecosystems is limited by the extensive use of insecticides. Predatory coccinellids are likely to be exposed to a great number of insecticides while foraging in the field. More specifically, they can be exposed to insecticides directly during pesticide applications or indirectly by contacting insecticide residues while foraging on sprayed surfaces or by consuming prey exposed to insecticides. Concerning the significant role of coccinellids and other natural enemies in aphid biocontrol, the use of more selective insecticides in integrated pest management (IPM) strategies is helpful to the conservation of natural enemies (4). The most suitable insecticides are those with high toxicity to pests and no toxic effects on natural enemies (18, 19). Thus, the impact of any compound used during crop protection on natural enemies should be part of the essential evaluation process.

The lethal (mortality) and sublethal (developmental time, fecundity, locomotory behavior) effects of insecticides on coccinellid predators have been studied extensively (2, 7, 15, 17, 22, 25). However, susceptibility of coccinellids to insecticides may vary significantly according to the insect species, its developmental stage and the type of the chemicals applied (21, 25). Although a great number of studies have addressed the toxic effects of insecticides against the coccinellid predators, there is no data concerning the effects of insecticides against some important coccinellid predators.

Ceratomegilla undecimnotata (Schneider) (Coleoptera: Coccinellidae) is a common European species that preys upon a high number of aphid species in cultivated and non-cultivated plants (10, 12, 13). *Propylaea quatuordecimpunctata* L. (Coleoptera: Coccinellidae), a widely distributed Palearctic aphidophagous species that has been established in Canada and has spread into the United States, preys upon numerous economically important aphids on a wide range of crops (6, 9, 16, 23). *Oenopia conglobata* L. (Coleoptera: Coccinellidae) is also a widespread coccinellid predator in Europe and

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Asia that feeds on numerous aphid species (1, 14, 24).

As there is lack of data on the toxic effects of insecticides against the above mentioned coccinellid predators, the aim of the present study was to evaluate the residual toxicity of one carbamate (methomyl) and two neonicotinoids (imidacloprid, thiacloprid) insecticides against the larvae (first and third instars) and adults of three common predacious coccinellids, *C. undecimnotata O. conglobata* and *P. quatuordecimpunctata*.

Material and methods

Insects

Laboratory cultures of *C. undecimnotata* were established from individuals collected in mid August 2006 from an alfalfa field in the area of Arta (Epirus, south-western Greece) and cultures *P. quatuordecimpunctata* and *O. conglobata* were established from adults collected during the same period from a corn field in the same area. In the area where the coccinellids were collected no insecticides had been applied for the last five years. Ladybird beetles were reared on broad bean plants (*Vicia faba* L.) infested with *Aphis fabae* Scopoli (Hemiptera: Aphididae) and held in a controlled-environment room at 23 ± 1 °C, 65±5% r.h. and a 16-h photoperiod. F_5 progeny were used for the experiments. Aphid colonies were established from individuals collected from a commercial bean crop (*Phaseolus vulgaris* L.) and reared on *V. faba* plants.

Insecticide application

Five broad bean seeds were sown in each pot (13.5 cm upper diameter, 7 cm base diameter, 12.5 cm height and 1 l volume) filled with a mixture of peat (black and white peat substrate, Floradur B fine, Floragard) and perlite (Perloflor, Protectivo Ltd) in a 6:1 weight proportion. When the plants reached 20 cm of height they were spayed with aqueous solutions of the insecticides at the recommended doses and leaves were collected 1 and 8 days after application. The insecticides tested were: imidacloprid (Confidor 200 SL, Bayer Hellas, 20% a.i., applied rate 0.5ml/l), thiacloprid (Calypso 480 SC, Bayer Hellas, 48% a.i., applied rate 0.3ml/l) and methomyl (Selanox 20 SL, SEGE SA Hellas, 20% a.i., applied rate 1.5 ml/l).

Bioassay

Four leaves from the sprayed plants were placed in each Petri dish (9 cm in diameter) and the appropriate developmental stage of the predator was left to walk on the sprayed leaves for 24 h. An opening was made to the lid of the Petri dishes, which was subsequently covered with fine muslin to allow ventilation and maintenance of humidity level inside the Petri dish similar to that of the laboratory. For the larval studies and in order to avoid cannibalism, first and third instar larvae were placed individually in the Petri dishes. Groups of 10 larvae were considered as one replicate. For the adult studies, five adults were placed in each Petri dish and two Petri dishes were considered as one replicate. First and third instar larvae were exposed to insecticides one day after their enclosure and adults five days after emergence. After exposure to insecticides, insects were placed in clean Petri dishes and were provided with *A. fabae* daily. Larvae were checked daily until they reached the adult stage. The adults were also checked daily for 20 days following exposure to insecticides and the dead individuals were counted. Three to five replicates were used for each insecticide and developmental stage of the predator. The same procedure was followed for the control plants which were sprayed with water.

Statistical analysis

The differences in mean mortality among treatments were determined by the Mann-Whitney U test using the statistical package SPSS 14.0 (20).

Results and Discussion

Insecticides were highly toxic to both larvae

and adults of *C. undecimnotata* exposed to 1- or 8-day insecticide residues (Table 1). Irrespectively of the insecticide used, almost all first instar larvae were found dead 24 h after their exposure to the residues. Similarly, all third instar larvae and adults exposed to methomyl residues did not manage to survive. Only a small proportion of the third instar larvae and adults survived the exposure to imidacloprid and thiacloprid residues. However, the small proportion of larvae that survived insecticide exposure did not manage to reach the adult stage. The longevity of the adult survivors was significantly lower compared to control. Similar were the effects of the tested insecticides on *P. quatuordecimpunctata* (Table 2) and *O. conglobata* (Table 3).

Imidacloprid has been exclusively tested against coccinellid predators and a broad range of its lethal and sublethal effects have been evaluated (11, 22, 25). However, the intensity of the toxic effects depends on the coccinellid species, as well as on the rate and methods of insecticides application (11, 25). For example, James (11) found that the direct application of imidacloprid at the recommended doses on *Stethorus punctum picipes* (Casey) and *Harmonia axyridis* Pallas was highly toxic to larvae of both coccinellid species. Nevertheless, Youn *et al*. (25) found that direct application of imidacloprid at the recommended doses against all developmental stages of *H. axyridis* was highly toxic to the egg stage as well as to the first, second and third instar larvae but not to the fourth instar larvae, pupae or adults. In our study, imidacloprid was highly toxic to both larval and adult stage of all three coccinellid species tested. In some cases, a small proportion of the third instar larvae or adults survived the exposure to imidacloprid, but they did not manage to reach adulthood or survive for long after their exposure to the insecticide, respectively. Although the studies on the effects of methomyl on coccinellid predators are limited, toxic effects due to its direct application have been reported on *H. axyridis* Pallas, *Coccinella repanda* Thunberg and *Harmonia octomaculata* (Fabricius) (3, 11).

Table 1. Residual toxicity of three insecticides to larvae (first and third instars) and adults of *Ceratomegilla undecimnotata* in a 24-h exposure bioassay. Mortality was assessed for larvae 1 day after exposure to insecticides, upon pupation and adult emergence and for adults 1, 10 and 20 days after exposure to insecticides.

Insecticides	% Mean mortality ± SE					
	1-day residues			8-day residues		
	First instar larvae					
	1 day	Pupation	Adult emergence	1 day	Pupation	Adult emergence
Imidacloprid	92.9 ± 7.1 a*	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a
Thiacloprid	100 ± 0.0 a	100 ± 0 a	100 ± 0 a	86.7 ± 13.0 a	93.3 ± 6.7 a	93.3 ± 6.7 a
Methomyl	100 ± 0.0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a
Control	14.0 ± 5.8 b	18.0 ± 4.6 b	18.0 ± 4.6 b	14.0 ± 5.8 b	18.0 ± 4.6 b	18.0 ± 4.6 b
	Third instar larvae					
	1 day	Pupation	Adult emergence	1 day	Pupation	Adult emergence
Imidacloprid	56.4 ± 9.1 b	90.8 ± 5.3 a	95.0 ± 5.0 a	84.3 ± 8.8 a	90 ± 5.0 a	95.2 ± 4.7 a
Thiacloprid	85.0 ± 15.0 ab	100 ± 0 a	100 ± 0 a	50.0 ± 18.8 b	100 ± 0 a	100 ± 0 a
Methomyl	100 ± 0.0 a	100 ± 0 a	100 ± 0 a	89.3 ± 10.7 a	96.4 ± 3.6 a	96.4 ± 3.4 a
Control	4.0 ± 4.0 c	4.0 ± 4.0 b	4.0 ± 4.0 b	4.0 ± 4.0 c	4.0 ± 4.0 b	4.0 ± 4.0 b
	Adults					
	1 day	10 days	20 days	1 day	10 days	20 days
Imidacloprid	72.9 ± 10.4 b	93.7 ± 6.2 a	100 ± 0 a	68.6 ± 15.6 b	91.7 ± 5.3 a	91.8 ± 5.3 a
Thiacloprid	100 ± 0.0 a	100 ± 0 a	100 ± 0 a	88.9 ± 11.1 ab	94.4 ± 5.5 a	100 ± 0 a
Methomyl	100 ± 0.0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a
Control	0.0 ± 0.0 c	5.0 ± 5.0 b	10 ± 6.1 b	0.0 ± 0.0 c	5.0 ± 5.0 b	10 ± 6.1 b

* Values within each column and developmental stage followed by the same letter do not differ significantly (Mann-Whitney U test, P≥0.05).

Table 2. Residual toxicity of three insecticides to larvae (first and third instars) and adults of *Propylaea quatuordecimpunctata* in a 24-h exposure bioassay. Mortality was assessed for larvae 1 day after exposure to insecticides, upon pupation and adult emergence and for adults 1, 10 and 20 days after exposure to insecticides.

Insecticides	% Mean mortality ± SE					
	1-day residues			8-day residues		
	First instar larvae					
	1 day	Pupation	Adult emergence	1 day	Pupation	Adult emergence
Imidacloprid	100 ± 0 a*	100 ± 0 a	100 ± 0 a	95.0 ± 5.0 a	95.0 ± 5.0 a	95.0 ± 5.0 a
Thiacloprid	100 ± 0 a	100 ± 0 a	100± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a
Methomyl	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a
Control	9.0 ± 5.6 b	13.0 ± 8.3 b	18.0 ± 4.6 b	9.0 ± 5.6 b	13.0 ± 8.3 b	18.0 ± 4.6 b
	Third instar larvae					
	1 day	Pupation	Adult emergence	1 day	Pupation	Adult emergence
Imidacloprid	75.0 ± 19.3 a	90 ± 10 a	90 ± 10 a	71.8 ± 18.1 a	88.7 ± 6.6 a	88.7 ± 6.6 a
Thiacloprid	79.2 ± 16.3 a	100 ± 0 a	100 ± 0 a	95.0 ± 5.0 a	95.0 ± 5.0 a	95.0 ± 5.0 a
Methomyl	95.8 ± 4.2 a	90 ± 4 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a
Control	5.0 ± 5.0 b	5.0 ± 5.0 b	16.5 ± 9.2 b	5.0 ± 5.0 b	5.0 ± 5.0 b	16.5 ± 9.2 b
	Adults					
	1 day	Pupation	Adult emergence	1 day	Pupation	Adult emergence
Imidacloprid	70 ± 20 b	90 ± 4 a	90 ± 4 a	60.7 ± 18.7 a	96.4 ± 3.5 a	96.4 ± 3.5 a
Thiacloprid	60 ± 20 b	100 ± 0 a	100 ± 0 a	82.2 ± 11.6 a	96.8 ± 3.1 a	100 ± 0 a
Methomyl	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a
Control	0.0 ± 0.0 c	15.0 ± 6.1 b	15.0 ± 6.1 b	0.0 ± 0.0 b	15.0 ± 6.1 b	15.0 ± 6.1 b

* Values within each column and developmental stage followed by the same letter do not differ significantly (Mann-Whitney U test, P≥0.05).

Table 3. Residual toxicity of three insecticides to larvae (first and third instars) and adults of *Oenopia conglobata* in a 24-h exposure bioassay. Mortality was assessed for larvae 1 day after exposure to insecticides, upon pupation and adult emergence and for adults 1, 10 and 20 days after exposure to insecticides.

Insecticides	% Mean mortality ± SE					
	1-day residues			8-day residues		
	First instar larvae					
	1 day	Pupation	Adult emergence	1 day	Pupation	Adult emergence
Imidacloprid	96.7 ± 3.3 a	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0a	100 ± 0a
Thiacloprid	100 ± 0 a	100 ± 0 a	100 ± 0 a	71.6 ± 13.3 a	100 ± 0a	100 ± 0a
Methomyl	100 ± 0 a	100 ± 0 a	100 ± 0 a	91.1 ± 12.2 a	100 ± 0a	100 ± 0a
Control	0 ± 0 b	0 ± 0 b	9.0 ± 5.5 b	0 ± 0 b	0 ± 0 b	9.0 ± 5.5 b
	Third instar larvae					
	1 day	Pupation	Adult emergence	1 day	Pupation	Adult emergence
Imidacloprid	93.3 ± 6.6 ab	100 ± 0 a	100 ± 0 a	70.8 ±23.9 a	87.5 ± 12.5 a	87.5 ± 12.5 a
Thiacloprid	70 ± 15.2 b	95.0 ± 7.6 a	100 ± 0 a	93.7 ± 6.2 a	100 ± 0 a	100 ± 0 a
Methomyl	100 ± 0 a	100 ± 0 a	100 ± 0 a	75.0 ± 14.4 a	87.5 ± 7.2 a	100 ± 0 a
Control	0 ± 0 c	0 ± 0 c	9.5 ± 6.2 b	0 ± 0 b	0 ± 0 b	9.5 ± 6.2 b
	Adults					
	1 day	10 days	20 days	1 day	10 days	20 days
Imidacloprid	100 ± 0 a	100 ± 0 a	100 ± 0 a	65.0 ± 11.9 a	89.5 ± 6.2 a	89.5 ± 6.2 a
Thiacloprid	96.7 ± 3.3 a	100 ± 0 a	100 ± 0 a	80.3 ± 6.9 a	100 ± 0 a	100 ± 0 a
Methomyl	100 ± 0 a	100 ± 0 a	100 ± 0 a	81.2 ± 11.8 a	87.5 ± 12.5 a	87.5 ± 12.5 a
Control	5.0 ± 5.0 b	10.6 ± 6.0 b	10.6 ± 6.0 b	5.0 ± 5.0 b	10.6 ± 6.0 b	10.6 ± 6.0 b

* Values within each column and developmental stage followed by the same letter do not differ significantly (Mann-Whitney U test, P≥0.05)

Overall, all the tested insecticides were highly toxic to all three coccinellids and the toxic effects remained the same even when the coccinellids came into contact with residues 8 days after the application of the insecticides on plant foliage. However, the exposure techniques under laboratory conditions may not be representative of the predator exposure to insecticides under field conditions and consequently, the actual mortality in the field may be different than that obtained under laboratory conditions (11). Nevertheless, laboratory evaluation of insecticide toxicity against beneficial insects may be a relatively good indicator of the possible effects these insecticides might have to beneficials at field level. Thus, it can be concluded that the insecticides tested in this study may have significant toxic effects against these three coccinellid predators and, therefore, may not be compatible with IPM strategies.

Literature Cited

1. Aslan, M.M. and Uygun, N. 2005. The Aphidophagus coccinellid (Coleoptera: Coccinellidae) species in Kahramanmaraş, Turkey. T*urkish Journal of Zoology,* 29: 1-8.
2. Bozsik, A. 2006. Susceptibility of adult *Coccinella septempunctata* (Coleoptera: Coccinellidae) to insecticides with different modes of action. *Pest Management Science*, 62: 651-654.
3. Broadley, R.H. 1983. Toxicity of Insecticides to *Coccinella repanda* Thunberg and *Harmonia octomaculata* (Fabricius) (Coleoptera: Coccinellidae). *Queensland Journal of Agricultural Animal Science*, 40: 125-127.
4. Croft, B.A. 1990. Arthropod Biological Control Agents and Pesticides. New York: John Wiley and Sons, 723p.
5. Dixon, A.F.G. 2000. Insect Predator-Prey Dynamics: Ladybirds and Biological Control, Cambridge, MA: Cambridge University Press, 279 p.
6. Freier, B., Triltsch, H., Mowes, M. and Moll, E. 2007. The potential of predators in natural control of aphids in wheat: Results of a ten-year field study in two German landscapes. *BioControl*, 52: 775-788.
7. Galvan, T.L., Koch, R.L. and Hutchison, W.D. 2005. Effects of spinosad and indoxacarb on survival, development and reproduction of the multicolored Asian lady beetle (Coleoptera: Coccinellidae). *Biological Control*, 34, 108-114.
8. Hodek, I. and Honek, A. 1996. Ecology of Coccinellidae, The Netherlands: Kluwer Academic Publishers, 480 p.
9. Honek, A. 1985. Habitat preferences of aphidophagous coccinellids (Coleoptera). *Entomophaga*, 30: 253-264.
10. Iperti, G. 1999. Biodiversity of predaceous coccinellidae in relation to bioindication and economic importance. *Agricultural, Ecosystems and Environments,* 74: 323-342.
11. James, D.G. 2003. Pesticide Susceptibility of Two Coccinellids (*Stethorus punctum picipes* and *Harmonia axyridis*) Important in Biological Control of Mites and Aphids in Washington Hops. *Biocontrol Science and Technology*, 13: 253-259.
12. Katsoyannos, P., Kontodimas, D. and Stathas, G. 2005. Summer diapause and winter quiescence of *Hippodamia* (*Semiadalia*) *undecimnotata* (Coleoptera: Coccinellidae) in central Greece. *European Journal of Entomology*, 102: 453-457.
13. Kavallieratos, N.G., Athanassiou, C.G., Tomanovic, Z., Papadopoulos, G.D. and Vayias, B.J. 2004. Seasonal abundance and effects of predators (Coleoptera, Coccinellidae) and parasitoids (Hymenoptera: Braconidae, Aphidiinae) on *Myzus persicae* (Hemiptera, Aphidoidea) densities on tobacco: a two-year study from central Greece. *Biologia Bratislava*, 59: 613-619.
14. Khan, I., Din, S., Khalil, S.K. and Rafi, M.A. 2007. Survey of predatory Coccinellids (Coleoptera: Coccinellidae) in the Chitral District, Pakistan. 6pp. *Journal of Insect Science,* 7:07. available online: insectscience.org/7.07.
15. Lucas, É., Giroux, S., Demougreot, S., Duchesne, R-M. and Coderre, D. 2004. Compatibility of natural enemy, *Coleomegilla maculata lengi* (Col., Coccinellidae) and four insecticides used against the Colorado potato beetle (Col., Chrysomelidae). *Journal of Applied Entomology*, 128: 233-239.
16. Obrycki, J.J., Orr, D.B., Orr, C.J., Wallendorf, M. and Flanders, R.V. 1993. Comparative development and reproductive biology of three populations of *Propylaea quatuordecimpunctata* (Coleoptera: Coccinellidae). *Biological Control*, 3: 27-33.
17. Olszak, R.W. 1999. Influence of some pesticides on mortality and fecundity of the aphidophagous coccinellid *Adalia bipunctata* L. (Col., Coccinellidae). *Journal of Applied Entomology*, 123: 41-45.
18. Plapp, F.W. and Bull, D.C. 1978. Toxicity and selectivity of some insecticides to *Chrysopa carnea*, a predator of the tobacco budworm. *Environmental Entomology*, 7: 431-434.
19. Rajakwlendran, S.V. and Plapp, F.W. 1982. Comparative toxicity of five synthetic pyrethroids to the tobacco budworn, an ichneumonid parasite

Compoletis sonorensis and a predator *Chrysopa carnea*. *Journal of Economic Entomology*, 75: 769-772.

20. SPSS. 2004. SPSS 14 for Windows, User's Guide. SPSS, Chicago, IL, USA.
21. Theiling, K.M. and Croft, A.B. 1988. Pesticide side effects on arthropod natural enemies: a database summary. *Agricultural, Ecosystems and Environments*, 21: 191-218.
22. Vincent, C., Ferran, A., Guige, L., Gambier, J. and Brun, J. 2000. Effects of imidacloprid on *Harmonia axyridis* (Coleoptera: Coccinellidae) larval biology and locomotory behavior. *European Journal of Entomology*, 97: 501-506.
23. Wheeler, A.G.J. 1990. *Propylea quatuordecimpunctata*: Additional U.S. records of an adventive lady beetle (Coleoptera: Coccinellidae). *Entomological News*, 101: 164-165.
24. Yaşar, B. and Özger, S. 2005. Functional response of *Oenopia conglobata* (L.) (Coleoptera: Coccinellidae) on *Hyalopterus pruni* (Geoffroy) (Homoptera: Aphididae) in three different size arenas. *Turkiye Entomoloji Dergisi*, 9: 91-99.
25. Youn, Y.N., Seo, M.J., Shin, J.G., Jang, C. and Yu, Y.M., 2003. Toxicity of greenhouse pesticides to multicolored Asian lady beetles, *Harmonia axyridis* (Coleoptera: Coccinellidae). *Biological Control*, 28: 164-170.

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Τοξικότητα ορισμένων εντομοκτόνων σε τρία θηρευτικά είδη εντόμων της οικογένειας των Coccinellidae

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Περίληψη Διερευνήθηκε, σε συνθήκες εργαστηρίου, η τοξική επίδραση τριών εντομοκτόνων (imidacloprid, thiacloprid και methomyl) σε προνύμφες πρώτης και τρίτης ηλικίας καθώς και ενήλικα των θηρευτικών εντόμων *Ceratomegilla undecimnotata* (Schneider), *Oenopia conglobata* L. και *Propylaea quatuordecimpunctata* L. (Coleoptera: Coccinellidae). Τα έντομα εκτέθηκαν μία και οκτώ ημέρες μετά τον ψεκασμό και για 24 ώρες σε φύλλα κουκιών που είχαν ψεκαστεί με τις συνιστώμενες δόσεις των υπό μελέτη εντομοκτόνων. Η θνησιμότητα των προνυμφών μετρήθηκε 24 ώρες μετά την έκθεσή τους στα εντομοκτόνα καθώς και κατά τη νύμφωση και ενηλικίωσή τους. Η θνησιμότητα των ενηλίκων μετρήθηκε 1, 10 και 20 ημέρες μετά την έκθεσή τους στα εντομοκτόνα. Όλα τα εντομοκτόνα προκάλεσαν σημαντική θνησιμότητα (μέχρι και 100%) στις προνύμφες και τα ενήλικα των θηρευτικών εντόμων.

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