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Use of the nuclear region ITS2 for arthropods species identification

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QBOL-EPPPO Conference on DNA Barcoding and diagnostic methods for plant pests

Haarlem (NL)
2012-05-21/25

Programme, participant list
and summaries of presentations
and posters



Netherlands Food and Consumer
Product Safety Authority
Ministry of Economic Affairs, Agriculture and
Innovation



QBOL-EPPO Conference on DNA barcoding and diagnostic methods for plant pests

Haarlem, NL, 2012-05-21/25

Programme

QBOL final Conference combined with EPPO Conference on diagnostic methods for plant pests

Monday 21 May 2012– Friday 25 May 2012

Monday Evening 2012-05-21

18.00	<i>Reception</i>	
19.30	Welcome	Wim van Eck VWA, the Netherlands
19.35	QBOL, an EU project delivering Barcodes to support Plant Health	Peter Bonants PRI, the Netherlands
19.40	EPPO Diagnostic activities: serving the needs of plant pest diagnostic laboratories	Francoise Petter EPPO
19.50	Using DNA Barcoding for diagnostics and monitoring of plant pathogens and pests, a Canadian perspective	André Lévesque Agriculture and Agri-Food Canada, Canada
20.40	Culture Collections in relation to DNA Barcoding	Pedro Crous CBS-KNAW, the Netherlands

Tuesday 2012-05-22

Session 1 Invasive plants & Phytoplasmas

Session leaders: Assunta Bertaccini (Univ. Bologna, Italy) and Maja Ravnikar (NIB, Slovenia)

08.30-09.00	Interactive image-driven identification keys for invasive plants	Johan v Valkenburg, NRC-NPPO, the Netherlands
09.00-09.30	QBOL-WP 7: DNA Barcoding of phytoplasmas	Mogens Nicolaisen, Univ Aarhus, Denmark
09.30-09.50	Data on temporary and spatial distribution of microbes in plants helps to improve on-site detection	Maja Ravnikar, NIB, Slovenia
09.50-10.10	Phytoplasma detection and identification: from 16S ribosomal gene to multiple gene identification	Assunta Bertaccini, Univ Bologna, Italy
10.10-10.40	<i>Coffee break</i>	
10.40-11.00	A panel of LAMP assays for detection of flavescence dorée phytoplasma	Jennifer Hodgetts, Fera, United Kingdom
11.00-11.20	Deep amplicon sequencing for detection of mixed phytoplasma infections in plants	Nicoletta Contaldo, Univ Bologna, Italy

Session 2 Viruses

Session leaders: Neil Boonham (FERA, United Kingdom) and Ellis Meekes (Naktuinbouw, the Netherlands)

11.20-11.50	QBOL-WP 6: Next Generation Sequencing for Plant Virus Discovery and Diagnosis	Ian Adams, Fera, United Kingdom
11.50-12.10	Detection of Potato spindle tuber viroid and Tomato chlorotic dwarf viroid in seeds of tomato	Ellis Meekes, Naktuinbouw, the Netherlands
12.10-12.30	Small RNA sequencing and assembly as a DNA barcoding method from plant viruses	Jan Kreuze, CIP, Peru

Programme

12.30-14.00	<i>Lunch</i>	
14.00-14.20	Multiplex pathogen detection using microarrays	Neil Boonham, Fera, United Kingdom
14.20-14.40	Microarray-based methods for a multiple and simultaneous detection of viruses highlighting the need for a collection database	Antonio Tiberini, Agricultural Research Council, Italy
14.40-15.00	RT-LAMP for detection of plant pathogenic viruses including Cassava brown streak virus	Jenny Tomlinson, Fera, United Kingdom
15.00-15.20	Development and validation of a real-time RT-PCR assay for generic detection of pospiviroids	Marleen Botermans, NRC-NPPO, the Netherlands
15.20-15.50	<i>Coffee break</i>	
15.50-16.10	Comparison of methods for detection of pospiviroids infecting Solanaceae	Pascal Gentit, Anses, France
16.10-16.30	State of the art multiplex Luminex xMAP and xTAG -detection of plant viruses	René van der Vlugt, PRI, the Netherlands
16.30-16.50	Plant viruses and viroids in an aqueous environment: survival, water mediated transmission and detection	Natasa Mehle, NIB, Slovenia
16.50-17.10	LAMP as a tool for On-Site Confirmation and Monitoring of plant pathogens	Cor Schoen, PRI, the Netherlands
17.10-17.30	Quality control in bioassays for broad screening for plant viruses	Annelien Roenhorst, NRC-NPPO, the Netherlands

Wednesday 2012-05-23

Session 3 Insects & Mites

Session leaders: Alain Roques (INRA, France) and Antoon Loomans (NRC-NPPO, the Netherlands)

08.30-09.00	QBOL-WP 3: Barcoding Quarantine Arthropods	Jean-Yves Rasplus, INRA, France
09.00-09.20	Morphological and molecular identification of EU quarantine species of <i>Spodoptera</i> (Lepidoptera, Noctuidae)	Marja van der Straten, NRC-NPPO, the Netherlands
09.20-09.40	Combining DNA barcoding and morphological analysis for the diagnosis of alien pests. The case study of <i>Epitrix</i> potato flea beetles (Coleoptera: Chrysomelidae)	Astrid Cruaud, CBGP, France, presented on behalf of Jean-François Germain, Anses, France
09.40-10.00	Morphological identification of <i>Thrips palmi</i> in the frame of ISO/IEC 17025	Antoon Loomans, NRC-NPPO, the Netherlands
10.00-10.20	Molecular characterization of Leptomastidea spp. (Encyrtidae, Hymenoptera) populations from Puerto Rico, Barbados and Florida	Jose Carlos Rodrigues Univ Puerto Rico, Puerto Rico
10.20-10.40	Characterization of two haplotypes of <i>Haplaxius</i> (<i>Myndus</i>) <i>crudus</i> , vector of different phytoplasmas associated with palm diseases, using cytochrome oxidase I gene	Michel Dollet, CIRAD, France
10.40-11.10	<i>Coffee break</i>	

Programme

Session 4 Nematodes

Session leaders: Jeurg Frey (ACWm, Switzerland) and Géraldine Anthoine (Anses, France)

11.10-11.40	QBOL-WP 5: Barcoding as a new tool for identification of quarantine nematodes and their close relatives	Sebastian Kiewnick, ACW, Switzerland
11.40-12.00	Use of a phylum-wide SSU rDNA-based molecular framework for the detection of plant parasitic nematode species in complex DNA backgrounds	Hans Helder, Wageningen University, the Netherlands
12.00-12.20	Validation of morphological identification method - the example of a morphological key for <i>Bursaphelenchus xylophilus</i> species	Géraldine Anthoine, Anses, France
12.20-12.40	Evaluation of barcoding and phylogenetic potential of four genes in the genus <i>Meloidogyne</i>	Martijn Holterman, ACW, Switzerland
12.40-14.00	Lunch	
14.00-14.20	Diagnostic kits for the detection of plant parasitic nematodes	Renske Landeweert, Clear Detections, the Netherlands
14.20-14.40	Direct detection of plant parasitic nematodes by real time PCR : experience gained from development and validation of different tests	Sylvie Gamel, Anses, France

Session 5 Posters

Session leader: Johannes Hallmann (JKI, Germany)

14.50-15.40	Poster session: 5 posters to be presented briefly (10 mins each) by authors, then viewing of the other posters	Françoise Petter (EPPO), Barbara Piskur (Slovenian Forestry Institute), Joanna Zaluga (Univ. Ghent, Belgium), May Bente Bruberg (Bioforsk, Norway), Stefan Wagner (JKI, Germany)
15.40-16.10	Coffee break	
16.10-17.30	Continuation Poster session	

From 18.00	Conference Dinner	
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Thursday 2012-05-24

Session 6 Bacteria

Session leaders: Martine Maes (ILVO, Belgium) and Emilio Stefani (UNIMORE, Italy)

08.30-09.00	QBOL-WP 4: Barcode identification of quarantine bacteria, the QBOL strategy and results	Bart Cottyn, ILVO, Belgium
09.00-09.20	PCR-based assays for detecting <i>Xanthomonas axonopodis</i> pv. <i>allii</i> in onion seed.	Isabelle Robene, CIRAD, France
09.20-09.40	Invasion of <i>Xanthomonas arboricola</i> pv <i>pruni</i> (XAP) in the ornamental <i>P. lauroceraceus</i> (cherry laurel) in the Netherlands	Maria Bergsma-Valmi, NRC-NPPO, the Netherlands
09.40-10.00	Validation of real-time PCR for detection and identification of <i>Xanthomonas fragariae</i>	Marcel Westenberg, NRC-NPPO, the Netherlands
10.00-10.20	Phenotypic and genotypic characteristics of <i>Dickeya</i> strains isolated from potato in Poland	Ewa Lojkowska, Univ Gdansk, Poland
10.20-10.50	Coffee break	

Programme

10.50-11.10	<i>Clavibacter michiganensis</i> strains characterized as non-pathogenic by phylogenetic and polyphasic analyses are transmitted by tomato seeds and interfere with <i>C. michiganensis</i> subsp. <i>michiganensis</i> detection	René Mathis, Variety and seed study and control group, France
11.10-11.30	Is identification by bar-coding of Q-clavibacters in conflict with the existing bacterial taxonomy of this group of bacteria?	Paul de Vos, Univ. Gent, Belgium
11.30-11.50	Detection of <i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i> in seeds of tomato	Harry Koenraadt, Naktuinbouw, the Netherlands
11.50-12.10	Emergence of strains of <i>Pseudomonas syringae</i> pv <i>actinidiae</i> (Takikawa, 1989) in France: methods of characterisation	Francoise Poliakoff, Anses, France
12.10-12.30	An array based technology designed to detect the complex plant pathogen <i>Ralstonia solanacearum</i>	Gilles Cellier, Anses, France
12.30-12.50	Graft-transmission of 'Candidatus Liberibacter solanacearum', the causal agent of potato zebra chip disease, from greenhouse-grown latently-infected tomato	Xiang Li, Canadian Food Inspection Agency, Canada
12.50-14.00	<i>Lunch</i>	

Session 7 Fungi

Session leaders: Hans de Gruyter (NRC-NPPO, the Netherlands and Ana Maria Pérez Sierra (Univ. Valencia, Spain)

14.00-14.30	WP2 of QBOL: Barcoding fungi of Q importance	Ewald Groenewald, CBS-KNAW, the Netherlands
14.30-14.50	DNA barcoding of the fungal genus <i>Phoma</i> from New Zealand	Wellcome Ho, Ministry of Agriculture and Forestry, New Zealand
14.50-15.10	Barcoding <i>Mycosphaerella</i> species of quarantine importance to Europe	William Quaedvlieg, CBS-KNAW, the Netherlands
15.10-15.30	New SNP markers for the genetic characterization of the quarantine pathogen <i>Puccinia horiana</i> and their application in pathogen migration analysis	Mathias de Backer, ILVO, Belgium
15.30-16.00	<i>Coffee break</i>	
16.00-16.20	Molecular phylogeny of <i>Phoma</i> : tool for a validated real-time (TaqMan) PCR for detection of <i>Stagonosporopsis andigena</i> and <i>S. crystalliniformis</i> in infected leaves of tomato and potato	Hans de Gruyter, NRC-NPPO, the Netherlands
16.20-16.40	Real-time PCR detection of the quarantine pathogen <i>Melampsora medusae</i> f. sp. <i>deltoidae</i>	Anne-Laure Boutigny, Anses, France
16.40-17.00	A DNA-based macroarray for multiplex detection of soil-borne fungi	Anita Rose Haegi, Agricultural Research Council, Italy
17.00-17.20	Using real-time PCR to detect plant pathogens in soil	James Woodhall, Fera, United Kingdom

Programme

Friday 2012-05-25

Session 8 Validation & Databases and other Diagnostic Tools

Session leaders: Peter Bonants (PRI, the Netherlands) and Françoise Petter (EPPO)

08.30-09.00	QBOL-WP 10: DNA barcoding as identification tool for regulated plant pests: an international collaborative test performance study among 14 laboratories	Bart van de Vossenberg, NRC-NPPO, the Netherlands
09.00-09.30	QBOL and Q-bank data management and analysis system	Vincent Robert, CBS-KNAW, the Netherlands
09.30-09.40	Reference plant pathogenic bacteria in support of the European plant health policy: the Q-Bacco-net initiative	Martine Maes, ILVO, Belgium
09.40-09.50	Q-bank Plant Virus database and collections	Annelien Roenhorst, NRC-NPPO, the Netherlands
09.50-10.10	Biodiversity sequencing - next generation DNA barcoding	Rachel Glover, Fera, United Kingdom
<i>10.10-10.30</i>	<i>Coffee break</i>	
10.30-11.00	Research on and applications of DNA Barcodes for species identification	Ming-Fu Li, Chinese Academy of Inspection and Quarantine, China
11.00-11.20	How to prioritize the development of diagnostic tests for plant pests based on a risk ranking tool?	Benedicte Moignot, Anses, France
11.20-11.40	qPCR, low level detection and validation data	Tanja Dreo, NIB, Slovenia
11.40-12.00	Validation as a project: 2 laboratories, 24 organisms, 3 years	Mariette Edema, NRC-NPPO, the Netherlands
<i>12.00-13.30</i>	<i>Lunch</i>	

Session 9 Stakeholders views and needs

Session leaders: Eric Regouin (EUPHRESKO) and Alan Inman (EUPHRESKO)

13.30-13.50	Results of all Work Packages of QBOL, from DNA barcodes to Database and Validation	Peter Bonants, coordinator QBOL, PRI, the Netherlands
13.50-14.10	Barcoding, Multilateral Initiatives for Biosecurity Outcomes	Welcome Ho, QUAD, New Zealand
14.10-14.30	Role of diagnostics in the EU plant health management	Harry Arijs, EU commission DG Sanco, Belgium
14.30-14.50	IPPC diagnostic protocols: underpinning global phytosanitary systems	Brent Larson, IPPC Secretariat
<i>14.50-15.20</i>	<i>Coffee break</i>	

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15.20-15.40	Diagnostic tools in perspective; Netherlands policy and the need for robust phytosanitary chains	Hans Smolders, Ministry EL&I, the Netherlands
15.40-16.00	Q-bank: more than a database	Wim van Eck, Q-bank steering committee, the Netherlands
16.00-16.20	International trade of high graded plant material : the need for harmonized and validated detection techniques	John van Ruiten, Inspection Service Naktuinbouw, the Netherlands
16.20-16.40	The view of horticultural producers on diagnostic tools	George Franke, AIPH, the Netherlands
16.40-17.00	TESTA - Seed health: development of seed treatment methods, evidence for seed transmission and assessment of seed health	Christine Henry, coordinator TESTA, Fera, United Kingdom
17.00-17.30	Discussion / Concluding remarks	Eric Regouin (Euphresco, the Netherlands) and Alan Inman (Euphresco, the United Kingdom)
<i>17.30-18.30</i>	<i>Drinks and close</i>	

Opening Session

Abstracts of Presentations

Monday 2012-05-21

Peter Bonants

QBOL, an EU project delivering Barcodes to support Plant Health

Bonants PJM (1)

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Many DNA Barcoding campaigns have been launched since the introduction of Barcoding of Life to study the biodiversity of life.

QBOL (www.qbol.org), an EU project on DNA barcoding, started in 2009 to generate DNA barcoding data of plant pathogenic quarantine organisms and their taxonomically relatives to support plant health diagnostics. Within this project researchers from all over the world worked together in several work packages to generate DNA/RNA sequences to discriminate quarantine organisms from their closest relatives. The six work packages to generate the barcodes are on Fungi (WP2), Arthropods (WP3), Bacteria (WP4), Nematodes (WP5), Viruses (WP6) and Phytoplasmas (WP7). Within each groups of organisms genes were selected to be used as barcoding genes. Protocols are generated on how to extract nucleic acids, how to amplify and sequence the selected genes and how to search within a database. Also decision schemes are developed to help NPPO's and other laboratories in correct identification of the quarantine organisms. All generated data are included in a database, called Q-bank (www.Q-bank.eu).

Françoise Petter

EPPO Diagnostic activities: serving the needs of plant pest diagnostic laboratories

Françoise Petter¹, Suffert Muriel¹, Roy Anne Sophie¹, Griessinger Damien¹, McMullen Madeleine¹

1) EPPO, 21 Boulevard Richard Lenoir, 75011 Paris, France hq@epo.int

EPPO has established a work programme in the area of diagnostics to harmonize procedures across the region. The different activities conducted in the framework of this programme are presented.

Early warning. The EPPO Secretariat has established early warning systems to identify emerging risks. It mainly consists of

- The Alert List draws the attention to certain pests potentially presenting a risk to them.
- A free monthly newsletter (EPPO Reporting Service).
- The List of invasive alien plants.

Laboratories can then be alerted about potential new pest they may have to identify.

Diagnostic protocols. A programme to prepare diagnostic protocols for regulated pests of the EPPO region was initiated in 1998. The work is conducted by the different diagnostic Panels. The diagnostic protocols are written by assigned authors according to a common format and are then reviewed by the relevant diagnostic Panels. They are approved following the regular EPPO Standards approval procedure. The first EPPO Standards were published in 2001, and more than 100 Diagnostic Standards have been approved so far.

<http://archives.eppo.int/EPPOStandards/diagnostics.htm>

Accreditation and quality management. Two quality assurance standard for diagnostic laboratories have been adopted

- PM 7/84 Basic requirements for quality management in plant pest diagnostic laboratories
- PM 7/98 Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity.

EPPO database on diagnostic expertise. The EPPO database on diagnostic expertise is an inventory of the diagnostic expertise available in the EPPO region <http://dc.eppo.int/>

Conferences and Workshops. Since 1985 EPPO has a regular programme of Conferences and Workshops on diagnostics.

André Lévesque

Using DNA Barcoding for diagnostics and monitoring of plant pathogens and pests, a Canadian perspective

C. André Lévesque

Agriculture and Agri-Food Canada, Central Experimental Farm, Ottawa, Canada

DNA sequencing has been used to identify fungi pretty much since PCR became available. The need to develop internationally accepted identification standards supported by NCBI as well as databases housing information not available in GenBank became apparent. In 2003, the concept of DNA Barcoding proposed by Paul Hebert, Guelph University, Canada, addressed this issue. The success of DNA barcoding depends largely on the breadth of the reference databases which means that it must be an international endeavour. Shortly after the term DNA barcoding was coined, the Alfred P. Sloan Foundation supported the creation of the Consortium for the Barcode of Life to foster this activity internationally and more recently Genome Canada has supported the creation of the International Barcode of Life Initiative (iBOL).

The access to comprehensive DNA barcode databases is essential for accurate identification by Sanger sequencing and there has been significant progress made recently in DNA barcoding of both true Fungi and oomycetes. These sequence databases open up many technological possibilities that can be rapidly developed from analyses of this reference data. Quantitative PCR, DNA array hybridization and pyrosequencing are just a few examples of technologies for molecular detection based on DNA barcode data. The Genomics Research and Development Initiative (GRDI) is the counterpart to Genome Canada for Canadian Federal government scientists. In January 2012, GRDI approved a project on Quarantine and Invasive Species that is involving almost all science-based departments of the Canadian government. This project has a strong component on plant quarantine species of relevance to Canada. Further databases are planned for plant quarantine insects, nematodes, fungi, phytoplasmas and viruses as well as invasive plants and apply some of the next generation sequencing tools for direct detection. This new Canadian project will complement the seminal work done by QBOL

Pedro Crous

Culture collections in relation to DNA Barcoding

Crous Pedro W.

CBS-KNAW Fungal Biodiversity Centre, Uppsalalaan 8, 3584CT Utrecht, the Netherlands p.crous@cbs.knaw.nl

The CBS Culture Collection (1904) is in the process of generating DNA barcodes of its entire holdings. Faced with a great diversity of undescribed and cryptic taxa, taxonomic types have the prominent function of anchoring existing species names. Taxonomic type sequence information is rare, because few types have to date been subjected to DNA sequencing. The barcode sequences of the more than 8000 ex-type cultures will be of tremendous value for fungal taxonomy and its user community. Furthermore, the CBS yeast collection holds almost 9000 strains including the 2240 ex-type strains of all described species. The resulting barcode data set will be an invaluable reference source for yeast research. For more than 100 years, strains entering the CBS collections have been identified based on state-of-the-art techniques at the time of accession. DNA barcoding the holdings will allow identifications to be updated and the recognition of strains in need of further research, which will not only increase the value of the collection, but will aid the user community. The DNA barcoding approach and online database identification tool will also prove invaluable to confirm the identity of strains selected for whole genome analysis.

Session 1 Invasive Plants & Phytoplasmas

Abstracts of Presentations

Tuesday 2012-05-22

van Valkenburg Johan

Interactive image-driven identification keys for invasive plants

van Valkenburg Johan

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For some years work has been ongoing to compile information to facilitate identification of non-native plants that pose a (potential) threat to the biodiversity of the ecozone comprising Northern Germany, the Netherlands, Belgium and North-Western France or are regulated by third countries and are likely to be present as contaminant in commercial exports originating from the Netherlands. This information is now available at <http://www.q-bank.eu/Plants/>.

This information system also comprises a set of interactive image-driven identification keys for invasive plants at various growth stages in a range of 'habitats': seed identification of contaminants in birdfeed, weeds in bonsai plants, seedling identification, invasive terrestrial plants and invasive aquatic plants. The idea of image-driven identification, using a multiple entry system is to avoid misunderstandings in terminology and failure in identification in the absence of certain characters as is sometimes the case in dichotomous keys. The interactive keys are linked to the species information in the database, including datasheets, distribution maps, specimen level information, barcodes of selected species etc. In addition to the above mentioned species information, the results of the EUPHRESCO DeCLAIM (Decision support systems for the control of alien invasive macrophytes) project are also incorporated in the database and available at <http://www.q-bank.eu/Plants/> under the header 'control'.

Mogens Nicolaisen

QBOL – WP 7: DNA barcoding of phytoplasmas

Nicolaisen Mogens (1), Makarova Olga (1), Contaldo Nicoletta (2), Paltrinieri Samanta (2), Bertaccini Assunta (2)

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2) Alma Mater Studiorum, University of Bologna, Plant Pathology, Viale Fanin, 42, 40127 – Bologna, Italy, assunta.bertaccini@unibo.it

Phytoplasmas infect a high number of plant species. Based on the 16S ribosomal gene sequence, over 20 groups have been established within the '*Candidatus* Phytoplasma' taxon, some of which are of quarantine status. To date, no protocol for *in vitro* cultivation of phytoplasmas has been developed, making classical microbiological identification procedures impossible. Phytoplasma identification is currently based on RFLP analysis of a 1.2 kbp region of the 16S rDNA, however, this method is not easy to set up and it only considers a minor part of the available phytoplasma sequence information. It has been suggested that universally amplified, short, and highly variable DNA barcodes may help to rapidly identify organisms. As part of the QBOL project, a universal DNA barcoding based tool for phytoplasma identification was developed. Two sets of primers amplifying a fragment of the Tuf gene and a fragment of the 16S rDNA gene, respectively, were designed and the potential of these fragments as DNA barcodes was verified. Successful amplification and sequencing of more than 150 phytoplasma strains, and ability to separate various phytoplasma strains to '*Candidatus* species' level, 16S ribosomal group and sub-group level confirm that these barcodes are efficient phytoplasma identification tools.

Maja Ravnikar

Data on temporary spatial distribution of microbes in plants helps to improve their on-site detection

Maja Ravnikar (1), Nina Prezelj(1), Petra Nikolič(1), Nataša Mehle(1), Rok Lenarčič(1), Tanja Dreo(1), Dany Morisset(1), David Dobnik(1), Jennifer Hodgetts(2), Neil Boonham(2), Matt Dickinson(3), Marina Dermastia(1)

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3) School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, UK

Different isothermal methods such as loop-mediated isothermal amplification (LAMP), which shows potential to be used for on-site detection, were developed recently. While the LAMP method is simple and fast, selection of testing material remains crucial, particularly due to the low amount of material tested. Time and spatial distribution of the pathogens in plants needs to be taken into account to improve reliability of such testing. In this contribution, a temporary and spatial analysis of Grapevine Flavescence dorée (FDp) phytoplasma distribution in grapevines will be presented. Data was collected at two locations with diverse climatic conditions, during two growing seasons and testing of various plant parts allowed targeted sampling of phytoplasma, leading to their reliable detection from the time of flowering up to the end of growing season. Additionally, simultaneous isothermal detection of RNA and DNA microbes will be addressed for the case of the potato quarantine bacteria and viroid with data presented on sample preparation, sample homogenization and nucleic acid extraction and purification.

Assunta Bertaccini

Phytoplasma detection and identification: from 16S ribosomal gene to multiple gene identification

Bertaccini Assunta (1), Paltrinieri Samanta (1), Contaldo Nicoletta (1), Franova Jana (2), Nicolaisen Mogens (3), Duduk Bojan (4)

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4) Institute of Pesticides and Environmental Protection, 11080 Belgrade, Serbia

Phytoplasmas are bacteria lacking a cell wall that are located in the phloem of plants and in the hemolymph of insect vectors. The phytoplasma classification scheme is based on PCR/RFLP analyses of 16S rDNA: a reliable tool for the differentiation that has become the most comprehensive and widely accepted classification system. On the other hand, the 'Candidatus Phytoplasma' species description, recently under adoption, refers to 16S rRNA gene sequence with a threshold <97.5% similarity to that of any previously described 'Ca. Phytoplasma' species. However because of the highly conserved nature of the 16S rDNA, many biologically or ecologically distinct phytoplasma strains, which may warrant designation of a new taxon may fail to meet this requirement. Additional unique biological properties such as antibody specificity, host range and vector transmission specificity as well as other molecular criteria need to be included for speciation. Some additional tools for phylogenetic analyses and finer strain differentiation of phytoplasmas such as *rp*, *secY*, *tuf*, *groEL* genes, and the 16S-23S rRNA intergenic spacer region sequences are now used as supplementary tools and multilocus analyses is providing useful and reliable information in combination with 16SrDNA for phytoplasma strains differentiation.

Jennifer Hodgetts

A panel of LAMP assays for detection of flavescence dorée phytoplasma

Hodgetts Jennifer (1), Hall Jayne (1), Tomlinson Jenny (1), Boonham Neil (1), González-Martín Irene (2), Ravnikar Maja (3), Dermastia Marina (3), Nikolić Petra (3) and Dickinson Matt (4).

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The use of loop-mediated isothermal amplification (LAMP) as a faster more robust alternative to PCR is becoming more widely used for a range of target organisms. The simple and minimal equipment requirements and fast reaction time make LAMP particularly suitable for field-based diagnostic applications. Flavescence dorée (FD) phytoplasma is a quarantine listed organism causing devastating disease in grapevines across much of Europe. At present detection of FD phytoplasma is by either conventional or real-time PCR, both of which require a well-equipped laboratory, specialist staff and a minimum of several hours from sample extraction to results. The EU FP7 project VITISENS will develop an innovative hand-held device that integrates the three steps required for detection - extraction, amplification and detection - in a single platform. Evaluation of the target gene for FD LAMP assays has been undertaken using five genes as targets, and designing multiple assays to each target.

Nicoletta Contaldo

Deep amplicon sequencing for detection of mixed phytoplasma infections in plants

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Phytoplasmas within single plants can be considered as populations of individuals and as phytoplasmas are obligate parasites of plants, single clones cannot be obtained. For identification of phytoplasmas, PCR followed by RFLP, sequencing PCR products directly or sequencing cloned PCR products are standard procedures. These procedures will, however, not show the diversity of populations within single plants, as only the most frequent genotypes will be detected and identified. A number of grapevine samples, in which mixed phytoplasma infections were detected by a nested PCR, were used for deep amplicon sequencing on the Roche Genome Sequencer FLX system. 50,926 phytoplasma sequences from the 5' end of the 16Sr DNA gene were obtained from 16 phytoplasma-infected grapevine samples. After clustering and alignment to phytoplasma reference sequences it was shown that the phytoplasmas in the grapevine plants belonged to diverse 16Sr groups, mainly the same groups that were detected using nested/PCR/RFLP. Furthermore, a high number of single nucleotide polymorphisms were present in individual samples.

Session 2 Viruses

Abstracts of Presentations

Ian Adams

QBOL – WP 6: Next Generation Sequencing for Plant Virus Discovery and Diagnosis

Adams Ian(1), Glover Rachel(1), Hany Ummey(1), Vlugt Rene van der(2), Dullemans Annette(2), Kreuze Jan(3), Willmer Cuellar(3), Boonham Neil (1).

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The advent of next generation sequencing has revolutionised the sequencing of plant viruses. When previously it could take months or even years to sequence the genome of a novel virus it is now possible to carry this out in a matter of days. As part of the EU funded QBOL project a range of different methods have been optimised using Roche 454 FLX and Illumina HS2000 sequencers to allow the rapid and cheap sequencing of virus genomes. As part of the project, these methods have then been used to sequence over 50 plant virus genomes. The methods have also been used to identify novel viruses in diseased material. New viruses discovered include Piper DNA virus 1 & 2 found on pepper plants in India and Watercress white vein virus, a virus identified on diseased watercress plants. Using the newly developed sequencing techniques it is now possible to go from a diseased plant to a genome sequence of a novel virus and finally to a high throughput real-time PCR assay to detect the novel virus in a matter of weeks.

Ellis Meekes

Detection of *Potato spindle tuber viroid* and *Tomato chlorotic dwarf viroid* in seeds of tomato

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Potato spindle tuber viroid (PSTVd) and *Tomato chlorotic dwarf viroid* (TCDVd) are contagious pathogens of tomato and several other hosts. Phytosanitary regulations for seeds of tomato have led to the development of a PSTVd/TCDVd seed assay, although the significance of seed transmission of both viroids is still unclear. In the assay sub samples of 1000 tomato seeds are incubated in an extraction buffer overnight to soften the seed coat. Then the seeds are extracted in a mini bagmixer. After subsequent RNA extraction a real-time RT-PCR based on TaqMan® technology is carried out for detection of PSTVd/TCDVd. For each sub sample two independent PCR reactions are carried out. In one tube PSTVd/TCDVd is detected in a singleplex reaction. In another tube detection of PSTVd/TCDVd is combined with the detection of endogenous Nad5 RNA amplicons as an internal amplification control to monitor RNA extraction and PCR inhibition. For PSTVd/TCDVd positive seed lots additional RT-PCRs are performed in order to sequence the viroid genome for definite identification to the species level. A validation study was performed to determine several performance characteristics of the assay. It was shown that one PSTVd-contaminated seed could be detected in sub samples of 1000 tomato seeds. Results will be presented of > 3000 commercial seed lots that were tested in the routine laboratory.

Jan Kreuze

Small RNA sequencing and assembly as a DNA barcoding method for plant viruses

Kreuz Jan

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Novel and emerging plant viruses, arising from pathogen evolution, global trade, crop intensification and potentially climate change, pose a key threat to agriculture worldwide. Even apparently symptomless virus infections can cause considerable yield losses, which can be further exacerbated by synergistic interactions with other viruses. RNA silencing constitutes a fundamental antiviral defence mechanism in plants in which host enzymes cut viral RNA into pieces of 20-24 nt. When isolated, sequenced en mass and properly aligned these virus-derived small RNA (sRNA) sequences can reconstitute genomic sequence information of the viruses being targeted in the plant. This approach is independent of the ability to culture or purify the virus and does not require any specific amplification or enrichment of viral nucleic acids as it automatically enriches for small RNAs of viral origin by tapping into a natural antiviral defence mechanism. To date the method has been used to identify numerous new viruses including single and double stranded RNA, DNA and reverse transcribing viruses and viroids from hosts as divergent as plants and invertebrate animals. In several cases unexpected apparently symptomless viruses were also identified, providing an important reminder that there may be many more viruses infecting our crop plants than we have previously been aware of, and their potential impacts on agricultural productivity remain to be understood.

Neil Boonham

Multiplex pathogen detection using microarrays

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Screening for the presence of multiple pathogens in samples using generic microarray techniques has been in development for some time, yet using genomics based platforms (e.g. high density glass slide arrays) has failed to make any impact in the diagnostics arena. In addition development of probes that give the desired level of specificity and sensitivity has been a challenge, since predictions based on bioinformatics are often not borne out by empirical testing. Working with panels of pathogens efforts have been invested in understanding in greater detail factors affecting probe binding of pathogens and the impact this will have on testing real samples in terms of sensitivity and specificity. In addition by performing the work to a diagnostics focused platform several screening tests for EU quarantine pathogens are now close being transferable to the routine testing laboratory and making these tests more widely available. The paper will discuss the approaches used to design primers and probes, the lessons learned as well as specific examples of the tests becoming available.

Antonio Tiberini

Microarray-based methods for a multiple and simultaneous detection of viruses highlighted the need for a collection database

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Since the first reports in 2002 on the use of this tool to detect viruses, the microarray-based approaches offer an alternative to the routine methods, as microarray virus-specific probes are potentially capable of detecting an almost unlimited number of virus species in one assay. Among these approaches, developing different microarray platforms, the microarray-based diagnosis methods, aimed to identify crop specific viruses or to develop a “universal” microarray to identify all the known viruses reported in plants.

The Combimatrix platform, has recently shown its potential to detect a large number of viruses in different plant matrices as tomato (*Solanum lycopersicum L.*) and globe artichoke (*Cynara scolymus L.*), allowing the identification of 37 viruses and 1 viroid on tomato and 14 viruses on globe artichoke. The repeatability and specificity of the Combimatrix platform showed how the identification of plant viruses is limited only by the spectrum of the viruses probes present in microarray. The increasing number of plant viruses’ nucleotide sequences may allow a great number of probes to be designed which must be evaluated to recognize the greatest number of strains and isolates belonging to the species target, highlighting the need of the creation and maintaining of a wide microorganism collection databases.

Jenny Tomlinson

RT-LAMP for detection of plant pathogenic viruses including Cassava brown streak virus

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Loop-mediated isothermal amplification (LAMP) assays have been developed for detection of a range of plant pathogens, including fungal, bacterial and viral pathogens. Primers have been developed for the specific amplification of *Cassava brown streak virus* (CBSV) and *Ugandan cassava brown streak virus* (UCBSV) (genus *Ipomovirus*, family *Potyviridae*), by reverse transcriptase-LAMP (RT-LAMP). Performance of the RT-LAMP assays compared favourably with that of conventional RT-PCR and TaqMan real-time RT-PCR. LAMP-based detection is simple, rapid and flexible, and methods using this technology have the potential to be used in situations where PCR-based testing is too complex, time-consuming or expensive. Potential workflows can be compared in terms of equipment costs, cost per sample and suitability for use in the field, and include real-time fluorescence detection using a specialised instrument such as the OptiGene Genie II, as well as non-instrumented detection using modified primers and lateral flow devices for detection of incorporated labels. The development and characterisation of RT-LAMP assays for CBSV and UCBSV detection, in addition to assays for other plant pathogen targets, will be discussed.

Marleen Botermans

Development and validation of a real-time RT-PCR assay for generic detection of pospiviroids

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In many countries phytosanitary regulations apply to *Potato spindle tuber viroid*, because it can cause serious damage in potato and tomato crops. Because other pospiviroids can cause similar damage and appear to be widely spread in ornamental crops, there was a need for a reliable and cost-effective generic testing method. An assay was developed and validated that detects all known species of the genus *Pospiviroid*, using real-time RT-PCR based on TaqMan technology. This GenPosp assay consists of two reactions running in parallel, the first targeting all pospiviroids, except *Columnnea latent viroid*, the second specifically targeting the latter viroid. To monitor RNA extraction Nad5primers were included with a newly designed probe in each assay. Method validation on tomato leaves showed that the GenPosp assay detects all pospiviroids up to a relative infection rate of 0.13% (equal to a 770 times dilution). No cross reactivity was observed, either with other viroids and viruses, or with leaf material. Repeatability and reproducibility were 100% and the assay appeared robust in an interlaboratory comparison. The new GenPosp assay has been shown to be a suitable tool for large-scale screening for all known pospiviroids. The assay has been validated for tomato leaves but can potentially be used for any crop.

Pascal Gentit

Comparison of methods for detection of pospiviroids infecting *Solanaceae*

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Since the end of the 1980s, new *Pospiviroids* such as *Tomato Chlorotic Dwarf Viroid* (TCDVd) and *Columnnea Latent Viroid* have emerged in the European territory. Often they are latent on ornamental *Solanaceae* which become reservoirs of these pathogens and which facilitate their spread (Verhoeven *et al.*, 2004).

The method used at the plant health laboratory at Anses aims to detect PSTVd and TCDVd using RT-PCR developed by Shamloul *et al.* (1997). To estimate the presence of the other viroids on *Solanaceae*, different methods were evaluated based on 2 techniques of end-point RT-PCR (Verhoeven *et al.*, 2004; Spieker *et al.*, 1996) and one technique of real-time RT-PCR (Monger *et al.*, 2010) towards a panel of target samples (*Pospiviroids*) and non-target samples (*Solanaceae* and viruses). The criteria of relative sensitivity, relative specificity, repeatability and reproducibility allowed a scheme to be elaborated for detection of *pospiviroids* including confirmation by genetic characterization.

Reference: Shamloul AM, Hadidi A, Zhu SF, Singh RP, Sagredo B (1997) Sensitive detection of potato spindle tuber viroid using RT-PCR and identification of a viroid variant naturally infecting pepino plants. *Can. J. Pl. Pathol.* 19: 89-96.

René van der Vlugt

State of the art multiplex Luminex xMAP and xTAG - detection of plant viruses

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Multiplex detection of viruses in a single sample would be an elegant way to improve DAS-ELISA efficiency and reduce costs. The Luminex xMAP technology allows such multiplex serological virus detection while retaining the standard DAS-ELISA 96-well format and workflow. Different Luminex colour-coded xMAP beads, each specifically coated, capture virus particles. Virus-specific conjugated antibodies, in combination with the unique bead colour-code identify a unique combination, allowing specific detection of each individual virus. Sensitivity is comparable to DAS-ELISA, while detection is completed in less than 3 hours with higher specificity.

When specific antibodies are not available, viral or viroid nucleic acid is detected on the same platform using Luminex xTAG beads. Similar colour-coded bead sets, labelled with virus-specific oligonucleotide probes, capture specific (RT)-PCR fragments. Similar to TaqMan probes, Template Specific Primer Extension (TSPE) adds an extra level of specificity to the xTAG test format while allowing a much higher level of multiplexing than Taqman. Luminex xMAP and xTAG tests were developed for a variety of plant viruses and viroids that allow the multiplex detection of more than 10 analytes in a single sample. They offer a state of the art, true multiplex and cost-effective alternative to the current DAS-ELISA and TaqMan formats.

Natasa Mehle

Plant viruses and viroids in an aqueous environment – survival, water mediated transmission and detection

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Hydroponic systems and intensive irrigation in horticulture, are widely used, but can potentially lead to rapid and efficient spread of water-transmissible plant pathogens throughout the whole crop. Although numerous plant viruses have been detected in aqueous environments, for many of them, the survival in water and the potential for direct transmission through irrigation water are still unknown. Therefore it was decided to explore whether water can be a source of infection with PepMV, PVY and PSTVd, that are relatively stable and contagious viruses/viroid, and a serious threat to tomato and/or potato production.

In irrigation waters, the viruses are usually present in concentrations lower than the detection limit of classical methods, but that may be sufficient to infect plants; therefore the development of highly sensitive diagnostic methods is necessary. Small sample volumes may lead to non-representative testing, therefore, a concentration step allowing the handling of larger water volumes may improve water monitoring diagnostic scheme. A fast and efficient way to concentrate highly diluted viruses consists on using CIM monolithic chromatographic supports. The application of such a strategy to the above mentioned plant viruses/viroid and the results of survival in water at room temperature and transmission efficiency through nutrient solutions in hydroponic cultivation of tomatoes and potatoes will be presented.

Cornelis Schoen

LAMP as a tool for On-Site Confirmation and Monitoring of plant pathogens

Schoen Cor (2), Boonham Neil (1), Tomlinson Jenny (1), Michael Andreou (5), Zorovic Maja (3), Spinelli Francesco (5), Morisset Dany (3), Lenarčič Rok (3), Ravnikar Maja (3), Blasioli Sonia (5), Biondi Enrico (5), Simonato Mauro (5), Goransson Jenny (6), Buehlmann Andreas (4) and Li Ming-fu (7).

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Effective detection methods are critical for the efficient functioning of inspection services and National Plant Protection Organisations (NPPOs). Yet in many cases, inspection services in particular, lack techniques that will enable efficient detection of quarantine pests. In WP7 of Q-detect different detection methods are developed. LAMP primer-sets for the detection of the whitefly transmitted viruses, bacteria and spindle tuber viroid (PSTVd) were developed.

Applicability of different simple and fast extraction methods for isolation of both DNA and RNA were tested. The Quick extract RNA Extraction procedure from Epicentre, suitable for both ssRNA and ssDNA virus extractions, meet the criteria of being sensitive, fast, simple and portable. For bacterial extractions two other on-site nucleic acid extraction methods were tested. One method is dedicated to the extraction of DNA from bacteria and based on a simple boiling procedure. Another procedure that has been designed for the nucleic acid extraction relies on the lateral flow device (LFD) extraction approach published by Tomlinson *et al.* (Phytopathology, 2010, 100: 143-149). Both procedures have been shown to allow nucleic acid extraction from plant tissue in just a few minutes. Preliminary experiments with the Genie II as a LAMP stand-alone detection device, 'point-of-care' diagnostics could be demonstrated.

Annelien Roenhorst

Quality control in bioassays used for broad screening for plant viruses

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Test plants are often used for broad screening for plant viruses. Mechanical inoculation of a series of test plants enables generic detection of mechanically transmitted viruses in only one assay. Moreover, such an assay is suitable for known as well as unknown viruses and variants. In comparison to serological and molecular methods, however, quality control in bioassays has hardly been addressed. The system of positive and negative controls (1st line), blind samples (2nd line) and proficiency tests (3rd line) is applicable, provided that a broader interpretation of positive and negative controls is used. For validation, performance criteria can only be determined for individual viruses. However, results often can be extrapolated. Sensitivity is addressed by dilution and comparison with other methods. Specificity is considered of minor importance for screening. Selectivity depends on the plant species tested, because some hosts contain components that inhibit transmission. Repeatability, reproducibility and robustness are determined by general features, such as environmental conditions and expertise of the virologist. Therefore, these performance criteria should be considered more generally. This presentation demonstrates that quality control can be implemented in bioassays, although it is more complicated than in serological and molecular assays.

Session 3 Insects & Mites

Abstracts of Presentations

Wednesday 2012-05-23

Jean-Yves Rasplus

QBOL-WP 3: Barcoding Quarantine Arthropods

Rasplus J-Y

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Application of molecular diagnostic methods has greatly accelerated identification of arthropod pests. At the same time, DNA barcoding based on the mtDNA COI gene as well as nuclear markers, has shown great potential to improve the detection of pests. Through a series of examples the potential of DNA barcoding to detect quarantine arthropods efficiently will be illustrated, the potential drawbacks will also be discussed. From the author's experience, DNA barcoding may provide an efficient new tool in the biosurveillance armoury for detection of pests but may sometimes be misleading. Nevertheless, developing a worldwide DNA library of barcodes of quarantine species, possibly including their main natural enemies that could be used in biological control projects, is of strategic importance to enhance our ability to detect and manage future invasive pests for agriculture and forests.

Marja van der Straten

Morphological and molecular identification of EU quarantine species of *Spodoptera* (Lepidoptera, Noctuidae)

Marja van der Straten & Bart van de Vossenbergh

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The genus *Spodoptera* comprises 30 species of which four are enlisted on the EU quarantine list: *S. littoralis*, *S. litura*, *S. frugiperda* and *S. eridania*. Of the EU listed *Spodoptera*, *S. littoralis* is intercepted the most frequently, followed by *S. litura*. These quarantine species need to be distinguished reliably and quickly from other *Spodoptera* species of which *S. exigua* is most frequently intercepted. Morphological identification is straightforward when the adult life stage is concerned. However, in trade it is more common that the immature stages are intercepted, and identification cannot wait until the specimen is reared to the adult stage. Morphological characters for identification of larval stages of the four EU quarantine species are presented here as well as real-time PCR tests based on TaqMan technology developed for rapid molecular identification. These tests were found to be reliable, reproducible and repeatable tools, applicable for the identification of all developmental stages of the four *Spodoptera* quarantine species.

Astrid Cruaud, CBGP, France, presented on behalf of Jean-François Germain, Anses, France

Combining DNA barcoding and morphological analysis for the diagnosis of alien pests. The case study of *Epitrix* potato flea beetles (Coleoptera: Chrysomelidae)

Jean-François Germain (1), Isabelle Meusnier (2), Jean-Yves Rasplus (2) and Astrid Cruaud (2)

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Fast and reliable identification of newly introduced pest species is essential to allow NPPOs to implement appropriate control measures. Identification can be performed using morphological characters and/or molecular data. Based on the example of two American species of potato flea beetles recently introduced in Europe, *Epitrix cucumeris* (Harris) and *E. similaris* Gentner (Coleoptera: Chrysomelidae) the advantages and disadvantages of both methods for quarantine entomology will be presented.

Antoon Loomans

Morphological identification of *Thrips palmi* in the framework of ISO/IEC 17025

Vierbergen Bert, Koch Jeanette C., Loomans Antoon J.M.

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Morphological identification of larvae and adults of the EU quarantine pest *Thrips palmi* is one of the routine procedures of the National Reference Centre (NRC) of the Dutch NPPO. Every year many samples are analysed from national import inspections. The quality of a morphological identification depends on several technical requirements, the most important of which are laboratory facilities, the literature, the reference collection, the quality of the available specimen(s) and the skills of the specialist. To be able to consistently produce valid identification results, the NRC has implemented a documented quality management system within the laboratory since 2007. Since March 1st 2012 the laboratory has been accredited by the Dutch National Accreditation Body. For the accreditation process, ISO/IEC 17025 was used to demonstrate competence for *Thrips palmi* identification under a fixed scope, "Morphological identification of *Thrips palmi* adults". The EPPO Standard PM 7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* guidance for entomology sets specific requirements for this process to enable the validation of morphological identification of *Thrips palmi* adults. The performance characteristics validated were analytical sensitivity, specificity, repeatability and reproducibility, however selectivity was not considered as being relevant as a performance characteristic for morphological identifications in entomology. In this presentation the results and value of these performance characteristics are discussed in detail.

Jose Carlos Rodrigues

Molecular Characterization of *Leptomastidea* spp. (Encyrtidae, Hymenoptera) Populations from Puerto Rico, Barbados and Florida

Rodrigues Jose Carlos V (1), Galindo-Cardona Alberto (1), Ciompelink Matthew (2), Segarra Alejandro (3) and Amy Roda (4)

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All known *Leptomastidea* are parasitoids of mealybugs, and at least one species, *L. abnormis* (Girault) is commonly used in biological control programs against mealybugs worldwide. Parasitoid wasps of the genus *Leptomastidea* (Hymenoptera: Encyrtidae) have been identified as important natural enemies of the *Harrisia* cactus mealybug (HCM), *Hypogeococcus pungens* (Granara de Willink) (Hemiptera: Pseudococcidae) in the Caribbean region. Three populations of *Leptomastidea* spp. have been identified attacking HCM in the region, and individuals were collected from Barbados, Puerto Rico and Florida. Morphological examinations by Dr. J. S. Noyes of the British Natural History Museum have been unable to establish taxonomical differences among individuals from these three populations. Nevertheless, minor morphological differences suggest the possibility of taxonomical divergence, which could lead to distinctive biological control potential. Genomic sequences of individuals from these populations were compared to determine their identity and their degree of genetic difference. Haplotypes of the three populations were different and the population from Puerto Rico was not genetically diverse, in contrast with those of Barbados and Florida. This study was able to provide evidence of differentiation of *Leptomastidea* spp. in Puerto Rico from other Caribbean populations in order to support the classical biological control of HCM.

Michel Dollet

Characterization of two haplotypes of *Haplaxius (Myndus) crudus*, vector of different phytoplasmas associated with palm diseases, using cytochrome oxidase I gene.

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In the 1980's *Haplaxius (Myndus) crudus* (Cixiidae) was reported to be the vector of the phytoplasma disease "lethal yellowing" (LY) of coconut palm in Florida. Most recently, the LY phytoplasmas have been shown to belong to the group 16S rIV-A. Transmission of the 16Sr IV-D phytoplasma ("Texas *Phoenix* palm decline") by *H. crudus* to *Pritchardia pacifica* was obtained in Yucatan, however transmission to coconut was not demonstrable. The taxonomy of the Cixiidae is confounding owing to the often cryptic nature of closely related variants and species, particularly of the tribe Oeclini Muir, 1922 to which *Haplaxius* belongs. Recently several *Myndus* species were transferred to other genera. *Haplaxius* itself was moved to the genus *Myndus* and re-classified as *Haplaxius*. In 2008 it was shown that phylogenetic trees reconstructed using the cytochrome oxidase I gene (mtCOI) and the 18S rDNA sequences were congruent, and that the trees were consistent with morphological classification for Oeclini. The highly evolving mitochondrial mtCOI were used to ascertain if differences occurred between *H. crudus* populations from Mexico with respect to the transmission of group 16S rIV-A and 16S rIV-D. Results indicated that two haplotypes of *H. crudus* exist in Mexico, possibly involved in the transmission of one of the two phytoplasma groups.

Session 4 Nematodes

Abstracts of Presentations

Sebastian Kiewnick

QBOL WP 5: Barcoding as a new tool for identification of quarantine nematodes and their close relatives

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Identification of quarantine plant pests needs to be fast and accurate to enable timely plant protection measures. Incorrect diagnosis could cause serious financial losses for trade and producers. Genetically based diagnostics is a reliable alternative to the classical identification generally based on morphological features requiring expert taxonomic skills. Genetic diagnostics through the use of DNA-barcodes, stretches of DNA that contain taxon-specific information, can be performed by any skilled laboratory-worker.

The European Union 7th Framework project QBOL: “Development of a new diagnostic tool using DNA barcoding to identify quarantine organisms in support of plant health” aims to establish DNA-barcodes for all European quarantine organisms and their close relatives, including plant parasitic nematodes. For quarantine nematodes, several gene regions such as COI, COII, SSU, LSU and RNA polymerase subunit II have been evaluated for their barcoding potential. The results and protocols for successful use of DNA-barcoding of quarantine nematodes and their close relatives are available through an online database, www.Q-bank.eu, freely accessible to all interested users, such as national plant protection organizations. A group of curators will ensure that data incorporated into the Q-bank database are validated and linked to specimen or populations in reference collections across Europe.

Johannes (Hans) Helder

Use of a phylum-wide SSU rDNA-based molecular framework for the detection of plant parasitic nematode species in complex DNA backgrounds

Helder, Johannes (1), Landeweert, Renske (2), Sven van den Elsen (1), Paul Mooyman (1), Hanny van Megen (1) Winfried Mulder (2), Gerrit Karssen (3), and Jaap Bakker (1)

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Although the overwhelming majority of nematodes play essential roles in the soil food web, some nematode species are serious parasites of major crops such as potato, sugar beet and soybean. Among the most notorious ones are cyst (*e.g. Globodera rostochiensis* and *G. pallida*), root knot (*e.g. Meloidogyne chitwoodi*, *M. fallax* and *M. minor*), stem and bulb (*e.g. Ditylenchus dipsaci* and *D. destructor*), and foliar nematodes (various *Aphelenchoides* species). A number of these plant parasites have a quarantine status, and as a consequence substrates (often soil) and plant materials should be checked for the presence of certain plant parasitic nematode species. So far, most molecular tests offer "local solutions"; they enable for instance the detection of certain *Meloidogyne* species in a pre-selected pool of root knot nematodes. In an attempt to reconstruct the evolution of the phylum Nematoda, a phylum-wide molecular framework has been constructed, consisting of appr. 2,500 taxa. This framework was used to define unique DNA sequence signatures that enable "blind" identification of individual nematode species in highly complex DNA backgrounds. Over 20 relatively simple, (Q-)PCR-based diagnostic tests for species identification, and a number of examples will be presented during this conference.

Geraldine Anthoine

Validation of morphological identification method - the example of a morphological key for *Bursaphelenchus xylophilus* species

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Many diagnostic laboratories have now developed quality management systems in order to ensure the quality of their official analysis. One of the requirements of such a quality management system, especially for accredited laboratories, is the validation of internal or non-standard tests.

The validation process is often discussed either at national or regional level, e.g. within EPPO panels. This validation process is often considered as impossible for morphological tests.

This presentation will describe, beyond the results of the validation, the procedure applied for the validation of a morphological key for the identification of a regulated nematode, *Bursaphelenchus xylophilus*. The different performance criteria defined in the EPPO diagnostic protocol (PM 7/98), such as sensitivity, specificity and reproducibility, have been evaluated by using confusing *Bursaphelenchus* species, different stages of *B. xylophilus* and several operators.

This evaluation led to the description of the most accurate, reliable and simple morphological identification key.

Martijn Holterman

Evaluation of barcoding and phylogenetic potential of four genes in the genus *Meloidogyne*

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Identification of plant pests, in particular quarantine species, needs to be fast and accurate to enable timely plant protection measures. It is now well established that genetically based diagnosis is a reliable alternative to the classical identification procedures which are generally based on morphological features, requiring expert taxonomic skills. On the other hand, genetic diagnosis through the use of so-called DNA-barcodes, stretches of DNA that contain taxon-specific information, can be performed by any skilled laboratory-worker. Root-knot nematodes of the genus *Meloidogyne* are some of the most important plant parasitic nematodes world-wide. As part of the QBOL project, four potential barcoding regions were tested and evaluated in the genus *Meloidogyne*. These were the small subunit (SSU) ribosomal RNA gene, the large subunit (LSU) ribosomal RNA gene as well as the mitochondrial cytochrome oxidase c subunit 1 (COI) and subunit 2 (COII) genes. As well as evaluating their barcoding potential, results were compared with earlier phylogenetic studies of the genus *Meloidogyne*.

Renske Landeweert

Diagnostic kits for the detection of plant parasitic nematodes.

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The highly conservative morphology of nematodes means that identification requires expert knowledge. Current nematode analyses are mostly based on microscopic examinations with limited throughput, sensitivity and specificity. This hampers standardized control for quarantine nematode species in importing and exporting countries. A solution would be a radical switch to DNA barcode based identification. Previous studies demonstrate that the small subunit ribosomal DNA (SSU rDNA) gene harbours enough phylogenetic resolution to distinguish between nematode families, genera and often even species (Holterman *et al.* 2009, Van Megen *et al.* 2009). On the basis of a framework of ~ 2,500 full length nematode SSU rDNA sequences, diagnostic Q-PCR tests for nematodes have been developed and offered to agricultural laboratories either as a detection kit or as dedicated system.

Recently, many agricultural laboratories have invested in the set-up of a molecular laboratory, indicating that molecular identification and detection of plant pathogens will be the golden standard in the near future. The planned product range introduced here enables inspection laboratories to implement standardized and validated molecular tests and allows them to work according to the latest taxonomic insights. These tests enable high throughput, specific and sensitive detection of quarantine nematodes and provide a major means for durable disease control and risk management.

Sylvie Gamel

Direct detection of plant parasitic nematodes by real time PCR: experience gained from development and validation of different tests

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Plant parasitic nematodes cause important damage on many crops and this occurrence is expected to increase over the next few years due to pesticides bans, increasing trade etc.. Some nematodes are regulated in Europe to limit their dispersal over non-infested areas. As a result, the development of reliable screening methods to detect quarantine nematodes in different matrices is important in order to detect and manage new infested areas. Morphological analysis is commonly performed to identify nematode species. However, this technique is time-consuming and not suitable to analyse large series of samples. Over the last few years, several real time PCR tests were adapted for direct detection, after isolation of nematodes from matrix. The example of the *Bursaphelenchus xylophilus* and *Meloidogyne chitwoodi* and *M. fallax* detection in nematodes suspensions respectively from wood and soil will be presented. Assessment procedure for validation according to the EPPO Standard (PM 7/98) is presented; the key points of its evaluation for detection purpose are highlighted.

Session 5 Posters

Abstracts of Posters

Françoise Petter

POSTER PRESENTED BY THE AUTHOR

EPPO databases

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PQR (Plant Quarantine data Retrieval system)

PQR (Plant Quarantine data Retrieval system) is the EPPO database on quarantine pests. It provides detailed information on the geographical distribution and host plants of quarantine pests. Its search tools also allow the users to identify commodities which are able to act as pathways in international trade for the movement of pests and diseases. In recent years, the database has been extended to cover invasive alien plants. The new PQR version also includes world maps, pictures and active links to the EPPO Reporting Service. PQR can be downloaded free of charge from this web site.

<http://www.epo.int/DATABASES/pqr/pqr.htm>

EPPO database on Diagnostic expertise

This database provides an inventory of the diagnostic expertise available in the EPPO region. Its aim is to cover the expertise on regulated pests (i.e. pests of EPPO A1 and A2 Lists, pests mentioned in EPPO Standards PM4: Production of Healthy Plants for Planting), pests possibly presenting a risk to EPPO member countries (EPPO Alert List) and plants of the EPPO List of invasive alien plants. This database does not include common pests which are widely distributed in the EPPO region. The EPPO Secretariat is maintaining the database but note that all information included in the database is based on individual expert's own declarations of their expertise.

<http://dc.epo.int/>

Barbara Piskur

POSTER PRESENTED BY THE AUTHOR

Molecular data to unravel the Botryosphaeriaceae complex found in diseased European hop-hornbeams: a case study

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An extensive die-back of European hop hornbeam (*Ostrya carpinifolia*) has been observed in Slovenia since 1997, which coincided with unusually hot and dry weather conditions. Numerous fungal isolates, belonging to Botryosphaeriaceae were isolated from the diseased trees. Closely related Botryosphaeriaceae species cannot be easily resolved due to overlapping morphological characteristics. In order to unravel the obtained Botryosphaeriaceae complex from the diseased *O. carpinifolia* trees, the analyses of sequence data for the ITS rDNA and translation elongation factor 1- α were performed. The sequence data revealed four groups; the majority of isolates were classified as *Botryosphaeria dothidea*, but a small group of isolates was identified as *Dothiorella spp.*, with three subgroupings, of which at least one most likely represents an undescribed species. The implementation of molecular data proved to be an indispensable step in the identification of the Botryosphaeriaceae found in diseased tissues of *O. carpinifolia* and enabled further characterization of the pathogenicity and significance of resolved species in the disease development.

References: Jurc D., Ogris N., Grebenc T., Kraigher H. 2006. Plant Pathology, 55: 299

Piškur B., Pavlic D., Slippers B., Ogris N., Maresi G., Wingfield M.J., Jurc D. 2011. European Journal of Forest Research, 130: 235-249

Joanna Zaluga

POSTER PRESENTED BY THE AUTHOR

Diagnostics for quarantine plant pathogens: DNA barcoding and MALDI-TOF MS revealed a new group of non-pathogenic to tomato *Clavibacter* isolated from tomato seeds

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The genus *Clavibacter* contains mainly plant pathogenic bacteria. They have specific hosts and are mostly quarantine organisms. The genus *Clavibacter* contains pathogens of economically important crops. The QBOL project aims to develop reliable identification using DNA-barcoding. The work presented here focused on *C. michiganensis* subsp. *michiganensis* (Cmm) which is considered one of the most destructive diseases on tomato. Recently, many outbreaks of bacterial canker have been recorded. In the first part of this work 200 strains were subjected to sequencing of *gyrB* and 16S rRNA genes and MALDI-TOF MS. A 500bp *gyrB* sequence was found to be a discriminative barcode for *Clavibacter*. MALDI-TOF MS with specific biomarkers can be used as an alternative for a sequence-based identification. Data also revealed the existence of a new group of non-virulent bacteria isolated from tomato seeds. Because comparative physiological and phylogenetic characterization of this group versus genuine Cmm is generally unknown, tests were performed *in planta* as well as DNA-DNA hybridization. Their lack of pathogenicity was investigated by checking the presence of known molecular virulence determinants.

Furthermore, ALFP and ISSR-PCR are being tested to optimize a reliable typing method which helps to better understand the epidemiology of *Clavibacter*.

May Bente Brurberg

POSTER PRESENTED BY THE AUTHOR

Outbreak of apple proliferation phytoplasma in Norway

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Apple proliferation (AP), caused by *Candidatus* Phytoplasma mali, is one of the most serious plant diseases in apple production areas all over Europe, and losses are estimated to be very high. The phytoplasma induces a range of symptoms, such as witches' brooms, enlarged stipules, yellowing, growth suppression, and undersized fruits. *Candidatus* Phytoplasma mali spread by vegetative propagation (grafting) or they are naturally transmitted by phloem-feeding insects.

AP was first reported in Italy in 1950 and has since then been detected in several European countries where apple is grown commercially. *Candidatus* Phytoplasma mali was detected in Norway for the first time in 1996, but the situation was monitored carefully and the few infected trees detected in the following decade were eradicated. In September 2010, however, a new more serious out-break of AP occurred in one of the major apple production areas in western Norway. This led to the start-up of a survey with a particular focus on nurseries and their surroundings in four apple producing counties. Symptomatic and non-symptomatic samples were analyzed using real-time PCR. No infected trees were detected in the nurseries, but infected trees were detected in close surroundings of 70 % of the nurseries indicating the seriousness of the situation.

Stefan Wagner

POSTER PRESENTED BY THE AUTHOR

Lab-on-a-chip detection of *Phytophthora* species

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Phytophthora species are a worldwide threat to natural ecosystems and the nursery industry. To prevent the spread of these pathogens with infected plants, easy to handle diagnostic systems with high sensitivity and high throughput are demanded. Furthermore such methods should give results within a short time on site of inspection.

In a three year project a chip-formatted PCR system combined with a chip-based electrical microarray will be adapted for the detection of important *Phytophthora* species in plant tissue (EPPO A2 / Alert list). A stationary PCR chip with integrated microstructured heaters and temperature sensors has been developed for the amplification of specific *Phytophthora* DNA fragments. A microfluidic system connects the PCR chip with a microarray where the labelled DNA fragments are detected. The miniaturization of the chip formatted PCR and array system enables high portability, low input of energy and expensive analytic chemicals and very fast reaction times due to low reaction volumes.

Gian Luca Bianchi

POSTER

Multiplex real time RT-PCR in the diagnosis of grapevine viruses

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Diagnosis of grapevine viruses is very important in certification of grapevine propagation materials. The aim of this work was to develop two multiplex real time RT-PCR assays for the detection of some viruses of grapevine (ArMV, GFLV, GLRaV-1, GLRaV-3 and GVA). 6790 grapevine samples of woody mature canes were collected from 299 stock nurseries of propagating material and assayed in ELISA and multiplex real time RT-PCR using group analysis procedure. ELISA tests were done on samples of 5 canes according to official procedures adopted in Italy while multiplex real time RT PCRs were done on samples of 20 canes.

The health of 280 stock nurseries was evaluated using ELISA and was confirmed by multiplex real time RT PCR. 10 stock nurseries had positive results using the molecular test and negative results to serological tests while 9 stock nurseries with positive ELISA results did not have these results confirmed by the multiplex real time RT PCR. Relative accuracy of the multiplex real time RT-PCR in comparison to the ELISA test was 93.7 %. The multiplex real time RT-PCR developed in this work was shown to be a useful tool for the diagnosis of grapevine viruses in comparison to the ELISA test, being faster and cheaper than the official serological method.

Utku Yükselbaba

POSTER

Determination of the biotypes of *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) populations from Antalya Province of Turkey by sequence analysis of mitochondrial Cytochrome Oxidase I (mtCOI) gene region

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Cotton whitefly *Bemisia tabaci* (Gennadius) is a polyphagous pest that is widely distributed in tropical and subtropical regions. Many biotypes of this pest have been identified and reported from different regions of the world. In this study, the biotypes of *B. tabaci* populations collected from 7 different regions of Antalya province were identified by sequence analysis of the mitochondrial Cytochrome Oxidase I (mtCOI) region. DNA extracted from each individual whitefly was amplified using primers specific to mtCOI region. An amplicon of approximately 800 bp of which 770 were used in sequence analysis were obtained. The sequences were subject to alignment analysis. Results of the analysis revealed that all the populations of *B.tabaci* studied here were biotype B. These results also showed that some populations from some regions previously known to be biotype Q had changed to biotype B, leading to the assumption that biotype B could take over and become the dominant biotype in a short period when introduced into new areas.

Béatrice Courtial

POSTER

Use of the Nuclear Region ITS2 for Arthropods Species Identification

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During the last decade, many barcode projects were launched for species identification by DNA sequencing in order to improve taxonomic knowledge of biodiversity and to try to assign sample individuals to species. In the case of Arthropods the most often used region is a part of the mitochondrial gene Cytochrome Oxidase I, which is often insufficient to reliably delineate and name the species. To confirm the identification of specimens it is necessary to use another DNA sequence. This sequence is very often the nuclear ribosomal internal transcribed spacer 2 (ITS2). The data presented here show the preliminary results of ITS2 sequencing on 40 arthropods species belonging to different orders (Coleoptera, Lepidoptera, Hymenoptera). The results presented show the difficulty of defining universal primers for this target ITS2 and the large variability of obtained sequences (presence of deletion, repeats, etc.). An example of sequences obtained for the genus *Dendroctonus* (Coleoptera, Curculionidae) is also supplied.

Natalia Sherokolava

POSTER

Using DNA Sequencing for Diagnosis of Certain Pests of Quarantine Concern for the Russian Federation

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The possibility of Ypt-1 gene sequencing for diagnosis of *Phytophthora fragariae* var. *ruby* and *Phytophthora fragariae* var. *fragariae* was investigated. Sequencing the PPV coat protein gene enabled strains to be determined for 133 PPV isolates from various regions of the Russian Federation. PPV-W strain (Winona) was found to be widely spread in Russia. A new PPV strain was identified and preliminarily called PPV-CR (Cherry Russian). Sequencing the 12S rRNA gene DNA in 12 samples of bark beetles allowed identification of the variability between genera and species of beetles sufficient to develop methods for their diagnosis.

Sequencing amplification products allowed confirmation of detection of previously absent pathogens (*Ralstonia solanacearum*, Beet necrotic yellow vein virus, Impatiens necrotic spot virus) or poorly identified pathogens (Tobacco ringspot virus, *Phytophthora fragariae*).

A PCR-analysis for Andean potato viruses was developed. It was tested on the DSMZ virus isolates.

Françoise Poliakov

POSTER

Methods comparison for the detection of Pepino Mosaic Virus (PepMV) on tomato seeds

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To review the French official detection method for PepMV, three detection methods were assessed. The reference method which is a serological detection test (DAS ELISA) was compared to a conventional RT-PCR test (Ling *et al*, 2008) and a real time RT-PCR test (Ling *et al*, 2007). Results obtained allowed specificity, sensitivity and repeatability criteria to be calculated. In this study, the real-time RT-PCR test had the best performance criteria with 100% for the three performance criteria assessed.

DAS ELISA test will remain the initial test used because it has good sensitivity, repeatability, reproducibility and low cost. Because of its higher sensitivity, the real-time RT-PCR test will be used for the confirmation of undetermined results obtained with the serological test.

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Peter Bonants

POSTER

Multiplex detection of *Phytophthora*: Padlock probe based Universal detection Multiplex Array (PUMA)

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Phytophthora spp. are responsible for many diseases worldwide and can occur on a wide range of different crops and other plants. To detect and identify phytophthora species several molecular methods have been developed for single species only. A uniform method for detection of all *Phytophthora* species would be very useful for research and regulatory communities. Therefore a diagnostic method was developed to detect a range of *Phytophthora* species, including *P. ramorum*. The method includes a generic TaqMan PCR amplification method for all *Phytophthora* species combined with species specific padlock probe (PLP) detection on a dedicated universal micro-array. Twenty-three padlock probes for 22 *Phytophthora* species relevant for the Netherlands were developed based on sequence differences in the ITS-1 region. After point mutation specific ligation of a mixture of the 23 PLPs on the generic amplicon, exonuclease treatment to degrade the unreacted probes, amplification of the ligated probes and hybridization on a micro-array, a unique signature on the micro-array can be obtained for each *Phytophthora* species included in the test. Specificity and sensitivity of this padlock based diagnostic tool has been combined with a cost effective microtiter plate array detection device and has been evaluated using reference *Phytophthora* cultures as well as mixed infected material collected from field surveys, including air-, root-, water- and plant tissue samples.

Martijn Holterman

POSTER

Barcoding in the *Ralstonia solanacearum* complex

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Ralstonia solanacearum is an important plant pathogen worldwide with a wide host range. It causes wilting diseases in, amongst others, solanaceous crops. Furthermore, races 2 and 3 of *R. solanacearum* have quarantine status in Europe. As part of the QBOL project (barcoding of European quarantine organisms) 11 genes were examined for their potential use as barcoding regions in the genus *Ralstonia*. Three genes, the *mutS*, *egl* and *rpoB* genes, were selected as the most promising barcoding regions. *R. solanacearum* is a complex species, consisting of four phylotypes, one of which also includes *R. syzygii* and the Banana Blood Disease bacterium. Recently it was proposed to divide this complex into three new species. All three selected genes allowed the phylotype to be determined and supported the division of the *R. solanacearum* complex into these three new species.

Juliana Irina Spies Perminow

POSTER

***Dickeya solani* survey in Norway**

Perminow Juliana Irina Spies, Sletten Arild, Brurberg May-Bente and Akselsen Inger-Lise

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Potato blackleg and soft rot are bacterial diseases of potato. Symptoms include dark discoloration of the stem base and aerial stem rot. There are several bacteria that cause the symptoms described above: *Pectobacterium carotovorum*, *P. atrosepticum*, *Dickeya chrysanthemi*, *D. dadanthi*, and *D. dianthicola*. In 2005 a new, more aggressive *Dickeya* sp. was detected in the Netherlands, and in the following years it was also found in several other countries. It is reported that the bacterium is more aggressive than both *P. atrosepticum* and *D. dianthicola*, can be found in a wider range of climatic conditions, needs lower bacterial concentrations to cause disease and spreads faster and more efficiently. The name '*Dickeya solani*' has been proposed for this pest.

In Norway, *D. solani* has not yet been detected. Bioforsk Plantehelse has received potato samples with unusual blackleg symptoms in the past two years, some of which were found to be infected by virulent strains of *Pectobacterium wasabiae*. In 2011 a small, initial *Dickeya*-survey was carried out. More than hundred isolates have been collected from 20 arbitrarily gathered samples of symptomatic potato plants and tubers. These have been analyzed with realtime PCR assays using primers and probes for *Pectobacteria* and *Dickeya*. *Dickeya solani* was not detected.

Johannes Hallmann

POSTER

Q-bank – The nematological approach of offering ecological, morphological and molecular information for the identification of regulated and other relevant plant-parasitic nematodes

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Taxonomic expertise is dramatically declining in the scientific community. In an attempt to conserve existing knowledge but also provide modern tools for nematode identification, Q-bank was started. This database comprises ecological, morphological, physiological, and sequence data of properly documented strains of plant pests and diseases allowing plant protection organizations, inspection bodies and private laboratories an accurate identification. The main focus of Q-bank is on regulated plant pests and pathogens, however, for accurate distinction of regulated from non-regulated pests and diseases, descriptions of the latter are equally important. Different groups of pests and diseases require different information. For the field of nematology, the concept and structure of Q-bank is presented and discussed. The available information will be listed. Q-bank is supported by the Dutch Ministry of Economic Affairs, Agriculture and Innovation and by QBOL, an EU-project with partners from 20 countries.

Sebastian Kiewnick

POSTER

Development and validation of molecular diagnostic tools for detection and identification of the root-knot nematode *Meloidogyne enterolobii*, a new EPPO A2 pest recommended for regulation.

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Root-knot nematodes (*Meloidogyne* spp.) pose a significant risk to agricultural production systems all over Europe. *Meloidogyne enterolobii* is a polyphagous species and has been found on many host plants. It is considered to be a very aggressive root-knot nematode species that is able to reproduce on root-knot nematode-resistant plants. Several interceptions of ornamental plants infested with *M. enterolobii* by the Dutch and German authorities in 2008, lead EPPO (European and Mediterranean Plant Protection Organization) to add it to its list of A2 list of pests recommended for regulation in 2010.

One of the main questions raised during the pest risk analysis (PRA) for *M. enterolobii* was the possible source(s) and route(s) of introduction into Europe. To ensure that appropriate phytosanitary measures are at hand, reliable detection and identification tools are needed. This EUPHRESKO project will deliver new and validated protocols for sensitive detection and reliable identification of *M. enterolobii* and consequently improve diagnostic expertise in Europe. It will provide a test performance study and a workshop establishing DNA-barcoding and the use of the Q-bank database as a useful identification tool. It will also support the decision making process for eradication or integrated control strategies once the nematode has been detected and correctly identified.

Anne-Laure Boutigny

POSTER

An optimised duplex real-time PCR tool for highly sensitive detection of the quarantine oomycete *Plasmopara halstedii* in sunflower seeds

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Plasmopara halstedii, the causal agent of downy mildew of sunflower, is an obligate parasite that is subjected to phytosanitary regulation. This pathogen may be found in a quiescent state in seeds of sunflower and therefore may be transported by international commercial trade of these seeds. In order to prevent the spread or the introduction of potentially new genotypes or fungicide-tolerant strains, an efficient method to detect *P. halstedii* in sunflower seed samples is required. This work reports the optimisation of a novel real-time detection tool that targets the oomycete within sunflower seed lots, and provides statistically supported validation data for that tool. The test is shown to be specific and inclusive, based on computer simulation and *in vitro* assessments, and could detect as little as 45 copies of target DNA. A fully optimised DNA extraction protocol was also developed starting from a sample of 500 sunflower seeds, and enabled the final detection of less than one infected seed / 500. In order to ensure reliability of the results, a set of controls was used systematically during the assays, including a plant specific probe used in a duplex qPCR fashion that enabled the assessment of the quality of each DNA extract.

Astrid Cruaud, CBGP, France, presented on behalf of Jean-François Germain, Anses, France

POSTER

From information to diagnosis: management of an alien pest: a case study of *Drosophila suzukii* (Diptera: Drosophilidae)

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At the time of emergence of a new pest, it is important that information flows quickly to the plant protection services. This information must be complemented by identification tools. The identification should be accessible at all levels, for field workers and for researchers. The emergence of *Drosophila suzukii* (Diptera: Drosophilidae) in Europe in 2009 illustrated the management of this new pest.

Ian Adams

POSTER

Development of a System for High Throughput Screening of Planting Material for Cassava Brown Streak Disease Causing Viruses

Adams Ian(1), Abidrabo Phillip (2,6), Miano Douglas(3), Alicai Titus(2), Kinyua Zachary (3), J Clarke Jasper(5) Macarthur Roy(1), Weekes Rebecca(1), Laurenson Lynn(1), U Hany Ummey(1), Peters Dai(4), Potts Michael(4), Glover Rachel(1), Boonham Neil(1), Smith Julian(1)

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Cassava provides more than half the dietary calories for half of the population of sub-Saharan Africa. Cassava brown streak disease caused by the viruses *Cassava brown streak virus* and *Ugandan cassava brown streak virus* is currently spreading throughout sub-Saharan Africa and can cause up to 70% yield losses.

As part of the Great Lakes Cassava Initiative (GLCI), which is providing disease free planting material to African farmers, a diagnostic method was developed. Using next generation sequencing, isolates of CBSV and UCBSV were genome sequenced and real-time PCR assays were designed to detect both viruses.

The performance of these assays was validated using 493 cassava samples from Kenya, Uganda and Tanzania and on extracts of pools of diseased and healthy cassava leaves. Using this data, the appropriate sampling and pooling strategy was (300 leaves in 30 sub-samples) was developed to give a 95% confidence of detecting infected plants that make up 1% of the plants in a field. Next generation sequencing was also used to confirm the absence of any virus in samples which appeared to have visual symptoms but tested negative by the real-time assay.

The diagnostic method has now been used to screen 150000 leaf samples from GLCI multiplication sites.

Mathieu Rolland

POSTER

***Clavibacter michiganensis* subsp *michiganensis* : evaluation of efficiency of different detection methods**

Rene Mathis(1), Celine Fricot(1), Magali Larenaudie(1), Thomas Baldwin(2), Rodolphe Germain (2), Valerie Olivier(3), Anne Quillevere(1), Mathieu Rolland(1), and Valerie Grimault(1)

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Clavibacter michiganensis subsp. *michiganensis* (Cmm) is one of the most important tomato seed transmitted pathogens. In recent years the number of outbreaks has increased. Due to absence of chemical control on tomato plants, the only way of controlling the spread of this disease is seed testing.

The efficiency of the existing detection/identification methods were compared on a collection of 213 bacterial isolates including 141 Cmm strains, saprophytes, other *Clavibacter* and other genera pathogenic on tomato. Methods compared were immunofluorescence, isolation on media, PCR and pathogenicity. Sensitivity and specificity of each method were determined. Comparison of growth of bacteria on two different semi selective media showed that slow and fast growing isolates must be taken into consideration for seed analysis.

Extraction methods and efficiency of detection on treated/untreated seeds were studied on a naturally contaminated seed lot obtained by artificial contamination of a tomato culture and extraction of seeds produced and on commercial seed lots. A promising confirmation method, direct PCR on seed macerate used for immunofluorescence, was set up and is under evaluation.

This study contributed to the validation of a detection method on media of the International Seed Federation and to the EPPO Standard.

Session 6 Bacteria

Abstracts of Presentations

Thursday 2012-05-24

Bart Cottyn

QBOL – WP 4: Barcode identification of quarantine bacteria, the QBOL strategy and results

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Within QBOL, barcode sequences have been defined for a group of quarantine bacteria from the combined EU/EPPO lists, comprising the three *Clavibacter michiganensis* subspecies, *Ralstonia solanacearum*, *Xylella fastidiosa* and a selection of *Xanthomonas* pathogens.

The challenges were to compose a relevant collection of quarantine and closely related bacteria, to unravel their taxonomic and pathogenic identity, and to define representative barcode sequence regions.

A barcoding strategy had to be developed. For this it was not possible to use other barcoding of life (BOL) programs, which study existing diversity of eukaryotes, but not of prokaryotes. Moreover, several quarantine bacteria are named as pathovars, an infra-species division that refers to a pathological specialization and does not necessarily correlate with a taxonomic unit. Barcode identification of the quarantine bacteria uses several gene sequences in a step-wise approach following different flows in a decision scheme. The barcoding principle and process will be illustrated for a set of *Xanthomonas* pathovars.

Isabelle Robène-Soustrade

PCR-based assays for detecting *Xanthomonas axonopodis* pv. *allii* in onion seed.

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Bacterial blight of onion is an emerging disease threatening world onion production, and causing damage to other *Allium* crops. The causal agent, *Xanthomonas axonopodis* pv. *allii* (Xaa) has been listed on the EPPO A1 list of pests recommended for regulation as quarantine pests, since 2009. A duplex nested-PCR assay, targeting two markers specific to Xaa has been recently developed (Robène-Soustrade *et al.*, 2010). A triplex quantitative real-time PCR assay (Taqman® technology) was developed targeting the same Xaa-specific markers and an internal control chosen in 5.8S rRNA gene from Alliaceae. Xaa strains were detected by the amplification of one or both of the two specific markers. The internal control signal validates both the extraction process and the reaction itself. Several successive steps have to be performed before detection from seed: seed maceration for 48h at 4°C, followed by homogenization of the seed macerate with a stomacher® and DNA extraction using DNeasy® Plant mini kit (Qiagen). This assay is currently being validated following the European standard EN ISO 16140: 2003 and the EPPO standard PM7/98 (1). The performance of Nested-PCR and real-time PCR assays are discussed. These PCR-based tools could be useful for the international sanitary surveillance of seed exchanges.

Reference: Robène-Soustrade, I., Legrand, D., Gagnevin, L., Chiroleu, F., Laurent, A., Pruvost, O. (2010). Appl. Environ. Microbiol. 76, 2697-2703.

Maria Bergsma-Vlami

Invasion of *Xanthomonas arboricola* pv *pruni* (Xap) in the ornamental *P. lauroceracus* (cherry laurel) in the Netherlands

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Bacterial spot disease, caused by *Xanthomonas arboricola* pv *pruni* (Xap) is a serious disease of *Prunus*. In other European countries Xap infects stone fruits, however, in the Netherlands it has only been detected in ornamental cherry laurel. So far, Dutch Xap strains were isolated from symptomatic leaves. The identity of the Xap isolates was confirmed by Pagani- and Pothier-PCR. For typing, *gyrB* and BOX/ERIC were applied. Both demonstrated a high degree of homology among Xap isolates. The genotypic diversity of 25 Xap isolates was assessed by MLVA. These isolates could be divided into two groups, however, no correlation with their geographical origin was established. These results indicated the high discriminatory potential of the MLVA typing.

Additionally, 5 *Xanthomonas* spp. isolates reacted in the Pagani- but not in the Pothier-PCR. Their characterisation with BOX/ERIC differed significantly with Xap. AFLP data and *gyrB* typing confirmed this observation. Their sequence analysis on *gyrB* did not match any published Xap *gyrB* sequences but demonstrated high homology with *Xanthomonas dyei*, *X. arboricola* pv. *celebensis* and *Xanthomonas arboricola*.

In conclusion, MLVA is a promising technique to assess variation within Xap populations. Additionally, use of complementary methods for identification such as BOX/ERIC, *gyrB*, MLVA, AFLP and the Pothier-PCR, are strongly recommended in order to guarantee a reliable diagnosis of Xap.

Marcel Westenberg

Validation of real-time PCR for detection and identification of *Xanthomonas fragariae*

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Xanthomonas fragariae is a quarantine organism (EPPO A2 List of pests recommended for regulation/EU A2) causing bacterial angular leaf spot of *Fragaria* spp. (strawberry). The National Plant Protection Organization has recently validated a real-time PCR based on the *gyrB* gene (Weller *et al.* 2007, J.Microbiol.Methods 70:379-3) for detection of *X. fragariae* in leaves and rhizomes of *Fragaria* spp. and for identification of pure cultures, according to the Dutch validation guideline for plant pathogens and pests. Limit of detection (LOD), measuring range, trueness, analytical specificity, selectivity, robustness, repeatability and reproducibility of the assay has been assessed. The LOD for detection, as determined by spiking rhizome- and leaf-extracts of two *Fragaria* spp. varieties with five *X. fragariae* isolates, is 8.0×10^4 cfu/mL and for identification, determined with the same isolates, is 7.8×10^4 cfu/mL. These LODs are approximately 100 x lower than those for a conventional PCR, making it more suitable for detection of lower population densities on infected plants. The assay identified all *X. fragariae* reference isolates used and did not give any cross reactivity with either plant DNA or DNA of other *Xanthomonads*, including *X. arboricola* pv. *fragariae*. The repeatability and reproducibility were both 100%. The real-time PCR has been shown to be a valid tool for the detection and identification for *X. fragariae*.

Ewa Lojkowska

Phenotypic and genotypic characteristics of *Dickeya* strains isolated from potato in Poland

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Pectolytic bacteria from *Pectobacterium* and *Dickeya* genera, that cause blackleg and soft rot, cause substantial crop reduction. The genus *Dickeya* is a highly diverse group, which at the moment contains six species. However, *Dickeya* strains, with significantly distinct genetic and biochemical profiles, have recently been isolated from symptomatic potato plants in Poland and other European countries. The new species *Dickeya* 'solani' has been postulated (Toth *et al.* 2011).

The aim of the study was characterization of strains recently isolated from infected potato plants in Poland. Phenotypic features such as the ability to chelate iron, biosurfactant production, motility and activity of cell-wall degrading enzymes have been analyzed. Tested strains produce a vast amount of pectolytic enzymes, which facilitate the degradation of plant cell walls and maceration of the plant tissue. Significant differences between tested strains, in the ability to produce pectolytic enzymes in anaerobic conditions and at different temperatures, were observed. All tested strains indicated both identical sequences for the housekeeping genes (*dnaX*, *gyrA*, *recA*, *rpoS*) and identical REP-PCR and PFGE patterns.

Reference: Toth I, van der Wolf J, Saddler G, Lojkowska E, Hellas V, Pirhonen M, Tsror L, Elphinstone J. 2011 Plant Pathology, 60: 385–399.

René Mathis

***Clavibacter michiganensis* strains characterized as non-pathogenic by phylogenetic and polyphasic analyses are transmitted by tomato seeds and interfere with *C. michiganensis* subsp. *michiganensis* detection.**

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Clavibacter michiganensis subsp. *michiganensis* (*Cmm*), the agent responsible for bacterial wilt and canker of tomato, causes severe losses worldwide. This quarantine phytopathogen is under regulation in order to control its spread. The best way to fight this seed borne pathogen is based on seed control. Improving pathogen detection tools requires a precise knowledge of the pathogen diversity. Therefore the diversity of a worldwide *Cmm* strain collection was investigated to explore the genetic diversity and to establish the phylogenetic relationship between strains using a MLSA-MLST type analysis based on the partial sequences from six housekeeping genes. Working on a collection of more than 200 strains isolated from tomato seeds, it was possible to show differences in aggressiveness between *Cmm* strains and to identify non-pathogenic strains. Sequence polymorphism observed in a subset of this collection shows that *Cmm* is monophyletic and is distinct from its closest taxonomic neighbours. Few *Cmm* clonal complexes were identified in MLST. The taxonomic identity of the *C. michiganensis* saprophytic strains was confirmed by 16S rDNA sequencing. Based on MLSA, we show that these strains are phylogenetically distinct from the pathogenic strains. However, these strains cross-react with the detection tools we tested. Our data provide a large data set to study this pathogen and to develop new detection tools.

Paul De Vos

Is identification by bar-coding of Q-clavibacters in conflict with the existing bacterial taxonomy of this group of bacteria?

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The genus *Clavibacter* contains mainly plant pathogenic bacteria elaborating a host specificity that makes them of great economic importance and most of them are regarded as quarantine and/or q-alert organisms. Taxonomically they are known to form a well delineated clade in the Microbacteriaceae. Bacterial taxonomy concerns grouping, naming and identification of bacteria at the species or sub-species level. The molecular backbone of the taxonomic system is the 16S rRNA gene sequence that allows delineating groups of higher taxonomic ranks. Species and subspecies delineation is based on comparative analysis of overall genome relatedness coupled with phenotypic discriminatory characteristics. Recent developments in bacterial taxonomy reveal that species delineation can be achieved by the MLSA (Multilocus Sequence Analysis) based on a number of well-selected house-keeping genes. The number of genes to be sequenced for obtaining a reliable identification is probably depending on the group of organisms under study. Within the frame of QBOL (Quarantine Barcoding of Life) European Project it is aimed to develop a reliable identification based on DNA barcoding to significantly improve the current diagnostics. The question may be raised whether the bar-coding approach would conflict the existing *Clavibacter* taxonomy.

Two hundred named clavibacter strains were subjected to sequence analysis of 16S rRNA gene and *gyrB*. A 500bp sequence of the housekeeping gene – *gyrB* was found to be a discriminative barcode at the subspecies level for the included clavibacters. Including phylogenetically related species confirmed the robustness of the method at this taxonomic level. However, it is known that bacteria exchange part of their genomes horizontally and this may affect the long term validity of existing bar-coding schemes for identification.

In order to back up the results of the bar-coding, MALDI-TOF MS (matrix-assisted laser desorption ionization-time of flight mass spectrometry) patterning was introduced. It turned out that the MALDI-TOF profiles allowed selecting for subspecies specific biomarkers, herewith supporting the sequencing data. The existence within the *Clavibacter* group of a new group of seed-borne non-pathogenic bacteria is discussed elsewhere (see Zaluga et al.).

Harrie Koenraadt

Detection of *Clavibacter michiganensis* subsp. *michiganensis* in seeds of tomato

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In 2007 there were several worldwide outbreaks of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) in tomato. Amplified Fragment Length Polymorphism (AFLP) was initiated to investigate the genetic diversity within Cmm strains and closely related strains. It revealed extensive genetic variation and showed that two contaminated seed lots were responsible for some of the outbreaks of Cmm. At present, for Cmm and related strains there is a database with more than 750 entries. The outbreaks have intensified international research to improve the reliability of the seed assay. Extraction of Cmm from contaminated seeds and concentration of the bacteria were identified as critical steps to improve the sensitivity of the assay. New media were developed that allow good growth of Cmm while the growth of many saprophytes is inhibited. Spiking, a technique in which a marked Cmm strain is added to samples, showed that antagonistic micro-organisms could obscure Cmm presence. Spiked and non-spiked versions of seed extracts are now routinely plated. A valid test result is only obtained when the Cmm spike is recovered. The molecular identification procedure was also improved. The adaptations in the Cmm protocol resulted in the new ISHI 4.0 protocol. The outbreaks initiated cooperation between seed companies and transplant producers leading to the foundation of “Good Seed and Plant Practices” (GSPP). The impact of GSPP and the new validated ISHI 4.0 protocol will be discussed.

Françoise Poliakov

Emergence of *Pseudomonas syringae* pv. *actinidiae* (Takikawa, 1989) in France: methods of characterization

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Pseudomonas syringae pv *actinidiae* (Psa), causal agent of Kiwi canker, has been known since recent outbreaks in Italy in 2007 (Balestra, 2009) as an aggressive pathogen on Kiwi fruit, spreading rapidly in orchards and the environment. The first recordings of Psa were in 1984 in Japan (Takikawa *et al.*, 1989, Serizawa *et al.* 1989), then in 1992 in Korea (Lee & Koh, 1994) and Italy (Scortichini, 1994) where outbreaks had been eradicated.

This bacterium was first detected in France in July 2010 by isolation on nutrient agar media and identification. Its spread has been rapid since then, to Portugal (Balestra, 2010), New Zealand (2010, MAF Biosecurity Website) it was then reported in 2011 in Chile, Australia, Switzerland and Spain. The bacterial canker poses a serious threat to the cultivation of the yellow-fleshed kiwifruit in Italy (*Actinidia chinensis*), but also to green-fleshed kiwifruit (*Actinidia deliciosa* cv. Hayward). In France, this variety comprises about 95% of orchards.

The French strains isolated in 2010 were shown to belong to the same population as the strains from recent outbreaks in Italy. These genetics profiles were confirmed for most of the strains isolated during 2011 by using different biomolecular tools. The genetic structure of the population of strains isolated from French Kiwifruit is under investigation.

Gilles Cellier

Story of an array based technology designed to grasp and detect the complex plant pathogen *Ralstonia solanacearum*

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The ancient soil borne plant vascular pathogen *Ralstonia solanacearum* evolved and adapted to cause severe damage on an unusually wide range of plants. Based on the phylotype classification, it was questioned how genetically and phenotypically diverse strains of *Ralstonia solanacearum* may be, in an attempt to produce adapted diagnostic tools. A pangenomic microarray was first used to better describe and understand the phylogeny of *R. solanacearum*, especially three particular ecotypes in the phylotype II: (i) Brown rot strains from sequevars IIB-1 and IIB-2, historically known as race 3 biovar 2 and clonal; (ii) new pathogenic variants from sequevar IIB-4NPB that lack pathogenicity to Cavendish banana but can infect many other plant species; and (iii) Moko disease-causing strains from sequevars IIB-3, IIB-4, and IIA-6, historically known as race 2, that cause wilt on banana, plantain, and Heliconia. Results revealed a phylogeographic structure within Brown rot strains, allowing European outbreak strains of Andean and African origins to be distinguished, and also showed a close but distinct relationship between Moko ecotype IIB-4 and the emerging IIB-4NPB strains. Based on those results, it is proposed to develop new DNA-based diagnostic tools, including array and routine PCR, to ensure better detection of this complex plant pathogen.

Xiang (Sean) Li

Graft-transmission of '*Candidatus Liberibacter solanacearum*', the causal agent of potato zebra chip disease, from greenhouse-grown latently-infected tomato.

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In contrast to potato plants propagated in growth chambers that developed severe symptoms of zebra chip (ZC) when infected by '*Candidatus Liberibacter solanacearum*' (CLS), ZC-infected tomato plants served as a latent carrier of CLS. Tomato plants (varieties Money Maker and Roma) graft-inoculated with scions from latently infected tomato plants remained symptomless, but tested positive in a ZC-specific PCR assay while potato plants (cultivars Jemsam, Atlantic, Shepody, Frontier Russet, and Russet Norkotah) showed typical symptoms of purple top and leaf scorch four weeks after being grafted with scions from the same tomato plants. Tubers from the graft-inoculated potato plants also showed typical symptoms of brown discoloration in the vascular ring and medullary rays. CLS could not be detected in the aerial tubers of graft-inoculated greenhouse-grown plants, but the bacterium was readily detected in the stems and progeny tubers of the same plants. It was also considered important to differentiate CLS from the potato witches' broom phytoplasma because they cause similar foliage symptoms in potato. Therefore, a culture-independent approach was employed to clone and sequence the *rrn* operon of the two phytopathogens. Sequence analysis provided ample targets to differentiate the two pathogens as would be expected for phylogenetically different microorganisms.

Session 7 Fungi

Abstracts of Presentations

Johannes Zacharias Groenewald

WP2 of QBOL: Barcoding fungi of Quarantine importance

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Fungal organisms were selected from the EU Council Directive and EPPO A1/A2 lists based on the availability of cultures and/or taxonomic expertise and were sequenced for (potentially) informative barcode regions. The following fungi and their related organisms were selected for the QBOL Work Package 2 (Fungi): *Ceratocystis fagacearum*, *C. fimbriata* f. sp. *platani*, *C. virescens*, *Pseudocercospora angolensis*, *Ps. pini-densiflorae*, *Melampsora farlowii*, *Me. medusae*, *Monilinia fruticola*, *Mycosphaerella dearnessii*, *My. larici-leptolepis*, *My. populorum*, *Puccinia pittieriana*, *Septoria malagutii* and *Thecaphora solani*. The internally transcribed spacer (ITS) regions of the nrDNA operon were initially used to confirm the taxonomic identity of all strains and to evaluate the resolution for species resolution of this commonly used region. One or more additional house-keeping genes, e.g. actin, beta-tubulin and translation elongation factor 1-alpha, were screened for almost all species to supplement data for species with poor ITS resolution. A molecular decision scheme was developed to support decision making of which loci to use for which species. This presentation will give an overview of the obtained results and highlight the more important findings.

Wellcome Ho

DNA barcoding of the fungal genus *Phoma* from New Zealand

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The New Zealand Ministry of Agriculture and Forestry, Plant Health & Environment Laboratory (PHEL) is responsible for the identification of new and exotic plant pests and diseases that may be damaging to the country's primary industries and environment. The PHEL has developed a DNA barcoding system using the fungal group *Phoma* as a model for the identification of pests and diseases of biosecurity concern. The barcode database comprises DNA sequences from the ITS regions, the actin and beta-tubulin genes. Around 800 sequences were generated from 320 *Phoma* isolates that were collected from culture collections in New Zealand. In addition, over 800 authentic sequences from type or representative specimens have been selected from around 2,000 published sequences.

Using the barcode database, 80% of the 320 isolates were identified to species level. Among these, 32 isolates were identified as belonging to 14 *Phoma* or closely-related species that had not been reported in New Zealand previously. The barcode database complements the morphological identification technique used for plant pathogenic fungi and has been demonstrated to accurately identify surveillance samples. The barcoding process has been extended to other groups of plant pests and diseases for rapid and accurate identification.

William Quaedvlieg

Barcoding *Mycosphaerella* species of quarantine importance to Europe

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The EU 7th Framework Program funded by the Quarantine Barcoding of Life (QBOL) project, aims to develop a quick, reliable and accurate DNA barcode-based diagnostic tool for *Mycosphaerella* species on the European and Mediterranean Plant Protection Organization (EPPO) A1/A2 lists of pests recommended for regulation as quarantine pests. In order to determine which barcoding loci would be best suited for identifying *Mycosphaerella* and/or its anamorph species on these A1/A2 lists, seven nuclear genomic loci (β -tubulin (Btub), internal transcribed spacer (ITS), 28S nrDNA (LSU), Actin (ACT), Calmodulin (CAL), Translation elongation factor 1-alpha (EF1) and RNA polymerase II second largest subunit (RPB2)) were tested on their Kimura-2-parameter-based inter- and intraspecific variation, their PCR amplification success rate and their ability to distinguish between quarantine species and their closest known neighbours. The research showed that none of the test loci was ideally suited individually to serve as a reliable barcoding locus; however, a combination of a primary and secondary barcoding loci can compensate for individual weaknesses and give reliable identification results. A combination of either ITS and EF1 α or Btub would be an ideal combination to serve as barcoding loci for EPPO A1/A2-listed *Mycosphaerella* species of quarantine importance.

Mathias De Backer

New SNP markers for the genetic characterization of the quarantine pathogen *Puccinia horiana* and their application in pathogen migration analysis

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The quarantine pathogen *Puccinia horiana*, causal agent of chrysanthemum white rust, is one of the most important diseases in chrysanthemum-producing regions worldwide. During this study the genetic variability of forty five isolates originating from North and South America, Asia, and Europe was determined based on neutral Single nucleotide polymorphism-markers. Together with information on collection year and geographical origin this allowed questions of migration, survival, and recombination to be addressed. A high level of genotypic diversity was observed and in most cases phylogenetic clustering was related to the geographic origin. Local survival was demonstrated based on multi-year findings of specific clones in a given geographic area. Recent long distance migration events were demonstrated but were limited in number. Exclusive clonal propagation of this microcyclic rust was disproved given clear indications of recombination between specific genotypes. The genotype data were also combined with the pathotype data of specific isolates but pathotype-specific markers were only observed in one of the clades. In combination with a fast SNP detection system, this technique could allow rapid characterization of intercepted or local isolates, which may help in questions relating to the need for quarantine action.

Hans de Gruyter

Molecular phylogeny of *Phoma*: tool for development of validated real-time (TaqMan) PCR assays for detection of *Stagonosporopsis andigena* and *S. crystalliniformis* in infected leaves of tomato and potato

de Gruyter Hans (1) van Rijswijk Patricia (1) Bonants Peter (2) van Gent-Pelzer Marga (2) Crous Pedro (3) Woudenberg Joyce (3)

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Species-specific real-time (TaqMan) PCR assays are presented for the detection of *Stagonospora andigena* and *S. crystalliniformis* in leaves of potato or tomato cultivars. Both species, originally described in the anamorph genus *Phoma*, are serious foliage pathogens and have been recorded only in the Andes area so far. *Stagonosporopsis andigena* is listed as an A1 quarantine organism in Europe.

The specificity of the TaqMan PCR assays was determined on genomic DNA extracted from strains of *S. andigena*, *S. crystalliniformis* and closely related species of *Stagonosporopsis*, *Phoma* and *Boeremia*. The validation of the methods developed included the DNA extraction and the TaqMan PCR assays. The specificity, analytical sensitivity, reproducibility, repeatability and robustness of the TaqMan PCR assays are demonstrated. Both TaqMan PCR assays were tested on symptomatic leaves of potato or tomato obtained after artificial inoculation with both pathogens under quarantine conditions.

Anne-Laure Boutigny

Real-time PCR detection of the quarantine pathogen *Melampsora medusae* f. sp. *deltoidae*

Anne-Laure Boutigny, Cécile Guinet and Renaud Ioos

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Melampsora medusae Thümen is one of the causal agents of leaf rust of poplars and is an EPPO A2 listed pest recommended for regulation as a quarantine pest. Two *formae speciales* have been described within *M. medusae*: *M. medusae* f. sp. *deltoidae* and *M. medusae* f. sp. *Tremuloidae*, on the basis of their preference for cultivated poplars (e.g. *Populus deltoides*) or wild poplars (e.g. *Populus tremuloides*), respectively. Given the host preference, it is assumed that only *M. medusae* f. sp. *deltoidae* was reported to be present in Europe so far. In this context, a *M. medusae* f. sp. *deltoidae* specific real-time PCR assay was developed. A set of primers and hydrolysis probe was designed based on sequence polymorphisms in the large ribosomal RNA subunit (28S). The real-time PCR assay was optimized and sensitivity, specificity, repeatability, reproducibility and robustness of the detection method were assessed. This assay was very sensitive as it allowed the consistent detection of one single urediniospore of *M. medusae* f. sp. *deltoidae* in a mixture of 2 mg (corresponding approximately to 800000 urediniospores) of other *Melampsora* species. This test can be used for specific surveys in nurseries and phytosanitary controls, in order to avoid introduction and spread of this pathogen in Europe

Anita Rose Haegi

A DNA-Based Macroarray for Multiplex Detection of Soil-Borne Fungi

Haegi Anita, Belisario Alessandra, Infantino Alessandro, Pucci Nicoletta, Tizzani Lorenza, Vitale Salvatore, Riccioni Luca

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A multiplex diagnostic method, based on macroarray, has been set up for the detection in soil of the following fungi: *Verticillium dahliae*, *V. albo-atrum*, *Armillariella mellea*, *Nectria galligena*, *Phytophthora cactorum* and *Chondrostereum purpureum*. The method has been set up to provide a diagnostic tool for the production of certified pome fruit propagation materials as regulated by the Italian Ministry of Agriculture following the European Directives. The macroarray method is an open system which enables a high level of multiplexing and allows new oligonucleotides detectors to be added for new target identification and/or to change sensitivity/specificity of detection. With this technology, oligonucleotides detectors are immobilized on a nylon membrane and used as target for microorganism detection. The DNA to be tested is amplified, labelled and then hybridized to the membrane. In this system, DNA is directly labelled with Alkaline Phosphatase which reacts with CDP-Star giving a chemiluminescent signal. Oligonucleotides detectors have been designed on the ITS regions of the rDNA, because of its high sequence variability and availability in gene bank databases. Species and genus-specific oligonucleotide detectors have been designed. The system has been validated using DNA extracted from pure fungal cultures and from infested soil. The characteristics of the macroarray system are discussed.

James Woodhall

Using real-time PCR to detect plant pathogens in soil

James W Woodhall, Ian P Adams, Jeff C Peters & Neil Boonham

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Soil borne diseases are a major constraint to crop production worldwide. Detection of a soil-borne plant pathogen prior to planting could therefore inform grower decisions concerning planting and soil treatment. Real-time PCR is a robust detection method and combined with a suitable DNA extraction method, could be used to quantify a specific pathogen in a particular soil sample. However, the detection of a pathogen in soil presents several challenges. These include the ability to extract DNA from biologically relevant sample sizes, effective sampling strategies and techniques to cope with inhibition due to the presence of humic compounds within a sample. Measures to overcome these challenges are discussed and several examples of successful diagnostic assays for important soil-borne plant pathogens are presented.

Session 8 Validation & Databases and other Diagnostic Tools

Abstracts of Presentations

Friday 2012-05-25

Bart van de Vossenberg

DNA barcoding as identification tool for regulated plant pests: an international collaborative test performance study among 14 laboratories

B.T.L.H. van de Vossenberg (1), Jean-Yves Rasplus (2), Martine Maes (3), Ewald Groenewald (4), Juerg Frey (5), Mogens Nicolaisen (6), Neil Boonham (7), Peter Bonants (8)

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DNA barcoding is a generic diagnostic method that uses a short standardised genetic marker in an organism's DNA to aid species identification. DNA barcoding protocols for arthropods, bacteria, fungi, nematodes and phytoplasmas were developed within the Quarantine organisms Barcoding Of Life (QBOL) project financed by 7th framework program of the European Union. The use of a single barcode region was found to be insufficient for the identification of the majority of regulated plant pests. Therefore, several short standardised genetic markers have been appointed as “barcodes” for the identification of plant pest groups. To determine the usability of the developed tests for diagnostics, an international collaborative test performance study among 14 laboratories was organised. Participants were provided with the protocols (presented as a draft EPP0 standard), interactive forms, primers, DNA extraction kits, test material and positive amplification controls in order to perform the tests and a USB-key to report the data. USB-keys were collected by PPS and the data was analysed to determine the diagnostic sensitivity, diagnostic specificity, reproducibility, repeatability and robustness of the tests.

Vincent Robert

QBOL and Q-bank data management and analysis system

Robert V., Szoke S., Jabas B., Vu D., Chouchen O. and Amor, A.

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For the Quarantine Barcoding of Life (QBOL, EU project) as well as the Q-bank (international consortium mainly supported by the Dutch government) projects a complete scientific data management system based on the BioMICS software has been developed. Morphological, physiological, chemistry, ecological, molecular, geographical, administration, bibliography and taxonomy data etc. can each be stored, handled and analyzed in the most appropriate ways. The system includes all relevant biological data related to Bacteria, Fungi, Insects, Nematodes, Phytoplasmas, Plants and Viruses associated with the quarantine problematic. The system not only allows data to be saved but also to be analysed (polyphasic identification, classification, statistics, automated curation, etc.) but also to be published online. The system is completely dynamic in the sense that new characters or fields, tables and records can be added to the system within minutes without the intervention of software developers.

Martine Maes

Reference plant pathogenic bacteria in support of the European plant health policy: the Q-Bacco-net initiative

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Bacterial plant pests cause economically important losses in agricultural crops.

In order to guarantee the long-term accessibility, the quality and authenticity of reference bacterial, three well-established public bacteria collections (BCCM/LMG, CFBP and NCPPB) have associated within Q-Bacco-net, stimulated by QBOL Bacteria WP leader (ILVO) and supported by EPPO. It was decided to accept and maintain the bacterial reference strains in at least two of these partner collections, a measure comparable to that in place for type strains upon new species description. A common standardized protocol to check authenticity and identification has been agreed. The list of selected reference strains with checked authenticity form the Q-Bacco-ref collection. Apart from the QBOL bar coded *Xanthomonas*, *Clavibacter*, *Ralstonia* and *Xylella*, this list contains type strains, pathovar reference strains and additional genome sequenced strains, referring to selected EPPO-listed quarantine pathogens. StrainInfo (www.straininfo.net/) will interlink these different resources with the Q-bank that contains the curated sequence bar coded data retrieving relevant information at the strain level. Finally, it is also important that the pathogenicity of the reference strains can be verified in planta when needed, a function which the Q-Bacco-net members will perform either directly or through existing partnerships with their National Reference Laboratories.

Annelien Roenhort

Q-bank Plant Virus database and collections

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The availability of characterised reference isolates is of crucial importance for research and test laboratories. The Q-bank Plant Virus database contains information on (regulated) plant viruses and viroids, with the unique feature that it is linked to specimens present in publicly available physical collections. The Q-bank database aims to share information on the availability of virus and viroid species between interested laboratories. Currently the database focuses on regulated virus species. In future more plant viruses and viroids will be included to provide a comprehensive information system. The curators invite virologists to participate in this international initiative by making available their data and isolates via Q-bank (see <http://www.q-bank.eu>).

Rachel Glover

Biodiversity sequencing - next generation DNA barcoding

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The application of next-generation sequencing technologies to environmental samples is not new, with 16S bacterial metagenomic studies being commonplace. However, recent improvements in the 454 pyrosequencing chemistry have resulted in sequence lengths of 600-700bp which enables the expansion of metagenomic amplicon sequencing to other loci, in particular those currently being used for DNA barcoding (COI, ITS, rbcL, 18S). This presentation will discuss these recent developments and their application to biodiversity studies and large-scale diagnostics in the near future.

Ming-Fu Li

Research on and applications of DNA Barcodes for species identification

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Along with the development of China's economy and trade, Import and export of goods and staff exchanges have become increasingly frequent in 2011. More than 500 thousand batches of goods were imported and exported, and thousands of harmful biological species intercepted. Facing more and more biological safety issues, the department of inspection and quarantine is looking for more effective methods to identify the species, and deal with the risks such as invasive alien species, genetic resources losses, economic fraud etc. With the birth of DNA barcode technology, a lot of research have been carried out in China , its application has been explored actively and some scientists have been involved in relevant international cooperation, So far, more than 20 different flora and 15 kinds of fauna were investigated and identified with DNA barcode technology and some important gene barcodes were acquired and verified; A few of specific barcode databases were built, and applied preliminary for inspection and quarantine, the preservation of Chinese medicine resources and the conservation of biodiversity. Meanwhile, several nationally important DNA barcode research projects have been funded in the twelfth five-year plan, that will be expected to further improve the technology system of inspection and quarantine in China, and play an important role in performing the related international treaty, realize the objectives of the Convention on Biological Diversity (CBD) by promoting conservation, sustainability, and the equitable sharing of benefits arising from use of genetic resources.

Bénédicte Moignot

How to prioritize the development of diagnostic tests for plant pests based on a risk ranking tool?

Moignot Bénédicte (1), Ouvrard Florian (1), Mouttet Raphaëlle (1), Suffert Frédéric (2), Reynaud Philippe (1)

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Many regulated and exotic plant pests threaten crops in the European Union. While inspections and surveys using diagnostic tests are useful to mitigate the risk of their introduction, prevent their permanent establishment and limit their subsequent spread, resources are dramatically limited to cover all regulated pests. Therefore detection efforts should be performed in accordance to the assessed pest risks. A pragmatic way to address this objective is to prioritize the pests according to their potential phytosanitary impact. Thus, a ranking system was developed using explicit data-driven criteria. It allows a rapid risk assessment which result in a pest prioritization in the French context (climate, crops, trade...). This tool has been applied for 100 selected pests. We compared the pests ranking first and the pests for which validated diagnostic protocols are available. Diagnostic protocols already exist for top priority pests, which confirmed that this tool gives similar results to expert judgment used so far to set priorities within the French NPPO. As a consequence, this system is an opportunity to prioritize needs to develop new diagnostic tests for emerging pests compared to already regulated pests ranked with a medium or low phytosanitary risk profile.

Tanja Dreo

qPCR, low level detection and validation data

Dreo Tanja (1), Nataša Mehle(1), Petra Nikolić (1), Manca Pirc(1), Marina Dermastia(1), Kristina Gruden (1) and Maja Ravnkar(1)

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Generation of validation data has become more systematic with the adoption of EPPO standards on assuring quality in detection and diagnosis of plant pathogens (PM 7/76, PM 7/84, PM 7/98) In the case of qPCR its use and validation data has led to the realization that due to its high sensitivity there is usually a range of low target concentrations that can only be detected as positive using q PCR and not by other less sensitive methods or methods based on different biological principles. This can give rise to difficulties in reporting results and their interpretation; particularly in cases of zero-tolerance quarantine pathogens when usually at least two methods with positive results are required to report a sample as suspicious and a pure culture is needed for final confirmation (e.g. for *Ralstonia solanacearum*). Several approaches to addressing this issue will be presented and discussed including use of several qPCR targets and determination of a so called 'cut-off value'.

Mariette Edema

Validation as a project: 2 laboratories, 24 organisms, 3 years

Session 9 Stakeholder views and needs

Abstracts of Presentations

Results of all Work Packages of QBOL, from DNA barcodes to Database and Validation.

Peter Bonants

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The rate of introduction and establishment of damaging plant pests and diseases has increased steadily over the last century as a result of expanding globalisation of trade in plant material, climate change, EU expansion, and by a recognised decline in the resources supporting plant health activities. Furthermore there is a constant decline in the number of taxonomic specialists in the different disciplines (mycology, bacteriology, etc.), capable of identifying plant pathogens, and funds to support this kind of work are very hard to obtain. In addition the number of other specialists in phytopathology and other fields who are vital for sustaining sound public policy on phytosanitary issues are diminishing. These problems affect all members of the EU and other nations. In this context QBOL (www.qbol.org), an EU project on DNA barcoding, started in 2009 to generate DNA barcoding data of quarantine organisms and their taxonomically relatives to support plant health diagnostics. The data are included in a database, called Q-bank (www.Q-bank.eu).

The six work packages (WP) to generate the barcodes on Fungi (WP2), Arthropods (WP3), Bacteria (WP4), Nematodes (WP5), Viruses (WP6) and Phytoplasmas (WP7) and also WP9 on the Database and WP 10 on Validation will be presented in an overview.

Wellcome Ho

Barcoding, Multilateral Initiatives for Biosecurity Outcomes

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The Plant Health Quads (PHQuads) is an international partnership in which senior regulators from the national plant protection organizations of Australia, Canada, New Zealand and the United States may engage in collaborative projects and discussions of plant health issues of mutual concern. In 2006, the PHQuads established the Quads Scientific Collaboration Working Group (SCWG). The purpose of the SCWG in support of the PHQuads is: to share and exchange technical information; to foster cooperation and collaboration in phytosanitary programs; and to advance plant health knowledge, science, and technology in areas of mutual interest.

The missions of the respective PHQuads national plant quarantine/ biosecurity organizations are to safeguard plant resources from invasive plant pests/pathogens. When a pest is detected it is imperative that the identity of that pest or pathogen is established for appropriate action to be taken. Misidentification of a pest may result in unnecessary actions that hinder trade.

The PHQuads Diagnostic Tools project team was formed to specifically, identify and coordinate the development of diagnostic tools for plant pests. To facilitate PHQuads understanding and application of DNA barcoding technology, a project on the topic was initiated in 2010. The PHQuads barcoding project has a number of aims that include: Identifying opportunities for cooperation on DNA barcoding projects between Quads members; Discussing and evaluating barcode libraries and protocols for pests of concern to Quads members; and Exploring the regulatory implications at the international level, especially the issue of species delimitation and its implications for regulators. Details of this project will be presented on behalf of the project members.

Harry Arijs

Role of diagnostics in the EU plant health management

Brent Larson

IPPC diagnostic protocols: underpinning global phytosanitary systems

Brent Larson, IPPC Secretariat, viale delle Terme di Caracalla, Roma, Italy, brent.larson@fao.org

National plant protection organizations worldwide rely on diagnostic protocols to detect and identify pests. Diagnosis serves various purposes, in relation to helping ensure safe trade and prevent pest introductions and spread, or provides support for such basic phytosanitary activities such as surveillance or determining pest status. This presentation will cover the aims and content of IPPC diagnostic protocols, the process for their development, and challenges in their implementation. The Commission on Phytosanitary Measures has established the Technical Panel on Diagnostic Protocols (TPDP) to facilitate the development of IPPC diagnostic protocols. The TPDP work programme focuses on pests for which there is a specific need for harmonized protocols. IPPC diagnostic protocols aim at providing a range of options for detection and identification (including morphological and molecular methods and methods based on biological properties). The methods are selected on the basis of their sensitivity, specificity and reproducibility, as well as practicability (for example ease of use, speed and cost). IPPC diagnostic protocols need to also be suitable for various levels of capabilities in IPPC contracting parties, in terms available expertise and equipment. Three IPPC diagnostic protocols were adopted so far, and 29 are currently under development. Additional expertise is needed to contribute to the development of these protocols, as well to suitable ways of integrating the new validated methods as they become available, such as barcoding. Finally, in order to implement diagnosis, contracting parties need access to laboratories, reference material and taxonomists, and capacity development programmes will be necessary.

Hans Smolders

Diagnostic tools in perspective; Netherlands policy and the need for robust phytosanitary chains

Hans Smolders,
Ministry of Economic Affairs, Agriculture and Innovation, the Netherlands

Agricultural trade, both import and export, is a highly relevant segment of the Netherlands agro-economy. Annual export on planting materials alone was valued at 1.6 billion Euros in 2009, representing a 75% increase since 2001; growth figures on imports are similar. Approximately 40% of total world trade for seeds, 60% for seed potatoes, and 80% for ornamental bulbs originates from the Netherlands. Such a high contribution to worldwide trade of agriculture products requires a high responsibility to both the Dutch government and private stakeholders to manage phytosanitary risk and to avoid the spread of harmful organism. Moreover, any interception or outbreak can be very damaging for the sector(s) involved.

In view of future growth of global trade, there is a need to strengthen the phytosanitary aspects of the international trade. Besides a strong role of governments, a higher involvement of primary stakeholders in the supply chain is required, which implies closer collaboration in the production chain with producers and traders upstream, including those in third countries. To build robust international phytosanitary chains, availability of new rapid and cost-effective diagnostic tools for traders and producers as well as the NPPO are vital. However, to keep risk control in the chain manageable, there is a need to combine new diagnostic tools with smart knowledge management, other technical innovation and non-regulatory measures to promote stakeholders willingness to take effective measures during growth, sorting, packing and transport. Some examples are provided of research and measures initiated by the Netherlands government to strengthen these phytosanitary aspects of the agriculture production and supply chain.

Wim van Eck

Q-bank: more than a database

Wim van Eck
Netherlands Food and Consumer Product Safety Authority, Chairman Steering Committee Q-bank

Q-bank is a tool for the identification of quarantine plant pests and invasive plant species. It is a web-based database. It contains data, amongst others on DNA sequences, linked to reliable reference collections. Q-bank is complementary to other databases, e.g. those of EPPO. A network of internationally recognized experts, the curators, ensures that only validated data are stored in the database. When QBOL was launched, it was agreed that data on DNA sequences generated through QBOL project would be stored in Q-bank. The link with reference collections and the validation of data by curators are key features of Q-bank. Q-bank therefore is not only a useful diagnostic tool but it contributes to strengthen national and international plant health systems. Q-bank is originally a Dutch initiative. The enhanced rate of the introduction of plant pests, mainly as a result of the globalization of the trade of plant products, called for mechanisms to rapidly disclose and share information on quarantine pests. This is the more important as resources in the area of plant health are limited, including the number of specialists able to identify this type of organisms. Q-bank is funded by the Dutch government until the end of 2013.

Q-bank is governed by a steering committee. Recently senior persons from amongst the member states of EPPO (Germany, France, United Kingdom and the Russian Federation) have joined this committee. This transformation into an international steering committee is with the view to strengthen the governance of Q-bank and to enhance the international profile of the database to make it a really international diagnostic data base.

Challenges for the future are to ensure that Q-bank will remain a living and attractive database, encouraging the phytosanitary community to forward new data to the database whenever these data become available. To ensure the high quality of the database an active international network of curators is indispensable as is the link with reference collections. Sustainable funding is a prerequisite for the future of Q-bank. The ultimate objective is to find a international home for Q-bank.

John van Ruiten

International trade of high graded plant material: the need for harmonized and validated detection techniques.

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Growers of agricultural and horticultural products are faced with many challenges. There is a great necessity to increase production and productivity and at the same time decrease use of fossil fuels and crop protection compounds. To achieve this goal it is required that high graded, absolutely disease free seeds and young plants/tubers are available for starting growing a crop.

Companies, inspection agencies and phytosanitary services have to develop better (even more sensitive) techniques/protocols for producing, testing and monitoring healthy seeds and plants. As material is produced and marketed globally it is vital that sampling and testing techniques are much better harmonized than at present. An initiative such as Q-bank offers the conditions and connections amongst specialists to develop (DNA-based) diagnostic and detection methods that are validated (and are therefore proven to be effective against occurring strains of a wide range of pathogens).

George Franke

The view of horticultural producers on diagnostic tools

Christine Henry

TESTA - Seed health: development of seed treatment methods, evidence for seed transmission and assessment of seed health

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The TESTA initiative plans to develop a range of novel methods to underpin the control of seed-borne diseases and pests, including faster, more accurate methods to assess the mode of seed transmission, economic and practical sampling approaches for the detection of low levels in large seed lots, novel and efficient generic detection methodologies, non-destructive testing methods and improved, effective and sustainable disinfection methods. Target crop and disease/pest combinations have been identified in consultation with EPPO, ISHI-ISF and ISTA. Outcomes from the project will include a comprehensive electronic database of seed transmitted diseases and pests, validated detection methods for target species, a validation protocol for assessing the efficacy of disinfection, as well as many key scientific publications. These will provide supporting methods and sources for the EU seed testing laboratories and plant health services.

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Discussion /concluding remarks

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