

VÉGÉPHYL – 12ÈME CONFÉRENCE INTERNATIONALE SUR LES RAVAGEURS ET AUXILIAIRES EN
AGRICULTURE
26, 27 ET 28 OCTOBRE 2021 - MONTPELLIER SUPAGRO

AT THE FOREFRONT AGAINST REGULATED PESTS: THE EURL FOR INSECTS AND MITES

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RÉSUMÉ

L'introduction et la colonisation d'espèces exotiques envahissantes (EEE) constituent l'une des principales menaces pour l'environnement et la biodiversité. Les insectes comptent parmi les envahisseurs les plus efficaces et constituent une préoccupation majeure pour la protection des cultures et la sécurité alimentaire dans le monde. Selon la législation européenne actuelle, 16 espèces d'insectes sont classées comme organismes de quarantaine prioritaires pour l'Union européenne (UE), en raison de leur impact économique, environnemental ou social potentiel. Le 27 mars 2019, la Commission européenne a désigné le consortium entre Anses (Montpellier) et AGES (Vienne) comme Laboratoire de référence de l'Union européenne (EURL) pour les Insectes et les Acariens, afin de soutenir les capacités de diagnostic des États membres dans ce domaine et d'apporter un appui scientifique et technique aux institutions européennes. Une identification rapide et fiable des EEE potentielles est cruciale car elle déclenche l'application de mesures phytosanitaires pour empêcher leur introduction et leur propagation.

Mots-clés : espèces exotiques envahissantes (EEE), Laboratoire de référence de l'Union européenne (LRUE), santé des plantes, arthropodes, identification

ABSTRACT

The introduction and settlement of invasive alien species (IAS) is one of the main threats to the environment and biodiversity. Insects are amongst the most successful invaders and major concerns for crops protection and food security worldwide. According to the current EU legislation, 16 insect species are listed as priority quarantine organisms for the European Union (EU), due to their potential economic, environmental or social impact. On 27 March 2019, the European Commission designated the consortium between ANSES (Montpellier) and AGES (Vienna) as the European Union Reference Laboratory (EURL) for Insects and Mites, in order to support the diagnostic capabilities of Member States in this field and to provide scientific and technical assistance to the European institutions. A quick and reliable identification of potential IAS is crucial since it triggers the application of phytosanitary measures to prevent their introduction and spread.

Keywords: Invasive Alien Species (IAS), European Union Reference Laboratory (EURL), plant health, arthropods, identification

Introduction

The introduction and settlement of invasive alien species (IAS) is one of the main threats to the environment and biodiversity, but also to human, animal and plant health. During the last 200 years the global rate of first records of newly established species has kept growing without any sign of reaching a saturation point (Seebens *et al*, 2017). Among those taxa, insects represent one of the more successfully invaders and one of the major concerns for crops protection and food security. Due to some of their ethological features, such as a small size, cryptic habits, trophic plasticity and opportunistic behavior, the accidental, usually men assisted, introduction of allochthonous insects can pass overlooked at the countries borders.

International trade represents a primary source of introduction of IAS as stowaways and contaminants of goods and packing materials. For invasive insect species, the trade of plants and plants products is widely acknowledged as a primary mechanism responsible for their transfer outside their native range. (Early *et al*, 2016; Seebens *et al*, 2017). Between 1950 and 2010, stowaways in planes and boats accounted for approximately the 5-10 % of introductions of terrestrial arthropods species to Europe and the increasing global connectivity, growing volumes and rates of movements of goods is causing the accelerating rate at which biological invasions occur (Esslet *et al*, 2015; Garnas *et al*, 2016).

In addition, the increasing frequency of extreme climatic events, the changing composition of native communities resulting from climate change (biome shifts) and the expansion of poorly biodiverse agro-ecosystems, represent factors of disturbance that enhance the dispersal and promote the establishment of IAS out of their native range (Diez *et al*, 2012; Bradshaw *et al*, 2016; Early *et al*, 2016).

The preparedness to face the introduction of an alien organism goes through an extensive surveillance of the territory, strict controls at the points of entries such as ports and airports, as well as a growing collaboration of professionals and citizens. The National and Regional Plant Protection Organizations are committed all over the world to implement territorial surveillance and inspection measures in order to promptly identify potential threats to crops and natural habitats. In this respect, the ability to identify suspicious organisms is clearly a crucial factor in responding to a potential invasion. A strong reactive capacity of a country requires the prompt and reliable identification of IAS at the required taxonomic level.

By means of Regulation (EU) 2016/2031, also known as the "Plant Health Law", and implementing regulations, the European parliament and the council establish the new plant health regime of the European Union. Within this framework, the Regulation (EU) 2017/625 defines the need of European Union Reference Laboratories (EURLs) to enhance and support Member States national plant health laboratories in their diagnostic capabilities, as it is already the case for diagnostic laboratories in the medical and animal health fields.

On 27 March 2019, by means of the implementing Regulation (EU) 2019/530, the European Commission designated the EURLs in the area of plant health, including the EURL for Insects and Mites. Born from the partnership between the Entomology and Invasive Plants Unit of ANSES Plant Health Laboratory (Montpellier, France) and the Institute for Sustainable Plant Production of AGES (Vienna, Austria), the EURL has consolidated experience in the identification of regulated and invasive pests for plants. The consortium can rely on a broad knowledge in the morphological and molecular identification of pests belonging to the main pest taxa, such as Coleoptera, Lepidoptera, Diptera, Thysanoptera, Hemiptera and Acari.

The two laboratories conduct their diagnostic activities under quality assurance regime and are accredited according to the ISO/IEC 17025 standard for testing and analysis.

With the European Food Safety Authority's scientific and technical input, the European institutions have established lists of regulated pests, identifying species that pose a real threat to the Union territory. Annex II of the Commission Implementing Regulation (EU) 2019/2072 includes the

list of Union quarantine pests, *i.e.* those species that are not present or, if present, not widely distributed in the territory, that are potentially capable of entering into, becoming established in and spreading within the territory. This list includes 98 species and genera of insects and mites that are not known to occur in the Union territory and whose introduction and spread should be prevented. Among these, a sub-group is selected as a priority for the EU. It includes twenty species whose potential economic, environmental or social impact is the most severe in respect of the Union territory (Annex of Commission Delegated Regulation (EU) 2019/1702). Of these, sixteen species are insects (80%).

The mission of the EURL for insects and mites mainly targets priority pests (Table I), but its scope can potentially include all EU regulated or unregulated species that can represent a potential threat to the Union territory.

Table I: List of insect species included in the EURL for insects and mites work programme 2019-2020				
Species	Order: Family	Quarantine pest (Regulation (EU) 2019/1702)	Priority pest (Regulation (EU) 2019/1702)	Emergency measure
<i>Agrilus anxius</i>	Coleoptera: Buprestidae	Annex II Part A ¹	yes	-
<i>Agrilus planipennis</i>	Coleoptera : Buprestidae	Annex II Part A	yes	-
<i>Anoplophora chinensis</i>	Coleoptera : Cerambycidae	Annex II Part B ²	yes	Decision 2012/138/EU
<i>Anoplophora glabripennis</i>	Coleoptera : Cerambycidae	Annex II Part A	yes	Decision (EU) 2015/893
<i>Aromia bungii</i>	Coleoptera : Cerambycidae	Annex II Part B	yes	Decision (EU) 2018/1503
<i>Bactrocera dorsalis</i>	Diptera: Tephritidae	Annex II Part A	yes	-
<i>Bactrocera zonata</i>	Diptera: Tephritidae	Annex II Part A	yes	-
<i>Epitrix</i> spp.	Coleoptera : Chrysomelidae	-	no	Decision 2012/270/EU
<i>Popillia japonica</i>	Coleoptera : Rutelidae	Annex II Part B	yes	-
<i>Rhagoletis pomonella</i>	Diptera: Tephritidae	Annex II Part A	yes	-
<i>Spodoptera frugiperda</i>	Lepidoptera: Noctuidae	Annex II Part A	yes	Decision (EU) 2018/638
<i>Thaumotibia leucotreta</i>	Lepidoptera: Tortricidae	Annex II Part A	yes	-
<i>Thrips palmi</i>	Thysanoptera: Thripidae	Annex II Part A	no	-

¹ Annex II A - Union quarantine pests **not** known to occur in the Union territory

² Annex II B - Union quarantine pests known to occur in the Union territory

Highlight on EURL's activities

This EURL mission is carried out through different activities planned in two-year work programmes and based on the Regulation (EU) 2017/625, specific instructions of the European Commission and the needs of the European Union National Reference Laboratories (EU NRLs) network. The work programme is structured around 4 main axes, which in turn include several subactivities:

- (1) to ensure availability and use of high quality methods and to ensure high quality performance by EU NRLs; this axis includes the validation and the selection of reference protocols, the provision of reference material (both as specimens and DNA) and the organization of inter-laboratories proficiency tests;
- (2) to provide scientific and technical assistance to EU NRLs; this axis includes the organization of training courses, the provision of relevant up-to-date scientific and technical information to the EU NRLs network through a dedicated website, a newsletter and workshops;
- (3) to provide scientific and technical assistance to the European Commission and other organisations, such as the European Food and Safety Authority (EFSA) and laboratories in third countries; this axis includes the provision of confirmatory analysis at request of the European Commission or EU NRLs;
- (4) to establish a list of recommended reagents for molecular biology and reference collections of specimens and DNA.

Some of the subactivities carried out in the first year are highlighted in more detail in the following paragraphs.

Proficiency Testing and training sessions: *Spodoptera frugiperda*

Spodoptera frugiperda (J.E. Smith) is a moth of the Noctuidae, native from tropical and subtropical regions of the Americas (Pogue, 2002). It is a polyphagous pest with a definite preference for the Poaceae (Casmuzet *al*, 2010) such as maize, rice, sorghum and sugarcane, but also feeding on Asteraceae and Fabaceae (Montezano *et al*, 2018). It was reported for the first time from the African continent in 2016 (Goergen *et al*, 2016). Since then, it has been rapidly spreading through the continent and it is now established in almost all the African countries south of the Sahara desert, causing tremendous losses to the crops and posing a major risk for food security and livelihoods. Recently, the parasite moved further north, being found in Egypt in 2019. Africa is not the only continent affected by the spread of the pest outside its native range: by 2018 the moth had already reached Asia and in early 2020 it was officially declared as present in Australia (CABI, 2020, EPPO, 2020). To date, the pest has only been intercepted occasionally in Europe on imported plant material (Europhyt, 2020).

The species is listed among the EU priority pests. The Commission Implementing Decision (EU) 2018/638 establishing emergency measures against this pest, requires Member States to conduct annual survey for the presence of the pest, including the use of pheromone or light traps and the collection of suspicious samples to be identified. With this respect, the EURL considered it a priority to assess the ability of EU NRLs to correctly identify *S. frugiperda*. In 2019 two proficiency tests (PTs) were organized. The first had the objective to verify the competence in the identification of adult specimens of the moth through morphological analysis of male genitalia. The second PT aimed at evaluating the ability to identify *S. frugiperda* by molecular testing. More than 300 slides were prepared in Canada Balsam, including both the target species and non-target species. Each of the 20 participants received a panel of 12 slides, including a variable proportion of target and non-target. The morphological PT was conducted according to standard ISO/CEI 17043 "Conformity

assessment-General requirements for proficiency testing". An online platform was used to facilitate the management of the PT documents and communications with EU NRLs (<https://leila.anses.fr/>).

All the participating EU NRLs used the EPPO PM7/124 Diagnostic standard (EPPO, 2015) to perform the identification and 90% of them obtained conforming results.

Regarding the molecular testing, DNA was extracted from the same specimens used for the dissection and slide-mounting of genitalia. More than 180 DNA samples have been prepared and 15 NRLs participated, either using a real time PCR test (EPPO, 2015) or a DNA barcoding approach (EPPO, 2016). The percentage of proficient participants reached 93.3%.

In order to meet the needs of EU NRLs who could not participate in the PTs due to lack of experienced staff and for EU NRLs who did not achieve compliant PT results, the EURL provides training sessions. The trainings cover the general technique of moth dissection and slide mounting of genitalia, the morphology of immature stages and the use of molecular techniques for identification.

Validation of available official protocols: *Bactrocera dorsalis*

One of the EURL tasks is to generate validation data for the available identification protocols for EU priority pests in order to be able to recommend their use to the EU NRLs network.

For many regulated arthropods, identification protocols are made available by international organizations such as EPPO (European and Mediterranean Plant Protection Organization) and IPPC (International Plant Protection Convention). These protocols, or standards, are drafted by specialists of the taxonomic group in question, are generally based on articles published in peer-reviewed journals and are examined, amended and validated by experts who form working groups for diagnostics in entomology. The protocols can include description of morphological characters, dichotomous keys, photographic material and illustrations to guide in the morphological identification of the pest, as well as molecular tests for a DNA-based identification (i.e. DNA barcoding, real-time PCR, Loop-mediated isothermal amplification). However, those protocols frequently are not accompanied by data on their performance criteria, i.e. sensitivity, specificity, repeatability, reproducibility and accuracy, which objectively state the reliability of the protocol itself.

In the year 2020, the focus was on the validation of the IPPC ISPM 27 Diagnostic protocols for regulated pests DP: 29 *Bactrocera dorsalis* (IPPC, 2019). The protocol includes morphological keys, tables of characters and molecular tests. Furthermore, the validation of the EPPO barcoding standard PM7/129 for the identification of *Bactrocera* spp. was included in the EURL activities.

Bactrocera dorsalis (Hendel) (Diptera: Tephritidae) is endemic and widespread in tropical areas of Southeast Asia and is part of a group of closely related species that are recognised as a complex of 85 described species (Drew & Hancock, 1994; Drew, 2004; Clarke *et al*, 2005; Vargaset *al*, 2015). Due to its high reproductive and biotic potential, a rapid dispersal ability and a broad host range, it is considered a species with a high invasive capacity. Since the first report in Kenya in 2003, the species has rapidly colonized almost the entire African continent. It is locally present in the United States and the recent, repeated interceptions in Italy (2018, 2019), Austria (2019) and France (2019) keep the European Plant Protection Organisations on alert (CABI, 2020; EPPO, 2020).

The validation plan involves a working team composed by supervisors and operators. Supervisors are in charge of the preparation of a panel of blind-coded specimens including some *B. dorsalis* and some morphologically similar species, selected from the available reference collections. Three operators perform independently the morphological and molecular identification strictly following the protocol under evaluation. Based on the results obtained by the operators, supervisors compute the performance characteristics for the selected criteria (sensitivity, specificity, repeatability, reproducibility and accuracy) and compare them with the *a priori*-defined, expected values.

Extensive Systemic Literature Search (ELS) for *Agrilus planipennis*, *Agrilus anxius*, and *Rhagoletis pomonella*

In order to provide EU NRLs with details and guidance on high quality methods of laboratory diagnosis, including reference methods for the identification of priority quarantine pests, an Extensive Systemic Literature Search (ELS) was performed to collect information applying an approved scientific procedure. The ELS was carried out to collect and compile comprehensive information about methods to identify, detect and diagnose *Agrilus planipennis*, *Agrilus anxius*, and *Rhagoletis pomonella*, which are listed as priority quarantine pests (Regulation (EU) 2016/2031; Regulation (EU) 2019/1702). Additionally, information about the occurrence, distribution and reported outbreaks of the three arthropod species was retrieved. Literature sources such as online databases (Ovid, Scopus), pertinent websites (EPPO Global Database, EPPO Reporting service, CABI Crop Protection Compendium, EFSA scientific opinions and EFSA survey cards) and scientific standard literature (identification keys) were selected and a search strategy defined. The ELS was carried out stepwise by applying different search terms sets and their combinations and inclusions/exclusion criteria as data filters. Search terms and search term sets for synonyms of pest species names, morphological and molecular identification/detection/diagnostic methods, occurrence, outbreak and distribution as well as exclusion terms were developed, pre-tested, finalized and applied. One EndNote® library was established for the ELS for the three species to archive the retrieved records in a systematic folder structure and for further processing (deduplication, labelling, selection, upload pdfs etc.). Additional literature was added manually to the EndNote library. The first analysis of the retrieved records showed that for *Agrilus planipennis* 88 records out of 343, for *Agrilus anxius* 17 records out of 69 and for *Rhagoletis pomonella* 63 out of 246 records provided relevant information. Thus 25.5% of the retrieved records fulfilled the defined inclusion criteria. From the included records, 31 dealt with morphological identification, 67 with molecular identification *sensu lato* and the remaining 70 with outbreak, occurrence and distribution.

Establishment of a quality assured reference and working collection

The EURL maintains an extensive collection of reference material (dry specimens, specimens stored in ethanol, slide-mounted specimens). For molecular diagnostics, a reference collection is constantly being increased with DNA samples and reference sequence data from voucher specimens. The aim is to build a high quality reference collection of nearly all EU regulated insects to make reference materials (reference collection and working collection) available for EURL needs (*i.e.* testing protocols or organization of proficiency tests) and to provide such material to EU NRLs upon request. To fulfill this task all available reference material from both partners was compiled and the information will be provided on the EURL website. Reliable identifications require appropriate reference material. Therefore, criteria for reference material, which were determined in the frame of the EU project Q-collect, were applied to increase the traceability and reliability of the EURL collection. DNA barcoding was used to confirm the morphological identification of voucher specimens. Literature search was the first step to optimize non-destructive DNA extraction approaches, essential for testing voucher specimens. A reference database software ('Specify'), accessible to the entire EURL team, is being tested to ensure the management and traceability of all information related to the reference specimens, including DNA barcodes.

Taxonomic study: *Epitrix* spp. from their native geographic range

Some groups of pests, such as *Epitrix* potato flea beetles, pose challenges for their identification. *Epitrix* (Coleoptera: Chrysomelidae: Alticinae) is a genus that has a worldwide distribution and consists of nearly 180 species (Bieńkowski & Orlova-Bienkowskaja, 2017). They are

tiny pubescent beetles with elytral punctures arranged into striae. Most of them are morphologically highly similar which makes them very difficult to identify. In many cases, identification requires that male and female genitalia be extracted and examined. Several species of *Epitrix* are reported to feed on foliage and/or tubers of potatoes. In the EU, emergency measures have been introduced to prevent the introduction and spread of four *Epitrix* species: *E. tuberis*, *E. subcrinita*, *E. cucumeris* (originating from North and Central America) and *E. papa* (of unknown origin thus far). *Epitrix cucumeris* and *E. papa* have been introduced in Spain and Portugal where they cause damage on potato tubers (Boavida & Germain, 2009). Since the introduction of invasive *Epitrix* in Europe, progress has been made in species identification via morphological methods and DNA barcoding focusing on EU regulated and European species (EPPO, 2017). However, the work done to date has overlooked South American species of *Epitrix*. Very little is known of the identity and biology of the many neotropical species even if some of them are significant potato pests in South America and could also be of concern for European phytosanitary authorities. The EURL is involved in a project aiming at better characterizing South American *Epitrix* species. Within this context, a trip to Peru was organised in collaboration with the National Agrarian University - La Molina and resulted in the collection of more than 3,000 *Epitrix* specimens. Specimens were collected in 30 potato-growing localities ranging from 2500 to 4000 m above sea level in the provinces of Cajamarca, Anta, Cusco, Paucartambo and Quispicanchi. Specimens will be studied with both morphological and molecular methods and the results should contribute to develop a deeper knowledge on *Epitrix* potato pests in South America.

Concluding Remarks

Worldwide, the introduction, settlement and spread of arthropod plant pests outside their original geographical range can have a very significant environmental, economic and social impact, resulting in increased costs for governments, public institutions and economic operators (Bradshaw *et al*, 2016). In developing countries, food security and livelihoods themselves can be put at risk. The importance that the European institutions attach to measures against the introduction of non-native arthropod plant pests into the Union territory is clearly highlighted by the long lists of these organisms included in the recent legislation. Within this regulatory framework, the ability to promptly and reliably identify a potential invasive pest to the specified taxonomic level plays a major role in countering new introductions.

Starting its activities in August 2019, the EURL for insects and mites aims at providing scientific and technical support to the network of National Reference Laboratories of the Member States in the identification of regulated insects and mites species, as well as of other non-regulated invasive species.

The selection and validation of best identification protocols, the provision of high quality reference materials to be used as a comparison during morphological analysis or as a positive control in the case of molecular analyses, together with the selection of DNA extraction reagents and kits, aim at harmonising testing practices in EU-NRLs laboratories. Where identification protocols are not available, the EURL engages in extensive literature studies and actively contributes to the production of protocols in working groups promoted by organizations such as EPPO and IPPC.

Molecular diagnostics (e.g. DNA barcoding and pest-specific PCR assays) complement the morphological analysis and allow the accurate identification, especially of life stages that are difficult or impossible to identify by morphological features. Provision of training on these techniques will enhance the ability of the EU NRLs to confirm their skills on identification, enabling a rapid deployment of sound results for stakeholders like national plant protection services.

The organization of inter-laboratories proficiency tests allow the participating laboratories themselves to test their skills in the identification of a particular pest and enable the EURL to identify any training needs, filled through the provision of specific training courses conducted by EURL staff or external qualified experts. It is worthy to emphasize that accreditation bodies usually require a

regular participation in PTs for those testing laboratories that are accredited, or aiming at accreditation, according to ISO/CEI 17025. *Ad hoc* training courses are also provided for EUNRLs that do not have the necessary skills or experience to deal with certain taxonomic groups of pests.

A dedicated EURL website, together with the regular issue of a newsletter and the organization of yearly workshops, allows EUNRLs to be updated on recommended protocols and reagents, availability of reference materials, ongoing or forthcoming activities and events promoted by the EURL, ongoing research projects on regulated pests at Union and international level, facilitating the exchange of scientific information and connections within the EUNRLs network.

Improving identification capability of EUNRLs is a way for the European Union to better anticipate future insect and mite invasions.

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