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REVIEW ARTICLE

Targeting injectisomes of virulence: Benefits of thirty years of research on bacterial secretion systems, to crop protection

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Summary The discovery in the mid-80s of contiguous gene clusters that were later shown to code for a novel protein secretion system has been a milestone in plant pathology and the study of plant-bacterial interactions. This system, named type III secretion system (T3SS), is dedicated to the translocation of virulence-related proteins, called effectors, from the pathogen to the host and is common among phytopathogenic, zoopathogenic and symbiotic bacteria. Bacterial pathogens overcome multiple layers of plant defense, both preformed barriers and inducible mechanisms. To accomplish this they deploy sophisticated molecular devices to secrete selected sets of proteins either to the intercellular spaces or directly inside the plant cell. Such proteins enable pathogens to avoid recognition, block induction of immune responses and/or interfere with the defense signaling network. Recent developments in molecular biology facilitated research on these interactions and rendered the inter-kingdom trafficking of proteins a key element of pathogenicity, virulence and host specificity of Gram-negative bacterial pathogens. While basic research on phytobacterial pathogens has progressed, little has changed on the crop protection schemes against bacterial infections. In this review, we summarize the latest findings on the different levels at which contact-dependent protein secretion from Gram-negative bacteria subverts and/or manipulates plant immunity. Additionally, we focus on the biotechnological applications which have emerged from basic research on phytobacterial T3SS and its cognate effectors, ranging from bacterial diagnostics, plant resistance and anti-virulence chemicals to toolkits for gene targeting in plants.

Additional keywords: Diagnostics, effector protein, *hrp*, resistance, type III secretion

Introduction

Bacteria have evolved different strategies to adapt and colonize specific niches, including the plant phyllosphere and rhizosphere. Dependence on the host for survival has lead bacterial species to adapt to a symbiotic or a pathogenic lifestyle, the latter including strategies to proliferate, disperse and overcome plant defenses in order to be successful pathogens. For this purpose, pathogens deploy an arsenal of molecular weapons, of which toxins, phytohormones, cell wall degrading enzymes, lipo- and exopolysaccharides were identified and characterized during the 60s and 70s. In the same period, scientists studied the various lines of plant defense, focusing on preformed physical barriers, phytoalexins and other antimicrobial compounds that pre-existed or were formed post-infection (reviewed in Hückelhoven, 2007). However, with few exceptions, plant defense, pathogen virulence and host range (especially race-cultivar specificity) could not be adequately explained on such bases (reviewed in Hückelhoven, 2007) but instead was thought to rely largely upon induction of a series of host immune responses. Flor's seminal analysis of the complemen-

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tary genetic systems of the flax rust fungus (*Melampsora lini)* and its host, uncovered the complementary genetic systems of the pathogen and the plant, determining the outcome of race-cultivar interaction (either compatible or incompatible) and led him to formulate the "gene-for-gene hypothesis", which explained in genetic terms the basis of race-cultivar specificity (Flor, 1971).

In the early 60s, Klement and his associates discovered that phytopathogenic bacteria, like fungi, were able to induce a rapid plant cell death at the infection site, also known as the plant hypersensitive response (HR), which was associated with restriction of pathogen proliferation (Klement and Goodman, 1967). Against this background, several parallel discoveries in the mid-1980s and the decade that followed led to the unified concept that bacterial virulence on plants relies heavily on sophisticated molecular machines, known as bacterial secretion systems or injectisomes, which enable them to target host defense systems at various levels. Key discoveries included the cloning of phytobacterial avirulence (*avr*) genes (Staskawicz *et al*., 1984), the discovery of gene clusters including the *hrp* genes (phonetic "Harp") (Panopoulos *et al*., 1984; Panopoulos and Peet, 1985; Lindgren *et al*., 1986), and the identification of virulence genes in animal pathogens (Isberg and Falkow, 1985). These were later shown to share homologies with *hrp* genes and to mediate secretion of virulence-related proteins lacking a canonical signal peptide (rev. in Tampakaki *et al*., 2010). The boost in molecular functional genetics of bacteria that followed confirmed this concept and revealed the injectisomes' organization and function. The latter is to help pathogens transfer (inject) directly into the plant cytosol proteins that affect immunity and are known as effector proteins. Today, the products of pathogen *avr* genes are collectively referred to as effectors, since in the absence of cognate plant immunity receptors they promote pathogen virulence by directly or indirectly interfering with plant defense mechanisms (Ritter and Dangl, 1996, Grant *et al*., 2006; Jones and Dangl, 2006; Guo *et al*., 2009). These findings helped explain the enigma why phytopathogens carry avirulence genes (Dangl, 1994; Gabriel, 1999).

Plant immunity consists of different layers of defense, including a series of immune responses that are triggered post-infection by a variety of elicitors. One group of elicitors that are conserved among different bacterial pathogens includes the so-called pathogen-associated molecular patterns (PAMPs), which elicit PAMP-triggered immunity (PTI). Another group includes effectors, which as previously mentioned are often coded by classical avirulence genes. Molecular recognition of PAMPs by the host is mediated by proteins that recognize molecular structures conserved across a broad range of pathogenic species and are known as pattern recognition receptors (PPRs). Recognition of effectors on the other hand by the host is mediated by resistance proteins that are plasma membrane or intracellularly located.

It is now widely recognized that in their interactions with eukaryotes, Gram-negative bacteria use a variety of molecular devices, including extracellular appendages, to deliver diverse proteins and other molecules to the host cell interior (Charova *et al*., 2012). With the rapid accumulation of bacterial genome sequences, our knowledge of the complexity of bacterial protein secretion systems has expanded and numerous biochemical studies have revealed the existence of at least six major mechanisms of protein secretion (Type I, II, III, IV, V and VI), which are often highly conserved among the Gram-negative bacteria species. This review focuses on the type III and type VI protein secretion systems (T3SS, T6SS) that mediate secretion and/or translocation of bacterial virulence-related proteins inside the host cell in a host contact dependent manner and under specific *in vitro* conditions (Hayes *et al*. 2010). Emphasis in this review is given on the biotechnological applications that emerged from the study of these systems, and range from plant pathogen diagnostics to antibiotic development. The review does not cover the Agrobacterial type IV secretion system, which has provided a basis for biotechnolog-

ical applications in the field of genetic engineering of crops on a commercial scale – the subject has been extensively reviewed elsewhere (Gelvin, 2010; Van Montagu, 2011).

T3SS function as vehicles of protein trafficking

T3SS are molecular nanomachines found in many Gram-negative proteobacteria (α, β, γ and δ subdivisions, also found in some non-proteobacteria such as *Chlamydiae)* that establish intimate relationships (pathogenic, symbiotic or commensal) with plant, human, insect, nematode or animal hosts and are evolutionarily related to bacterial flagella (Dale and Moran, 2006; Tampakaki *et al*., 2004; Troisfontaines and Cornelis, 2005; Charova *et al*., 2012). They function as "injectisomes", translocating diverse repertoires of proteins to extracellular locations and into eukaryotic cells. Many T3SS-secreted proteins (T3SS effectors) modulate the function of host regulatory networks, which determine the outcome of recognition and biological accommodation with the eukaryotic host. T3SS have evolved into seven phylogenetic families (Troisfontaines and Cornelis, 2005). Some bacteria may harbor more than one T3SS, which usually belong to different phylogenetic families (non-orthologous), an indication of relatively recent horizontal gene transfer.

In plant pathogenic bacteria, the T3SS forms a long pilus that equals or exceeds in length that of the bacterial cell and has a central channel through which the secreted proteins are transported from the prokaryotic cytoplasm across the inner and outer bacterial membrane as well as the plant cell wall, into the host cytosol. The proteins that build and regulate the assembly and function of this secretion apparatus are coded by three classes of genes (reviewed in Tampakaki *et al*., 2010). Firstly, the *hrc* genes (*hrp* conserved), which also have homologs in the bacterial flagellum and whose protein products are conserved at the sequence level among animal and plant pathogenic bacteria. They express proteins known as the T3SS core components, including HrcJ, HrcU, HrcV, HrcN, HrcR, HrcT, HrcS and HrcC, which form the basal body of the apparatus that is embedded in the inner and outer bacterial membrane. HrcC forms a ring-like structure in the outer membrane. HrcN and its homologs catalyze ATP hydrolysis providing energy to the system and are involved in protein unfolding and transport (Lorenz and Büttner, 2009). Secondly, transcriptional regulators of the T3SS operons and effector genes, such as a) the alternative sigma factor (σ^L) HrpL, the master regulator of the secretion system in *P. syringae* pathovars*, Pantoea, Pectobacterium* and *Erwinia/Dickeya* species, and b) the AraC-type regulators HrpB and HrpX in *Ralstonia* and *Xanthomonas* species, respectively (Büttner and Bonas U., 2009). Importantly, *avr/*effector genes and *hrp/hrc* operons are co-regulated, a finding that provided an early clue about their functional linkage. The third class of genes codes for the extracellular T3SS components (the pilus subunits, HrpA in *P. syringae* and *Erwinia amylovora*, HrpY in *R. solanacearum*, HrpE in *X. campestris*), proteins serving as chaperones assisting in injectisome assembly, secreted proteins with extracellular enzymatic function, proteins forming pores in the plant cell wall or assisting translocation activity (HrpK, HrpF; reviewed in Büttner and He, 2009) and proteins such as harpins (HrpZ, HrpW, PopA; He *et al*., 1993) that are able to elicit the HR in purified form.

The *hrp/hrc* genes are generally located in pathogenicity islands (PAIs) and are primarily found on the bacterial chromosome rather than on plasmids. Typical *hrp* clusters, such as those of *P. syringae* pathovars, consist of core regions of 6 operons and 27 open reading frames (ORFs), flanked by two effector rich loci, the conserved effector locus (CEL) and the exchangeable effector locus (EEL; Alfano *et al*., 2000). New *in silico* analysis revealed multiple T3SS clusters in the same strain, found not to be non-orthologous and functionally non-redundant but serving bacterial virulence in different ways. For example, in case of *E. amylovora,* one T3SS mediates interaction with the plant host, while the second might be serv-

ing dissemination by insect vectors (Triplett *et al.*, 2006).

The type VI secretion system

The recently discovered T6SS is typically encoded by clusters of 13 genes thought to constitute the minimal set needed to produce a functional secretion apparatus. Our current knowledge concerning T6SS regulation of gene expression and apparatus components has been obtained basically from studies on human and animal bacterial pathogens (Boyer *et al.*, 2009). T6SS occur mostly in α-, β-, and γ-proteobacteria (about 25% of the sequenced genomes; Bingle *et al.*, 2008) and in *Helicobacter hepaticus* (Chow and Mazmanian, 2010), a member of the ε-(epsilon) group, which is an intestinal microbiote. Multiple copies of these clusters (often referred to as T6SS loci in the literature) are frequently found in the same genome and are non-orthologous, indicating that they did not originate from internal duplication in the bacterial host but were probably acquired independently by horizontal transfer (Sarris *et al.*, 2010). In few such cases that have been studied, each T6SS assumes a different role in the interactions of the bacterial organism with others.

At present, only few T6SS substrates have been verified experimentally, but others may merely await identification. Some of the T6SS core component proteins are structurally related to the cell-puncturing devices of tailed bacteriophages, and at least in some well-studied cases, the system has been shown capable of translocating effector proteins into the host cell cytoplasm, and thus is implicated in virulence of certain plant pathogenic bacteria, including *Agrobacterium tumefaciens, Pectobacterium atrosepticum* and *Xanthomonas oryzae*, as well as in the multi-host pathogen *Pseudomonas aeruginosa* strain PA14 (reviewed in Sarris *et al.*, 2012). However, the T6SS is not only implicated in pathogenesis, as several reports attribute a fundamental role in efficient root colonization/nodule formation by the nitrogen-fixing plant symbionts/mutualists *Mesorhizobium loti*, *Rhizobium leguminosarum* and *Cupriavidus taiwanensis* (Bingle *et al.*, 2008).

The diverse role of Bacterial effectors–not the last words

Bacterial T3SS effectors constitute a large and diverse group of virulence proteins with a wide range of cellular-subcellular targets and biochemical functions. A prominent feature of these proteins is their modular architecture, comprised by domains or motifs that confer an array of biochemical functions within the eukaryotic cell. They are in essence molecular chimeras of functionally distinct sequence domains, distributed in different ways along the effector sequence properly but with some common rules. For example, sequence motifs required to direct secretion from the bacterial cell, translocation into the host cell and membrane localization, are generally located to the N-terminus of the protein, while functional domains and motifs mediating subcellular targeting and interactions with host targets typically localize to the central or C-terminal portions. This modularity has been exploited for construction of reporters to monitor secretion/translocation and in genetic screens designed to identify new effectors. An impressive variety of structural motifs are found in various combinations in T3SS effectors (Dean, 2012), which may be viewed as "genetic jugglery" to borrow a term from Davis and Davis (2010).

The motifs required for T3SS are located in the first 15-25 N-terminal residues, but without discernible consensus, and a chaperone binding domain 50 to 150 residues downstream. Subcellular targeting signals include mitochondrial targeting motifs, membrane targeting motifs nuclear localization signals, caspase processing sites and G-protein regulatory domains, a variety of motifs involved in protein–protein interactions, and a long list of catalytic activities (Dean, 2012). These features form the bases of the diverse cellular roles of effectors in bacteria-host interactions.

Particular effectors have been found to control bacterial proliferation on the plant

surface, prevent stomata closure and inhibit enzymatic degradation of bacterial peptidoglycans in the leaf apoplast. Moreover, they mask elicitor recognition and block signaling receptors. They are able to downregulate, suppress and/or degrade defense mechanisms such as callose deposition and papillae formation, kinase phosphorylation and dephosphorylation, resistance protein activation, and block the hypersensitive response triggered by other effectors (reviewed in Jones and Dangl, 2006; Göhre and Robatzek, 2008). Moreover, they modulate plant transcription, proteosomal degradation machinery and hormone-signaling networks (involved in defense signaling) by down regulation of microRNAs in the RNA silencing mechanism (Navarro *et al*., 2008; Zhang *et al*., 2011). The plant defense-suppression functions of *P. syringae* effectors are summarized in Table 1.

Large scale genome sequencing and subsequent effector prediction, answered the riddle of effector redundancy and revealed the rich effector repertoire of different pathogens: Studies by Sharkar *et al*. (2006) on host specificity showed that low conservation of T3SS effector repertoires among different bacterial pathovars and species underlies their differences on host specificity, while high conservation of T3SS effectors can explain pathogenicity and a broad host range. Further on, acquisition of new T3SS effectors by HGT or other genetic mechanisms can widen a pathogen's host range (Jones and Dangl, 2006). It is therefore evident that the effector repertoire of a pathogen largely determines its infection strategy as well as its host range.

Biotechnological applications

Mining for effectors reveals new potential tools in plant breeding

Following Flor's hypothesis and using Klement's technique (see introduction), different panels of scientists determined that plant resistance was associated with HR elicitation to specific pathogen races. This led to the characterization of many avirulence (effector) genes and their matching plant resistance (*R)* genes, which in some cases enabled the full characterization of resistance due to *avr-R* interacting gene pairs in several pathosystems at the race/cultivar or species level. One of the first resistance genes to be cloned was *Bs1* from pepper accession PI163192, which interacted with the protein effector encoded by the *avr* gene *avrBs1* from *X. campestris* pv. *vesicatoria* (Minsavage *et al*., 1990). Backcross programs were initiated to transfer the resistance gene into commercially valuable cultivars (Early Calwonder). Similar efforts will help the development and/or identification of several cultivars of pepper (Bs1-4) and tomato (rx1-3, Xv3, Xv4) with resistance towards bacterial spots caused by *Xanthomonas* spp. (reviewed in Stall *et al*., 2009). Indeed, several pepper varieties resistant to three or more of the six bacterial leaf spot races are now commercially available (Sweet Bell, Sweet Italian, Hot peppers etc.). Also in the early nineties, Martin *et al*. (1991) isolated the Pto kinase *R* gene, which confers resistance to bacterial speck disease in tomato by recognition of the corresponding *avrPto* avirulence gene, in the pathogen *P. syringae* pv. *tomato*. Similarly, breeding efforts with rice *R* genes such as *Xa1-26* that confer resistance to bacterial blast caused by *Xanthomonas oryzae* pathovars, are now implemented in control measures along with cultural practices, chemical and biological control, and disease forecasting (Niño-Liu *et al*., 2006). Several other efforts have been reported on different pathosystems. Genome sequencing programs and protein motif comparison and function prediction analysis facilitate similar efforts and will provide breeders and geneticists with a significant number of resistance genes in the near future.

TAL Effectors: Novel tools for gene targeting and genome engineering

Transcription activator–like effectors (TALEs) are T3SS-secreted avirulence proteins found in *Xanthomonas*. These proteins can bind promoter sequences in the host plant

Table 1. Biochemical functions of Type III secretion system effector proteins of *Pseudomonas syringae* bacteria and role in HR or/and the plant innate immunity mechanisms.

Effector	Function	Ref. strain	Arabidopsis	*N. tabacum*	Yeast*	*N. benthamiana*[b]			Pea/Bean	Tomato	Avirulence
						I	II	III			
HopAB2 (avrPtoB)	Suppresses cell death & HR triggered by other effectors, suppress FRK1/SIRK alters ethylene responses; targets Pto/Fen/PAMP & RLKs/BAK1	*P.s.t* DC3000	v	v	v					v	Tomato
HopX1 (avrPphE$_{pto}$)	Induces JA -responsive genes; suppress HopA1-dep. HR & RAP2.6	*P.s.t* DC3000	v	v	v						Bean
HopAM1 (avrPpiB$_{pto}$)	Enhances virulence on water-stressed plants	*P.s.t* DC3000	v	v	x						Tomato
HopD1 (HopPtoD1)	Intermediate ability to suppress HR	*P.s.t* DC3000	v								
HopE (HopPtoE)	Inhibits HR & Arabidopsis WNK Kinase	*P.s.t* DC3000	v	v	v						Lettuce
HopF2 (HopPtoF)	Suppressing the flagellin-induced *NHO1* expression; targets R proteins RIN4/MKK5	*P.s.t* DC3000	v	v	v	v	v	v			
HopF1 (avrPphF)	Suppresses R-gene induced cell death	*P.s.ph* 1449B								v	Bean
HopK1 (HopPtoK)	Intermediate ability to suppress HR; suppress RAP2.6	*P.s.t* DC3000	v		x						Lettuce
HopG1 (HopPtoG)	Targets mitochondria, alters plant development & suppresses innate immunity	*P.s.t* DC3000	x		v	v	v	x			Tomato
HopAO1	Suppresses cell death Induce JA-resp. genes	*P.s.t* DC3000	v[a]					v			
AvrRps4	Suppression of PTI	*P.s.pi* 151		v							Arabidopsis
AvrPto1	Suppressing the flagellin-induced NHO1& FRK1/SIRK; interacts with Rab-GTPases; targets Pto/Fen/PAMP	*P.s.t* JL1065	x	v		v	v	v		v	Tomato
AvrB1	Suppresses basal defences & RAP2.6; Induce JA-responsive genes targets R prot. RIN4/RAR1/MPK4	*P.s.gl* R4 & R0		v							Soybean

HopM1 (HopPtoM)	Degradation of AtMIN7 to suppress PTI & SA-regulated immunity; targets MIN7 and others	*P.s.t* DC3000			v	v	x		Tomato
AvrE1	Suppress defences by perturbing host's G-protein regulation	*P.s.t* DC3000			v	v	x		Arabidopsis
HopC1 (HopPtoC)	Inhibit flagellin-induced NHO1 expression	*P.s.t* DC3000			x	x	x		Lettuce
HopN1 (HopPtoN)	Is localized to chloroplasts, degrades PsbQ & inhibits PSII	*P.s.t* DC3000		v			v		
HopAB1 (VirPphA)	Suppression of HR	*P.s.ph* R7 1449B					v	v	
AvrB2 (avrPphC)	Suppress cell death triggered by other effectors	*P.s.ph* R7 1449B						v	Bean, Pepper
HopZ3 (HopPsyV)	Suppress isoflavone biosynthesis	*P.s.s* B728a					v		Cotton, Lettuce, Tomato, Pepper, *N. benthamiana*
AvrRpt2	Suppresses basal defences, RPM1-induced HR; suppresses RAP2.6; targets RIN4	*P.s.t* JL 1065	v						Arabidopsis, Pepper, Tomato
AvrRpm1	Suppresses basal defences; target RIN4	*P.s.ma* M6	v						Soybean, Arabidopsis
HopZ2 (avrPpiG1)	Targets host *SUMOylated* proteins; Degradation of PBS1	*P.s.pi* 151						v	
HopAR1 (avrPphB)	Induces JA -responsive genes Degradation of PBS1; Suppresses RAP2.6; targets PBS1/BIK1/PBLs	*P.s.ph* R4 & R3	v						Bean, Arabidopsis Tomato, Cotton, Lettuce
HopAI1 (HolPtoAI)	Targets MAPK-signalling & flagellin-induced NHO1 expr.	*P.s.t* DC3000	v						Lettuce
HopS1 (HolPtoZ)	Suppressing the flagellin-induced *NHO1* expression	*P.s.t* DC3000	v						
HopAF1 (HopPtoJ)	Suppressing the flagellin-induced *NHO1* expression	*P.s.t* DC3000	v						
HopT1-1 (HopPtoT1)	Suppressing the flagellin-induced *NHO1* expression	*P.s.t* DC3000	v						Arabidopsis, Tomato, Lettuce
HopT1-2 (HolPtoU2)	Suppressing the flagellin-induced *NHO1* expression	*P.s.t* DC3000	v						
HopAA1 (HopPtoA1)	Suppressing the flagellin-induced *NHO1* expression	*P.s.t* DC3000	v						*N. benthamiana*, Tomato, Lettuce, Cotton
HopI1	Causes chloroplast thylakoid structure remodelling and suppresses SA accumulation; targets Hsp70	*P.c.al* ES4326	v						
HopU1	Suppresses plant innate immunity has mono-ADP-ribosyltransferase activity; targets GRP7 and other	*P.s.t* DC3000	v	v					

Effector nomenclature in parenthesis refers to their original names. Table depicts effector suppression function in different hosts tested, original avirulence phenotypes to certain host cultivars (mentioned first) and new avirulence functions found. Abbreviations mentioned in effector function correspond to plant resistance proteins and proteins related to plant defense signaling. HR: Hypersensitive response. PAMP: pathogen-associated molecular pattern. JA: Jasmonic acid. P.s.: *Pseudomonas syringae* pathovars; t: tomato; ph: *phaseolicola*; ma: *maculicola*; pi: *pisi* gl: *glycinea*. P.c.al.: *Pseudomonas cannabina* pv. *alisalensis*.

v: Suppression function

x: No suppression function

*: Ability to suppress Bax-induced killing of Yeast

a: HopAO1 in Arabidopsis suppresses callose deposition elicited by the Pst DC3000 hrpA mutant, and allows the normally non-pathogenic hrpA mutant to multiply within the leaf tissue. HopAO1 also suppresses resistance to Pst DC3000 induced by flg22, a PAMP. However, HopAO1 does not suppress the HR triggered by several classical avirulence genes

b: I. Challenge HR electrolyte leakage, II. Vascular dye accumulation, III. Suppression of hypersensitive response (HR)

Table Adapted from:

1. *Mol. Plant Micr. Interact.,* 17: 1328–1336, 2004. *Pseudomonas* Type III Effector AvrPto Suppresses the Programmed Cell Death Induced by Two Non-host Pathogens in *Nicotiana benthamiana* and Tomato.
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and activate the expression of plant genes that aid in bacterial infection. They recognize plant DNA sequences through a central domain consisting of a variable number of tandem, 33–35 amino acid repeats, followed by a single truncated repeat of 20 amino acids. The native function of these proteins is to directly modulate host gene expression. Upon delivery into host cells via the bacterial T3SS, TAL effectors enter the nucleus, bind to effector-specific sequences in host gene promoters and activate transcription (Boch and Bonas, 2010; Voytas and Bogdanove, 2011). The DNA binding code of TAL effectors is fairly simple: a hypervariable pair of adjacent residues at positions 12 and 13 in each repeat, the 'repeat-variable di-residue' (RVD), specifies the target, one RVD to one nucleotide, with the four most common RVDs each preferentially associating with one of the four bases in the DNA target. In naturally occurring TAL proteins the recognition sites are uniformly preceded by a T nucleotide that is required for TAL effector activity (Voytas and Joung, 2009; Moscou and Bogdanove, 2009; Boch *et al*., 2009).

These straightforward sequence relationships between the variable amino acids in TAL effector repeats and DNA bases in their target sites both allow the prediction of TALEs and their target sites and enable the redesigning of effectors to selectively bind to DNA targets of choice. These proteins are interesting to researchers both for their role in disease of important crop species and the relative ease of retargeting them to bind at pre-chosen DNA sequences. Similar proteins can be found in the pathogen *Ralstonia solanacearum*.

Genome sequencing generates opportunities to strategically manipulate selected genes in DNA targeting for a wide range of applications: understanding gene function in model organisms, reprogramming the regulation of selected loci in higher eukaryotic genomes through novel transcription factors, site directed mutagenesis and other techniques. Potential applications are envisioned in treating human genetic disorders, improving traits in crop plants, genome engineering and synthetic biology. At present, a serious technical limitation of such applications is the difficulty of altering nucleotide sequences and expression of genes in living cells in a targeted fashion. The TAL effectors of plant pathogenic *Xanthomonas* have provided researchers with a new tool to meet this challenge. Fusions of these effectors to rare-cutting restriction endonucleases (called TALENs, TALE nucleases), or other programmable nucleases, to customizable arrays of polymorphic amino acid repeats, direct the nuclease to particular DNA sites which they subsequently cleave (Bogdanove and Voytas, 2011; Morbitzer *et al*., 2010). In a recent plant-related application (Li *et al*., 2012) a TAL effector of *Xanthomonas oryzae* pv. o*ryzae* (Xoo), which transcriptionally activates a specific rice disease-susceptibility (*S*) gene, was fused to the DNA cleavage domain of *Fok*I to create a TALEN able to edit the particular *S* gene, altering the susceptibility response and thereby engineering resistance to bacterial blight.

Harpins

T3SS proteins of phytopathogenic bacteria, such as harpins, have been studied for their use in crop protection. The first example was the use of the harpin protein HrpN from *E. amylovora* overproduced in a heterologous bacterial system (*Escherichia coli*) (Wei *et al*., 1992). Harpins elicit a complex set of metabolic responses in the treated plant, which result in promotion of plant growth, induction of defense responses against different types of pathogens and insects, and tolerance to drought stress (reviewed in He *et al*., 2004). Harpins or harpin-like proteins such as HrpZ from *P. syringae* pv. *syringae* (Strobel *et al*., 1996), PopA from *Ralstonia solanacerum* (Belbahri *et al*., 2001) also elicit similar reactions on non-host plants. Transgenic cotton lines expressing $Harpin_{Xoo}$ from *X. oryzae* pv. *oryzae* accumulate harpin along the plant cell wall where it likely acts as an endogenous elicitor (Miao *et al*., 2010), leading the plant to a primed state which improves resistance against *Verticillium* wilt. Similarly, transgenically expressed

HrpZ protein fused to a plant signal peptide enhanced "rhizomania" resistance both in a model plant (*Nicotiana benthamiana*) and sugar beet, the natural host of BNYVV (Pavli *et al*., 2011). However, the exact action site of harpins remains unknown. Tampakaki and Panopoulos (2000) suggested that the harpin receptor could be extracellular in transgenic tobacco expressing HrpZ of *P. syringae pv. phaseolicola*, while other groups proposed the plasma membrane as the location of harpin receptor using immune-cytological experiments (Lee *et al*., 2001).

Harpin's potential culminated from basic and applied research that led to the registration and placing in the market plant protection products with various harpins as the active ingredients, by companies such as EDEN Bioscience™ which launched Messenger™ and ProAct™ as biopesticides for yield enhancement and disease management. This novel crop management technology offers the advantages of being environmentally friendly as it does not leach, bioacccumulate, or persist in the environment, and leaves no detectable residues on treated crops. Nevertheless, its small share in the global market indicates that there are limitations to this technology and probably space for improvement.

Hrp-based Diagnostics

Conventional methods for detection of bacterial plant pathogens relies on culture-based methods, biochemical and serological typing (immunofluorescence, enzyme-linked immunosorbent assay - ELISA), protein profiling (SDS-PAGE), fatty acid methyl-ester (FAME) profiling and pathogenicity confirmation testing. However, detection is increasingly depending on polymerase chain reaction (PCR) - based techniques, which mainly focus on amplification of the 16S rDNA gene and the 16S-23S internal transcribed spacer by genera/species specific primers, combined occasionally with simple restriction fragment length polymorphisms (RFLPs) and repetitive-sequence-based PCR (REP-PCR) analysis. A list of internationally agreed diagnostic protocols for regulated pests is published by the European and Mediterranean Plant Protection Organization (EPPO, 2012). Other primer target genes usually include genes involved in basic cellular functions, as for example the *Rhs* gene family (Park *et al*., 2006; Albuquerque *et al*., 2012) and gyrase B (*gyrB*) (Weller *et al*., 2007). Moreover, several specific primers have been designed on genera and species specific genes, such as the genes controlling production of bacterial phytotoxins, such as phaseolotoxin or coronatine for the detection of the bean halo blight (Schaad *et al*., 1995) and the bacterial speck pathogen of tomato (Cuppels, 2006). The main drawbacks so far of PCR-based methods are sensitivity, cross reaction with other bacteria, and false negatives/positives, usually due to the DNA extraction method and/or the plant tissue. Real time, multiplex and competitive PCR protocols have been developed to overcome such limitations (Pastrik, 2000; Berg *et al*., 2006; Chen *et al*., 2010).

The use of primers targeting functional systems that only pathogenic bacteria possess, such as the T3SS, could offer an alternative tool to overcome the aforementioned limitations. In this direction, there have been reports for the detection of Gram-negative pathogens by PCR amplification of *hrc* genes by means of genus, species or pathovar specific primers. In their pioneering work, at a time when full annotations of the *hrp* cluster were not available, the Stall and Bonas group (Leite *et al*., 1994) had foreseen the potential of *hrp* genes for diagnostic purposes. They have used genomic DNA of a *X. campestris* pv. *vesicatoria* strain and DNA from plasmids that contained cloned parts of the *hrp* cluster to develop oligonucleotide primers specific for different *hrp* regions, which amplified DNA from *X. fragariae* and 28 pathovars of *X. campestris*. More recently, other groups have also used the same set of primers for *Xanthomonas* species either *per se* or as a basis for designing new primer sets against additional *Xanthomonas* species and pathovars, based on the load of available sequences. Indeed, Obradovich *et al.* (2004) differentiated the *X. campestris* pv.

vesicatoria groups in isolations from pepper and tomato with the same primers as Leite *et al.* (1994), while Park *et al.* (2010) designed new primers that specifically amplified a 243-bp product from genomic DNA of *X. arboricola* pv. *pruni* strains, and not from the 21 other strains of *Xanthomonas* and two epiphytic bacterial species. In addition, Zaccardelli *et al.* (2007) proposed a rapid detection method for *X. campestris* pv. *campestris* in crucifer seeds and plants, based on amplification under stringent conditions of an internal *hrcC* fragment of 519 bp from a number of isolates and by means of pathogen-specific primers.

Recently, conventional PCR assays were adapted to real-time PCR to develop more sensitive and rapid techniques. Even if most of these studies target gene sequences other than those involved in T3SS function, as for example those coding for putative members of the ATP-binding cassette (ABC) transporter family in *X. arboricola* pv. *pruni* (Palacio-Bielsa *et al.*, 2011), there have been pilot reports on real-time PCR diagnostic assays based on *hrp* genes. Berg *et al.* (2006) for example, used fluorescently labeled probes to develop a multiplex PCR amplifying a 78-bp segment of the *hrpF* gene from different *X. campestris* pathovars and a 100-bp segment of the *Brassica* spp. 18S–25S internal transcribed spacer. The latter provided an internal control for the amplification process to prevent false negatives. Nevertheless, several real-time PCR protocols have been published in the last few years based on T3SS gene sequences of mammalian pathogens. Most of these PCR protocols target gene sequences such as *hilA* and *ssaN*, a T3SS transcriptional regulator and a putative T3SS ATP synthase gene of *Salmonella enterica* serovars, respectively, of pathogens borne in food and dairy products (McCabe *et al.*, 2011; Chen *et al.*, 2010). Moreover, similar assays target genes coding for T3SS secreted effectors such as *tccP* and *tccP2* from pathogenic *Escherichia coli* (Madic *et al.*, 2011). Considering the fact that both basic and applied research on bacterial secretion system progress since the very beginning hand in hand for plant and mammalian pathogens, one should only expect that use of real time PCR with sensitive and efficient probes (such as the TaqMan technology), will also expand rapidly in the next few years for phytopathogen detection. This approach offers the advantage that detection can be completed within a few hours with a sensitivity of 10^2 CFU ml^{-1}, thus surpassing the sensitivity of the existing conventional PCR.

The microarray technology offers a promising tool for transcriptional profiling, which has also been exploited for T3SS expression profiling and regulation (Ferreira *et al.*, 2006). Moreover, microarrays have recently been exploited for microbial species detection, community profiling on specific niches and/or hosts. High density microarrays for example, based on gene sequences encoding mainly for the small ribosomal subunit (16S) rRNA, have been developed for microbe/pathogen monitoring (Brodie *et al.*, 2007). Such tools could complement the currently available methods for pathogen detection, which are time consuming, and specific, but still lack economic feasibility. The approval of DNA microarray technology by governmental authorities (US Food and Drug Administration) has paved the way for new applications to clinical diagnostics and pathogen detection in foods and crops.

Microarray applications for the detection of plant pathogens have already been developed, mainly targeting a specific host plant. Probe designing on most cases has been based on the 16S rRNA gene region with the complementation of pathogenicity islands containing known virulence genes (Aittamaa *et al.* 2008) or housekeeping genes such as *rpoB*, *groEL*, *ftsZ* (Pelludat *et al.*, 2009) and *gyrB* (Kostic *et al.*,2007). The diagnostic ability could extend from genus and species to pathovar level. However, such diagnostic tools are still in their infancy with plant pathogens as they have been applied to pure culture lysates rather than infected plants and cross hybridization between probes often occurs. The use of *hrp* genes could address the latter problem, as although they are conserved, they still in-

clude regions of high polymorphism. Based on pilot studies from the authors of this manuscript (Skandalis *et al.*, 2010), we suggest that microarray target probe designing on *hrc* genes could be a valuable tool for detection of a wide range of Gram-negative plant pathogens as well as for simultaneous infections. In support, Garrido *et al.* (2006) already developed a diagnostic oligonucleotide array for the detection of shiga toxin-producing and enteropathogenic *E. coli* strains based on the genetic variability present at the enterocyte effacement (LEE) pathogenicity locus, which contains genes encoding for the T3SS and T3SS effector proteins.

Are secretion systems the key to a new generation of antibiotics?

Development of resistance to antibiotics that target bacterial viability, urged for new antimicrobial development strategies that target various pathways related to virulence, including toxin function, toxin delivery, regulation of virulence expression and bacterial adhesion (reviewed in Clatworthy *et al.*, 2007). Inhibition of these virulence factors would render hazardous bacteria non-infectious only within the host, thereby reducing the selective pressure for resistance development, and would not perturb the nontarget microflora. In this regard, T3SS apparatus components that are linked to the outer membrane or exposed, offer an attractive substrate for inhibitor (antibiotic) development. Hence, screening for T3SS inhibitors has involved four distinct approaches, yielding inhibitors of T3SS transcription, assembly and specific effector protein secretion (reviewed in Lee and Kessler, 2009). In particular, small molecules termed Innate Parmaceuticals AB (INPs), and in particular acylated hydrazones of different salicylaldehydes were found to prevent molecule translocation for *Yersinia pseudotuberculosis* (Nordfelth *et al.*, 2005), inhibit intracellular growth but not invasion of *Chlamydia trachomatis* into host cells (Muschiol *et al.*, 2009), inhibit the T3SS and virulence of *Salmonella enterica* serovar T*yphimurium* (Hudson *et al.*, 2007) and finally inhibit secretion assembly of *Shigella flexneri* T3SS. Effector protein inhibitors were identified either –in the case of pseudolipasin A - on the basis of inhibition of host cell lysis by the *P. aeruginosa* ExoU cytotoxin effector or alternatively, by screening for compounds, such as exosin and 0433YC1-2, that permitted the growth of yeast with induced expression of cytotoxic effectors of *P. aeruginosa* (ExoS) and *Chlamydia pleumoniae* (CopN). Determination of 3-D structures of T3SS components and effectors provide unexplored options for designing antivirulence chemicals, including next generation virulence inhibitors binding to adjacent, functionally relevant binding pockets of T3SS components to reduce the chances of resistance development.

Conclusions

Historically, the genes coding for the T3SS were identified in phenotypic screens of mutants altered in their interaction with other organisms (higher eukaryotes). The identification of effectors in plant pathogens also had a similar starting point (transfer of whole genome libraries from avirulent to virulent strains and carrying out HR screens on *R* gene differentials; Staskawicz *et al.,* 1984; Table 1). The discovery of the T6SS followed the opposite route: its existence emerged from bioinformatic sourcing of genomic data. It is conceivable that future sourcing of genomic, proteomic, transcriptome and structural database data may point to new potential candidates, particularly for effectors. Possibly, new secretion systems and new effectors may even be predicted, "the way Mendeleev had anticipated characteristics of yet unknown elements", as is already the case with TAL effector DNA binding specificities.

Large scale analysis of interactions of effectors with different hosts by means of *Agrobacterium* transient expression (Wroblewski *et al.*, 2009; Lindeberg *et al.*, 2012) confirmed that their suppression and avirulence function varied significantly, espe-

cially among distant plant families, some of which are highlighted in Table 1. This might reveal new potential in genetic engineering towards phytoprotection, as exploitation of a certain effector function in a specific crop could lead to priming of defense responses upon challenge with a pathogen. Moreover, the genomics era for plant pathogens, which began with the sequencing of *Xylella fastidiosa* genome in year 2000 (Simpson *et al*., 2000) and the new comparative analysis of genomes (Hamilton *et al*., 2011), which allows for identification of conserved and divergent features, thus leading to species, pathovar and isolate-specific genes, supports diagnostics research towards new molecular kits based on such targets, where both *hrp* genes and T3SS effectors might be in the centerfold.

With regard to applications, it is perhaps significant that various uses of bacterial effectors have been the subject of several patents, to enable possible applications in a number of areas, including plant disease and pest control. In addition, new frontier areas are likely to emerge from the developments in high-throughput DNA sequencing (next-generation sequencing technologies). Very high-throughput DNA sequencing platforms are commercially available at affordable costs and new more cost effective techniques are under development. Rapid genome-wide characterization and targeted capture of genomic subsets and polymorphisms from many phytopathogen strains at a time, and public access to genome, transcriptome and metagenome data will likely reshape many areas of "phytopathogenics" and will enable applications in disease diagnosis, epidemiology, effectoromics and comparative pathogenomics and the development of resistance strategies in the future. Characteristic of this trend are two recent publications (Baltrus *et al.,* 2012; Bart *et al.,* 2012), which concern the sequencing of new 14 and 65 phytobacterial genomes, respectively.

Research on antibacterial compounds for phytoprotection could follow the mammalian research initiatives. Specificity of T3SS virulence inhibitors could render field application feasible, in contrast to conventional antibiotics. However, development of such molecules would face drawbacks similar to those of the mammalian pathogens: post-infection usage, broad range activity, targeting of infected tissue and non-target organism safety issues. In addition, the lack of an adaptive immune system would render T3SS therapeutics more complicated and disqualify methods such as usage of secreted effectors as vaccines and T3SS mutants as live vaccines, which are under investigation for mammalian bacterial pathogens (see Coburn *et al*., 2007).

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ΑΡΘΡΟ ΑΝΑΣΚΟΠΗΣΗΣ

Τριάντα χρόνια έρευνας στα εκκριτικά συστήματα των βακτηρίων: Βιοτεχνολογικές εφαρμογές και οφέλη στη φυτοπροστασία

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Περίληψη Η ανακάλυψη στα μέσα της δεκαετίας του ‘80 ενός εκκριτικού συστήματος πρωτεϊνών, που ονομάστηκε εκκριτικό σύστημα τύπου III (T3SS), κοινού σε παθογόνα φυτών και ζώων καθώς και σε συμβιωτικά βακτήρια, αποτέλεσε ορόσημο στην φυτοπαθολογία και συγκεκριμένα στις αλληλεπιδράσεις φυτών-βακτηρίων. Τα φυτοπαθογόνα βακτήρια πρέπει να καταβάλουν τα πολλαπλά επίπεδα άμυνας του φυτού που είτε προϋπάρχουν είτε επάγονται μετά τη μόλυνση. Για να το επιτύχουν, χρησιμοποιούν σύμπλοκα πρωτεϊνών που δημιουργούν δομές κατάλληλες για την έκκριση πρωτεϊνών τόσο στον εξωκυτταρικό χώρο του βακτηρίου, όσο και απευθείας στο κύτταρο του φυτού ξενιστή. Οι πρωτεΐνες αυτές εμποδίζουν την αναγνώριση των παθογόνων από τον ξενιστή, αποτρέπουν την επαγωγή αμυντικών μηχανισμών και παρεμβαίνουν στην σηματοδότηση που οδηγεί στην ενεργοποίησή τους. Πρόσφατα επιτεύγματα στη μοριακή βιολογία βοήθησαν την έρευνα στον τομέα των αλληλεπιδράσεων και έχουν καταστήσει τη μεταφορά πρωτεϊνών με το T3SS ως θεμελιώδη στην παθογένεια, τη μολυσματικότητα και τον καθορισμό του εύρους των ξενιστών των gram- φυτοπαθογόνων βακτηρίων. Σε αντίθεση με τη πρόοδο στη γνώση μας στη βιολογία των παθογόνων, λίγα πράγματα έχουν αλλάξει στις μεθόδους και τον τρόπο καταπολέμησής τους. Στην παρούσα ανασκόπηση παρουσιάζεται συνοπτικά η λειτουργία και ο τρόπος δράσης του T3SS. Η έμφαση δίνεται στις βιοτεχνολογικές εφαρμογές που έχουν προκύψει με άξονα τη βασική έρευνα στο T3SS, από την ανάπτυξη νέων διαγνωστικών μεθόδων και την βελτίωση της ανθεκτικότητας των φυτών σε παθογόνα, στη δημιουργία αντιβιοτικών και τεχνολογιών για γονιδιακή στόχευση στα φυτά.

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SHORT COMMUNICATION

Records of *Aedes albopictus* and *Aedes cretinus* (Diptera: Culicidae) in Greece from 2009 to 2011

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Summary *Aedes* (*Stegomyia*) *albopictus,* an invasive mosquito species of great medical importance, was first recorded in Athens, Greece, in 2008. Its presence raised awareness among people and as a consequence numerous "tiger-like" mosquito specimens were sent to Benaki Phytopathological Institute for identification and relevant information. The results of the adult mosquito specimens, collected for three years (2009-2011), revealed that *Aedes albopictus* occurs in many municipalities around Athens, in Attica Prefecture, and in many parts of the country. The mosquito samples confirmed also the presence of the native species *Aedes* (*Stegomyia*) *cretinus*. Our data, based on people awareness and annoyance, suggest the need to implement appropriate surveillance programs for monitoring the presence and population densities of *Stegomyia* mosquitoes in the frame of an integrated mosquito control program.

Additional key words: Aedes albopictus, *Aedes cretinus*, Attica, Greece, *Stegomyia*

The Asian tiger mosquito *Aedes* (*Stegomyia*) *albopictus* (Skuse 1894) is a species native to tropical and subtropical regions of South-East Asia, which has undergone an astonishing expansion of its range within the last few decades (3, 9). The international trade of used tires has primarily facilitated *Ae. albopictus* spread around the world via transportation of its eggs (14).

In Europe, it was recorded for the first time in Albania in 1979 (1). Ever since the species has been reported in 11 other countries, including Italy (1990), France (2000), Switzerland (2003), Spain (2004), Croatia (2004) and the Netherlands (2005) (24), with Italy to be considered the most heavily infested European country nowadays (8).

The geographic position and climatic conditions of Greece have been considered highly suitable for a potential invasion and establishment of Asian tiger mosquito (15, 18). Hence, *Ae. albopictus* was first detected in Greece in the North-western part of the country near Albania and Italy in the Prefectures of Thesprotia and Corfu in 2003 (22). Subsequently, it was found in northern Greece in the Prefecture of Serres in 2007 (25) and the following year in central-west Greece in Aitoloakarnania Prefecture (16).

Aedes albopictus is an anthropophilic species that causes serious nuisance problems and can play an important role in the transmission of a wide range of human pathogens. This mosquito is a laboratory-competent vector of at least 22 arboviruses, notably Dengue virus, the most important arboviral disease in humans (13). The recent cases of autochthonous transmission of

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Dengue in France (2010) and Croatia (2010) and Chikungunya virus in Italy (2007) and France (2010) justify the awareness of *Ae. albopictus* related potential risk for introduction and spread of serious diseases in continental Europe (11, 12, 17, 19).

The presence of *Ae. albopictus* in the capital of Greece, Athens, was confirmed for the first time in 2008 from mosquito specimens originated from the Rizoupoli area in Municipality of Athens (16). After the media announcement on finding this invasive mosquito in Athens, and the description of its medical importance, the public awareness in Greece was raised resulting in more than 100 specimens of "tiger-like" adult mosquitoes, which have been sent to Benaki Phytopathological Institute (BPI) for identification within the following three years (2009-2011). Most of these specimens were sent to BPI by civilians, pest control companies or official authorities with the note for "aggressive day-time biting mosquitoes".

The majority of the specimens were mosquitoes of subgenus *Stegomyia* (Diptera: Culicidae) although other mosquito species or mosquito resembling dipterous insects (e.g. Tipulidae, Chironomidae) were also recorded. Mosquito species identification was performed using appropriate dichotomous keys (6, 20). Most mosquito samples were identified as *Ae. albopictus*, whereas some of them belonged to the indigenous *Aedes* (*Stegomyia*) *cretinus*. This species is closely related to *Ae. albopictus* and has a limited distribution across the world (Greece, Cyprus, Georgia and Turkey) (2, 7). In Greece, it has been reported to exist in Crete, Macedonia and some parts of Attica (21). *Aedes cretinus* is described as an aggressive day-time biting mosquito causing considerable irritation to humans (7, 21). It is a container-breeding mosquito but little appears to be known about its biology (2).

For all the specimens an official answer was produced by the BPI experts or advisory services via telephone communication were provided, including a synopsis of the biology of the identified species, medical importance and appropriate protection measures especially for *Ae. albopictus*.

Herein, we review the results of records of *Ae. albopictus* and *Ae. cretinus* in Greece based on samples sent to BPI during a 3-year period (2009-2011). The results were recorded on maps referring to the specimen incidence and the origin sites for both Stegomyia species in chronological order for Attica Prefecture and in the rest Prefectures of Greece, indicating the first records. The administrative units, either municipalities of Attica or Prefectures of Greece, where samples came from, were color marked in the aforementioned maps, even in case of single mosquito specimens. We consider that information about the presence of *Stegomyia,* especially for *Ae. albopictus*, although limited, is very important for the committees of Municipalities and Prefectures, which are mainly responsible for the regional mosquito management programs in Greece. In Figure 1 the first specimen of *Ae. albopictus* in 2008 is also presented and mentioned as "first point".

In a total of 76 *Stegomyia* specimens, 71 were in good condition for identification and had been sent from 35 Municipalities of the Attica Prefecture (including Municipality of Athens). During the 3-years period, *Ae. albopictus* was found in 24 Municipalities, which are located mainly in central and south-east part of Attica. *Aedes cretinus* adults were collected from 15 Municipalities located primarily in central and northern areas of Attica, while in 4 cases (localities) both *Stegomyia* species were present. Overall, *Ae. albopictus* was recorded from 7 Municipalities in 2009, 11 in 2010 and 13 in 2011. According to Figure 1, its presence was recorded for the first time in 6, 8 and 9 different Municipalities in the years 2009, 2010 and 2011, respectively. Accordingly, *Ae. cretinus* specimens were sent in a total of 12 Municipalities in 2009, 8 in 2010 and only 2 in 2011, while first reports of this species were declining (11 in 2009, 3 in 2010 and only 1 in 2011) (Figure 2).

With regards to the rest of Greece, a total of 35 *Stegomyia* specimens were collected from 15 out of 52 Prefectures, during the 3-years period, mostly from southern Greece (Peloponnese and Crete) and the northern parts of the country (Macedonia). Overall,

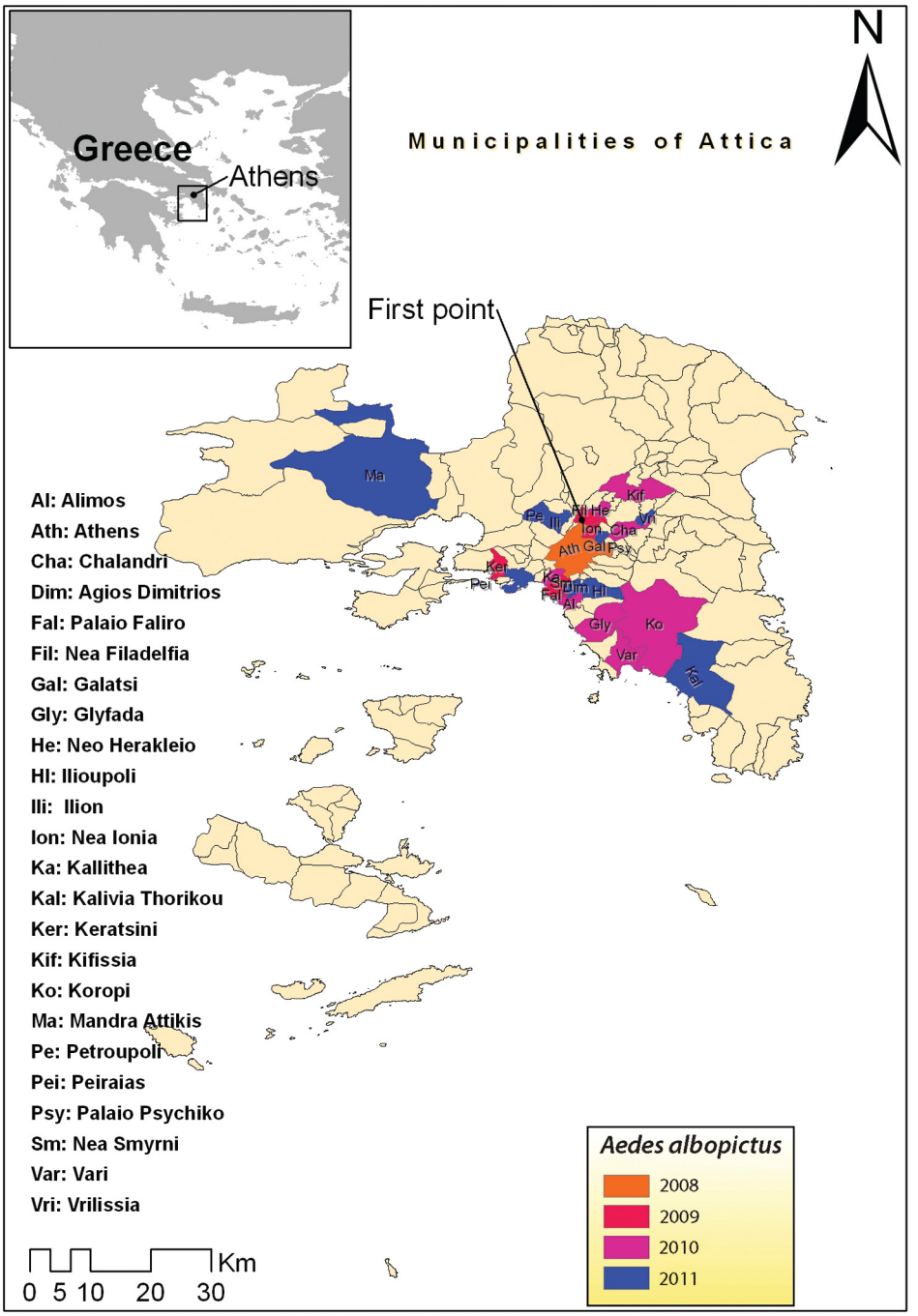

Figure 1. Municipalities* of Attica where *Ae. albopictus* was first reported over a 3-year period (2009-2011). The first specimen of *Ae. albopictus* in 2008 is also presented and mentioned as "first point".

*Municipalities are displayed according to the Greek administrative unit system until 2010.

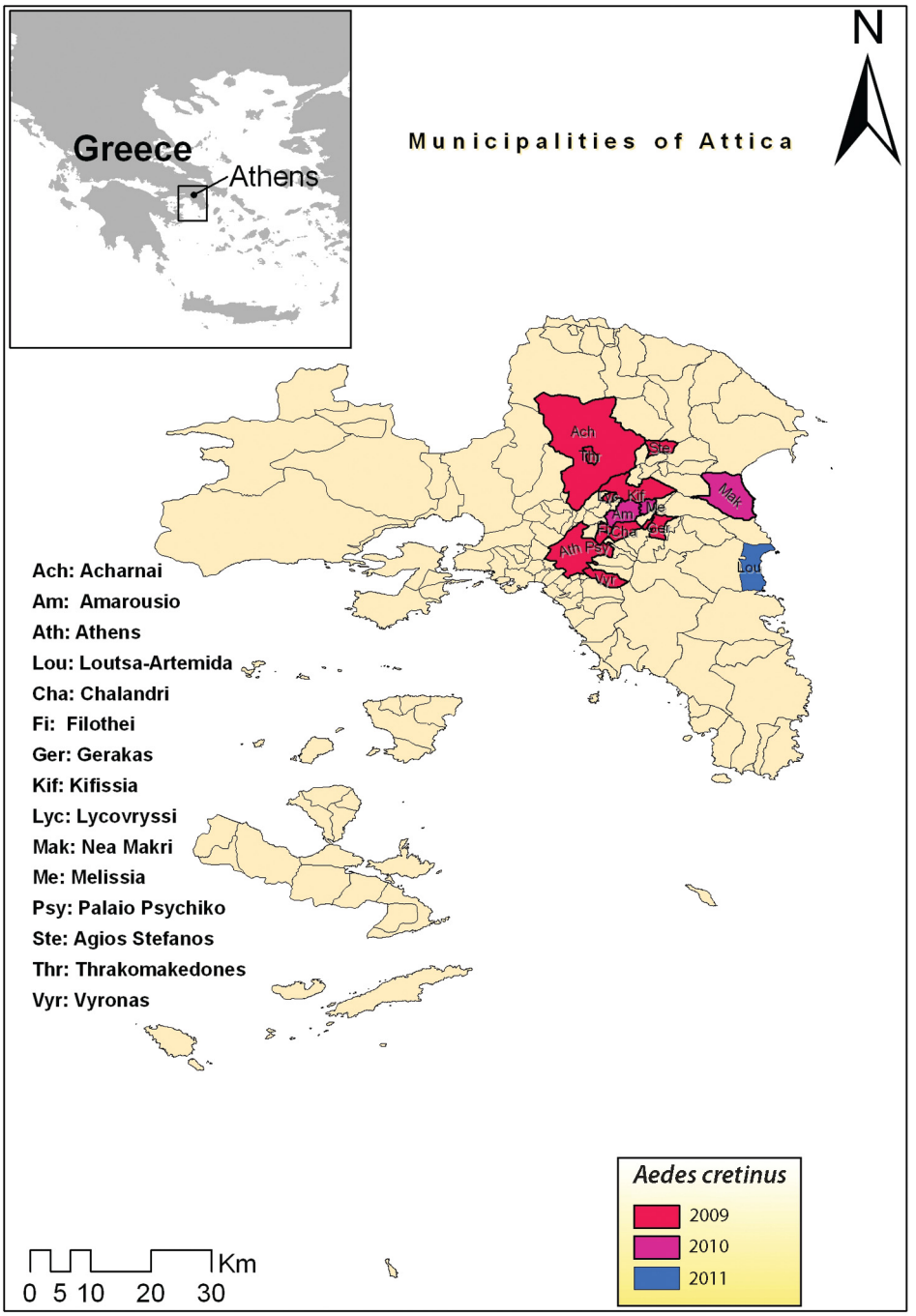

Figure 2. Municipalities* of Attica where *Ae. cretinus* was first reported over a 3-year period (2009-2011).

*Municipalities are displayed according to the Greek administrative unit system until 2010.

Ae. albopictus and *Ae. cretinus* adults were recorded from 8 Prefectures each. *Aedes albopictus* specimens were sent from 5 Prefectures in 2009 and 2010 and 2 in 2011. *Aedes cretinus* was detected in 6 Prefectures in 2009, 2 in 2010 and 2 in 2011. Figures 3 and 4 show that *Ae. albopictus* was first found in 4 and 3 Prefectures, while *Ae. cretinus* was first found in 5 and 1 Prefectures for the years 2009 and 2010, respectively. In 2011, there was no first record of *Ae. albopictus*, whereas *Ae. cretinus* was recorded for the first time in

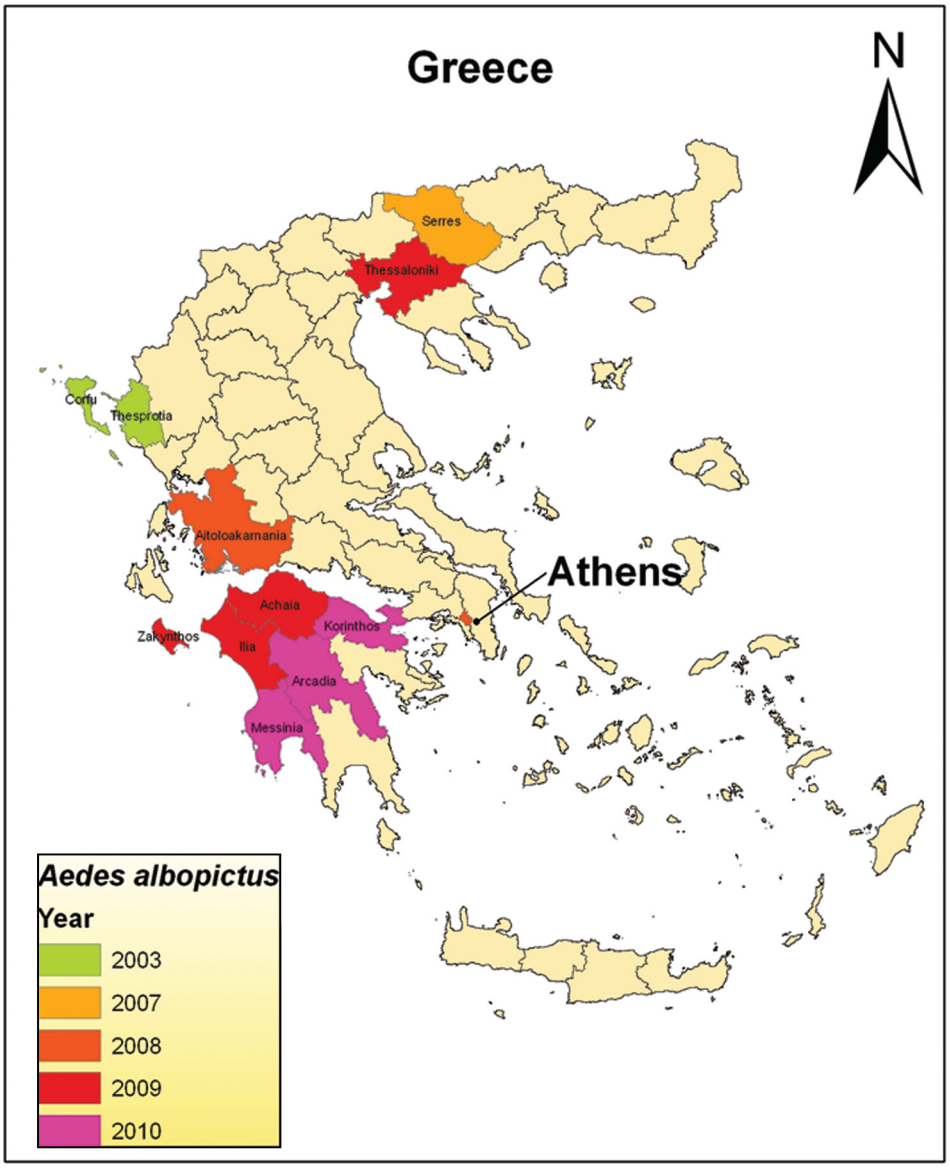

Figure 3. Prefectures* of Greece where *Ae. albopictus* was first reported over a 3-year period (2009-2011). Previous literature reports, concerning *Ae. albopictus* presence, are also presented for 2003, 2007 and 2008.

*Prefectures are displayed according to the Greek administrative unit system until 2010.

1 Prefecture. In Figure 3, except the records for the 3-year period, previous literature reports concerning *Ae. albopictus* presence are also presented (2003, 2007 and 2008).

Our results revealed that 8 years after the first detection of *Ae. albopictus* in northwestern Greece, this species is present in many parts of the country and in many suburbs in and around Athens, where almost half of the population of Greece live. The current review of *Ae. albopictus* and *Ae. cretinus* samples in Greece, based on people awareness and annoyance, indicates the presence of these two *Stegomyia* species and com-

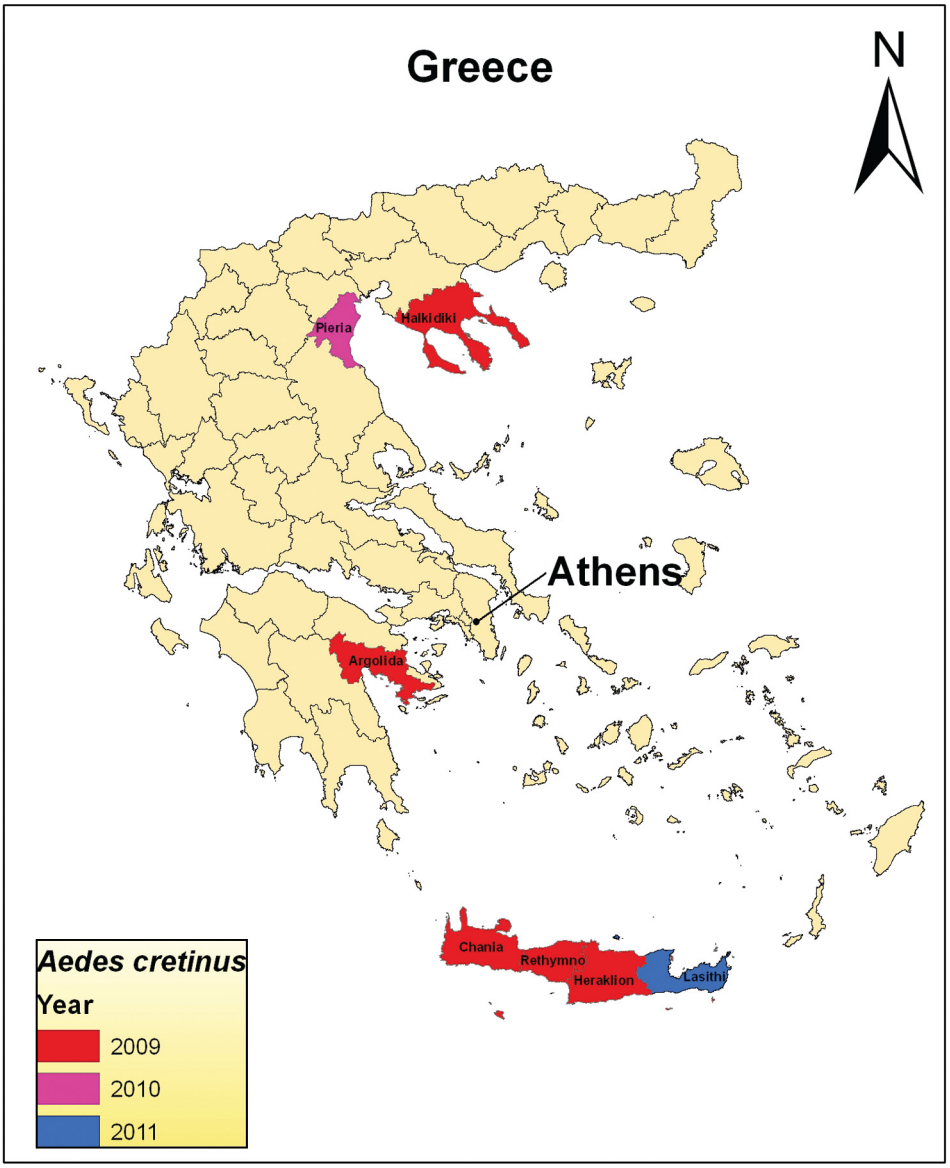

Figure 4. Prefectures* of Greece where *Ae. cretinus* was first reported over a 3-year period (2009-2011).

*Prefectures are displayed according to the Greek administrative unit system until 2010.

prises useful information while organizing mosquito surveys. Although it is generally believed that the public awareness may result in exaggeration, fear and unreasonable reactions and behavior, we also have to take into account that members of the public can also be a great source of information by reporting new invasions, recording phenological changes associated with invasions or disease outbreaks, and finally can participate in management efforts (5).

A recent mosquito surveillance in Athens, using ovitraps, verified the presence of both *Stegomyia* species and revealed that *Ae. albopictus* has currently developed considerably high populations, displaying a trend of increase over time, in the urban environment of the Greek capital city (10). Moreover, the co-occurrence of *Ae. albopictus* and *Ae. cretinus*, mainly in some Municipalities of Attica, arises the need for a better study on the biology of *Ae. cretinus* and suggests the implementation of surveillance programmes using ovitraps and other surveillance methods in order to investigate the population dynamics of each species and the possible interspecific competitive interactions.

Surveillance programs will be also useful to prevent the introduction and establishment of other invasive container-breeding *Aedes* species with medical importance such as *Ae. aegypti, Ae. japonicus, Ae. atropalpus, Ae. koreicus* and *Ae. triseriatus*. These species have been reported the recent years as introduced into various European countries (4, 23).

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Στοιχεία της παρουσίας των *Aedes albopictus* και *Aedes cretinus* (Diptera: Culicidae) στην Ελλάδα για την τριετία 2009-2011

Α.Κ. Γιατρόπουλος, Α.Ν. Μιχαηλάκης, Γ.Θ. Κολιόπουλος και Κ.Μ. Ποντικάκος

Περίληψη Το *Aedes* (*Stegomyia*) *albopictus* είναι ένα επεκτατικό είδος κουνουπιού με μεγάλη υγειονομική σημασία, του οποίου η παρουσία καταγράφηκε για πρώτη φορά στην Αθήνα το 2008. Τα επόμενα 3 έτη (2009-2011) πολυάριθμα δείγματα κουνουπιών από την Αττική και την Ελλάδα γενικότερα εστάλησαν στο Μπενάκειο Φυτοπαθολογικό Ινστιτούτο προς αναγνώριση και παροχή σχετικών πληροφοριών, ως αποτέλεσμα της όχλησης των κατοίκων ορισμένων περιοχών καθώς και της ανησυχίας που προκλήθηκε από την παρουσία και υγειονομική σημασία του συγκεκριμένου είδους κουνουπιού. Από την εργαστηριακή εξέταση των δειγμάτων προέκυψε ότι το *Ae. albopictus* δραστηριοποιείται σε αρκετούς Νομούς της χώρας και Δήμους της Αττικής. Επιπλέον, επιβεβαιώθηκε η παρουσία και του συγγενούς είδους κουνουπιού *Aedes* (*Stegomyia*) *cretinus*. Αν και τα δεδομένα βασίζονται κυρίως σε δείγματα πολιτών, καταδεικνύουν την ανάγκη εφαρμογής κατάλληλων μεθόδων μελέτης της παρουσίας των συγκεκριμένων ειδών στα πλαίσια της ολοκληρωμένης αντιμετώπισης του γενικότερου προβλήματος των κουνουπιών.

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Validation of single residue methods for the determination of dithiocarbamates and inorganic bromide residues in plant products

E. Bempelou, K.S. Liapis and G.E. Miliadis

Summary The use of pesticides in agricultural practice is still inevitable. However, public concern on food safety has made the surveillance of plant products for the determination of pesticide residues absolutely necessary. Multiresidue methods are usually applied for this purpose, although certain compounds require specific methods, the single residues methods. Being focused on those compounds in the present study, we validated two analytical methods for the determination of dithiocarbamate fungicides and bromide ion, in plant products using gas chromatography with flame photometric detector (FPD) with sulphur filter and electron capture detector (ECD), respectively. The determination of dithiocarbamates was based on the release of carbon disulfide after heating of the sample with aquatic solution of tin (II) chloride, while the corresponding for bromide ion was achieved after derivatization to 1-bromopropanol-2 and 2-bromopropanol-1 in an acidic propylene oxide solution, and the use of 3-bromo-1-propanol as internal standard for quantification. Validation parameters, namely trueness, precision, sensitivity and linearity were evaluated for both methods and validation procedures were carried out at 2 to 3 concentration levels with 5 replicates each according to the requirements of SANCO Document 10684/2009. The highest level of validation was that of the EU MRL, while the limit of determination was the successful lowest validated level. The two methods are characterized by good accuracy, precision and sensitivity and are considered as suitable for routine analysis in accordance to the requirements of the European Commission.

Additional keywords: dithiocarbamates, inorganic bromide, residues

Introduction

The widespread consumer concern for food safety and quality has highly increased nowadays and pesticide residues in agricultural commodities have been the subject of strict regulations. Certainly, the use of pesticides according to good agricultural practices (GAPs) is not expected to cause problems of public concern in health and environmental areas. However, in the case of inappropriate treatments undesirable residues can be detected on vegetables or other plant products after harvest.

Pesticide detection in plant products is most usually perfomed by application of multiresidue methods. However, there are certain compounds that are not suitable for multiresidue determination, as they have physicochemical properties significantly differing from the majority of pesticides. These compounds need specific methods for their determination, the single residue methods (SRMs). The validation process for each of those methods in representative matrices provides the evidence that the method is fit for purpose. Bromide in agricultural products is appearing as the degradation product of various fumigants, mainly methyl bromide ion. It is therefore required to determine by a SRM the total bromide residues in its inorganic form, i.e. bromide ion. Dithiocarbamates on the other hand are not stable at the extraction step used in multiresidue analysis and therefore require an SRM.

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Dithiocarbamate fungicides are pesticides extensively used worldwide in agricultural practice on a wide range of crops. Due to their low acute toxicity combined with strong action, low cost production and low environmental persistence, the use of these compounds is still increasing worldwide [16].

The determination of dithiocarbamate (maneb, mancozeb, metiram, propineb, thiram and ziram) residues is based on the detection of their common degradation product, which is carbon disulfide (CS_2). Maximum residue limits (MRLs) set by Regulation 396/2005/EC are also referred to CS_2, with evident, however, the inability to distinguish the actual active compound (of dithiocarbamates) sprayed in the field.

The methods of analysis for the determination of dithiocarbamates are mainly based on the use of headspace gas chromatography (GC) [12], photospectrometric techniques of absorption [14], GC with electron capture detector (ECD) or flame photometric detector (FPD) [4] or mass spectrometry (MS) [1, 4, 7], while their determination as parent compound has been reported by LC/APCI/MS [3] and LC/ESI/MS [6]. The official method of the European Community Reference Laboratory for single residue methods is based on the determination of dithiocarbamates as CS_2 by GC/ECD [9].

Inorganic bromide (Br) atom [10097-32-2] has atomic number 35, atomic mass 79.904, and belongs to Group 17 (VIIA) of the Periodic Table, the halogens. It is the degradation product of fumigants, mainly methyl bromide, used for soil disinfection as well as post-harvest treatment of plant products. It can be absorbed by plants from treated soils or it may be contained in fumigated products. Furthermore, the natural bromide content of most fresh plant materials is up to 5mg/kg [10]. Regarding that application of methyl bromide is not further allowed in agriculture, the determination of inorganic bromide in plant products is imperative to detect illegal uses.

Different analytical methods have been published for the determination of the bromide ion. They are based on x-ray fluorescence, spectroscopic [2], GC [14] and HPLC techniques [14] with gas chromatography being the most widely used. Recently, the determination of inorganic bromide in plant products was achieved by the use of GC/ECD with the addition of internal standard in the final extract [10].

In the present study our goal was to validate two GC robust methods for the determination of dithiocarbamates and inorganic bromide in plant products with FPD with sulphur filter and ECD detectors, respectively, according to the requirements of the SANCO Document 10684/2009.

Materials and methods

Chemicals and reagents

Analytical standard of thiram (99%) used for the fortification experiments of the dithiocarbamates method was obtained from Dr Ehrenstofer (Augsburg, Germany), a mixture of 1-bromo-2-propanol and 2-bromo-1-propanol analytical standard was used for the identification of the bromide derivatives and 3-bromo-1-propanol (99.5%) as internal standard were obtained from Chem-Service (West Chester, UK). Carbon disulfide (CS_2) (density 1260mg/mL at 25°C) was purchased by Merck, as well as potassium bromide and were used as analytical standards. Ethyl acetate, isooctane, ethanol and acetone were of pesticide residues grade and water of HPLC grade, all obtained from Lab Scan (Dublin, Ireland).

Standard Solutions

Analytical standard stock solutions of thiram and 3-bromo-1-propanol (internal standard) at 1000μg/L were made in acetone and ethyl acetate and stored at -20°C. Stock solution of carbon disulfide at 1000μg/L in isooctane was prepared anew every working day, while the corresponding of potassium bromide was prepared in water and stored at 8°C. Working standard solutions were prepared in ethanol for thiram and in water for bromide for the validation procedures. The quantification of CS_2 residues was based on

the calibration curve at 9 concentration levels (0.05-0.1-0.2-0.5-1-2-5-10 and 20μg/L). Bromide ion residues were also quantified by calibration curve using 5 solutions equivalent to 5, 25, 50, 100 and 150μg Br.

Thiram was used for the fortification of the blank samples during the validation of dithiocarbamates, as it is the only compound of the group that can be diluted in organic solvents.

Preparation of fortified samples

Control samples were prepared from organically produced apples, leek, potatoes, strawberries, wheat, tomatoes, lettuce and rice and analysed in order to certify that no interfering peaks with the analytes appeared in chromatograms. Aliquots of each product blank sample were fortified at two or three levels as presented in tables 1 and 2, ranging from 0.1 to 1mg/kg for the lowest level and 1 to 50mg/kg for the corresponding highest. The highest level of fortification was that of the MRL set for dithiocarbamates or bromide ion by the European Union; while the limit of determination was set as the lowest validated level. The validation procedures were in accordance to the SANCO Document10684/2009.

Sample preparation

The extraction procedures for the determination of inorganic bromide and CS_2 are described below. As far as **inorganic bromide** is concerned, 5g portion of homogenated sample (or 1g for dry products) was weighted into a 100mL Erlenmeyer flask and 3 (or 8mL respectively) of water were added. 5mL of propylene oxide solution and 1mL of sulphuric acid were added for the derivatization of inorganic bromide to 1-bromopropanol-2 and 2-bromopropanol-1. The flask was closed and shacked briefly and the mixture stand at room temperature for 60 min. 50mL ethyl acetate and 4g of ammonium sulphate were added to the suspension and the flask was shaken by hand first vigorously for 1 min and then occasionally for 20 min. The upper organic phase was decanted and dried through anhydrous sodium sulphate, 1 mL of the organic extract was transferred to an autosampler vial and 100μL of internal standard solution (10μg/mL) was added. The calibration solutions followed the complete method procedure (derivatization, partitioning etc.)

As for the **dithiocarbamates**, their residues are typically located superficially. Thus, sample comminuting (e.g. cutting, milling, grinding) is only to be performed where this is necessary to obtain acceptable sub-sampling variability, which is also a function of the analytical portion size. The bigger the portion, the smaller the sub-sampling variability becomes. In most cases opposite segments of each unit are cut out. A defined portion of 50g was taken for analysis into a cleavage vessel and 25mL isooctane was added. Then 150mL of the hydrolysis reagent tin (II)-chloride in hydrochloric acid was added and the vessel was immediately closed with a screwcap with septum. The samples were put into a shaking water bath for 2 hours at 80°C. The reaction mixture was cooled down and 1mL of the isooctane-phase was pipetted into a GC vial for analysis.

Gas- chromatographic analysis

Determination of carbon disulfide was performed by the use of a Shimadzu GC-2010 chromatographic system with a splitless injector and a DB-5 column (5%- phenylmethylpolysiloxane, 50m, 0.32mm i.d. and 1μm film thickness) connected to a flame photometric detector (FPD) with sulphur filter. The oven temperature programme started from 45°C (1 min), increased to 250°C at a rate of 20°C/min, and held there for 5min. The injection volume was set to 1μL.

Determination of inorganic bromide was based to a 6890N Agilent gas chromatographic system with an electron capture detector (ECD), a splitless injector and a DB-Wax 52 CB column (chemically bonded polyethylene glycol, 30m, 0.25mm i.d. and 0.25μm film thickness). The oven temperature programme started from 50°C (1 min), increased to 150°C at a rate of 2.5°C/min, increased to 200°C at a rate of 10°C/min and held there for 10min. The injection volume was also set to 1μL.

Results and discussion

In the present study the determination of carbon disulfide and bromide ion was carried out in plant products using gas chromatography with FPD and ECD detectors, respectively. The two methods were found to be effective for the extraction of the tested compounds.

The retention time of the CS_2 proved to be very stable at 4.5 ± 0.2% min (Figure 1). Confirmation can be obtained by GC/MS or GC/ECD, but the clarification of the initial compound used in the field is not possible. The determination of inorganic bromide was based on the use of the internal standard 3-bromo-1-propanol. The ratio of the areas of 1-bromo-2-propanol and 2-bromo-1-propanol (Figure 2) to the area of the internal standard was used for the calculation of the regression curve of the derivatized standard solutions.

Validation results

The methods were evaluated by assessing the basic validation parameters, namely trueness, precision, sensitivity and linearity. Trueness was estimated by the calculation of the attained recovery, whereas precision by assessing the relative standard deviation (RSD) values. The linearity of the methods was assessed from the parameter of the calibration lines. The limits of quantification (LOQs) were also estimated.

Recoveries were calculated after the fortification of blank plant matrices from apples, leek, potato, strawberry, tomato and wheat for dithiocarbamates and tomato, lettuce and rice for bromide ion. The obtained values for CS_2 ranged from 71% to 110% (Table 1), while the corresponding values for inorganic bromide were 80% to 99% (Table 2), all in the required acceptable range of 70-120% [5]. The relative standard deviation values ranged from 4 to 15.5% for CS_2 and

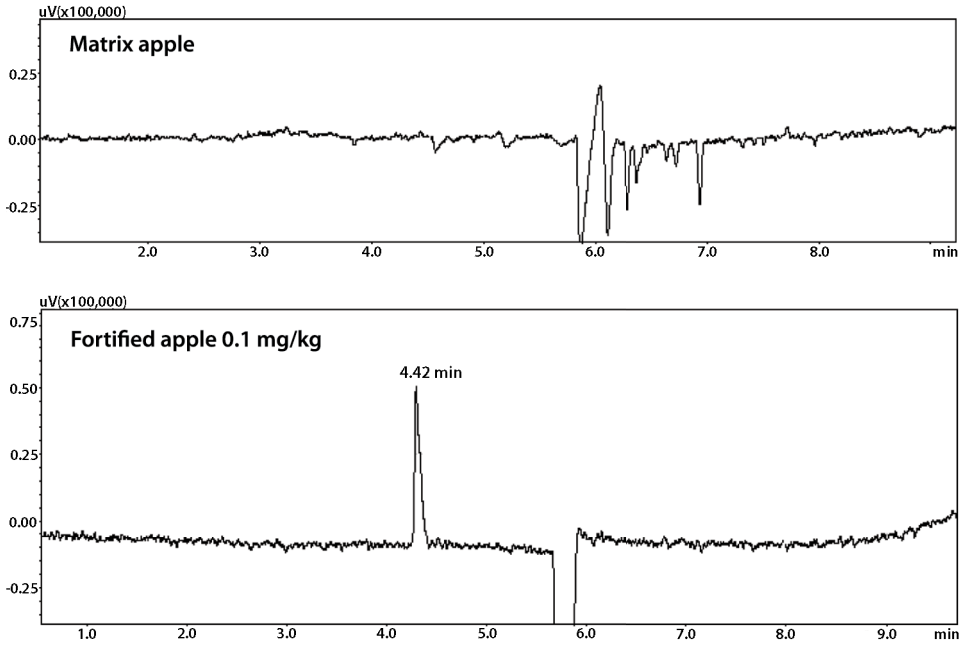

Figure 1. Determination of carbon disulfide (CS_2) in apples. Chromatograms of apple samples, matrix and fortified with 0.1mg/kg of CS_2.

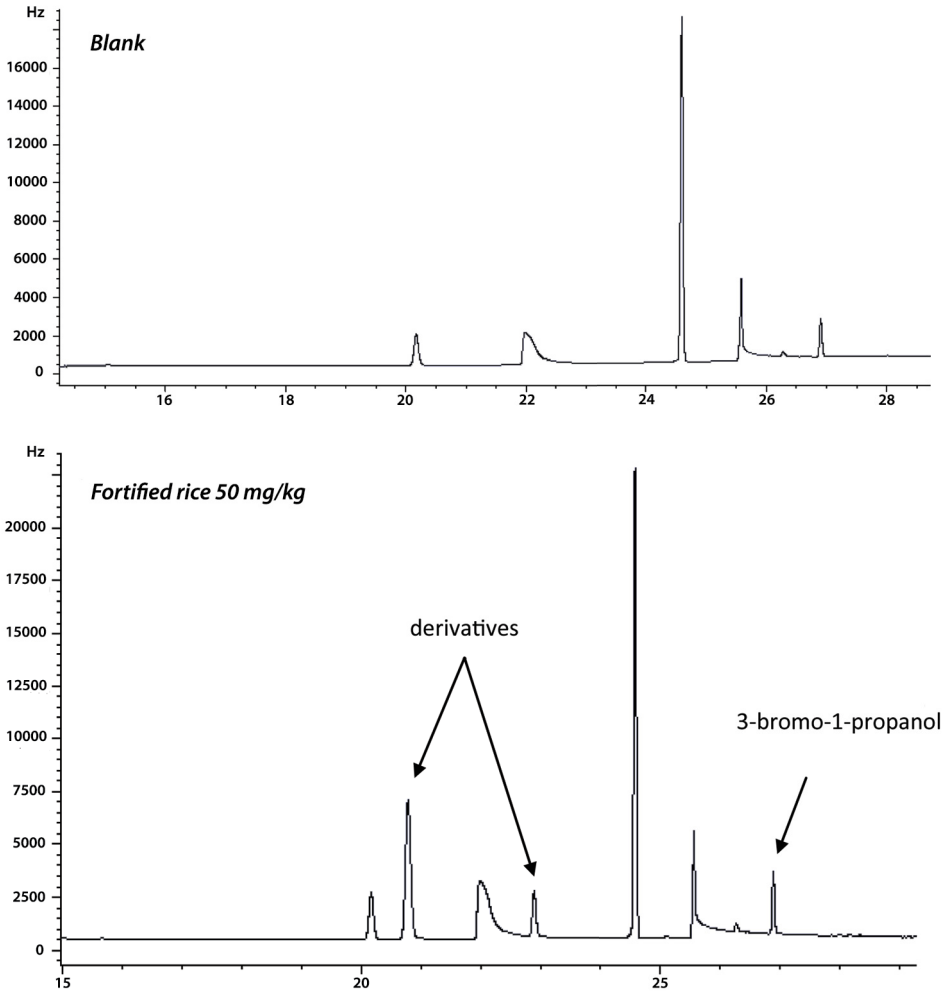

Figure 2. Determination of inorganic bromide in rice. Chromatograms of rice samples blank and fortified with 50mg/kg of bromide. The quantification is based on the ratio from the sum of the area of the two derivatives (1-bromopropanol-2 and 2-bromopropanol-1) and the internal standard (3-bromo-1-propanol).

4.6 to 18.5% for inorganic bromide, meeting the requirement of RSDs ≤20% [5]. These results are acceptable and indicate good accuracy and precision for both methods.

Validation was performed at two or three fortification levels (Tables 1 and 2), at five replicates each. The highest level was selected to be at the maximum residue limit (MRL) as set by EU Regulation 396/2005 for each product (Tables 1 and 2). Limit of quantitation (LOQ), is defined from a regulatory perspective as the lowest concentration tested at which an unambiguous identification of the analyte can be proven and at which an acceptable mean recovery with an acceptable relative standard deviation (RSD) is obtained [5, 11].

Linearity

Concerning CS_2, calibration lines were constructed from data acquired after injecting calibration standards in isooctane

Table 1. Mean recoveries (R, %), number of replicates (n), relative standard deviations (RSD, %) for the determination of CS_2 in plant products at 2 fortification levels.

Matrix	1st level (n=5)			2nd level (n=5)		
	C (mg/kg)	R (%)	RSD (%)	MRL (mg/kg)	R (%)	RSD (%)
Apples	0.1	101	11.6	5	90	9.6
Leek	0.1	83	6.9	3	84	4.0
Potato	0.1	110	9.1	1	104	5.8
Strawberry	1.0	112	5.3	10	104	6.4
Wheat	0.2	89	15.5	1	71	6.7
Tomato	0.3	93	10.1	3	84	5.6

Table 2. Mean recoveries (R, %), number of replicates (n), relative standard deviations (RSD, %) for the determination of inorganic bromide in plant products in 3 fortification levels.

Matrix	1st level (n=5)			2nd level (n=5)			3rd level (n=5)		
	C (mg/kg)	R (%)	RSD (%)	MRL (mg/kg)	R(%)	RSD (%)	C (mg/kg)	R (%)	RSD (%)
Tomato	10	89	9.3	50	94	6.2			
Lettuce	10	95	9.0	50	89	4.7	0.5	82	6.7
Rice	10	90	18.5	50	80	4.6			

at nine levels of concentration, i.e. 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20μg/mL, and linearity parameters were found acceptable, with correlation coefficients (r) being higher than 0.998. The contribution of the uncertainty of the calibration line on the total uncertainty of the method was calculated at the middle concentration (0.5μg/mL) of the calibration curve and was estimated to be 31.4%, based on the equation

$$S_u = \frac{S_{y/x}}{b}$$

In the case of inorganic bromide, as already mentioned, the calibration solutions followed the complete method procedure using five final solutions in ethyl acetate equivalent to 5, 25, 50, 100 and 150μg Br. Linearity parameters were found also acceptable, with correlation coefficients (r) to be higher than 0.99. The contribution of the uncertainty of the calibration line on the total uncertainty of the method was calculated at the middle concentration (10μg/mL) and was estimated to be 26.3%, based on the equation mentioned above.

The suitability of the present analytical methods for the determination of dithiocarbamate fungicides and inorganic bromide in plant products was further proved by the successful participation of our laboratory in two proficiency tests EUPT-SRM 5 and EUPT-SRM 6 (European Proficiency Tests for Single Residue Methods 5 and 6) organized by the European Reference Laboratories. The test materials of these proficiency tests were apple and rice, respectively. The method of dithiocarbamates was evaluated with the acceptable z scores of 1.42 for apples and 0.69 for rice, while the one of inorganic bromide participated only in the proficiency test of rice and was evaluated with the very good z score of -0.2.

The described methods were applied for the analysis of domestic and imported samples, 150 for dithiocarbamates and 45 for inorganic bromide, 12% of which gave positive results for CS_2 but not above the MRLs set by legislation. In the case of inorganic bromide, 92% of the tested samples gave positive results, since bromide is a natural component of plant tissues. There were, however, only two samples of rice in which very high con-

centrations of Br were determined, almost twice the MRL of 50mg/kg.

Conclusions

Dithiocarbamate fungicides belong to the most extensively used fungicides in agriculture. Because of poor solubilities of dithiocarbamates in common organic and aqueous solvents and, additionally, the lack of stability during homogenization of plant samples, the acid treatment of the whole sample (or large segments), evolving CS_2 as analyte in the process of gas chromatography determination with flame photometric detector (FPD) with sulphur filter seems to be most effective.

Inorganic bromide is the degradation product of fumigants or other pesticides used for post-harvest treatment. Not many laboratories analyse plant products for this compound. High bromide concentrations were determined in rice samples obtained from the market. Rice is a product that has been through processing before reaching the shelf and the detection of bromide residues in a processed product justifies the necessity for elaborating the validation of such a specialized analytical method.

The two methods are characterized by good accuracy, precision and sensitivity and are considered as suitable for routine analysis in accordance to the requirements of European Commission. Our findings contribute to our goal to develop and validate the vast majority of single residue methods in order to monitor domestic and imported samples for analytes that are not able to be detected with the multi residue methods usually applied by most laboratories and thus ensure food safety and quality.

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Επικύρωση εξειδικευμένων μεθόδων προσδιορισμού υπολειμμάτων διθειοκαρβαμιδικών μυκητοκτόνων και ανόργανου βρωμίου σε φυτικά προϊόντα

Ε. Μπεμπέλου, Κ.Σ. Λιαπής και Γ.Ε. Μηλιάδης

Περίληψη Η χρήση φυτοπροστατευτικών προϊόντων στη γεωργική πρακτική είναι μέχρι σήμερα αναπόφευκτη. Η αυξημένη, ωστόσο, κοινωνική ευαισθησία σε θέματα ασφάλειας τροφίμων καθιστά αναγκαίο τον έλεγχο των φυτικών προϊόντων σχετικά με την ύπαρξη υπολειμμάτων φυτοπροστατευτικών προϊόντων. Οι έλεγχοι αυτοί πραγματοποιούνται συνήθως με πολυϋπολειμματικές αναλυτικές μεθόδους, υπάρχουν, όμως, περιπτώσεις αναλυτών που δεν προσδιορίζονται με τις πολυϋπολειμματικές μεθόδους και απαιτούν εξειδικευμένες μεθόδους προσδιορισμού. Έχοντας ως στόχο τον προσδιορισμό ουσιών αυτής της κατηγορίας, στην παρούσα μελέτη επικυρώθηκαν αναλυτικές μέθοδοι προσδιορισμού διθειοκαρβαμιδικών μυκητοκτόνων και ανόργανου βρωμίου, ως ιόν, σε φυτικά προϊόντα με χρήση αεριοχρωματογραφίας σε συνδυασμό με φλογοφωτομετρικό ανιχνευτή (FPD) με φίλτρο θείου, και ανιχνευτή σύλληψης ηλεκτρονίων (ECD), αντίστοιχα. Η μέθοδος προσδιορισμού των διθειοκαρβαμιδικών βασίστηκε στην απελευθέρωση διθειάνθρακα και τον προσδιορισμό του έπειτα από θέρμανση του δείγματος με ένυδρο διχλωριούχο κασσίτερο. Ο προσδιορισμός ανόργανου βρωμίου πραγματοποιήθηκε με παραγωγοποίηση και μετατροπή του βρωμίου σε 1-βρωμο -προπανόλη-2 και 2-βρωμο προπανόλη-1 μέσα σε οξινισμένο διάλυμα οξειδίου του προπυλενίου με τη χρήση εσωτερικού προτύπου. Οι επικυρώσεις πραγματοποιήθηκαν σε δύο ή τρία επίπεδα συγκεντρώσεων με πέντε επαναλήψεις στο καθένα, σύμφωνα με τις απαιτήσεις του SANCO Document 10684/2009. Το υψηλό επίπεδο εμβολιασμού αντιστοιχούσε στο ανώτατο όριο υπολείμματος (MRL), ενώ το χαμηλό στο όριο ποσοτικοποίησης. Οι δύο μέθοδοι χαρακτηρίζονται από αξιοπιστία και ευαισθησία και κρίνονται κατάλληλες για αναλύσεις ρουτίνας υπολειμμάτων διθειοκαρβαμιδικών ενώσεων και ανόργανου βρωμίου σε φυτικά προϊόντα, ώστε να πληρούνται οι απαιτήσεις της Ευρωπαϊκής Επιτροπής.

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SHORT COMMUNICATION

Tanymecus dilaticollis (Coleoptera: Curculionidae): First record on *Zea mays* seedlings in Greece

S.C. Papadopoulou

Summary *Tanymecus dilaticollis* Gyllenhal, 1834, was recorded for the first time infesting severely maize seedlings in the region Agios Athanasios, northern Greece in 2010. The weevil was observed to complete one generation per year on maize and high temperatures enhanced the feeding of the pest.

Additional Keywords: corn, genitalia, maize seedlings, *Tanymecus dilaticollis*

Maize is widely cultivated throughout the world, with its production per annum being greater than that of any other grain. In 2007, over 150 million hectares of maize were planted worldwide, with a yield of 4,970.9 kilograms/hectare (8). The United States account for almost half of the world's production (~42.5%). Other top producing countries include China, Brazil, Mexico, Argentina, India, Pakistan and France. The crop is very important for Greece, decidedly contributing to the country's Gross National Product (6).

The most significant insect infestation related to *Zea mays* cultivation in Greece is mainly attributed to moths i.e. the corn stalk borer, *Sesamia nonagrioides* (Lepidoptera: Noctuidae), and the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae). Furthermore, over the four past years, adults of the western corn rootworm, *Diabrotica virgifera* (Coleoptera: Chrysomelidae), were monitored in pheromone traps in some regions of northern Greece (4). *Diabrotica virgifera* is one of the major maize pests in neighbouring countries to Greece (Italy, FYROM and Bulgaria).

Tanymecus dilaticollis Gyllenhal, 1834 (Coleoptera: Curculionidae) has been recorded infesting sugar beet in Greece (1) and hereby it is reported for the first time on maize. Sorghum, wheat, barley, sunflower, peach, apple, and almond trees are other hosts of *T. dilaticollis* (2). In April 2010, a severe infestation by the maize leaf weevil (southern gray weevil), *T. dilaticollis,* was observed on seedlings of corn in a 6.15 ha field, in Agios Athanasios region of Thessaloniki (northern Greece).

The infestation was recorded in a field where corn was cultivated for over a period of 6 years continuously. In spite of successful germination, young plant development was not satisfactory and the growers were led to believe that the seed used was not of good quality (Figure 1 and Figure 2). Inspection of the entire plant as well as of the soil revealed that the culprit was a coleopteran species (Figure 3). Adult insects were collected and brought to the laboratory for identification based on morphological characteristics as well as examination of male genitalia. Based on the morphology, the species was thought to be *Mesagroicus pilifer* Boheman, 1833 (5), which strongly resembles *T. dilaticollis*. However, examination of the genitalia according to Snodgrass (7), combined with external morphological observation (3), allowed us to conclude that the genus was *Tanymecus*

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Figure 1. Young maize seedling totally devastated by *Tanymecus dilaticollis*.

Figure 2. Young corn plants damaged by *Tanymecus dilaticollis* adults.

Figure 3. *Tanymecus dilaticollis* infesting young maize plants.

Figure 4. Male genitalia of *Tanymecus dilaticollis* (~x 70).

(Figure 4). The finding was confirmed by the Natural History Museum in London, where the species was identified as *T. dilaticollis* by Richard Thompson (September 8th, 2010). The permanent preparations of the isolated genitalia of *T. dilaticollis* are kept in the Technological Educational Institute of Thessaloniki, School of Agricultural Technology, Laboratory of Entomology.

Tanymecus dilaticollis was observed to complete one generation per year on maize and overwintered as pupa in the soil. Personal observations showed that adults' emergence from the soil occurred in late April during the warmest hours of the day and lasted for several days. Also, adults attacked young corn seedlings, consuming the leaf edges and destroying apical meristems before the 4-leaf stage of the plant's development and higher temperatures during the day enhanced the feeding of *T. dilaticollis*.

Cultural methods can be applied in an effort to reduce the weevil population, including time of sowing, crop rotation (maize is necessary to alternate with cereals) and plant density. Growers who would have to sow in early April 2011 in the same fields as in 2010 were instructed to use seeds dressed with insecticides in order to protect the emerging corn seedlings from the pest.

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ΣΥΝΤΟΜΗ ΑΝΑΚΟΙΝΩΣΗ

Tanymecus dilaticollis (Coleoptera: Curculionidae): Νέα καταγραφή του εντόμου σε καλλιέργεια αραβοσίτου στην Ελλάδα

Σ. Παπαδοπούλου

Περίληψη Το είδος *Tanymecus dilaticollis Gyllenhal*, 1834, καταγράφηκε για πρώτη φορά να προκαλεί σοβαρή καταστροφή σε νεαρά φυτάρια αραβοσίτου, στην περιοχή του Αγίου Αθανασίου στη Βόρεια Ελλάδα, το έτος 2010. Παρατηρήθηκε ότι το έντομο συμπληρώνει μία γενεά το έτος στον αραβόσιτο και η προσβολή ευνοείται από υψηλές θερμοκρασίες.

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