JKI Berichte

21st International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops

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Kontaktadressen

Wilhelm Jelkmann, Julius Kühn Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Schwabenheimer Str. 101, D-69221 Dossenheim

Gabi Krczal, RLP AgroScience GmbH, AlPlanta - Institute for Plant Research, Breitenweg 71, D-67435 Neustadt

Falko Feldmann, The German Phytomedical Society, Managing Director, Messeweg 11/12, D-38104 Braunschweig

Der Forschungsbereich des Bundesministeriums für Ernährung, Landwirtschaft und Verbraucherschutz (BMELV) hat seit dem 1. Januar 2008 eine neue Struktur.
Die Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA), die Bundesanstalt für Züchtungsforschung an Kulturpflanzen (BAZ) sowie zwei Institute der Bundesforschungsanstalt für Landwirtschaft (FAL) wurden zum Julius Kühn-Institut - Bundesforschungsinstitut für Kulturpflanzen zusammengeschlossen. Das Johann Heinrich von Thünen-Institut (vTI) wurde aus der Bundesforschungsanstalt für Fischerei, der Bundesforschungsanstalt für Forst- und Holzwirtschaft und aus Teilen der Bundesforschungsanstalt für Landwirtschaft errichtet.
The research branch of the Federal Ministry of Food, Agriculture and Consumer Protection (BMELV) has been reorganized. The former Biological Research Centre for Agriculture and Forestry (BBA) has been merged with other institutions. The newly established Julius Kühn Institute (JKI), Federal Research Centre for Cultivated Plants, is working on plant protection, plant breeding, crop and soil science. The Johann Heinrich von Thünen Institute (vTI) was created from the German Federal Research Centre for Fisheries, the German Federal Research Centre for Forestry and Forest Products and part of the German Federal Agricultural Research Centre.

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Organizing Committee

Wilhelm Jelkmann, Julius Kühn Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Schwabenheimer Str. 101, D-69221 Dossenheim

Gabi Krczal, RLP AgroScience GmbH, AlPlanta - Institute for Plant Research, Breitenweg 71, D-67435 Neustadt

Falko Feldmann, The German Phytomedical Society, Managing Director, Messeweg 11/12, D-38104 Braunschweig

Scientific Committee

Marina Barba, Centro di Ricerca per la Patologia Vegetale, Via C. G. Bertero, 22, 00156 Rome, Italy

Assunta Bertaccini, Alma Mater Studiorum' University of Bologna, Dista, Plant Pathology, viale Fanin 42 -40127 Bologna, Italy

Walter Bitterlin, BIOREBA AG, Chr. Merian-Ring 7, CH-4153 Reinach BL 1, Switzerland

Kadriye Caglayan, Mustafa Kemal University, Plant Protection Department, Hatay, Turkey

Mariano Cambra, Centro de Protección Vegetal y Biotecnología, Instituto Valenciano deInvestigaciones Agrarias (IVIA), Carretera Moncada-Náquera km 5, 46113 Moncada, Valencia, Spain

Thierry Candresse, UMR GDPP, INRA et Université Bordeaux 2, Centre INRA de Bordeaux, Villenave d'Ornon, France

Luis Conci, Instituto de Fitopatologi-a y Fisiologi-a Vegetal, INTA – IFFIVE, Camino 60 Cuadras Km 5 ½, (5119) Córdoba, Argentina

Fiona Constable, Department of Primary Industries, Knoxfield, Private Bag 15 Ferntree Gully Delivery Centre, Victoria 3156, Australia

Ken Eastwell, Washington State University, 24105 North Bunn Road, Prosser, WA 99350, USA

Ricardo Flores, Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Universidad Politécnica de Valencia, 46022 Valencia, Spain

Pascal Gentit, Centre Technique Interprofessionel de Fruits et Légumes de Lanxade (Ctifl), 24130 Prigonrieux, France

Delano James, Canadian Food Inspection Agency, Sidney Laboratory, 8801 East Saanich Road, Sidney, BC, V8L 1H3, Canada

Gerard Jongedijk, Vermeerderingstuinen Nederland, Tienrayseweg 9a, 5961 NK Horst, The Netherlands

Gerardo Llacer, Instituto Valenciano de Investigaciones Agrarias IVIA, Apartado Oficial, 46113 Moncada (Valencia), Spain

Stuart MacFarlane, Plant Pathology Programme, SCRI, Invergowrie, Dundee DD2 5DA, Scotland, UK

Tadeusz Malinowski, Research Institute of Pomology and Floriculture, Pomologiczna 18, 96-100 Skierniewice, Poland

Robert Martin, USDA-ARS Northwest Center for Small Fruit Research, Horticultural Crops Research Lab, 3420 NW Orchard Ave., Corvallis, OR 97330, USA

Verena Müller, UNIVIVEROS - Sociedad Agricola Uniagri Copiapo Ltda, Av 18 de Septiembre 6578, 9540000, Paine, Chile

Svetlana Paunovic, Fruit Research Institute, 3200 Cacak, Kralja Petra I/9, Republic of Serbia

Josef Spak, Biology Centre v.v.i., Institute of Plant Molecular Biology, Czech Academy of Sciences, Branisovska 31, CZ-37005 Ceske Budejovice, Czech Republic

Nobuyuki Yoshikawa, Plant Pathology Laboratory, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan

21st "International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops"
July 5 - 10, 2009
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http://www.phytomedizin.org/icvf.html

Dear colleages,

The German Organizing Committee has the honour to welcome you to attend the XXIst International Conference on Virus other Graft Transmissible Diseases of Fruit Crops held in Neustadt an der Weinstrasse, Germany.

For the first time our current meeting is held under a new title covering virus and other graft transmissible diseases of tree fruit and small fruit crops. Therefore, we would like to briefly outline the history of the previous conferences and explain the decision of the scientific committee and the local organizers.

The topic of viruses and other graft transmissible diseases dates back to a meeting in 1954 in Wädenswil, Switzerland. This meeting was entitled “Symposium on Fruit Tree Virus Diseases” and was held by a small group of international plant pathologists interested in the few virus diseases of deciduous fruit trees then known to occur in Europe and North America. The subsequent meetings became progressively wider in scope and increasingly concerned with viruses per se and virus diseases of fruit trees and small fruit crops worldwide. Furthermore, the scope included diseases caused by phytoplasmas and viroids when these organisms were identified as new and serious pathogens, many of them in fruit crops.

A scientific committee responsible for organizing the meetings was founded from the first meeting on. It consisted of the local organizers of subsequent meetings and other leading scientists in the field.

In 1973 the organizing committee accepted the invitation of the International Society for Horticultural Science to become one of the working groups set up by its Plant Protection Commission. This meeting was entitled “IXth International Symposium on Fruit Tree Virus Diseases”.

Proceedings of the first eight meetings were published as supplements of different phytopathological journals. Extended abstracts were published for the meetings in 1954 and in 1958. With one exception, the proceedings since the 9th meeting were published by ISHS in Acta Horticulturae.

Although research on virus diseases of strawberry was already a topic at the 1954 meeting, a separate symposium title on virus diseases of small fruit crops was established at the 1976 conference in Heidelberg, Germany. In 2006 the XXth International Symposium on Virus and Virus-Like Diseases of Temperate Fruit Crops and the XIth International Symposium on Small Fruit Virus Diseases were held in Antalya, Turkey. The chronological orders of meeting places that have hosted the Symposia have been:

1954 Wädenswil, Switzerland
1955 Wageningen, the Netherlands
1956 East Malling, United Kingdom
1960 Lyngby, Denmark
1962 Bologna, Italy
1965 Belgrad, Yugoslavia
1967 Aschersleben, Germany
1970 Bordeaux, France
1973 East Malling, United Kingdom
1976 Heidelberg, Germany
1979 Budapest, Hungary
1982 Vancouver, Canada
1985 Bordeaux, France
1988 Thessaloniki, Greece
1991 Vienna, Austria
1994 Rome, Italy
1997 Bethesda, USA
2000 Canterbury, United Kingdom
2003 Valencia, Spain
2006 Antalya, Turkey

After discussions and approval by the current Scientific Committee it was decided to return to a meeting organization without the auspices of an international organization. Thus, the local organizers of the 2009 conference informed ISHS about this decision. At the same time it seemed appropriate to identify and establish a new title for the conference. The goal was to establish one name covering the Tree Fruit and Small Fruit Crops and to include viroid and phytoplasma diseases that were referred to as virus-like diseases when no or little information was available on these pathogens in the past.

The Scientific Committee therefore decided to hold future meetings under the title "International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops"

We are proud that the scientific community interested in the above fields of research will now meet again in Germany after 33 years and come together for the 21st time….

Wilhelm Jelkmann & Gabi Krczal

Conveners of the Conference

Jointly organized by*:*

Julius Kühn Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Schwabenheimer Str. 101, D-69221 Dossenheim

RLP AgroScience GmbH, AlPlanta - Institute for Plant Research, Breitenweg 71, D-67435 Neustadt

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We sincerely thank the sponsors of the 21st "International Conference on Virus and other Graft Transmissible Diseases of Fruit Crops"

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Oral Presentations

New drugs for acute and chronic Hepatitis B Virus (HBV) Infection: from HBV-entry inhibition to liver-specific drug targetting

Urban, Stephan
Department of Molecular Virology, Im Neuenheimer Feld 350, University of Heidelberg, 69120 Heidelberg, Germany

The currently approved treatment options for chronic hepatitis B either obstruct HBV genome replication (nucleoside or non-nucleoside reverse transcriptase (RT) inhibitors) or stimulate innate or adaptive immune responses (interferon α (IFNα)). However, although these drugs are capable of reducing serum titres by several logs, elimination of the virus is commonly not achieved. Furthermore, prolonged virus replication at reduced levels in the presence of RT-inhibitors results in the selection and amplification of resistant mutants. This relates to the reservoir function and the high stability of cccDNA, the template of HBV transcription which accumulates in the nucleus of infected hepatocytes4. Since reduction of virus titres lowers the risk of developing liver damage and hepatocellular carcinoma, prolonged treatment even without achieving sustained virological responses is recommended.

In addition to the recent successes in the development of potent nucleoside analogues showing better resistance profiles or INFs with improved pharmacokinetic properties and less side effects, there are several promising new compounds that target so far unaddressed replication steps of HBV. One of these steps is nucleocapsid assembly which can be efficiently inhibited by a family of small molecules called dihydroarylpyrimidines (HAPs)1. HAPs, at low concentrations bind yet unassembled HBV core proteins thereby preventing the formation of an RNA-containing nucleocapsid and redirect the complex into a proteasome-dependent degradation pathway. Another approach addresses the stability of HBV-specific transcripts. Adenoviral transfer of HBV-specific of shRNAs into hepatocytes of HBV transgenic mice lead to a profound and sustained reduction of HBV gene expression3.

We have recently shown that large envelope protein-derived lipopetides efficiently interfere with HBV entry into hepatocytes in vitro and in vivo2. The lead substance of these peptides (Myrcludex B) consists of the first 47 amino acids of the HBV L-protein and is N-terminally myristoylated. Its IC50 in vitro is ~200 pM. Pharmacokinetic studies showed that the peptide targets the liver of even non HBV-susceptible animals with extraordinary selectivity, suggesting the presence of a species-independent but hepatocyte-specific receptor. Mutational analyses revealed that both, myristoylation and a conserved seven amino acid sequence motif are necessary for liver-targeting. Preclinical toxicology studies of Myrcludex B are ongoing and will be presented. Considering the possibility that even under strong suppression of viral replication infection of naive hepatocytes takes place, it will be interesting to see whether an entry inhibitor is capable of reducing the viral cccDNA in chronically infected patients.

Beside their direct antiviral activity the strong liver-tropism of non-immunogenic HBV surface protein derived lipopetides variants can also target other drugs to the liver. One approach is the delivery of IFNα to avoid systemic side effects. First results on the feasibility of this concept will be presented.

Taken together there are promising new substances under preclinical development. In the near future these drugs will hopefully broaden the therapeutic spectrum to treat chronic hepatitis B with increased success rates.

Next Generation Sequencing offers new perspectives in Comparative Genomics of plant associated Bacteria

Kube, Michael
Max Planck Institute for Molecular Genetics, Ihnestr. 63, 14195 Berlin
Contact: kube@molgen.mpg.de

Next Generation Sequencing technologies have started to change genome research. Highly automated systems provided by companies like 454 Life Sciences or Illumina introduced a revolution in genome research. The power of the new technologies can be characterized by a few catchwords like: comparable low costs, high amount of data and short turn around time.

However, just a draft sequence can be provided by this approach for the majority of genome projects with de novo character in contrast to projects with re-sequencing character. Uncertainties resulting from the used technique and/or resulting draft sequences limit the benefit. In consequence, sequence quality has to be increased by the combination of new and old technologies. Examples and preliminary results from running projects will be presented to illustrate the use and benefit of the new techniques in the genome research of plant associated species from the Enterobacteriaceae and Acholeplasmataceae.

Oral Session I

Elucidation of the roles of blackcurrant reversion virus and phytoplasma in the etiology of full blossom disease in currants

Spak, Josef, Kubelkova, Darina, Pribylova, Jaroslava, Spakova, Vlastimila, Petrzik, Karel
Biology Centre v.v.i., Institute of Plant Molecular Biology, Academy of Sciences
Contact: pribyl@umbr.cas.cz

To determine the roles of phytoplasmas and Blackcurrant reversion virus (BRV) in the etiology of full blossom disease (FBD), we conducted graft and dodder transmission experiments. Scions from FBD affected Ribes rubrum were grafted onto red, white and black currants. Red and white cultivars revealed symptoms of FBD, whereas blackcurrant displayed symptoms of BRV infection. No differences in symptoms were observed between plants infected with BRV only and those infected with BRV and phytoplasma.

Aster yellows phytoplasma subgroup 16SrI-C was transferred from FBDinfected red currants to periwinkle, which symptoms of green and yellow petal were observed. Back transmission of phytoplasma to currant seedlings of red and black currant was not successful.

Scions of periwinkle infected with aster yellows phytoplasmas of subgroup 16SrI-C and 16SrI-B, which were bottle-, bark-, and approach-grafted onto seedlings of red and black currant resulted in positive but symptomless transmission of phytoplasma to red currant. We conclude that FBD symptoms are induced by BRV rather than by phytoplasma, which was originally described as the causal agent of FBD.

This work was supported by grants QH91224 from the Ministry of Agriculture and AV0Z50510513 from the Academy of Sciences of the Czech Republic and COST 863 Action.

The authors thank Prof. A. Bertaccini, University of Bologna, Italy, for recommendations regarding phytoplasma detection, Dr. M. Barba, ISPAVE, Rome, Italy, for dodder seeds, Dr. A. Lemmetty, MTT, Jokioinen, Finland and Dr. A.T. Jones, SCRI, Scotland for recommendations regarding BRV detection and †Ing. R. Karešová, RBIP, Holovousy, Czech Republic for the ´Jonkheer van Tets´ and ´Baldwin´ seeds.

Association of Tomato Rinsgpot Virus, Tobacco Ringspot Virus and *Xiphinema americanum* with a decline of highbush blueberry in New York

Fuchs, Marc
Cornell University, Department of Plant Pathology, 630 W. North Street, Geneva, NY 14456, USA Contact: mf13@cornell.edu

A survey of highbush blueberry (Vaccinium corymbosum L.), including some cultivars showing virus-like symptoms, i.e. stunted growth, chlorotic spots, purple lesions, distorted leaves and defoliated shoots, was conducted for the occurrence of viruses in New York. Leaves from symptomatic and nonsymptomatic plants were tested for virus infection by enzyme-linked immunosorbent assay using specific serological reagents (Bioreba, Reinach, Switzerland). Several samples reacted positively for Tobacco ringspot virus (TRSV) and Tomato ringspot virus (ToRSV), two virus species belonging to the genus Nepovirus in the family Comoviridae. The occurrence of TRSV and ToRSV was confirmed in blueberry leaf samples by reverse transcription-polymerase chain reaction (RT-PCR) or immunocapture-RT-PCR with appropriate primers to amplify a 310-bp and a 580-bp fragment of the RNA-dependent RNA polymerase gene, respectively. Comparative sequence analysis of the viral amplicons of New York isolates indicated moderate to high nucleotide sequence identities with corresponding ToRSV and TRSV reference strains. Also, analysis of soil samples collected from the root zone of blueberry bushes for the occurrence of nematodes indicated the presence of specimens from the Xiphinema americanum group.

Cucumber bait plants planted in soil samples containing X. americanumgroup nematodes became infected with either ToRSV or TRSV in a greenhouse. Altogether, our findings indicate that ToRSV and TRSV and their vector X. americanum sensu lato are associated with the decline of highbush blueberry in New York.

A new member of the family Reoviridae may contribute to severe crumbly fruit in 'Meeker' red raspberry

Quito, Diego[1], Jelkmann, Wilhelm[2], Alt, Simone[2], Leible, Svenja[2], Martin, Robert R.[3]
[1]Dept. of Botany and Plant Pathology, Oregon State University, Corvallis, OR, 97330 USA
[2]Julius Kühn Institute, Institute for Plant Protection in Fruit Crops and Grapevine, Dossenheim, Germany
[3]USDA-ARS HCRL, 3420 NW Orchard Ave., Corvallis, OR 97330 USA
Contact: bob.martin@ars.usda.gov

A virus induced crumbly fruit disease of considerable importance in 'Meeker' and other cultivars of red raspberry has been observed in northern Washington, USA and British Columbia, Canada. Raspberry bushy dwarf virus (RBDV), a pollenborne virus, has been attributed as the causal agent of the disease. Recent dsRNA extractions from symptomatic plants in northern Washington revealed the presence of additional viruses, as evidenced by more than 12 bands on agarose gels. All the bands, except those corresponding to RBDV (2.2 kb and 5.5 kb) were gel-purified and cloned for sequencing. Thus far, sequencing results showed the presence of at least two viruses in addition to RBDV. One has significant amino acid sequence identity (~40%) to 8 genome segments of Rice ragged stunt virus (RRStV), a ten-RNA-segmented oryzavirus that belongs to the family Reoviridae. The complete sequence for the segments that correspond to RNA S1 S4 and S7 of RRStV has been determined. Partial sequences of segments S2, S3, S5, S9, and S10 are also known and are being used to generate the complete genomes using poly A tailing of the 3' ends. In addition, Raspberry mottle virus (RMoV), a recently characterized member of the Closteroviridae, was also identified from raspberries with severe crumbly fruit. These findings along with the lack of severe crumbly fruit symptoms in 'Meeker' red raspberry singly infected with RBDV in Oregon, suggest the existence of a novel virus complex associated with severe crumbly fruit in red raspberries. The complex may involve RBDV, RMoV and/or this new reovirus. Transmission studies are underway to determine the affect of each of these viruses singly and in all combinations on crumbly fruit symptom development in 'Meeker' red raspberry.

Biology of *Cixius wagneri* the planthopper vector of '*Candidatus Phlomobacter fragariae*' in strawberry production tunnel, and its consequence on the epidemiology of strawberry marginal chlorosis

Salar, Pascal
UMR-1090, INRA et Université de Bordeaux 2, BP81, F-33883, Villenave d Ornon, France
Contact: foissac@bordeaux.inra.fr

«*Candidatus Phlomobacter fragariae*» is the prevalent agent of strawberry marginal chlorosis (SMC) transmitted by the planthopper *Cixius wagneri*. Because the insect biology was unknown, a field experiment was set up to determine if it was able to reproduce on strawberry plants, to determine the number of insect generations per year and the ability of nymphs to transmit SMC. During spring 2004, 80 *C. wagneri* adults were delivered into 4 small insect-proof tunnels containing 30 healthy plants. Fifteen percent of the delivered insect population was carrying the pathogen. At October 2004, only 3 young L1 instar nymphs were found in the first tunnel, demonstrating there was no new insect generation during summer. At April 2005, 430 *C. wagneri* of early L1 to late L5 nymph instars were collected at the roots of the plants. It clearly indicated that a single insect generation had overwintered as larvae and had emerged at the following spring. All instars were proved to carry '*Ca. P. fragariae*' (70 to 75% of the larvae) and were able to transmit SMC as assessed by transmission assays. An insecticide treatment was applied in March 2005 in a third tunnel and a fourth tunnel was kept as a control. More than two hundred *C. wagneri* adults were collected on the control tunnel 4 in June 2005 confirming that an insect generation arose in the tunnel, whereas no insects could be found in the treated tunnel 3. All plants were kept for two years, surveyed for symptoms expression and tested for '*Ca. P. fragariae*' infection by 16S-PCR. Results indicated a reduced mortality and SMC incidence in tunnel 3, and a higher mortality and SMC incidence in tunnel 2 than in tunnel 1, attesting that *C. wagneri* larvae had spread SMC and that an early insecticide treatment could control the disease.

Production of antisera and evaluation of serology-based techniques for the detection of Blackcurrant reversion virus

James, Delano[1], Croft, Heather[1], Malinowski, Tadeusz[2], Reed, Christopher[1]
[1]Sidney Laboratory, CFIA, 8801 East Saanich Road, Sidney, BC, V8L 1H3
[2]Research Institute of Pomology and Floriculture, Pomologiczna 18, 96-100 Skierniewice, Poland
Contact: Delano.James@inspection.gc.ca

Synthetic peptides based on the amino acid sequence of the coat protein of Blackcurrant reversion virus (BRV) were used as immunogens for the production of polyclonal and monoclonal antibodies. In preliminary studies the polyclonal antibodies were evaluated by plate-traped antigen ELISA, using both herbaceous and woody plants, to determine their affinity and specificity. Subsequently, the polyclonal antibodies were used in combination with the various monoclonal antibodies in a TAS-ELISA assay format for the detection of BCRV. Also, the polyclonal antibodies were purified and used for trapping in immunocapture RTPCR. Immunocapture RT-PCR was found to be more sensitive and more reliable when compared to standard RT-PCR for the detection of BRV

Molecular diagnostics for the detection of strawberry viruses in Australia

Constable, F.E.[1], Bottcher, C.[1], Kelly, G.[1], Milinkovic, M.[1], Persely, D.M.[2], Rodoni, B.C.[1]
[1]Department of Primary Industries, Knoxfield, Private Bag 15 Ferntree Gully, Delivery Centre, Victoria 3156, Australia
[2]Department of Primary Industries and Fisheries, 80 Meiers Rd, Indooroopilly, 4068, Queensland, Australia
Contact: fiona.constable@dpi.vic.gov.au

The supply of high-health, certified strawberry runners throughout Australia is dependent on the collections of nucleus plants maintained in Victoria and Queensland. Both collections are tested annually in spring for several virus associated diseases using a biological indexing method of petiole grafting candidate tissue onto sensitive indicator species. This method is reliable and sensitive only if done in spring or early summer. Biological indexing is also labour intensive, expensive and time consuming, taking 6-8 weeks to generate a result.

Advances in molecular techniques have been published for detection of Strawberry mottle sadwavirus (SMoV), Strawberry crinkle cytorhabdovirus (SCV), Strawberry mild yellow edge potexvirus (SMYEV), Strawberry vein banding caulimovirus (SVBV), Beet pseudos yellows crinivirus (BPYV), and Strawberry pallidosis associated crinivirus (SPaV). We have adopted these tests from international, peer-reviewed literature and trialled them on positive control plants, maintained 12 months of the year under glasshouse conditions. Each PCR test more accurately identified virus infection in graft inoculated indicator plants compared to visual observation for virus associated symptoms. Results of monthly testing of the glasshouse-grown, virus-infected strawberry plants over two years indicated that the best time to detect viruses using molecular methods was in spring and autumn.

The X-tractor Gene™, an automated system for the extraction of nucleic acid, was trialled for nucleic acid extraction from strawberry because of reduced labour costs and consumables. Unfortunately the X-tractor Gene™ was unreliable for nucleic acid extraction from strawberry compared to the RNeasy® Plant Mini Kit and further development is required.

We have partially validated these tests by surveying 100 strawberry plants from Queensland, Western Australia and Victoria. Our results indicate that the tests can detect viruses in a field situation and very few positive results were observed. These results suggest that strawberry viruses may not be widely distributed in Australia, indicating the success of the certification schemes in reducing the incidence of these pathogens.

Detection of phloem restricted bacteria responsible of strawberry marginal chlorosis (SMC) by real-time PCR in a single assay

Fimbeau, Sébastien
Hortis Aquitaine, Maison Jeannette, F-24140, Douville, France
Contact: danet@bordeaux.inra.fr

Two uncultured phloem restricted plant pathogens, the γ3 proteobacterium «*Candidatus Phlomobacter fragariae*» and the stolbur phytoplasma (group 16SrXII-A) are associated with SMC in France. As "*Ca. P. fragariae*" and stolbur phytoplasma induce identical symptoms, the only way to identify the pathogen infected a given diseased plant rely on molecular DNA tests such as conventional nested PCR techniques. Because the two nested–PCR techniques targeting each of the two bacteria must be separately performed and are time consuming, a new approach using triplex real time PCR was developed for the routine detection of "*Ca. P. fragariae* " and stolbur phytoplasma. The real time PCR brings the advantage of being faster and present reduced risks of producing false positives. Furthermore, real-time PCR techniques provide the possibility of multiplexing by using probes with different compatible fluorescent dyes. Here, we present a new sensitive Taqman® method which permits the amplification and the differenciation of three DNA targets in one test: the map gene of stolbur phytoplasma, the spoT gene of "*Ca. P. fragariae*" and the cox gene of strawberry chloroplast taken as an internal control. The specificity and the efficiency of this method were determined and its evaluation for routine detection on field samples was assessed.

Sequencing studies for the identification and characterization of new and old Rubus viruses

MacFarlane, Stuart, McGavin, Wendy
Plant Pathology Programme, SCRI, Invergowrie, Dundee DD2 5DA, Scotland
Contact: s.macfarlane@scri.ac.uk

In Europe, raspberry plants are commonly infected with a complex of aphidtransmitted viruses that together cause raspberry mosaic disease (RMD). During the previous 30 years, by grafting and vector transmission to a range of red and black raspberry cultivars, these viruses have been loosely characterized and identified as Raspberry leaf spot virus (RLSV), Raspberry leaf mottle Virus (RLMV), Black raspberry necrosis virus (BRNV) and Rubus yellow net virus. An additional, very widespread virus, Raspberry vein chlorosis virus (RVCV), is spread by a different aphid vector. Recently some sequence data have been obtained for RYNV, BRNV and Raspberry mottle virus (RMoV), a virus found in plants showing RMD symptoms.

We have carried out sequencing studies using random amplification and mass analysis approaches and will present information on the relationship between RMoV, RLSV and RLMV, as well as the first data for RVCV and a novel, possibly segmented minus-strand RNA virus infecting raspberry.

Emerging strawberry virus and virus-like diseases in the world

Tzanetakis, I.E.
Department of Plant Pathology, Division of Agriculture, University of Arkansas, AR, USA

Strawberry production is increasing annually, with the world production exceeding four million tons. Strawberry caused virus diseases are also increasing. A decade ago there were about a dozen viruses known to infect strawberry. Today this number has more than doubled with several new viruses being identified in declining plants in the United States. There are now five aphid transmitted viruses - Crinkle, Mottle, Mild yellow edge, Vein banding and Chlorotic fleck. The former four remain the most common viruses in temperate areas around the world, whereas chlorotic fleck is not as common although it is present in both the United States and Europe. A new group of viruses, members of the genus *Crinivirus* have emerged as a new threat to strawberry in areas where vectors are present. There have been four new criniviruses discovered, with Strawberry pallidosis associated virus being the most common of the four, present in both the Old and New World. The ilarviruses that infect strawberry include Strawberry necrotic shock, Apple mosaic, Tobacco streak and Fragaria chiloensis latent viruses. Strawberry necrotic shock is the predominant ilarvirus in the United States whereas Fragaria chiloensis latent has significant presence in Chile. Tobacco streak is not very common in strawberry - previous reports of Tobacco streak in strawberry were probably misdiagnosis of Strawberry necrotic shock. Modern strawberry cultivation

has minimized the impact of nematode transmitted viruses but the elimination of methyl bromide may lead to the reemergence of this group of viruses in the future. With the knowledge we have acquired over the last decade it is now possible to have robust certification systems, the cornerstone for minimizing the impact and spread of strawberry viruses. With the massive increase of strawberry production in China recently, it will be curious to see if new viruses endemic to the area will infect strawberry.

Viruses and virus-like diseases in blueberry

Martin, R.
USDA-ARS, Horticultural Crops Research Laboratory, Corvallis, OR, USA

Over the past 15 years, blueberry production has increased dramatically in hectares grown and in the number of countries producing blueberries. It should be expected that as blueberry cultivation continues to expand into new areas, the plants will become exposed to viruses that have not been observed in blueberry previously. Most blueberries are grown in North America and that is where most viruses of the crop have been described. Blueberry scorch, an aphid-transmitted Carlavirus, can be very devastating and is the virus of greatest importance, in terms of management and quarantine. Blueberry red ringspot (BRRSV), a Caulimovirus, has been spreading in the mid and southern atlantic states, but spread has not been observed in the Pacific Northwest. Blueberry shock, a pollen-borne Ilarvirus, is interesting in that it causes a 100% crop loss for one year, and then plants recover to full production in subsequent years with no apparent loss in yield or fruit quality. The nepoviruses Tomato and Tobacco ringspot can be important in some areas, but Peach rosette mosaic has only been observed in experimental blocks. Blueberry shoestring is still an important virus in Michigan, but less so in other areas. Blueberry leaf mottle appears to almost have vanished, or at least has not been detected in the past five years. A new ringspot of blueberry in the southeastern USA has been observed since 2008 and several viruses have been partially cloned from dsRNA extracted from symptomatic plants, a Tobamo, Poty and possible Cilevirus. Whether any of these viruses individually or a in combination cause the disease is still unknown. There is also a phytoplasma, Blueberry stunt that occasionally affects blueberry. Cranberry, another widely planted Vaccinium, has two additional viruses reported, Tobacco streak and Cucumber mosaic. At this point, CMV is associated with a funky flower disease in cranberry.

Viruses and virus-like diseases in European *Ribes*

Špak, J., Kubelková, D., Přibylová, J., Špaková, V., Petrzik, K.
Biology Centre, Institute of Plant Molecular Biology, Czech Academy of Sciences, Branišovská 31, 370 05 České Budějovice, Czech Republic
Contact: fax: +420 385 310 356

During the last decade there was a tremendous advance in the identification of viruses and phytoplasmas infecting currants. The breakthrough – identification of the *Blackcurrant reversion virus* (BRV) as the causal agent of black currant reversion disease (BCRD) (Lemmetty and Lehto, 1999), economically the most important disease in currants, was followed by BRV genome sequencing (Latvala-Kilby and Lehto, 1999; Pacot-Hiriart et al, 2001) and development of PCR- based detection protocol (Jones and McGavin, 2002) - a prerequisite for effective control of the disease. However, the existing the E- and R-forms of BCRD were not elucidated by the studies on BRV variability (Lehto et al., 2004; Přibylová et al., 2008) and remain obscure. The gall mite (*Cecidophyopsis ribis* Westw.) a vector of BRV, is the most serious pest of black currant causing the 'big bud' damage. Measures to control *C. ribis* in Europe are restricted to sulphur sprays, since previously available chemical agents are now withhdrawn for environmental reasons. Successful resistance-breeding programme against *C. ribis* in black currant continues at the Scottish Crop Research Institute, U.K. and has recently led to the development of a PCR-based marker linked to the resistance (Brennan 2008). Hot water treatment of *Ribes* cuttings is now more widely used for the elimination of *C. ribis* from propagation material of sensitive cultivars.

Phytoplasmas were identified in black currant currants with symptoms of the BCRD (Špak et al., 2004) and in red and white currants with symptoms of the Full blossom disease (Navrátil et al., 2007), always together with BRV infection, but seem to be of less economic importance.

Gooseberry vein banding virus, a badnavirus transmitted by aphids and causing gooseberry veinbanding disease in currants was identified and partially sequenced, which enables its PCR detection in germplasm and propagation materials (Jones at al., 2001). Other viruses, which are subjects to testing during the

production of nuclear stock according to the new EPPO Certification scheme for *Ribes* (Anonym 2008) - *Strawberry latent ringspot virus*, *Raspberry ringspot virus*, *Arabis mosaic virus*, and *Cucumber mosaic virus* seem to be of declining importance. Nevertheless, there are still challenges for further research on e.g. no-name rhabdovirus reported first from black currant in the U.K. (Roberts and Jones 1997) and later from the Czech Republic (Přibylová et al., 2002).

A new research programme aimed at the production of 6 certified *Ribes* cultivars fulfilling the criteria of the EPPO Certification scheme started in the Czech Republic in 2009. It is based on co-operation between the Institute of Plant Molecular Biology (IPMB) and Research and Breeding Institute of Pomology Ltd. (RBIP). IPMB´s experience in detection and identification of viruses and phytoplasmas infecting *Ribes* combined with the RBIP´s experience in tissue-culture virus elimination, propagation and maintenance of virus-free clones in technical and field isolates and check of trueness-to-type fruit quality are employed in the project, which is funded by the National Agency for Agricultural Research grant No. QH 91224 and supported by COST 863 Action.

Oral Session II

Disease detection in quality systems for production of nursery stock

Meekes, Ellis[1], Bakker, Daniël[1], Konings, Hans[1], Toonen, Marcel[1], Jongedijk, Gerard[2]
[1]Naktuinbouw, P.O. Box 40, 2370 AA Roelofarendsveen, The Netherlands
[2]Progation Nuseries, Tienrayseweg 9a, 5961 NK Horst, The Netherlands
Contact: e.meekes@naktuinbouw.nl

Growers of nursery stock aim to produce a high quality product. Plants are selected and propagated, stocks are indexed and possibly certified through quality-plus systems. Detection tests for plant pathogens e.g. viruses are carried out in order to identify and remove infected plant material.

Although detection tests are reliable they cannot guarantee with a 100% certainty that the plant material is healthy. In recent years, laboratories like inspection services started to obtain accreditation for the detection tests. Validation is the key to describe the reliability of a detection test. It includes the determination of factors like detection limit, selectivity and specificity, repeatability, robustness etc. Besides the quality of the test other factors are important to consider. The successful detection of viruses in plants depends also on the right sampling procedures. Virus titres can vary with season, viruses of viruslike diseases may not be uniformly distributed throughout the plant, etc. Conditions under which plants are kept, disease pressure in the surrounding environment, presence of vectors will influence the disease status of the stock. For quarantine viruses a zero-tolerance is in effect, however, what will it mean when more sensitive detection methods are implemented?

In order to develop a certification system that includes disease testing these aspects need to be addressed. The paper will discuss these aspects using examples from detection of fruit viruses in current certification systems of Naktuinbouw, for instance Plum pox virus (PPV) in Prunus and virus diseases in strawberry.

Introduction of a certification program in a production of a plum planting material

Jevremović, Darko, Paunović, Svetlana
Fruit Research Institute, Kralja Petra I 9, Čačak, Serbia
Contact: darkoj@tfc.kg.ac.rs

Certification program for the production of a fruit planting material in not completely established in the Republic of Serbia. Despite this fact, Fruit Research Institute – Čačak started an introduction of a certification in a production of a plum planting material of cultivars developed at the Institute.

Propagate material from pomologically selected trees in commercial and experimental orchards was collected and grafted onto virus-free Myrobalan rootstock. A total of 89 plants of 15 plum varieties are included in this study: Čačanska lepotica, Čačanska rodna, Čačanska najbolja, Čačanska rana, Valjevka, Valerija, Čačanski šećer, Jelica, Timočanka, Boranka, Mildora, Krina, Pozna plava, Požegača, Stenley, and perspective hybrid 14/21.

All tests have been done according to the EPPO recommendations. Selected clones were tested on woody indicators Prunus tomentosa, *P. persica* and *P. serrulata* cv. Shirofugen. ELISA test was performed in

appropriate time for the detection of the following viruses: *Plum pox virus, Prune dwarf virus, Prunus necrotic ringspot virus*, Apple chlorotic leaf spot virus, Apple mosaic virus and Myrobalan latent ringspot virus. To increase the sensitivity of Plum pox virus detection, IC-RT-PCR was used. Material was also tested for the presence of '*Candidatus* Phytoplasma pruni' by nested-PCR method.

The presence of the viruses was found in 8 plants. Four plants of cv. Jelica were found to be positive in ELISA test with Apple chlorotic leaf spot virus. Latent infection with *Plum pox virus* was detected in 4 candidate clones (1 plant per cvs Valerija, Čačanska rodna, Čačanska lepotica and Požegača). The rest of the material was free of all other viruses. Not a single infection with '*Candidatus* Phytoplasma pruni' was found.

Susceptibility of a range of hazelnut cultivars to apple mosaic ilarvirus

Gentit, Pascal, Brans, Yoann, Ramat, Charlotte
Ctifl BP21 Lanxade 24130 LA force, France
Contact: gentit@ctifl.fr

The susceptibility of seven hazelnut cultivars to apple mosaic virus was compared (ApMV) for four years in an experimental orchard. Observations were carried out on different criteria regarding the symptoms severity on leaves as well as the yield and growth. All the cultivars were sensitive to ApMV with different level of symptoms. The yield and the growth were affected by ApMV infection.

Confirmation of the elimination of Apple stem grooving virus from apple trees by *in vitro* chemotherapy

Delano, James
Sidney laboratory, CFIA, 8801 East Saanich Road, Sidney, BC, V8L 1H3
Contact: Delano.James@inspection.gc.ca

Apple stem grooving virus (ASGV) is widespread in its distribution in apple trees. The virus causes tree decline and graft union necrosis in certain combinations of scion and rootstock, and attempts are made usually to control the virus where apples are grown commercially. ASGV is difficult if not impossible to eliminate by heat therapy. In 1996, in vitro cultures of apple infected with ASGV were grown for 9-12 weeks on media containing quercetin and ribavirin (10 ug/ml of each), then cultured on media free of these chemicals. Analysis by immunocapture (IC) RT-PCR failed to detect the presence of ASGV, while all untreated controls were positive. Treated and untreated cultures were subcloned, rooted, hardened, and eventually planted in the field. The plants were observed and tested regularly by IC/RT-PCR from 1998 - 2008. The treated plants were consistently negative by IC/RT-PCR, while untreated plants tested positive. After 10 years of testing by the sensitive IC/RT-PCR assay, it is safe to say that in vitro chemotherapy with quercetin and ribavirin is effective for the elimination of ASGV from apple plants.

Detection of four pome fruit viruses by ELISA and RT-PCR and cluster analyses of apple chlorotic leafspot virus (ACLSV) in apple and pear at the Canadian Clonal Genebank

Wang, L.[1,2], Michelutti, R.[1], Zunnoon-Khan, S.[4], Wang, G.[2], Hong, N.[2], Matić, S.[3], Myrta, A.[5]
[1]Greenhouse and Processing Crops Research Centre, Agriculture and Agri-Food Canada (AAFC), 2585 County Road 20, Harrow, Ontario, N0R 1G0, Canada
[2]National Indoor Conservation Center for Virus-free Germplasm of Fruit Crops
College of Plant Science and Technology, Huazhong Agricultural University, Wuhan,
Hubei 430070, P. R. China
[3]Plant Virology Group, ICGEB, Biosafety Outstation Ca'Tron, Via Piovega 23, 31050 Ca'Tron di Roncade (TV), Italy
[4] Federal Public Service Youth Intern Program, Agriculture and Agri-Food Canada, YMCA-AAFC.
[5] Certis Europe, Via A. Guaragna, 3 21047 Saronno (VA) Italy
Contact: micheluttir@agr.gc.ca; Fax: +1-519-738-2929

A survey of apple (*Malus domestica*) and pear (*Pyrus communis*) viruses was carried out at the Canadian Clonal Genebank (CCG), Greenhouse and Processing Crops Research Center (GPCRC), Harrow Ontario, Canada, during the fall/winter of 2007 and spring of 2008. In total, 438 accessions of apple and 122 of pear were tested using randomly collected samples of leaves and/or dormant cuttings and processed using Double Antibody Sandwich – Enzyme Linked

ImmunoSorbent Assay (DAS- ELISA). All samples were tested by ELISA for the presence of *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV) and *Apple mosaic virus* (ApMV). Specific infection rates for apples were ACLSV (48.1%), ASGV (10.0%), ASPV (6.6%) and ApMV (7.1%) and for pears ACLSV (42.6 %), ASGV (0 %), ASPV (91.8 %) and ApMV (90.1%). Seventeen of the apple accessions were also tested by multiplex RT-PCR and the results proved that RT-PCR is more sensitive than ELISA. One-step RT-PCR was used to amplify 22 ACLSV isolates (from apple and pear accessions originated in different countries) and to obtain the nucleotide sequence of the coat protein. Using these data a phylogenetic relationship among the ACLSV isolates was created. The nucleotide homology was 84%-100% among the 22 ACLSV isolates whereas the homology of amino acid was 91%-100%. The homology of nucleotide was 100% among the isolates of MAL0427, MAL0270, MAL0537, MAL0375, MAL0577, MAL0844, MAL0976, MAL0107, PYR0206, PYR0112, and PYR0129.

Keywords: *Malus*, *Pyrus*, DAS-ELISA, multiplex RT-PCR, one-step RT-PCR, cluster analyses.

Effects of thermotherapy temperatures on the growth of in vitro cultured pear plants and the elimination of three viruses

Hong, Ni, Wang, Guoping, Tan, Rongrong, Wang, Liping
College of Plant Science and Technology, Huazhong Agricultural University
Contact: whni@mail.hzau.edu.cn

In vitro-cultured plants of Pyrus pyrifolia cv. Huanghua, widely grown in central and southern China, were treated at different temperatures and the virus presence of regenerated plants was evaluated by ELISA and dot-blot hybridization. Results showed that treatment with a continue high temperature at 37 °C had a serious effect on the growth of in vitro pear plants and resulted in a high death rate, but virus-free plants could be regenerated from tips about 1 mm in length from survived plants. Treatment at 32 °C /38 °C changed every 8 h and 16 h had a mild effect on the survival and could improve the growth speed of in vitro pear plants. However, no virus-free plants were regenerated from shoot tips in size 0.5 mm to 1 mm after a treatment period for 50 days. Therefore, treatment temperatures were raised to 34 °C /42 °C changed every 8 h and 16 h. Although the death rates during the treatment were somewhat higher than that during the treatment at 32 °C /38 °C and no improvement on the growth speed compared to plants under a normal condition, a higher virus elimination efficiency (100%) was achieved by thermotherapy for more than 55 days combined with 0.5 mm to 1 mm shoot tip culture. The results were confirmed with three other pear varieties. Results also indicated that ACLSV was more sensitive to high temperature, which could be eliminated from shoot tips after a treatment period five-day shorter than ASGV and ASPV.

Keywords: thermotherapy, meristem culture, virus, pear

A one-Step Reverse transcription-polymerase chain reaction-based detection of olive trees viruses in Egypt

Sahar, A.Y.[1], Moawed, S.M[2], El-Sayed, M.[3], Shalaby, A.A.[1]
[1]Plant Pathology Res. Inst., ARC, Giza, Egypt
[2]General Administration of Seed Test and Certification (CASC), Giza, Egypt.
[3]Horticulture Res. Inst., ARC, Giza, Egypt
Contact: saharyoussef@link.net

Olive (Olea Europea) is a very important crop for the new reclamation land in Egypt. The estimated acreage of cultivated olive trees in Egypt is above 135.00 Feddens of which the total production is 500.000 tons per year (Ministry of Agriculture statistics, 2007). Olive trees are affected by several viruses and virus-like diseases. To date, 15 viruses that belong to eight genera have been isolated from olive trees. In a preliminary survey for the assessment of the sanitary status of olive tree in five different location in Egypt, leaf samples from 300 trees of 9 different cultivars were collected. Using virus-specific primers, a one-step RT-PCR assay was used to detect and identify each of the eight viruses most commonly found in olives. Namely, Cucumber mosaic virus (CMV), Olive latent ringspot virus (OLRSV), Olive latent virus-1 (OLV-1), Olive latent virus-2 (OLV-2), Olive leaf yellowing-associated virus (OLYaV), Strawberry latent ringspot virus (SLRSV), Cherry leaf roll virus (CLRV), and Arabis mosaic virus (ArMV). Among the eight viruses assayed , OLRSV (6.7%), OLV-1 (5.7%), CLRV (4.6%),

OLV-2 (2.7%), SLRSV (2.3%), OLYaV (1.3%) and ArMV (0.7%) were detected. The most common virus found was CMV which was prevailed with a high incidence of 24.7% in olive orchards. The use of one step RT-PCR has revealed efficient and reliable to detect most of the eight olive viruses found in Egypt. Surprisingly, the infection rate found is lower than the expected, if we take into consideratin previous surveys conducted in the Mediterranean area. Last, this technqiue could be useful to be applied in the decetion of olive viruses for production of certified plant along with the certification programs.

Oral Session III

The microarray detecting six fruit-tree viruses

Lenz, Ondrej, Petrzik, Karel, Spak, Josef
Biology Centre ASCR, v.v.i., Institute of Plant Molecular Biology
Contact: lenz@umbr.cas.cz

An oligonucleotide microarray for the detection of *Apple mosaic virus* (ApMV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV), *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV) and *Plum pox virus* (PPV) was developed. Specific fragment of viruses were PCR amplified by using Cy3-labelled primers and hybridized onto microarray and detected simultaneously. Advantages and drawbacks of the method will be discussed.

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Validation of a microarrays protocol for detection and genotyping of PPV reference samples

Pasquini, Graziella
CRA-PAV Centro di Ricerca per la Patologia Vegetale via C.G. Bertero, 22 00156 Rome, Italy
Contact: graziella.pasquini@entecra.it

A genomic strategy for PPV identification has been recently developed (Pasquini et al., 2008). The method is based on using a 70-mer oligonucleotide DNA microarray chip capable of simultaneously detecting and genotyping PPV strains. Universal and specific probes have been identified and used with a sensitive protocol of hybridization using an indirect fluorescent labelling of cDNA product with cyanine that enhanced the sensitivity of the virus detection without the use of the PCR amplification step About 30 samples belonging to a PPV isolates collection, including M, D, EA, and C strains, have been used for the validation and standardization of the protocol. The sensitivity, specificity, repeatability and reproducibility of the protocol have been tested.

Real time PCR quantitative analysis of plant viruses in stone fruit trees tissues

Jarosova, Jana, Kundu, Jiban Kumar
VURV, Drnovska 507, 16106 Prague
Contact: j.jarosova@vurv.cz

Real Time PCR assays aiming at quantifying the level of plant infection by pathogens have been increasing for the last few years. Within microbiology, the application of Real Time PCR has had the biggest impact upon the field of virology. However, Real-time PCR application in fundamental plant virology studies is still lagging behind. The use of relative and absolute quantification is discussed in this study. Also, case studies including Plum pox virus, Prune dwarf virus and Apple chlorotic leaf spot virus are presented.

Simultaneous detection of the main stone fruit viruses and viroids by non-isotopic molecular hybridization polyprobe

Sánchez-Navarro, Jesús[1], De la Torre, Rodolfo[2], Pallás, Vicente[1]
[1]Instituto de Biología Molecular y Celular de plantas, Universidad Politécnica de Valencia-CSIC, Avenida de los naranjos s/n, 46022 Valencia, Spain
[2]FES-IZTACALA-UNAM. Unidad de Biotecnología y prototipos (UBIPRO). Avenida De los Barrios 1, Los Reyes Iztacala, Tlalnepantla, Mexico. 54090
Contact: jesanche@ibmcp.upv.es

Stone fruit are affected by a large number of economically important and common viruses and viroids. Relevant pathogens include the Ilarvirus *Apple mosaic virus* (ApMV), *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) and *American plum line pattern virus* (APLPV); the potyvirus responsible of the 'Sharka' disease, *Plum pox virus* (PPV), the Trichovirus *Apple chlorotic leaf spot virus* (ACLSV), the Foveavirus *Apricot latent virus* (ApLV), the ampelovirus *Plum bark necrosis stem pitting associated virus* (PBNSPaV) and the viroids *Peach latent mosic viroid* (PLMVd) and the *Hop stunt viroid* (HSVd). A critical step in the sanitary status of the crop is the detection of the virus/viroid in the early stage of the infection. In this sense, the efforts in the diagnostic methods have been addressed to improve the sensitivity but also to reduce the processing time/cost of the analysis by performing the simultaneous detection of several pathogens in a single assay. The multiple detection of several pathogens by using non-radioactive molecular hybridization technique and a unique riboprobe 0 'Polyprobe' carrying partial sequences of different viruses fused in tandem has probed to be very attractive (Herranz et al., 2005. J. Virol. Methods 124, 49-55). In the present study we have used the polyprobe technology to detect eight viruses and two viroids affecting the stone fruit crop.

Detection of cherry leafroll virus, prune dwarf virus and prunus necrotic leafroll virus in prunus pollen

Horner, Mary[1], Van den Brink, Roy[2], Hough, Ellena[1], Eastwell, Kenneth[3], Howell, William[3]
[1]Plant and Food Research, Hawke's Bay, Private Bag 1401 Havelock North, New Zealand
[2]Plant and Food Research, Palmerston North, Private Bag 11600 Palmerston, North New Zealand
[3]Washington State University- IAREC, Prosser 99350
Contact: mhorner@hortresearch.co.nz

Current protocols requires that pollen imported into New Zealand and used in breeding programmes needs to undergo lengthy testing of progeny in a Level 3 Quarantine Facility for potential transmission of viruses. A reliable method was developed to directly test the pollen material by Reverse transcription polymerase chain reaction (RT-PCR). Experiments testing various maceration methods on pollen material and the effect of various associated antioxidant agents upon Ribonucleic acid (RNA) extraction and of polyphenolic inhibition of RT-PCR were investigated. An optimum protocol for RNA extraction from pollen was developed using a ball bearing silica extraction method in association with a modified extraction buffer. This protocol reliably detected the presence of *Prunus necrotic ringspot virus, Prune dwarf virus* and *Cherry leafroll virus* in pollen or anther material. *Cherry leafroll virus* was reliably detected in pollen samples containing 0.01 % infected pollen. If adopted by Biosecurity New Zealand, this method, which could be used to directly analyze the pollen as it enters New Zealand, should simplify testing protocols and reduce compliance costs and time associated with the importation of pollen material.

Reverse transcription loop-mediated isothermal amplification (RTLAMP): A novel method for the detection of *Peach latent mosaic viroid* (PLMVd)

Boubourakas, I.N.[1], Fukuta, S.[2], Fagioli, F.[3], Luigi, M.[3], Barba, Â.M.[3], Kyriakopoulou, P.E.[1]
[1]Agricultural University of Athens, Department of Plant Production Science, Plant Pathology Laboratory, 11855 Athens, Greece
[2]Biotechnology Group, Aichi Agricultural Research Center, 1-1 Sagamine, Yazako, Nagakute, 480-1193 Aichi, Japan
[3]CRA - Centro di Ricerca per la Pathologia Vegetale, Via C.G. Bertero 22, 00156 Roma, Italy

A reverse transcription loop-mediated isothermal amplification method (RTLAMP) for the detection of Peach latent mosaic viroid (PLMVd) was developed. LAMP is a novel nucleic acid amplification method, quite simple, preformed under isothermal conditions (60-65ºC), with high specificity, efficiency and rapidity. It is characterized by the use of four different primers: F3 (forward outer primer), B3 (backward outer primer), FIP (forward inner primer), BIP (backward inner primer), specifically designed to recognize 6 distinct regions on the target sequence. Four primer sets (OLD, OLD1, NEW and FUKUTA'S) were designed. Based on preliminary experiments, the set OLD1 was selected for further evaluation and proved to be highly specific. Simple and accelerated RT-LAMP was preformed using non-degenerate and degenerate F-Loop and B-Loop primers respectively. In the case of the accelerated protocol, the viroid could be detected within only 31 min using as template T-RNA or trace of plant tissue, taken by sterilized toothpick, compared to 180 min with a RT-PCR assy. In addition, the RT-LAMP method was found to be 100 times more sensitive than the RT-PCR. Using RT-LAMP

PLMVd was detected in naturally infected peach, plum apricot, pear, wild pear and quince plants as well as in an Italian peach calico infected plant.

Sensitive detection and strain discrimination of plum pox virus using Rt - Real Time Pcr - Fret Method

Malinowski, Tadeusz
Research Institute of Pomology and Floriculture, Pomologiczna 18, 96-100 Skierniewice, Poland
Contact: tmalin@insad.pl

A new method of Plum pox virus detection and strain identification / discrimination, based on real time PCR with fluorescence resonance energy transfer (FRET) probes, has been developed. One 'universal' donor probe as well as D and M (Rec) specific acceptor probes labelled with different fluorophores are utilised for one-tube detection and typing of amplicons during the reaction. Two different channels are used for simultaneous detection of D and M (Rec) type isolates. Post-reaction melting curve analysis provides further data and allows also positive identification of EA type isolates, based on different melting curve profiles. The method is fast, sensitive and provides a way of quantification of PPV types in mixed infections.

Application of scanning electron microscopy for diagnosis of phytoplasmas in single and mixed infections in papaya

Lebsky, Vladimir[1], Poghosyan, Arevik[1], Silva-Rosales, Laura[2]
[1]Centro de Investigaciones Biologicas del Noroeste (CIBNOR), Mar Bermejo 195, Col. Playa Palo de Sante Rita, A.P.128, La Paz, BCS, 23090, Mexico
[2]Centro de Investigacion y de Estudios Avanzados del IPN, Campus Guanajuato, Km. 9.6 Lib. Nte. Carr. Irapuato-León, Irapuato, Gto. 36500, Mexico
Contact: arevik04@cibnor.mx

Virus and virus-like diseases are a major threat to papaya production in Mexico, where it is a leading commercial crop in the States of Michoacan, Oaxaca, Jalisco, Nayarit, Yucatán, Veracruz and Chiapas. Additionally, the State of Baja California Sur is the main producer of organically grown papaya destined for USA market. Infection with papaya ringspot virus (PRSV), papaya mosaic virus (PMV) and phytoplasma has been reported from different Mexican states. Some symptoms of yellow type diseases such as mosaics, stunting, bunchy top and leaf chlorosis, necrosis and malformations are somewhat similar in appearance, but provoked by distinct pathogens. A set of complex diagnostic procedures are needed for correct and precise diagnosis of pathogens so that timely and correct control measures can be taken.

Using scanning electron microscopy (SEM) technique phytoplasmas were detected in the phloem tissues of field and greenhouse-indexed papaya plants from Baja California Sur. The 32 regional varieties as well as cv. Maradol with numerous symptoms of dieback, mosaics, bunchy top and yellow crinkle were analyzed. The pathogen was detected in stems, leafstalks, roots, axillary leaflets, leaf veins and in germinated seeds within the fruit. No viral infection was revealed by test-plants and molecular techniques. Analysis by a SEM technique of papaya samples from Veracruz and Irapuato, both field-grown and mechanically infected with PRSV and PMV in various combinations, revealed phytoplasmas in phloem of most of the tested samples. In some cases, along with phytoplasma, rod-shaped bacteria were also distinguished. Further investigations would determine phylogenetic relations between phytoplasmas from samples collected from different states and provide more information about mixed infections and disease epidemiology.

Oral Session IV

New viruses identified in fig trees exhibiting fig mosaic disease

Tzanetakis, Ioannis[1], Martin, Robert[2]
[1]University of Arkansas, Dept. of Plant Pathology
[2]USDA-ARS, Corvallis, OR
Contact: itzaneta@uark.edu

Though fig mosaic disease has been known for decades but the causal agent has been elusive. Here we present data on the incidence of at least four new viruses isolated from fig trees exhibiting mosaic

symptoms. One of the viruses is closely related to the recently identified European mountain ash ringspot-associated virus. The second is a member of the genus *Badnavirus* and is related most closely to *Citrus yellow mosaic virus*. The other two viruses belong to the family Closteroviridae. Detection protocols have been developed for each of the four viruses and a limited survey of fig mosaic trees carried out. The first agent was present in all samples while the rest were found in a subset of the samples. The incidence of the European mountain ash ringspot-associated virus-like agent in all tested material and its morphology that is identical to the fig mosaic agent indicates that the virus is indeed the causal agent of the disease. The provisional name Fig mosaic associated virus has been given to the virus. Transmission trials are under way to identify vectors of the latter three new viruses and better understand their involvement in disease development.

Fig latent virus 1, a new putative member of the family flexiviridae

Gattoni, Giuliano[1], Minafra, Angelantonio[1], Castellano, Maria Antonietta[1], De Stradis, Angelo[1], Boscia, Donato[1], Elbeaino, Toufic[2], Digiaro, Michele[2], Martelli, Giovanni Paolo[1]
[1]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale del CNR, Unità Organica di Bari, Via Amendola 165/A, 70126 Bari, Italy.
[2]Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano, Bari, Italy.
Contact: ggattoni_99@yahoo.com

A virus with filamentous particles ca. 700 nm long, denoted Fig latent virus 1 (FLV-1) is widespread in Apulian (southern Italy) fig orchards, in trees showing or not symptomts of mosaic disease. FLV-1 was consistently identified in different batches of symptomless seedlings, which prompted its naming. It was transmitted by sap inoculation to a very restricted range of herbaceuos hosts without inducing apparent symptoms and was transmitted through fig seeds to a very high percentage (80 to 100%). FLV-1 was succesfully purified from root tissues of infected figs. A virus-specific antiserum raised in rabbits, proved useful for its detection in fig leaf dips by immune electron microscopy. Bundles of filamentous particles were observed in the cytoplasm of parenchyma cells of infected fig trees and seedlings. The viral genome is a single stranded positive sense RNA about 8,000 nt in size, 6,500 of which have been sequenced starting from the polyadenylated 3' terminus. Genomic RNA consists of four open reading frames encoding, in the order from the 5' to the 3' end, the replicationassociated protein (ORF1), a 42 kDa putative movement protein (ORF2), the 46 kDa coat protein (ORF3), and a 12 kDa protein with nucleic acid binding properties. The genome structure and organization resembles that of members of genus *Trichovirus*, family *Flexiviridae* and, indeed, FLMaV-1 clusters with trichiviruses in phylogenetic trees constructed with the coat protein and the movement protein sequences. However, a distinct difference with all members of the genus rests with the size of the coat protein subunits (46 versus 22-27 kDa) and the presence of ORF4, which is not represented in all trichoviruses.

Keywords: *Ficus carica*, *Flexiviridae*, FLV-1, RACE-PCR, sequencing

Molecular characterisation of viruses from kiwifruit

Pearson, Michael[1], Cohen, Daniel[2], Chavan, Ramesh[1], Bluin, Arnaud[2]
[1]School of Biological Sciences, The University of Auckland, Private Bag 92019, Auckland, New Zealand
[2]The New Zealand Institute of Plant and Food Research Ltd., Auckland, New Zealand
Contact: m.pearson@auckland.ac.nz

In 2003 Apple stem grooving virus was discovered in Actinidia accessions from China, being held in quarantine in Auckland. Subsequent examination of kiwifruit germplasm from the same source has detected several additional viruses, including a ~300 nm rigid rod related to Ribgrass mosaic virus (*Tobamovirus*) and a 700-750 nm flexuous virus related to Citrus leaf blotch virus (*Flexiviridae*). Currently these viruses have not been reported from commercial kiwifruit crops in New Zealand or elsewhere. The biological properties of the viruses from kiwifruit and their phylogenetic relationships with similar viruses from other plants will be described, and the possible implications for the international movement of Actinidia germplasm will be discussed.

Towards the elucidation of the taxonomic position of Prunus-infecting viral agents belonging to the Foveavirus genus and their relationship with Apple stem pitting virus

Marais, Armelle[1], Youssef, Fater[1], Faure, Chantal[1], Barone, Maria[2], Maliogka, Varvara[3], Katis, Nikos[3], Foissac, Xavier[1], Gentit, Pascal[4], Candresse, Thierry[1]
[1]UMR GD2P, IBVM, INRA, Université Bordeaux2, BP81, 33883 Villenave d'Ornon Cedex, France
[2]Facolta di Agraria, Universita di Napoli "Federico II" 80055 Portici (NA), Italy
[3]Faculty of Agriculture, Aristotle University of Thessaloniki, 541 24 Thessaloniki, Greece
[4]Ctifl, Centre de Lanxade, 24130 La Force, France
Contact: amarais@bordeaux.inra.fr

Surveys in Greece, Italy and Iran on pome and stone fruit trees identified viral agents closely related to one another and belonging to the Foveavirus genus. In the 289 bp RdRp fragment generated by PDO RT-PCR (Foissac et al., 2005), the amino acid genetic distance between *Apple stem pitting virus* (ASPV) and *Apricot latent virus* (ALV) is around 12.5%. Divergence values in the same range were observed between the uncharacterized agents and ASPV or ALV, impeding their taxonomic assignment. Complete genomic sequences were determined for one of these agents (apricot isolate A18) and for isolates of ALV (PAS LA2 isolate) and of the closely related *Peach sooty ringspot virus* (PSRSV, Caserta 12 and SB12452 isolates). The preliminary data obtained from the comparisons of the sequences showed two different situations depending on the genomic regions considered. In the region comprising the RdRp gene and the two first genes of the triple gene block (ORF2 and ORF3), there is a close relationship between the *Prunus*-infecting *Foveaviruses* and ASPV, with a genetic divergence at the amino acid level below the species demarcation criteria for the Foveavirus genus. However, in the region comprising the ORF4 and the CP gene, a much higher divergence level is observed. In particular, the region of the first 211 amino acids of the CP seemed to be highly variable (up to 74% of divergence between ASPV and ALV-like sequences). Interestingly, the C-terminal part of the CP is on the contrary very conserved (between 97.3% and 88.6% of identity). These findings suggest the existence of recombination in the evolutionary history of these agents or, conversely, the adaptation of ASPV-related viruses to a new type of host, such as *Prunus*. (Foissac et al., 2005. Phytopathology, 95,617-625)

Oral Session V

Peach latent mosaic viroid: further dissection of the molecular determinant inducing peach calico disease

Navarro, Beatriz[1], Delgado, Sonia[2], Rodio, Maria Elena[1], Flores, Ricardo[2], Di Serio, Francesco[1]
[1]Consiglio Nazionale delle Ricerche, Istituto di Virologia Vegetale, 70126 Bari, Italy,
[2]Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Universidad Politécnica de Valencia, 46022 Valencia, Spain
Contact: f.diserio@ba.ivv.cnr.it

Peach calico (PC) disease is a severe chlorosis induced by Peach latent mosaic viroid (PLMVd) (1). This symptomatology is exclusively elicited by PLMVd variants containing a specific insertion of 12-13 nt that, in the proposed secondary structure of the viroid RNA, folds into a hairpin composed by a short stem of four base pairs capped by a U-rich loop. In previous works, we showed that both the loop and the stem of this structural element play critical pathogenic roles (2-4). Here we have further dissected this structural element by sitedirected mutagenesis, bioassay of the mutated variants and molecular analysis of the progenies, confirming that not only the size but also nucleotide composition of the stem strongly affect the pathogenic properties. We have also observed an uneven distribution of PLMVd variants in the chlorotic and adjacent green leaf sectors of GF-305 seedlings inoculated with in vitro dimeric transcripts of PC-inducing variants: new variants, most likely non-pathogenic and with a modified stem of five base pair capped by a U-rich loop, were generated during the infection and accumulated preferentially in the green sectors. Our data support the use of PLMVd as model system for studying the pathogenesis and evolution of non-coding RNAs.

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3. Rodio et al. (2006). J. Gen. Virol. 87: 231-240; 4. Rodio et al. (2007). Plant Cell 19: 3610-3626.

Towards dissecting the structural determinant of peach latent mosaic viroid inducing mosaic symptoms

Delgado, Sonia[1], Navarro, Beatriz[2], Gentit, Pascal[3], Di Serio, Francesco[2], Flores, Ricardo[1]
[1]Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Universidad Politécnica de Valencia, 46022 Valencia
[2]Istituto di Virologia Vegetale del CNR, 70126 Bari, Italy
[3]Centre Technique Interprofessionel de Fruits et Légumes de Lanxade, 24130 Prigonrieux, France
Contact: rflores@ibmcp.upv.es

Most isolates of *Peach latent mosaic viroid* (PLMVd) (1,2) do not incite foliar symptoms, but a few number cause peach mosaic (PM) or peach calico (PC), an extreme albino phenotype. The structural determinant of PC maps at an insertion of 12-13 nt folding into a hairpin capped by a U-rich loop (3,4). However, the molecular determinant of PM remains unidentified because, not being associated with a specific insertion, it could reside in one or more domains of the branched PLMVd conformation. Moreover, cloning of latent and PM-inducing PLMVd isolates has revealed that they are formed by complex populations of variants, and bioassays on GF-305 peach seedlings of individual variants have shown that the biological properties of PLMVd isolates depend on the complexity of their populations and on the presence of specific variants. From the variants recovered from a typical PM isolate (GDS), we have selected for further dissection one (gds6) that is very infectious and elicits consistently a characteristic PM. We have initially focused on G339, a position that appears associated with PM in multiple alignments with other variants. To determine the role of G339 in infectivity and symptoms, blocks of GF-305 seedlings were inoculated with in vitro transcripts of recombinant plasmids containing dimeric tandem inserts of PLMVd-cDNAs with all possible changes at this position introduced by site-directed mutagenesis. Deletion of G339 reduced the number of plants showing symptoms or their severity, while substitutions to A, U or C had moderate effects. Sequencing of the resulting progenies should provide hints on the feasibility of this approach for identifying the PM determinant.

1. Hernández and Flores (1992). PNAS USA 89: 3711-3715; 2. Flores et al. (2006). Mol. Plant Pathol. 7: 209-221 ;
3. Malfitano et al. (2003). Virology 313: 492-501 ; 4. Rodio et al. (2007). Plant Cell 19: 3610-3626.

Deep sequencing of the viroid-derived small RNAs accumulating in peach infected by two symptomatic variants of peach latent mosaic viroid

Di Serio, Francesco[1], Giesel, Andreas[2], Navarro, Beatriz[1], Delgado, Sonia[3], Martínez de Alba, Angel-Emilio[3], Donvito, Giacinto[4], Flores, Ricardo[3]
[1]Istituto di Virologia Vegetale del CNR, 70126 Bari, Italy
[2]Istituto di Tecnologie Biomediche del CNR
[3]Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC)
[4]Istituto di Nazionale di Fisica Nucleare, 70126 Bari, Italy
Contact: rflores@ibmcp.upv.es

Northern-blot hybridization of nucleic acid preparations from plants infected by representative members of both viroid families has revealed the presence of 21- 24 nt viroid-derived small RNAs (vd-sRNAs) with the structural properties of the small interfering RNAs (siRNAs), the characteristic effectors of RNA silencing (1). These results strongly support that viroids are elicitors and targets of the RNA silencing machinery of their hosts. Moreover, conventional sequencing of the vdsRNAs from two members of the family Pospiviroidae (2) and one of the *Avsunviroidae* (3) has confirmed that they are predominantly of 21 and 22 nt and (+) polarity. This approach, however, generates incomplete datasets and, therefore, the ensuing conclusions may be biased. To overcome this limitation we have subjected to deep sequencing the vd-sRNAs accumulating in GF-305 peach seedlings infected by two molecular variants of Peach latent mosaic viroid (PLMVd), one causing peach mosaic and the other peach calico (4). The analysis of the resulting PLMVd-sRNAs permits to draw inferences about the dicer-like enzymes catalyzing their genesis and the RNA substrates upon which they act, as well as to assess whether some specific PLMVd-sRNAs containing the pathogenicity determinant of peach calico may be involved in symptom development.

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Molecular characterization and variability analysis of Apple scar skin viroid

Walia, Yashika, Kumar, Yogesh, Rana, Tanuja, Hallan, Vipin, Ram, Raja, Zaidi, A.A
Plant Virology Lab, Institute of Himalayan Bioresource Technology, CSIR, Palampur, 176061, Himachal Pradesh, India
Contact: yashikawalia@gmail.com

Apple scar skin viroid (ASSVd) infection is a major limitation to apple fruit quality and causes huge economic losses. In orchard surveys of apple growing regions of Himachal Pradesh (H.P), ASSVd was detected and molecularly characterized from apple plants showing dappling symptoms. Ten clones were sequenced out of which seven new sequence variables of ASSVd were found. The clones showed significant sequence variability (94-100%) with each other. Variability was more common in the pathogenic domain of the viroid genome. Four of the clones were 330 nucleotides (nt) long and the other six that of 331 nt. Multiple alignment and phylogenetic analysis of the present isolates was carried out taking different ASSVd sequences available in EMBL database reported from different countries. In phylogenetic study, the present clones showed close affinity with some Chinese and Korean isolates. The study reports seven new variables of ASSVd and also gives a first molecular evidence of a viroid infection (ASSVd) in apple from India. Infectious clones of the ASSVd were constructed for in vitro mutagenic studies.

The molecular characterization of hop stunt viroid isolates associated to dapple fruit and fruit rugosity in plum seedlings suggests a possible role of the breeding in viroid dissemination

Luigi, Marta[1], Faggioli, Francesco[1], Barba, Marina[1], Giunchedi, Luciano[2]
[1]CRA-Centro di Ricerca per la Patologia Vegetale, 00156 Rome, Italy
[2]Dipartimento di Scienze e Tecnologie Agroambientali - Università di Bologna
Contact: francesco.faggioli@entecra.it

Hop stunt viroid (HSVd) has been found in a wide range of hosts where the infection appears to be latent, whereas in others (hop, cucumber, citrus, Japanese plum, peach) it is frequently pathogenic. In this work, the presence of HSVd has been found to be associated to symptoms of dapple fruit and occasionally fruit rugosity in plum seedlings 4 years old obtained from cross breeding. In particular, the plum samples have been analyzed for the presence of viroids (HSVd and PLMVd) and viruses (PPV, ACLSV, ApLV, PDV, PNRSV, ApMV). HSVd was found in all symptomatic samples, whereas no other viruses or viroids were found in the analyzed samples with the exception of ACLSV, rarely and sporadically detected. All the HSVd isolates have been cloned and sequenced and a high percentage of homology (98-100% with the exception of one clone obtained from the isolate F20) has been obtained among them, making it possible to hypothesize a potential unique origin of the infection. For this purpose, the 'Black Sunrise' and 'Black Glow' plum trees, the cultivars used in the breeding as pollen donors, have been analyzed. The results showed the presence of HSVd in parental plants and the obtained sequences resulted similar (95-100 %) to the isolates found in the seedlings, suggesting a possible role of the breeding in the dissemination of the viroid. In the next months, pollen from infected parental plants will be collect and analyzed to confirm the possible presence of HSVd. Moreover, these results confirm the hypothesis that HSVd could be the cause of the dapple fruit and fruit rugosity diseases.

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Two novel variants of hop stunt viroid associated with yellow corky vein disease of sweet orange and split bark disorder of sweet lime in Iran

Bagherian, Seyed Ali Akbar, Izadpanah, K.
College of Agriculture, Shiraz Univ., Shiraz, Iran
Contact: izadpana@shirazu.ac.ir

Yellow corky vein was reported as a graft-transmissible disease of lime in India. It was attributed to infection by Hop stunt viroid (HSVd)and citrus exocortis viroid (CEVd). Recently similar symptoms have been observed in Washington navel orange in Jahrom and Darab in the Fars province of Iran. It is characterized by yellowing and suberization of veins followed by tree decline. In the case of sweet lime, split bark is a disorder of increasing importance in the Fars province. It is characterized by cracks in the bark of the main stem which may spread to branches of the tree. Since these symptoms resembled those of certain viroids, a study was undertaken to determine possible association of viroids with the disorders. Reverse transcription polymerase chain reaction (RT-PCR) followed by cloning and sequencing of PCR products and dot-blot hybridization were used to identify the viroids associated with the diseases. Comparison of molecular properties (nucleotide composition, primary and secondary structures, molecular weights, phylogenetic relationships, percent nucleotide similarity and difference and restriction sites) of viroid variants were carried out. It was found that, a novel variant of hop stunt viroid (HSVd-sycv) was associated with yellow corky vein disease of Washington navel and another new variant (HSVd-sb) with split bark disorder of sweet lime. No other viroids were constantly detected. HSVdsycv was closely related to noncachexia variant of hop stunt viroid (HSVd-cit) but only with 93.7% homology with HSVd-lycv. It differed in a single nucleotide from HSVd-cit, in the variable domain in the so-called "cachexia expression motif". HSVd-sb had only 94.8% homology with a noncachexia variant of hop stunt viroid (CVd-IIa-117) which causes mild bark-cracking symptoms on St. Michael orange-Wakayama rootstocks. According to the performed molecular comparisons, HSVd-sb differed from CVd-IIa-117 in "cachexia expression motif" and probably severe cracks induced by HSVd-sb occurred because of variation in this motif.

Oral Session VI

Infectious uncloned full-lenght cDNAs as a tool for the study of the etiology of fruit tree viral diseases

Youssef, Fater, Marais, Armelle, Faure, Chantal, Candresse, Thierry
UMR GDPP, IBVM, INRA, Université Bordeaux2, BP 81, 33883 Villenave d'Ornon Cedex, France
Contact: fyoussef@bordeaux.inra.fr

Frequent mixed viral infections make it difficult to understand the respective contribution to the observed symptomatology of individual viruses present in fruit trees. As a tool for the study of the etiology of fruit tree viral diseases, we have developed a simple and generic method to separate viral complexes through the use of uncloned infectious full-length cDNAs, thus bypassing several tedious or limiting steps (need for an herbaceous host, problems of viral purification or for cloning of infectious cDNAs...). As a model system we are using *Apricot pseudo-chlorotic leaf spot virus* (APCLSV) and the closely related *Apple chlorotic leaf spot virus* (ACLSV). In the first strategy tested, the 7.6 kbp full-length viral cDNA is amplified from crude total nucleic acid extracts using a forward primer containing the bacteriophage T7 RNA polymerase promoter. Viral RNAs are then produced by in vitro transcription and directly inoculated to host plants. In the second strategy (Fakhfakh et al., 1996), megaprimers linking signals for transcription in planta (CaMV 35S promoter and Nopaline synthase terminator) to the ends of the viral genome are used for the synthesis of a long PCR product integrating the promoter, the full-length viral cDNA and the terminator, which is biolistically inoculated to host plants.

The preliminary results obtained with the first strategy show infection rates of the inoculated herbaceous hosts of between 10 and 50%. Results of inoculation of GF 305 peach seedlings with the same transcripts and of herbaceous and woody hosts with the PCR products developed using the second strategy will also be presented. These techniques should find wide application for the definite association of specific symptoms to a given agent, even when it is only available as mixed infections. (Fakhfakh et al. 1996. Journal of General Virology, 77, 519-523.).

Expression of the coat protein genes of PNRSV and PDV in the synergistic disease peach stunt.

Kim, B.T.[1], Gibson, P.G.[2], Scott, S.W.[3]
[1]Genetics & Biochemistry, Clemson University, Clemson, SC 29634, USA
[2]Gwinnett Technical College, Bioscience, Lawrenceville, GA 30043, USA
[3]Entomology, Soils & Plant Sciences, Clemson University, Clemson, SC 29634, USA
Contact: sscott@clemson.edu

Simultaneous infections of peach (*Prunus persica* Batsch L.) with the two ilarviruses, Prunus necrotic ringspot virus (PNRSV) and Prune dwarf virus (PDV), produce a synergistic disease referred to as “peach stunt”. Previous work showed significant differences in the expression of the coat protein (CP) genes. In the presence of PNRSV, an up to 17-fold reduction in the amount of (+) strand RNA 3 of PDV, as compared to similar trees infected with PDV alone, was observed. However, the presence of PDV had no effect on the concentration of (+) strand RNA 3 of PNRSV (Scott et.al., 2001. Acta Hort. 550:229-236). This work re-examines and extends these observations using multiplex real-time PCR. Probes to both the plus and minus strands of the RNA 3 of each virus were designed and synthesized. The comparative CT method (ΔΔCT) was used for relative quantitation of gene expression. A reduction in the amount of (+) strand RNA 3 of PDV observed in the earlier work was not seen using real-time PCR. However, in a time course experiment with samples collected at 14-day intervals for 6 weeks, there was a substantial increase in the concentration of the (+) strand of RNA 3 of PDV after 14 days irrespective of whether the virus was present as a sole infecting agent or as a co-infection with PNRSV. At this same point in time there was a decrease in concentration of (+) of the RNA 3 of PNRSV. By the next sample date the concentrations of the (+) strand of RNA 3 of both viruses had returned to “normal”. The results are discussed in relation to the most extensively studied plant viral synergism synergism (PVX and PVY) and to known changes in concentration of PNRSV and PDV based on earlier observations made using ELISA.

Investigations on virus occurrence in different tissues throughout the year and sequence variability of Apple stem pitting virus

Arntjen, Anja, Jelkmann, Wilhelm
Julius Kühn Institute, Institute for Plant Protection in Fruit Crops and Viticulture, Schwabenheimer Str. 101, 69221 Dossenheim, Germany
Contact: Anja.Arntjen@jki.bund.de

Apple stem pitting virus (ASPV) is a latent virus of apple and belongs to the genus Foveavirus in the family Flexiviridae. Mixed infections with *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem grooving virus* (ASGV) can lead up to 60% yield loss.

Virus RNA extractions were made from buds or leaves, phloem and roots from four different trees at least once a month throughout the year. A Real- Time PCR assay was evaluated and the virus titer was determined.

The complete sequence of the ASPV isolate PB 66 was obtained. It is 9363 bp in size and consists of 5 open reading frames. Comparison with the isolate PA 66 of the coat protein sequence showed identity of 82% in the sequence and 81% amino acid similarity. The whole sequence showed 80% sequence identity to isolate PA 66. Selected portions of the newly analyzed genotype were compared to database entries to analyze the variability of ASPV.

Close similarities between Cherry chlorotic rusty spot disease from Italy and Cherry leaf scorch from Spain

Barone, M.[1], Covelli, L.[2], Di Serio, F.[3], Garcia Becedas, M.T.[4], Ragozzino, A.[1], Alioto, D.[1]
[1]Dipartimento di Arboricoltura, Botanica e Patologia Vegetale, Università di Napoli, 80055 Portici, Italy
[2]Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC), Universidad Politécnica de Valencia, 46022 Valencia, Spain
[3]Istituto di Virologia Vegetale del CNR, 70126 Bari, Italy
[4]Junta de Extremadura, Servicio de sanidad vegetal, 10600 Plasencia, Spain
Contact: alioto@unina.it

Cherry chlorotic rusty spot (CCRS), a disease affecting sweet and sour cherry in southern Italy was regularly found associated with an unidentified fungus and with a complex pattern of viral-like double-stranded RNAs as well as with two small circular RNAs (cherry small circular RNAs, cscRNAs). Further studies revealed that i) the ds-RNAs correspond to the genome of different micoviruses belonging to the genera *Chrysovirus*, *Partitivirus* and *Totivirus* and ii) the two viroid-like RNAs consist of two groups of variants with similar sequence but differing in size (394–415 and 372–377 nt for cscRNA1 and cscRNA2, respectively). Here we report that the dsRNAs of *Chrysovirus* and Parti*t*ivirus have been detected by RT-PCR analysis with CCRS specific primers in nucleic acid preparations from cherry leaves affected by cherry leaf scorch (CLS) in Spain, a disease whose etiological agent is the ascomycetes *Apiognomonia erythrostoma*, order *Diaporthales*. Moreover, Northern-blot hybridization assays showed that a viroid-like RNA co-migrating and sharing high sequence similarity with the cscRNA1 previously reported in Italy, accumulate in leaves from CLS affected trees in Spain. These data, together with other evidences showing similar symptoms, disease cycle and fungal fructifications in CCRS and CLS affected trees, suggest a close relationship between the two cherry disorders.

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Vertical transmission of Prunus necrotic ringspot virus: hitch-hiking from gametes to seedling.

Amari, Khalid[1], Burgos, Lorenzo[1], Pallas, Vicente[2], Sanchez-Pina, M. Amelia[1]
[1]Dpto. de Biología del Estrés y Patología Vegetal. CEBAS-CSIC. Campus Universitario de Espinardo. P.O. Box 164. 30010 Espinardo-Murcia. Spain.
[2]Instituto de Biología Celular y Molecular de Plantas. UPV-CSIC. Avda. de los Naranjos s/n. 46022 Valencia. Spain; e.mail: vpallas@ibmcp.upv.es.
Contact: vpallas@ibmcp.upv.es

The aim of this work was to follow infection by Prunnus necrotic rigspot virus (PNRSV) in apricot reproductive tissues and transmission of the virus to the next generation. PNRSV was detected in repoductive tissues during gametogenesis. The virus distribution was studied at diferent developmental stages such as at the gloular, torpedo, bent cotyledon and mature stages. The results obtained shed light on the PNRSV vertical trasnmission from gametes to seedlings.

Molecular characterization of a new Prunus-infecting Flexiviridae member

Marais, Armelle[1], Faure, Chantal[1], Gentit, Pascal[2], Foissac, Xavier[1], Candresse, Thierry[1]
1UMR GD2P, IBVM, INRA, Université Bordeaux2, BP81, 33883 Villenaved'Ornon Cedex, France
2Ctifl, Centre de Lanxade, 24130 La Force, France
Contact: amarais@bordeaux.inra.fr

During a survey in Iran, the occurrence of Trichoviruses, Foveaviruses, and Capilloviruses was investigated in symptomatic stone fruit trees, by the PDO nested-RT-PCR technique, which allows the detection of viruses belonging to these three genera of the family *Flexiviridae* (Foissac et al., 2005). The PDOamplified cDNAs obtained from a *Prunus persicae tree* displaying mottle symptoms was cloned and sequenced, revealing two different sequences, one belonging to an isolate of Apricot pseudo-chlorotic leaf spot trichovirus, and the other showing its highest identity (only 73%) with Citrus leaf blotch virus, a member of the tentative Citrivirus genus in the family *Flexiviridae*. The nucleotide sequence of the 3' end of the genome (3055 bp) of this second agent was determined. The molecular organization is similar to that of some trichovirus members such as *Peach mosaic virus* (PcMV), with 4 open reading frames coding respectively for a replication-associated protein, a movement protein, a capsid protein, and a nucleic acid-binding protein. However, the identity levels between the PcMV proteins and those of the new agent are very low (at the most, 51.5% for the partial replicase). Phylogenetic reconstructions for the three complete ORFs and the partial replicase suggest that this agent should be considered as a new virus defining a new genus in the *Flexiviridae* family. The biological characterization of this virus is underway. Interestingly, a second isolate of this new agent was recovered from a Japanese plum collected in France but of unknown origin and test-inoculated on GF305 Prunus persicae seedlings in greenhouse. After two or three months the peach seedlings displayed red marbling symptoms on leaves (co-infected with Prunus necrotic rusty mottle virus, PNRSV). This second isolate has a similar genomic organization and over 90% nucleotide identity in the sequenced region. (Foissac et al., 2005. Phytopathology, 95,617-625).

Widespread occurrence of Tomato ring spot virus in deciduous fruit trees in Iran

Moini, A.A.[1], Roumi, V.[2], Masoumi, M.M., Izadpanah, K.[2]
[1]Plant Protection Research Institute, Tehran, Iran
[2]Plant Virology Research Center, Shiraz Univ., Shiraz, Iran
Contact: izadpana@shirazu.ac.ir

Despite long precedence of fruit tree growing in all provinces in Iran, the information on tree viruses in this country is scant. In the present study, presence of tomato ring spot virus (ToRSV) was surveyed in various woody plants in this country by mechanical transmission to herbaceous hosts, ELISA using a commercial antiserum, and PCR with specific primers. ToRSV was identified in the following plant-symptom combinations: Walnut with mottling, deformation, necrosis, and yellowing of main veins from Tehran Province; plum with yellowing of main veins, peach with yellowing of main veins and marginal necrosis, and hazelnut with interveinal chlorosis and marginal necrosis from Ardabil Province; apple with yellowing of main veins, mosaic and necrotic lesions, quince with large necrotic spots, and almond with leaf deformation and rossetting from Khorasan Province; and raspberry with marginal necrosis of leaf and necrotic lesions from Mazandaran Province. Mechanical inoculation from walnut,

plum, peach, hazelnut, apple, quince, almond, and raspberry to *Nicotiana tobaccum* cv. Samsun resulted in systemic infection. The virus isolates induced local lesions, leaf deformation, and necrosis in N. rustica, chlorotic local lesions on *Chenopodium quinoa*, and large local lesions on Gomphrena globosa. All samples were ELISA positive. PCR with specific primers resulted in the amplification of expected fragment (490 bp). This study shows extensive occurrence of ToRSV in Iran.

Virus diseases of stone fruit trees in Belarus

Kukharchyk, Natalia, Semenas, Svetlana
Kovaleva Street 2, Samokhvalovichy, Minsk region,
Institute for fruit growing, Kovaleva Street 2, Samokhvalovichy, Minsk region, 223013 Republic of Belarus
Contact: Kychnataly@tut.by

The purposes of the work were studying of plum and cherry orchards viral contamination, selection of virus free trees of varieties and vegetative propagated rootstocks of *Prunus L.*, monitoring of the nuclear rootstocks collection. Serological test (DAS-ELISA) was used for virus disease detection. For this time investigation of viruses distribution of stone fruit trees have been carried out and the first collection of industrial cultivars nuclear has been created. Sanitary selection didn't allowed to find virus free plants of rootstocks for sour cherry and sweet cherry. Virus elimination *in vitro* (meristem culture and chemotherapy) was successful for 5 forms of rootstock.

ApMV, ACLSV and PDV were often detected in plum orchard (20,63 %, 30% and 41,79 % accordingly). As in many European countries, the presence of Sharka is the major threat for the stone fruit orchard. In Belarus Sharka is not included in the list of quarantine objects. In 2000 year contamination by PPV was revealed for the first time (1 tree from Grodno region), in 2005 PPV was registered in 3 trees, in 2007 – in 30.

High contamination of all sour and sweet cherry plantings by PNRSV (86,23 %) was established. Other viruses were detected in the industrial orchards of sour and sweet cherry considerably below (ACLSV, ArMV, PDV, ApMV, RRV – no more then 13 %), than in propagation plantation. All tests for CLRV were negative and only one positive result was established at testing PPV. From the most widespread viruses of fruit cultures in the adjacent countries, in our researches only CLRV was not revealed, all other viruses in different quantity were presented in Belarussian orchards and nurseries.

Detection, monitoring and characterization of LChV-1 isolates from southern italy

Matic, S.[1*], Minafra, A.[1], Sánchez-Navarro, J.A.[2], Pallás, V.[2], Myrta, A.[3], Martelli, G.P.[1]
[1]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale, Sezione di Bari, Via Amendola 165/A, 70126 Bari, Italy
[2]Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas-UPV, CPI 8E, C/ Ingeniero Fausto Elio s/n, 46022 València, Spain
[3]Certis Europe, Via A. Guaragna 3, 21047 Saronno (VA), Italy
*Present address: Plant Virology Group, ICGEB Biosafety Outstation, Via Piovega 23, 31056 Ca'Tron di Roncade, Italy
Contact: Fax: +39 0422 789730; E-mail: matic@icgeb.org

Little cherry virus 1 (LChV-1), one of the causal agents of the Little Cherry Disease in cherries, has unknown impact in other stone fruit hosts. LChV-1 was diagnosed by different molecular techniques (one-step RT-PCR, dot-blot hybridization, tissue-printing hybridization), serological technique (ELISA), and serological-molecular techniques (IC RT-PCR and IC one-step RT-PCR). Different LChV-1 Prunus isolates from Southern Italy were assayed from leaf samples collected randomly during late summer and early autumn 2007. The molecular techniques described here showed to be more reliable in virus detection than the serological and serological-molecular assays. LChV-1 was monitored also during different seasons over the year by the use of tissue-printing hybridization from different tissues (leaf petioles, branches, dormant cuttings and buds). LChV-1 was detectable throughout the year in all types of tissues tested, with the most reliable detection during the summer and autumn. Virus-specific primers were used to amplify fragments of ORF1a, ORF4, CP and CPm of selected LChV-1 isolates. Italian LChV-1 isolates showed high sequence identity among them (90% or more) in all sequenced genomic regions, even though they came from different stone fruit species and orchards.

Keywords: stone fruits, diagnosis, monitoring, sequence analyses

Oral Session VII

Pathogen-derived technologies for improving Plum Pox Virus resistance of transgenic plum (*Prunus domestica* L.)

Mikhailov, Roman[1], Serova, Tatyana[2], Shulga, Olga[3], Firsov, Alexey[4], Dolgov, Sergey[4]
[1]Branch of Shemyakin Institute of Bioorganic Chemistry RAS, Pushchino, Russia
[2]Branch of Shemyakin Institute of Bioorganic Chemistry RAS, Pushchino, Russia;Pushchino State University, Pushchino, Russia
[3]Institute of Agriculture Biotechnology RAAS,Timiryazevskaya,42, Moscow, Russia
[4]Branch of Shemyakin Institute of Bioorganic Chemistry RAS, Pushchino, Russia; Institute of Agriculture Biotechnology RAAS,Timiryazevskaya,42, Moscow, Russia
Contact: t_serova@rambler.ru

Stone fruit trees are valuable owing to quality of their fruits and as the source of hard wood in all world. Virus and fungus diseases are main dangers for cultivatione of the stone fruit culture. Plum Pox Virus (PPV), causal agent of Sharka disease, is currently being considered as the most infectious pathogen in peaches and nectarine, apricots, plums and cherry. Considering the severity of the disease, complicated control of its spread, and lack of resistant varieties, the necessity of resistant cultivars is apparent.

There are some approaches to improving plant resistance to viral pathogens in modern biotechnology. Pathogen-derived resistance (PDR), based on virus resistance by sense (co-supression) and antisense virus gene expression, or expression of self-complementary fragments of virus gene (RNA-silencing), is widespread at the last time. PDR are divided by two types: transgenic proteinmediated resistance and RNA-mediated resistance. Protein-mediated resistance is occurred due to accumulation of viral protein in plant cell. Post-transcription gene silencing (PTGS), or RNA-silencing, is realized at RNA-mediated virus resistance. RNA-silencing mechanism is cleaving of double-stranded RNAs (dsRNA) result in small interfering RNAs (siRNA), which are template for cleaving homologous molecules of mRNAs.

For improving the plants resistance to plum pox virus (PPV) two technologies were based on co-supression gene and RNA-silencing. Binary vector pCamPPVcp which contained the selective hpt gene and ppv-cp gene in sense-orientation (driven by double 35S promoter). Vector pCamPPVRNAi contained selfcomplementary fragments of gene ppv-cp driven by double 35S promoter and the hpt and gus genes.

Seven independent transgenic lines with ppv-cp gene and five transgenic lines with a inverted repeats of ppv-cp gene fragment were produced in our laboratory. Stable integration of genes into genome of plants was confirmed by PCR analyses. The accumulation of coat protein was evaluated by Western blot assay.

The transgenic shoots were rooted and acclimatized to the greenhouse.

The hairpin structure of the Plum pox virus coat protein gene in HoneySweet C5 plum is responsible for PPV resistance

Scorza, Ralph[1], Georgi, Laura[2], Callahan, Ann[1], Petri, Cesar[1], Hily, Jean-Michel[1], Dardick, Chris[1], Damsteegt, Vern[3], Ravelonandro, Michel[4]
[1]USDA-ARS-Appalachian Fruit Research Station, Kearneysville, WV USA
[2]Clemson University, Clemson, SC USA
[3]USDA-ARS-FDWSRU, Ft. Detrick, Maryland USA
[4]UMR GDPP INRA-Bordeaux, France
Contact: ralph.scorza@ars.usda.gov

The genetically engineered plum ‘HoneySweet’ (aka C5) has proven to be highly resistant to Plum pox virus (PPV) for over 10 years in field trials. The original vector used for transformation to develop ‘HoneySweet’ carried a single sense sequence of the full length ppv-cp gene, yet the resistance mechanism of ‘HoneySweet’ was found to be based on post-transcriptional gene silencing (PTGS). Sequencing of the transgene insert revealed an inverted repeat of the PPV-CP sequence in ‘HoneySweet’. We hypothesized that this structure, acting as a hairpin (hp), was responsible for PTGS of the transgene and of viral CP which resulted in a high level of resistance to PPV infection. In order to test this hypothesis the hpPPV-CP insert was cloned from ‘HoneySweet’ and transferred into ‘Bluebyrd’ plum seedlings through Agrobacterium tumefaciens transformation of hypocotyl slices. Two versions of the hpPPV-CP insert were tested. One spanned the CP inverted repeat only (minimal construct) and the other

spanned the inverted repeat plus 304 bp of plum DNA upstream and 591 bp downstream of the inverted repeat. Transgenic plum plants containing single or multiple copies of these hp inserts were inoculated with PPV D isolated from Pennsylvania, USA. PPV infection was evaluated through three cycles of coldinduced dormancy (CID) by symptom expression and by at least two and up to five ELISA and PCR tests. Of 24 plants evaluated nine were never infected, six in some tests showed weak infection, and nine plants were consistently infected. Most of the resistant lines contained a single copy of the minimal hp construct. These data strongly suggest that the hp portion of the PPV-CP insert in 'HoneySweet' plum is responsible for PPV resistance.

The hypersensitivity resistance of European plum to Plum pox virus and its potential impact on the epidemiology of the virus

Neumüller, Michael[1], Hartmann, Walter[2], Treutter, Dieter[1]
[1]Technische Universität München, Fachgebiet Obstbau, Dürnast 2, 85354 Freising
[2]Universität Hohenheim, Fachgebiet Obstbau
Contact: nm@wzw.tum.de

Since the early report on the Sharka disease in 1935, breeding for Sharka (Plum pox) virus resistance is considered to be the most promising way of minimising the outstanding economic damage caused by the disease in stone fruit production. Several resistance breeding strategies are embarked by breeders throughout the world. Those used in European plum (*Prunus domestica*) breeding were compared concerning their potential use in both minimizing the economic losses and avoiding the dissemination of PPV in orchards and over long distances.

Varieties tolerant to PPV are able to prevent the decline of plum production in areas where PPV is prevalent. However, they contribute to the dissemination of PPV into regions up to know free from PPV via latently infected plant material. For a long time, breeding for PPV resistance has been limited due to the lack of sources of resistance. Up to know, two different types of natural resistance have been found in *P. domestica*: The so called quantitative resistance and the hypersensitivity resistance. The latter inhibits the viral replication and dissemination within the plant. Hypersensitive genotypes are, under natural inoculation conditions, no source of PPV. Therefore, they do not contribute to the dissemination of PPV both via insect vectors within an orchard or via infected plant material. On the contrary, quantitatively resistant genotypes, even if they have low virus titer after inoculation with PPV, are sources of infection and hold the same disadvantages than tolerant varieties concerning the spread of PPV. It was shown that the hypersensitivity trait can be found in interspecific hybrids between *P. domestica* and $^{P.\ salicina}$ and between *P. domestica* and *P. cerasifera*. These genotypes can be used as rootstocks. The use of hypersensitive genotypes as rootstocks for Prunus species opens a new dimension in avoiding the dissemination of PPV via latently infected plant material.

Transient expression as a method to evaluate effectiveness of SCFV fragments to interfere with plum pox virus infection

Gil, M.[1], Esteban, O.[1], García, J.A.[2], Peña, L.[1], Cambra, M.[1]
[1]Centro Protección Vegetal y Biotecnología. Inst. Valenciano de InvestigacionesAgrarias (IVIA). Carretera de Moncada-Náquera Km 5, 46113 Moncada (Valencia), Spain.
[2]Dpto. Genética Molecular de Plantas, Centro Nacional de Biotecnología (CNB), CSIC. Campus de la Universidad Autónoma, 28049 Cantoblanco (Madrid), Spain
Contact: maitegil@ivia.es

The constitutive expression of single chain Fv fragments (scFv) specific to different viral proteins in transgenic plants has been used to immunomodulate or interfere with viral infection. However in most of the referenced reports the level of protection was only partial. Probably the success of this approach relies on efficient scFv expression and accumulation and on the election of a relevant target protein. Transient expression mediated by agroinfiltration is an alternative method to the stable transformation to evaluate the stability and accumulation level of scFv fragments in the plant cell. Co-agroinfiltration experiments were carried out in Nicotiana benthamiana. Plants were agroinfiltrated with a co-cultures of recombinant Agrobacterium tumefaciens (carrying different versions of the scFv2A fragment-specific for the NIb replicase or the scFv5B fragment-CP specific) and an infective PPV-GFP clone. Agroinfiltrated leaves were excised after three days post-agroinfiltration, observed under stereomicroscope with UV light and photographed. A significant reduction on the number and size of the fluorescent foci were

observed with both scFv fragments and viral accumulation (estimated by DASI-ELISA) was also reduced.

The use of PPV-GFP allowed viral infection monitoring at early stages, and supplied valuable information about the immunomodulation (interference effects) produced by both scFv fragments against structural and non-structural PPV proteins.

Natural deletion is not unique in the coat protein (CP) of recombinant Plum pox virus (PPV) isolates in Hungary

Szathmáry, Erzsébet, Palkovics, László
Corvinus University of Budapest, Faculty of Horticultural Science, Department of Plant Pathology,
H-1118 Budapest, Ménesi Road 44. HUNGARY
Contact: laszlo.palkovics@uni-corvinus.hu

Plum pox virus (PPV) is the economically most important viral pathogen of stone fruit trees in Europe. PPV isolates characterized by different serological, molecular and epidemiological properties are grouped into three major (PPV-D, PPV-M, PPV-Rec) and three minor (PPV-EA, PPV-C, PPV-W) groups. During our study apricot (Prunus armeniaca L.) leaves showing typical symptoms of PPV infection were collected from Hungary in 2008. Samples were tested for the presence of PPV by RT-PCR using a Potyvirus-specific primers (PolyT2 and Poty7941), which amplified the 3'NIb–CP–3'UTR region form the viral genome. The PCR fragments were sequenced, and then sequence comparisons and phylogenetic analysis were done.

The multiple alignments of the nucleotide and deduced amino acid sequences corresponding to the 3'NIb-CP-3'UTR region showed that the isolate PPVGodollo2 bearing a 33-nucleotid (11-amino acid) natural deletion in frame in the N-terminal part of the coat protein (CP), downstream to the DAG amino acid motif. Currently we have reported on a Hungarian PPV-Rec isolate (PPV-B1298) collected from plum bearing a much larger, 135-nucleotide (45-amino acid) natural deletion at similar position to that of the PPV-Godollo2 (Szathmáry et al., Archives of Virology 154: 141–145, 2009).

According to the phylogenetic analysis of the nucleotide sequence of the 3'NIb– 5'CP region PPV-Godollo2 isolate clustered together with the previously characterized PPV-Rec isolates.

Our present study and previous data suggest that presence of natural deletion in the N-terminal part of the CP in the Hungarian recombinant PPV population is not unique.

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Symptomatic and real-time PCR scoring of Plum Pox Virus resistance in two apricot (*Prunus armeniaca* L.) segregating populations

Vera-Ruiz, Elsa María[1], Soriano, José Miguel[2], Martínez-Calvo, José[1], Llácer, Gerardo[1], Badenes, María Luisa[1], Romero, Carlos[3]
[1]IVIA, Apartado Oficial, 46113 Moncada (Valencia), Spain
[2]Wageningen UR Plant Breeding, Wageningen University, P.O. Box 16, 6700 AA Wageningen, The Netherlands
[3]Unidad Fundación Agroalimed-IVIA, Apartado Oficial, 46113 Moncada (Valencia), Spain
Contact: llacer_ger@gva.es

Resistance to Sharka disease, caused by the *Plum Pox Virus* (PPV), is one of the main aims in all the apricot breeding programmes currently in progress. Nevertheless, PPV resistance screening means a bottleneck for the programmes since it is very time consuming and requires a large greenhouse space. To date our lab performed the evaluation according to a biological test confirming the virus presence by ELISA-DASI. This procedure, although trustworthy, only allowed to classify seedlings into resistant (1) or susceptible (0). To get a more detailed picture of PPV resistance in segregating populations, we have follow the same biological test but implementing a symptomatic evaluation in which all leaves were scored individually. In addition, real-time PCR was performed on symptomatic and asymptomatic leaf samples to increase PPV detection sensitivity. For this purpose, two families, with different PPV resistance sources, have been evaluated, an F1 (`Goldrich' x 'Currot') and an F2 ('Lito' x 'Lito'). Results from this new scoring might be helpful to discern among the different hypothesis about the genetic control of apricot PPV resistance already reported.

Serological and molecular screening of Plum pox virus in the F1 and F2 romanian apricot genotypes

Ion, Ligia[1], Hoza, Dorel[1], Isac, Maria[2], Preda, Silvia Ana[2], Topor, Elena[3], Neagu, Tudora[1], Moale, Cristina[1], Poenaru, Silvia[2], Nicolae, Constantin[1]
[1]USAMV Bucuresti
[2]Fruit Growing Research & Extension Station Valcea
[3]Fruit Growing Research & Extension Station Constanta
Contact: mariaisac10@hotmail.com

Sharka (plum pox) is considered one of the most devastating diseases of stone fruits in terms of agronomic impact and economic importance (Dunez & Sutic, 1988; Nemeth, 1994). The disease is very detrimental in apricot, peach and plum trees because it produces reduced quality and premature dropping of fruits. The dynamics of virus infection in F1 and F2 romanian apricot cultivars (Prunus armeniaca) were analyzed in the natural infection conditions. The F1 and F2 romanian apricot progenies were serologically tested by DAS-ELISA using PPV-D and PPV-M specific monoclonal antibodies. Molecular tests were done by RT-PCR using P1/P2 primers (Wetzel et all., 1991). PPV detection by conventional serological and molecular methods was performed during the dormant period (wintertime). This period is important drawbacks due to false negative (sensivity) but also false positive (specificity). Romanian apricot F1 and F2 progenis evaluated were initially classified into three groups; susceptible to PPV, partially resistant to PPV and resistant to PPV. After that the most important genotypes were grafted onto infected GF305 in greenhouse conditions. The goals of the work presented in this communication are to introduce and to develop the marker-assisted selection (MAS) in PPV resistances F1 and F2 progenis in apricot Romanian cultivars.

Tracking Plum pox virus in Chile throughout the year by three different methods and molecular characterization of Chilean isolates

Fiore, Nicola[1], Araya, Carolina[2], Zamorano, Alan[1], González, Flor[1], Mora, Roxana[2], Sánchez-Navarro, Jesús[3], Pallás, Vicente[3], Rosales, Inés Marlene[2]
[1]Fundación Facultad de Ciencias Agronómicas de la Universidad de Chile. Av. Santa Rosa 11315, La Pintana, Santiago, Chile.
[2]Unidad de Biotecnología, Instituto de Investigaciones Agropecuarias (INIA), Santiago, Chile. Av. Santa Rosa 11610, La Pintana, Santiago, Chile.
[3]Instituto de Biología Molecular y Celular de Plantas, CSIC-UPV, CPI 8E, C/Ingeniero Fausto Elio s/n, 46022 Valencia, España.
Contact: nfiore@uchile.cl

During 2007 a survey was performed to detect and identify Plum pox virus (PPV) in Chilean stone fruits commercial orchards. Using primers P4b and P3D described by Candresse et al. (1998, Phytopathology 88,198-204) a fragment of 467 bp was amplified corresponding to the replicase-coat protein (Nib-CP) region from the virus genome. The fragment sequences obtained enabled to characterize all isolates as PPV-D, confirming that this is the only serotype present in Chile. To optimize the virus detection, 27 PPV-positive trees were selected, and sampled monthly from December 2006 until December 2007, collecting plant tissues available at the time of sampling (leaves, cuttings, buds and flowers). Each sample was analyzed by three different techniques: ELISA, RT-PCR and non-isotopic molecular hybridization (MH). For ELISA, the kit "Realisa Reforzado" (REAL, Spain) was used. The RT-PCR was carried out by using PPV universal primers P1/P2, whereas the probe used for HM corresponded to the CP region. The results showed that RT-PCR was more sensitive for detection in all months excepting January 2007, when the three techniques showed the same sensitivity. In general, MH showed a better sensitivity compared with ELISA. The best plant materials for analysis were: leaves, in February, March, September and October; phloem from cuttings in June; buds in July, and flowers in August. These results suggested that the use of RT-PCR should be considered for routine control of PPV in Chile, taking into account two major sampling windows during the year: the first, from July to September; and the second with slightly lower sensitivity, during the months of February and March. MH is shown as a clear alternative to the serological test for large-scale surveys.

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Oral Session VIII

First insights into the Genomes of the rich equipped Acholeplasma species highlight the Genome Condensation of the related Phytoplasmas

Migdoll, Alexander M.[1], Seemüller, Erich[2], Reinhardt, Richard[1], Kube, Michael[1]
[1]MPI for Molecular Genetics, Ihnestr. 63-73, 14195 Berlin
[2]Julius Kühn-Institut (JKI), Federal Research Centre for Cultivated Plants,Institute for Plant Protection in Fruit Crops and Viticulture Schwabenheimer Straße 101, 69221 Dossenheim
Contact: migdoll@molgen.mpg.de

Acholeplasmas are thought to be the closest related group of the phytoplasmas. In contrast to the host-depending phytoplasmas Acholeplasma species can be cultivated in established Mollicutes-growth media and several organisms show an ubiquitary distribution.

We determined high-quality draft genome sequences of *Acholeplasma brassicae* str. O502 and Acholeplasma palmae str. J233. These species were selected because of their isolation from plants, their detection in phytoplasma vectors and the phylogenetic proximity to phytoplasmas. The draft genome of A. brassicae, which should be extended to a finished state, contains ca. 1.9 Mb with estimated 1800 protein coding sequences while the genome of *A. palmae* is approx. 1.6 Mb in size with 1500 protein coding sequences. In comparison to *Acholeplasma laidlawii*, whose complete genome sequence is available, both posses a considerable higher amount of coding sequences for transposases/integrases as well as possible paralogs, which all may indicate duplication or insertion events. Besides sequences for DNA-replication and –repair, transcription and translation most identified protein coding sequences are related to the carbohydrate metabolism, the amino acid- and nucleotide-synthesis as well as transport systems for corresponding precursors and products and suggest a nonspecialized and probable opportunistic lifestyle. However, many pathways show analog or differing losses in smaller to larger amount, probably indicating a beginning genome condensation. The content of the protein coding genes of the complete sequenced *Candidatus* Phytoplasma species was compared to *A. laidlawii, A. brassicae* and *A. palmae*. The determined highly interconnected metabolic networks in the *Acholeplasma* species indicate an unexpected distance to the phytoplasma species which is impressive and weakly depicted by the current phylogeny. Typical candidates for virulence associated proteins are missing in both plant-associated groups. Nevertheless, from earlier studies it is indicated that *Acholeplasma* and *Candidatus* Phytoplasma species use comparable insect vectors and therefore probably the same mode of transmission.

Identification of host genes potentially implicated in the *Malus pumila* and *Candidatus* Phytoplasma mali interactions

Aldaghi, Majid[1], Massart, Sebastien[1], Bertaccini, Assunta[2], Jijakli, M. Haissam[1], Lepoivre, Philippe[1]
[1]Plant Pathology Unit, Gembloux Agricultural University (FUSAGx), 13 Avenue Marechal Juin, 5030 Gembloux, Belgium
[2]Department of Agro-environmental Science and technology, Plant Pathology, Alma Master Studiorum, University of Bologna, Bologna, Italy
Contact: aldaghi.m@fsagx.ac.be

Apple proliferation (AP) is one of the most serious diseases of apple trees in Europe. It is caused by a phytoplasma, '*Candidatus* Phytoplasma mali'. The goal of the present study was to analyze transcriptional profiles of *Malus pumila* during infection by 'Ca. P. mali' using cDNA-AFLP technique. A rootstock of apple (MM106) susceptible to 'Ca. P. mali' to maximise the range of the potential host responses, and two strains (AP and AT) of the pathogen were used. Gene expression comparisons were studied in 3 categories of plant materials: healthy sample versus infected samples, symptomatic versus non-symptomatic sample, and AP-infected sample versus AT-infected sample. Forty-five genes whose steady-state levels of expression significantly changed in response to phytoplasma infection were isolated and identified. Of 45 partial cDNA sequences, twenty-seven showed similarity to international DNA or protein data bases. Of these, 18 were previously characterized in plants (the rest was related to unknown or hypothetical proteins). Eighteen out of 45 did not show any similarity with sequences in data bases, and so may be present novel genes. The majority of fragments were differently expressed between healthy sample and infected samples (fewer differences between symptomatic and non-symptomatic samples, or between the samples infected by different strains of phytoplasma). Quantitative Real-time

RT-PCR (qRT-PCR) was used to confirm differential expression of sequences isolated by cDNA-AFLP. Consequently, qRT-PCR showed the similar profile expression as primary elucidation technique (cDNA-AFLP) for 11 known genes (between 18) and 13 unknown, hypothetical or novel genes (between 27). Changes in gene expression involved a wide spectrum of biological functions, including processes of metabolism, cell defence, senescence, photosynthesis, transport, transcription, signal transduction and protein synthesis. The possible effect of phytoplasma infection on these processes and their relationships with disease development, symptom appearance and probably plant defence system is discussed. A model is proposed to explain the mode of action of the 'Ca. P. mali' in its host plant, apple tree.

In vitro screening of interspecific hybrids (*Malus* spp.) for resistance to apple proliferation

Bisognin, Claudia[1], Ciccotti, Anna Maria[1], Salvadori, Antonella[1], Jarausch, Wolfgang[2], Grando, Stella[1]
[1]Fondazione Edmund Mach, IASMA Research Centre - Via Mach 1, 38010 San Michele
[2]Institute for Plant Research, RLP AgroScience, Breitenweg 71, 65435 Neustadt/W.
Contact: claudia.bisognin@iasma.it

A breeding program was started in 2001 in order to develop apple proliferation (AP) resistant apple rootstocks exploiting the natural resistance found in *Malus sieboldii*.

Twenty-six hybrids generated from the crossings of *Malus sieboldii* and its hybrids with *Malus x domestica* were micropropagated and studied under standardized conditions in vitro. An in vitro screening system for AP resistance previously established for the parental genotypes of the crosses was adopted and further improved. Specific symptoms of the disease i.e. height and basal proliferation of the shoot, and size of the leaves were recorded in vitro at 3 months post inoculation. At the same time phytoplasma concentration was determined in the whole shoot by quantitative PCR. Ex vitro plants were obtained from each culture line and were graft-inoculated *in vivo* in triplicates with two different phytoplasma strains. Phenotype and phytoplasma titre were evaluated in the roots the year after infection.

Preliminary results indicated that the resistance trait segregates in the progenies. The resistant genotypes had lower phytoplasma concentration than the susceptible controls, did not show AP-specific symptoms and their growth was not affected by infection. By comparing the resistant behaviour of the same genotypes, the *in vitro* screening seems to be more severe than the *ex vitro* system thus allowing a quick selection of genotypes that are worth being evaluated in the field for agronomic traits.

Experimental transmission trials by *Cacopsylla pyri,* collected from pear decline infected orchards in Turkey

Caglayan, Kadriye, Gazel, Mona, Ulubas Serce, Cigdem, Can, Feza
Mustafa Kemal University, Plant Protection Department, Hatay/Turkey
Contact: kcaglayano@yahoo.com

A study was carried out on the experimental transmission efficiency of the Pear Decline (PD) phytoplasma by *Cacopsylla pyri* (L.), collected from two naturally infected orchards from Bursa and non-infected orchard from Hatay province of Turkey. *C. pyri* adults were collected for the phytoplasma transmission to healthy periwinkle plants (*Vinca rosea*). Groups of five psyllids per plant were used for transmissison tests and the study was replicated three times. The presence of PD phytoplasma in psyllid transmitted *V. rosea* plants was investigated by using P1/P7 and U3/U5 primer pairs for nested PCR. Although *C. pyri* has limited host range, they were able to survive up to 20 days on periwinkles. Insects collected from the Bursa province survived 16-20 days whereas second group from Hatay, which were fed on PD infected pear for a week, were survived 7-12 days on periwinkles. Symptoms consist of a yellowing or clearing of the veins in newly infected leaves and shortening of the internodes of the main stem. They also remain stunted and flowers were small. All periwinkle plants from the first group showed phytoplasma symptoms whereas only one plant was found symptomatic in the second group. According to the RFLP analysis of Bursa samples, the experimental infection rate of periwinkle plants and psyllids was 33.3% and 16.6%, respectively. No infected periwinkle was found in second group including symptomatic plant but psyllids were 33.3 % infected . Transmission trials under controlled conditions showed the capability of *C. pyri* to transmit PD to healthy test plants and prooved as vector of *Ca. P. pyri* in Turkey.

Analysis of the acquisition and multiplication efficiency of different strains of Ca. Phytoplasma mali by the vector *Cacopsylla picta*

Jarausch, Barbara, Fuchs, Annette, Krczal, Gabi, Jarausch, Wolfgang
AlPlanta-IPR, RLP AgroScience, Breitenweg 71, D-67435 Neustadt
Contact: barbara.jarausch@agroscience.rlp.de

Based on previous observations during long-term acquisition and transmission trials, studies were carried out under standardized conditions in order to analyse the acquisition and multiplication efficiencies of different strains of *Ca. P. mali* by different developmental stages of *C. picta*. The acquisition of *Ca. P. mali* from micropropagated plants infected with different strains was tested for nymphs, larval stages and new adults of *C. picta*. When born on infected plants a nearly 100% acquisition was achieved for all strains of *Ca. P. mali* by *C. picta*. Differences in acquisition efficiency were observed for new generation adults which acquired the phytoplasma as imagines. The multiplication efficiency of the different *Ca. P. mali* strains inside the insects was analysed by quantitative realtime PCR. Despite high acquisition rates only few subsequent transmission events to healthy test plants could be recorded.

Oral Session IX

Strain differentiation of *Candidatus* Phytoplasma Mali by SSCP- and sequence analyses of the HFLB gene

Schneider, Bernd[1], Seemueller, Erich[1], Jarausch, Wolfgang[2]
[1]Julius Kühn Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, Schwabenheimer Str. 101, 69221 Dossenheim, Germany
[2]AlPlanta - Institute for Plant Research, RLP AgroScience GmbH, Breitenweg 71, 67435 Neustadt/W, Germany
Contact: bernd.schneider@jki.bund.de

Apple proliferation caused by *Candidatus* Phytoplasma mali is an important disease in Central and Southern Europe and reports from Greece and Turkey reflect the spread of the disease. The pathogen itself is known for almost forty years but due to a lacking in vitro culture little biological information is available on the causal agent. Field trials, however, have demonstrated that differences, expressed in virulence, are present. Although, the complete genome sequence of one Ca. P. mali strain is known, the search for genes related to pathogenicity is ongoing. A number of putative candidate genes have been identified, amongst others the *hflB* gene. This gene is present in multiple copies which might reflect its importance for the organism. To explore the *hflB* gene of pathogen strains and isolates in more detail a 530 bp PCR fragment from 42 German, Italian and French accessions were examined. The amplimers were analyzed by singlestrand conformation polymorphism (SSCP) analysis and revealed an unexpected variability. More than 20 different profiles could be discerned. Sequencing of the PCR fragments showed that the nucleic acid homology of the strains ranged from 94.2% to full identity. Although the differences in gene sequence could not yet be related to pathogenicity traits the fragment is predestined as molecular marker for tracking strains in epidemiological studies as well as defining and monitoring inoculum strains in resistance breeding projects.

Molecular characterization of *Candidatus* Phytoplasma mali strains in outbreaks of apple proliferation in north eastern Italy, Hungary, and Serbia

Paltrinieri, Samanta[1], Duduk, Bojan[1], Dal Molin, Federica[2], Mori, Nicola[3], Comerlati, Giovanni[2], Bertaccini, Assunta[1]
[1]DiSTA, Plant Pathology, viale Fanin, 42 - 40127 Bologna, Italy
[2]Servizio Fitosanitario Regione Veneto, viale dell'Agricoltura 1/A, Buttapietra, 37060, Verona, Italy
[3]Dipartimento di Agronomia Ambientale e Produzioni Vegetali, Legnaro, Padova
Contact: ipwg2007@gmail.com

During 2005-2008 apple plants showing proliferation symptoms were observed in diverse cvs in different areas of north eastern Italy (Veneto and Trentino), Hungary and Serbia. Leaves and young shoots were collected from June to October in orchards with epidemic presence of apple proliferation, and in others where the symptomatic plants were present in a scattered way. PCR/RFLP amplification carried out on R16F2/R2 amplicons showed that all the samples were infected with 'Candidatus Phytoplasma mali'. Further strain characterization was carried out using RFLP analyses with HpaII and FauI on almost full ribosomal DNA plus spacer region, *Alu*I on *rpl22-s3* genes, and RcaI and HincII on AP13/AP10

amplicons from representative samples collected in these geographic areas. Analyses of 16S plus spacer region distinguished two phytoplasma profiles (P-I and P-II). P-I was detected in reference strains AP, AT1, AT2, in samples from Serbia, and in the majority of samples from Trentino. P-II profile was prevalent in samples from Veneto, and both profiles were identified in samples from Hungary, in the majority of the cases together. The analyses of *rpl22-s3* genes allow to identify in all the samples showing P-I profile the presence of phytoplasmas belonging to rpX-A subgroup, while in samples showing P-II profile it was possible to distinguish the four described rp subgroups. In the majority of samples from Veneto region phytoplasmas belonging to rpX-D subgroup were identified, while rpX-B and X-C subgroups were identified in a few samples from Trentino and Veneto regions respectively. Further RFLP characterization on AP13/AP10 amplicons differentiates among strains belonging to rpX-A subgroup: the Serbian samples show AP profiles, while those from Trentino show AT-2 profiles. In the samples from Hungary the presence of AT1, AT2, and AP profiles was identified. The combined use of these molecular markers allows differentiating 'Ca. P. mali' strains.

Breeding of rootstocks resistant to apple proliferation disease

Seemüller, Erich[1], Bisognin, Claudia[2], Grando, Stella[2], Schneider, Bernd[1], Velasco, Riccardo[2], Jarausch, Wolfgang[3]
[1]Julius Kuehn Institute, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, D-69221 Dossenheim, Germany
[2]Fondazione Edmund Mach, IASMA Research Centre - Via Mach 1, I-38010 San Michele
[3]RLP AgroScience, AlPlanta-IPR, Breitenweg 71, D-67435 Neustadt
Contact: wolfgang.jarausch@agroscience.rlp.de

Apple proliferation (AP) is caused by the wall-less bacterium *Candidatus* Phytoplasma mali and is spread by psyllids. Previous work indicated that, due to the colonization behavior of the causal agent, the disease could be controlled by the use of resistant rootstocks. Unfortunately, extensive screening revealed no satisfactory resistance in established rootstocks. In contrast, substantial levels of resistance were identified in experimental rootstocks derived from crosses of the apomictic species *M. sieboldii* and genotypes of $^{M.\ x\ domestica}$ and *M.* x *purpurea*. However, these hybrids were more vigorous and less productive than standard stock M 9. For this reasons, a program was initiated to reduce vigor and improve yield by crossing and backcrossing M. sieboldii and its apomictic hybrids with M 9 and other dwarfing stocks. From 2001 through 2006 a total of 36 crosses were made. However, only 23 progenies consisted of a substantial number of seedlings while the other crosses largely failed due to pollen incompatibility. The 3.500 seedlings obtained were DNA-typed using codominant SSR markers to distinguish apomicts and recombinants in the progenies. A total of 1.800 seedlings consisting of all recombinants and a representative number of apomicts were screened for AP resistance by graft inoculation followed by observation in the nursery and under commercial growing conditions. Several progenies showed a good inheritance of resistance. In two of them (4608 x M 9 and D2212 x M 9) more than 50% of the individuals never developed symptoms.

Influence of apple stem grooving virus on *Malus sieboldii*-derived apple proliferation resistant rootstocks

Liebenberg, Annerie, Wetzel, Thierry, Kappis, Alexander, Herdemertens, Michelle, Krczal, Gabriele, Jarausch, Wolfgang
AlPlanta-IPR, RLP AgroScience, Breitenweg 71, D-67435 Neustadt
Contact: wolfgang.jarausch@agroscience.rlp.de

Apple stem grooving virus (ASGV, Capillovirus) is widely spread in apple growing regions. As it causes no symptoms on most cultivated apple varieties and rootstocks it is considered latent in *Malus* x *domestica*. In Asia, however, ASGV has been found associated with topworking disease of apple rootstocks originating from *Malus sieboldii*. Recently, *M. sieboldii* and its hybrids have gained new interest in Europe as they confer resistance to apple proliferation (AP) disease. A new breeding program aiming to develop AP-resistant rootstocks of agronomic value for modern apple culture, reported unexpected tree decline and found it to be associated with ASGV. As little information is available on the variability of ASGV isolates in Germany, the complete genome of a German isolate of ASGV associated with tree decline was cloned and sequenced. Sequence comparisons with available ASGV isolates revealed two regions of high variability in the genome. The genetic variability of additional isolates from Germany and other countries were collected and the variable areas characterised. In addition ASGV was successfully maintained in micropropagated apple trees and could be transmitted by

in vitro grafting to various genotypes, making it possible to study the effect of the virus and virus/phytoplama combination on *M. sieboldii*-derived genotypes.

Identification of host plants for *Candidatus* Phytoplasma prunorum and of his vector *Cacopsylla pruni* in SPAIN

Sabate, J., Laviña, A., Batlle, A.*
IRTA, Ctra de Cabrils s/n 08348 Barcelona. Spain
Contact: assumpcio.batlle@irta.es

Candidatus phytoplasma prunorum is the causal agent of the "European stone fruit yellows" disease. This phytoplasma infects different wild and cultivated Prunus species that are tolerant to the disease, favouring a natural cycle: vector- Prunus-pathogen, making the disease unnoticed. The introduction of a more sensitive species like the Japanese plum-tree (*Prunus salicina*) in an agroecosystem with many several inoculum sources, like the Baix Llobregat area (Barcelona), and the presence of a very efficient vector in the acquisition and the transmission, has caused a quick dissemination of the disease.

Cacopsylla pruni was identified for the first time in Spain in the Baix Llobregat (Catalonia) in 2003 and later in other Spanish regions. The cycle of *C. pruni* has been studied during four years in this area. The insect population was evaluated in two geographical areas of wild Prunus (*P. mahaleb*) and in two commercial orchards of *P. salicina* with a high incidence of the disease. Adults appeared in the middle of February, and the populations reached two maximums, at the end of March and at the beginning of June. In the commercial plantations the species followed a similar evolution with interannual fluctuations. *C. pruni* was captured also in specific samplings on *P.spinosa* in different areas. The percentage of individuals of *C.pruni* carrying the phytoplasma was about 15 %, the high number of positives were identified in the re-immigrant adults (January - March) whereas in the new generations (April - June) the percentage of positives is lower. The phytoplasma was detected both in wild and cultivated Prunus. The fact that the vector might accomplish his life cycle on wild infected hosts, and emigrate to the commercial plots, despite the direction of the plantations, points out that wild Prunus has an important role in the epidemiology of the ESFY and therefore in his potential control.

Infection rates of natural psyllid populations with *Candidatus* 'Phytoplasma mali' in South Tyrol (northern Italy)

Baric, Sanja, Oettl, Sabine, Dalla Via, Josef
Research Centre for Agriculture and Forestry Laimburg, I-39040 Auer/Ora (BZ), Italy
Contact: sanja.baric@provinz.bz.it

Apple proliferation is a severe disease of apple trees spreading in many European apple growing areas. It is caused by *Candidatus* 'Phytoplasma mali' that was shown to be transmitted through infected grafting material, via natural root grafts and by sap-sucking insects. Two psyllid species, *Cacopsylla picta* and *C. melanoneura*, that are recognised as the vectors of the disease occur in orchards of South Tyrol (northern Italy). The aim of the study was to assess the infection rates of natural populations of these insect species with *Ca.* 'Phytoplasma mali'. Two additional psyllid species (*C.* mal*i* and *Trioza urticae*), which are frequent in apple orchards of South Tyrol, were also investigated. A total of 800 specimens from 18 orchards were analysed using a real-time PCR procedure. While no specimen of T. urticae was found to be infected with *Ca.* 'Phytoplasma mali', the mean infection rate of *C. melanoneura* and *C.* mali was below 1%. The highest infection rate was found for *C. picta*, with a mean value of 11%. Based on these results, it can be concluded that *C. picta* plays the major role as the vector of apple proliferation in South Tyrol.

Comparison of European stone fruit yellows phytoplasma strains differing in virulence by multi-gene sequence analyses

Marcone, Carmine[1], Schneider, Bernd[2], Seemüller, Erich[2]
[1]Dipartimento di Scienze Farmaceutiche, Università degli Studi di Salerno, Via Ponte Don Melillo, I-84084 Fisciano (Salerno), Italy
[2]Julius Kuehn Institute (JKI), Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Fruit Crops and Viticulture, D-69221 Dossenheim, Germany
Contact: cmarcone@unisa.it

Twenty strains of the ESFY phytoplasma, which on the basis of graft-inoculation experiments greatly differ in aggressiveness, were examined by sequence analyses of several PCR-amplified non-ribosomal genes in order to identify molecular markers linked to virulence. These strains, which were maintained in various stone fruit genotypes, were indistinguishable with techniques for routine phytoplasma differentiation and characterization such as sequence and RFLP analyses of PCR-amplified rDNA. Also, the virulent ESFY strains maintained in periwinkle, namely GSFY1, GSFY2 and ESFY1 as well as an avirulent strain of the same phytoplasma, maintained in apricot, which was identified in recovered apricot trees, in France, and used there as a cross protecting agent, were included in the work for comparison. For PCR amplification, primers were designed from a number of genes distributed over the chromosome of strain AT of the apple proliferation phytoplasma. Visible PCR products were only obtained with primer pairs derived from the tuf gene which encodes the elongation factor Tu (EF-Tu), *rps*C (*rps*3) gene encoding the ribosomal protein S3, tlyC gene which encodes hemolysins, a membrane-damaging agents that serve as important virulence factors for many bacteria, the imp and fol genes encoding an immunodominant membrane protein and an enzyme involved in the folate biosynthesis, respectively. Nucleotide sequence comparisons revealed that the highest genomic variability occurred within the imp gene sequence with dissimilarity values ranging from 0.2 to 4.6%. For the remaining genes, the strains examined proved to be identical o nearly identical. Within the tuf gene, the presence of an additional TaqI restriction site, which had already known to occur in the strain GSFY1, was not identified for the other strains. The genetic differences observed among the strains examined are neither suitable markers for strain differentiation nor linked to pathological traits.

Hypo and hyper-virulence in apricot trees infected by European stone fruit yellows

Ermacora, Paolo, Loi, Nazia, Ferrini, Francesca, Loschi, Alberto, Martini, Marta, Osler, Ruggero, Carraro, Luigi
Via delle Scienze 208 Udine Italy
Contact: paolo.ermacora@uniud.it

An apricot orchard, located in an area of northeast Italy under high European stone fruit yellows (ESFY) infection pressure, has been monitored starting from the year of planting (1990). By time, most of the plants showed symptoms or resulted infected by PCR analyses. Particularly interesting resulted two groups of apricots: some asymptomatically infected and others recovered from the symptoms but not from the pathogen. With the aim to isolate strains of the phytoplasma characterised by different virulence, each group was used as mother plants and propagated. The new plants were used to constitute experimental orchards, where the plants were observed for the presence of symptoms and in part tested by PCR starting from 2003. The obtained results confirmed the presence of strains of the pathogen characterised by different virulence. The strains originally present in infected apricots recovered from the symptoms of ESFY resulted hypo-virulent; all the propagated infected plants never showed symptoms of the disease. Surprisingly, the strains present in asymptomatic mother plants of apricot resulted hyper-virulent and the propagated plants always showed severe symptoms. In the propagated plants, the transmission of the pathogen resulted higher for the hyper-virulent strains in comparison with the hypo-virulent ones. A graft transmission trial carried out in the greenhouse using some of the identified hypo and hyper-virulent strains, confirmed the results obtained in open field. Real time PCR analyses showed that in the plants infected by hypo-virulent strains the colonisation of the pathogen was lower than in the plants infected by the hyper-virulent ones. It is possible to affirm that the hypo-virulent strains were present in the originally recovered mother plants of apricot. The research will continue with the aim to verify the possibility of cross protection among the identified hypo and hyper-virulent strains.

Poster Presentations

First survey on blueberry viruses and phytoplasma in the Czech Republic

Spak, Josef, Kubelkova, Darina, Pribylova, Jaroslava, Spakova, Vlastimila, Petrzik Karel
Biology Centre, Institute of Plant Molecular Biology, Czech Academy of Sciences, Branisovska 31,
370 05 Ceske Budejovice, Czech Republic
Contact: spak@umbr.cas.cz; fax: +420 385 310 356

First survey on the occurrence of viruses and phytoplasma in blueberry Vaccinium corymbosum (highbush blueberry) and naturally occurring Vaccinium species is currently conducted in the Czech Republic. Plantations, germplasma collections, propagation materials and wild plants are monitored for

symptoms, assayed on differential host plants, by commercial ELISA kits for *Blueberry scorch virus* (BBScV), *Blueberry shock virus* (BlShV), *Blueberry shoestring virus* (BSSV), *Blueberry leaf mottle virus* (BLMoV), and by PCR for *Blueberry red ringspot virus* (BRRV) and phytoplasma. First results will be presented. Granted by the Czech Ministry of Education OC09022.

Tomato ringspot nepovirus (ToRSV) in wild blackberry (*Rubus fruticosus* L.) in Hatay province of Turkey

Sertkaya, Gülsen
Department of Plant Protection, Faculty of Agriculture, University of Mustafa Kemal, 31034 Antakya/Hatay-Turkey
Contact: gsertkaya@mku.edu.tr

During observations of virus-like symptoms in wild blackberry (*Rubus fruticosus* L., Rosaceae) some stunted plants growing in the border of stone fruit orchards in Hatay were found showing severe yellow blotching and deformity of the leaves. Samples (shoots and leaves) were collected in September 2008 from wild blackberries plants growing at the border of apricot orchards and neighboring stone fruit nurseries in Hatay province in Eastern Mediterranean Region of Turkey. Each of 12 wild blackberry samples taken from 7 symptomatic and 5 symptomless plants was tested for virus by mechanical inoculation of sap to herbaceous plants. Sap was inoculated on *Chenopodium amaranthicolor, C. quinoa, Cucumis sativus, Gombherena globosa, Nicotiana benthamiana, N. clevelandii, N. glutinosa, N. rustica* and *Petunia hybrida*. Sap from six symptomatic plants induced symptoms as necrotic or chlorotic lesions, ring spots on test plants. No symptoms were induced in these test plants by sap from symptomless blackberry plants. A sap-transmissible virus was obtained from each of symptomatic plants and identified as Tomato ringspot nepovirus (ToRSV) by double-antibody sandwich enzyme-linked immunosorbent assay (DASELISA). Results of biological indexing were also confirmed by serological assays (DAS-ELISA). Cuttings of symptomatic plants were rooted in pots and kept in insect-proof growing room for symptom observations and other studies. Investigations on the virus in wild and cultivated *Rubus* spp. and its vector/s have been in progress. Further studies are necessary to investigate distribution and natural transmission of the main virus diseases in cultivated *Rubus* spp. due to its recent increasing numbers of plantations and economical importance in Hatay.

To our knowledge, ToRSV were reported for the first time in wild blackberry in Turkey.

Detection of Blueberry red ringspot virus in highbush blueberry cv. Coville

Mavric, Plesko Irena , Virscek, Marn Mojca, Koron, Darinka
Agricultural Institute of Slovenia, Hacquetova 17, Ljubljana, Slovenia
Contact: irena.mavric@kis.si

Blueberry red ringspot virus (BRRSV) is a member of genus Soymovirus in the family Caulimoviridae. It is known to infect Vaccinium corymbosum, V. formosum, V. australe and probably V. macrocarpon. Many blueberry cultivars are sensitive to BRRSV, cv. Bluecrop is reported to be field-resistant and cv. Jersey is field-immune. BRRSV causes red ringspots or red blotches on one year old stems or older. In mid- to late summer reddish-brown spots develop on older leaves. The spots are prominent on the upper surface of the leaf. In some cvs. fruit symptoms, circular areas of light colour and/or fruit deformations can be seen. The disease can significantly reduce yield. The disease is present in the USA. On one plant of highbush blueberries in introduction plantation at Brdo pri Lukovici, symptoms indicating BRRSV infection were observed. Red rings appeared on some of the stems and also red rings or spots were observed on some leaves. At the harvest time spots of light colour were observed in ripening fruits. DNA was isolated from symptomatic tissue in spring 2008. Primers RR13 and RR14 (Glasheen et al., 2002) were used in subsequent PCR. Obtained amplification product of approximately 490 bp was sequenced and the sequence confirmed the infection of blueberry plant with BRRV.

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Comparison of Raspberry bushy dwarf virus isolates from Hungary and Slovenia

Virscek, Marn Mojca[1], Mavric, Plesko Irena[1], Gorsek, Janja[1], Nyerges, Klára[2], Lázár, János[3], Tökés, Ágnes[4]
[1]Agricultural Institute of Slovenia, Hacquetova 17, 1001 Ljubljana, Slovenia
[2]Agricultural Office of County Fejér, Plant Protection and Soil Conservation Directorate, Orszag ut 23, 2481 Velence, Hungary
[3]Research Institute for Viticulture and Enology, Miklostelep, Urihegy 5/A, 6000 Kecskemet, Hungary
[4]Central Agricultural Office, Keleti Károly u. 24, 1024 Budapest, Hungary
Contact: mojcavm@kis.si

In 2006 and 2007 samples of wild and cultivated *Rubus* species were collected on 7 locations in Hungary and on 16 locations in Slovenia in the frame of a bilateral project. 7 varieties of raspberry from one Hungarian collection orchard were found to be infected with *Raspberry bushy dwarf virus* (RBDV). In Slovenia the presence of RBDV was confirmed only in 3 samples of wild *Rubus* out of 43 samples collected in the woods. Serological characterisation with three monoclonal antibodies (R2, R5 and D1) was performed on positive samples. Selected positive samples were partially sequenced. The results of serological and molecular analyses were compared with the results of raspberry and grapevine isolates obtained in Slovenia in the frame of other projects.

Occurrence of small fruit viruses in Belarus

Valasevich, Natallia, Kolbanova, Elena
Department of Biotechnology, Institute for Fruit Growing Kovalev str., 2, Samokhvalovichi, 2 23013, Belarus.
Contact: NataVolosevich@yahoo.com; Kolbanova@tut.by

Phytosanitary state of small fruit plantations was studied in Belarus by DASELISA. Objects of research were cultivars of Rubus idaeus L. and Ribes sp. L. According to “Statute of fruit plant material production in Belarus” following viruses were identified: CMV, ApMV, SLRV, RRV, RBDV, ArMV, TBRV and ToRSV. A high level of virus infection in commercial raspberry cultivars (Alyonushka, Balsam and Meteor) was detected: 52.9% of SLRV, 41.0% of RRV, 38.2% of RBDV, 32.3% of ApMV, and 20.5% of ArMV. CMV, TBRV and ToRSV were absent from all tested plants. Upon the average only 5.8% of samples were free from viruses tested, 32.3% were infected by one virus, 35.2% had two viruses, 23.5% contained three viruses and 2.9% of samples had four viruses. The phytosanitary inspection of black currant collection plants determined the high level of virus infection: 100% of RRV,

97.5% of TBRV, 100% of SLRV and 81.8% of ArMV, and only 5.8% of plants were infected by CMV. All black currant cultivars tested were 100% infected with RRV and SLRV, as well as with TBRV, except cultivar Katyusha (83.3% of the tested plants were infected). Not less intensive infection was detected in black currant mother plantations: RRV – 100%, TBRV – 93.3%, SLRV – 71.1%, CMV – 62.2%. The most common red currant viruses appeared to be RRV (47.9%) and TBRV (34%). CMV was detected in 2.1% of tested plants, and ArMV - in 4.3 of ones correspondingly. Only 28.7% of samples were free from viruses tested (RRV, TBRV, SLRV, ArMV, CMV, TomRSV), 47.9% were infected by one virus, 20.2% had two viruses, 3.2% contained three viruses.

Virus survey in strawberry production fields in the United States and Canada

Martin, Robert, R.[1], Tzanetakis, Ioannis, E.[2]
[1]USDA-ARS HCRL, 3420 NW Orchard Ave., Corvallis, OR USA 97330
[2]Department of Plant Pathology, University of Arkansas, Fayetteville, AR, USA 72701
Contact: bob.martin@ars.usda.gov

In an effort to determine the incidence of viruses in strawberry production fields in the US and Canada, approximately 1500 samples were collected and either brought back or shipped to the USDA-ARS laboratory in Corvallis, Oregon between 2002 and 2007. RNA was extracted from leaf tissue and archived at - 80C for subsequent uses. During the same time, RT-PCR tests were developed for most known strawberry viruses. For this study a subset of 275 samples, representing the major strawberry production areas in the US and Canada were tested for: Beet pseudo yellows (BPYV), Fragaria chiloensis latent (FClLV), Strawberry crinkle (SCV), Strawberry latent ringspot (SLRSV), Strawberry mottle (SMoV), Strawberry mild yellow edge (SMYEV), Strawberry necrotic shock (SNSV), Strawberry pallidosis (SPaV) and Strawberry vein banding (SVBV) viruses, as well as a housekeeping gene as an internal control by RT-PCR. The Pacific Northwest had the highest rates of infection with the aphid-borne viruses but was virtually free of the whitefly transmitted viruses. In contrast, California, southeastern US, northeastern US, midwestern US and Ontario had aphid and whitefly transmitted viruses in about equal numbers. The midwestern US had the lowest incidence of virus infection. BPYV was only found in samples from CA and southeastern US, but has been detected from Maryland in previous studies. In the Pacific Northwest, fields with aphid control had very low incidence of virus infection compared to nearby fields without aphid control. Also the disease pressure was much lower in Oregon than in northern Washington and British Columbia. As a result of this information, management strategies can be designed for the major viruses and vectors that occur in a given area. As an example, management efforts in the Pacific Northwest should be targeted toward control of aphids, whereas in other areas, whiteflies are important vectors of a number of viruses.

Infectious agents associated whit strawberry decline in Italy

Ratti, Claudio[1], Terlizzi, Federica[1], Credi, Rino[1], Pisi, Annamaria[1], Babini, Anna Rosa[2], Vicchi, Valerio[2], Rubies, Autonell Concepcion[1]
[1]DISTA - Plant Pathology, University of Bologna, Via G. Fanin, 40 – 40127 Bologna, Italy. e-mail: concepcion.rubies@unibo.it
[2]Servizio Fitosanitario Regionale, SFR, Regione Emilia Romagna, Bologna, Italy. e-mail : ababini@regione.emilia-romagna.it
Contact: claudio.ratti@unibo.it

Symptoms of decline have been observed in different strawberry cultivated areas and since 2003-2004 they have spread in north Italy and especially along the cost of the Emilia-Romagna region, where most of the nurseries are present. In the Spring the plants showed loss of vigour, small and distorted leaves, yellowing and/or light yellow edge. In the Summer and Fall groups of plants showing decline, reddish and curled leaves, poor development of the central crown leaves with an evident edge chlorosis, distributed in patches in the fields, were observed. Plant samples with the most representative symptoms were collected, and analyses, based on molecular diagnostic protocols, have been applied to detect viruses transmitted by aphids, by whitefly or without known vectors and prokaryotes transmitted by planthoppers. The results obtained from the analyses done in 2007 and 2008 on plants with decline and chlorosis symptoms, have showed the presence of *Strawberry mottle virus* (SMV), *Strawberry mild yellow edge virus* (SMYEV) and *Strawberry crinkle virus* (SCV), already known to be present in our cultivated areas, and *Strawberry vein banding virus* (SVBV) and *Strawberry chlorotic fleck virus*, (SCFV), never found before in Italy. On a total of 86 tested samples, 35 (40%) were infected with one or

more of the above mentioned viruses. Fifty plants with evident decline symptoms in the Fall, were collected and 28 (56%), after molecular analysis, were found to be affected by two different prokaryotes: *Candidatus phytoplasma solani* (Stolbur) and a γ 3-proteobacterium similar to that identified on sugarbeet in France. The presence of these viruses and prokaryotes had quite often a spot-like distribution. However it should be controlled, since these pathogens, due to their transmission mode, can spread in a short time causing epidemies with significant economic losses.

Characterisation of mixed virus infections in *Ribes* species in Switzerland

Besse, Sébastien, Gugerli, Paul, Ramel, Maria-Elena, Balmelli, Carole
Swiss Federal Research Station of Agroscope Changins-Wädenswil ACW, Department of Virology,
CH-1260 Nyon 1, Switzerland
Contact: sebastien.besse@acw.admin.ch

Various symptoms of viral infections are frequently observed in Ribes sp. in Switzerland. Although viruses infecting *Ribes* sp. were described in several countries, the situation in Switzerland remains poorly documented. Therefore, symptomatic plants from diverse origins were analysed by electron microscopy (EM), immuno-precipitation electron microscopy (IPEM), Western blot and (RT)- PCR. By EM, at least four different particles types, often in combination, were observed. (1) Bacilliform particles were typical for the *Badnavirus* genus with dimensions of 145 x 28 nm. This wirus was further identified by PCR as the *Gooseberry vein banding associated virus* (GVBaV). (2) Filamentous particles were mainly observed on black currants with downward rolling of leaves with interveinal reddening in late summer and fall. We tentatively named this unknown virus *Blackcurrant leafroll-associated virus 1* (BCLRaV-1). In phylogenetic analysis of HSP70h nucleotide sequences, BCLRaV-1 felt in the *Closterovirus* genus. In Western blot analysis, one dominant protein with an estimated molecular weight of about 28 kDa was detectable. Nevertheless, this virus was shown to be different from the *Raspberry mottle closterovirus* (RMoV) by IPEM and RT-PCR. (3) RT-PCR and sequencing of products also clearly demonstrated the presence in our *Ribes* samples of *Rubus chlorotic mottle virus* (RuCMV), a *Sobemovirus* recently descriebed in Scotland. This finding correlates with the presence of the 30 nm particles observed by EM. (4) Still another entity with isometrical particles of 60 nm could not yet be attributed to a particular genus. Altogether, our data suggest the presence of multiple virus infections in *Ribes* sp. in Switzerland and emphasize the need for an efficient sanitary selection process.

Evaluation of the reliability of lateral flow immunochromatography strips for detection of *Plum pox virus*

Malinowski, Tadeusz
Research Institute of Pomology and Floriculture, Pomologiczna 18, 96-100 Skierniewice, Poland
Contact: tmalin@insad.pl

The need for fast (preferably cheap) and reliable diagnostics of plant virus and virus-like disease is especially high in the case of vector-transmitted pathogens like *Plum pox virus*. For some applications, fast diagnostics 'in the field' may be critical for effective control of the pathogen. Diagnostic kits based on lateral flow immunochromatography are offered by several companies for detection of *Plum pox virus* as well as many other pathogens. The result of such test is usually based on visual checking for the presence of two bands developed on the strip. The question of sensitivity and reliability of such assay performed by untrained users was addressed in this simple study. Plum and peach samples containing different amounts of PPV (estimated by ELISA) were tested with AgriStrip (Bioreba) assay. The set of strips have been presented to a number of persons with the task of selecting them into three groups: 'positive', 'negative', 'don't know'. Although there were differences in the evaluation of the strips corresponding to lower amounts of the virus, consistent evaluation results were observed for relatively high and medium concentrations of the virus. These results indicate that immunostrip assay may be a valuable tool in the hands of nurserymen/nurserywomen and farmers for rapid checking the suspicious plants. On the other hand, some samples with very low amount of virus may need verification performed with other techniques in the specialised laboratory.

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Transient expression of the coat protein of Apple chlorotic leaf spot virus inhibits the viral RNA accumulation in *Nicotiana occidentalis*

Yaegashi, Hajime[1], Isogai, Masamichi[2], Yoshikawa, Nobuyuki[2]
[1]Plant Pathology Research Team, Apple Research Station, National Institute of Fruit Tree Science, National Agricultural and Food Research Organization (NARO), Morioka 020-0123, Japan
[2]Plant Pathology Laboratory,Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan

The coat protein of Apple chlorotic leaf spot virus(ACLSV-CP) plays a crucial role in infectivity and efficient viral RNA accumulation in host cells (J. gen. Virol, 88, 2007). In this study, the effect of ACLSV-CP on viral RNA accumulation in *Nicotiana occidentalis* was investigated. The wild type CP (wtCP), CPm40 (an amino acid (aa) substitution of Ala to Ser at aa position 40), CPm75 (a substitution of Phe to Tyr at aa position 75) (both CPm40 and Cpm75 are fatal to viral infectivity and RNA accumulation), and CPm40m75 (two aa substitutions at positions 40 and 75) of ACLSV-P205 were transiently expressed in *N. occidentalisleaves* by agroinfiltration. Immunoblot analysis showed that wtCP and CPm40m75 were stably accumulated in infiltrated tissues, in contrast to proteins of CPm40 and CPm75 which were not detected, indicating that the stable accumulation of CP is important for effective viral RNA accumulation. However, co-agroinfiltration of an infectious cDNA clone of ACLSV (pBICLSF) or pBICLSF-based CP mutants (pBICLCPm40, pBICLCPm75, pBICLCPm40m75) with wtCP showed that no viral genomic RNA accumulations were found in any tissue infiltrated with pBICLSF, pBICLCPm40m75, pBICLCPm40, or pBICLCPm75. The inhibition of ACLSV-RNA accumulation was found only in leaves co-expressed with CP protein, but not with a frame-shift mutant of CP or P50 movement protein of ACLSV. These results suggested that the stable accumulation of ACLSV-RNA may be regulated by the level of CP accumulation and/or the timing of CP expression.

Highly efficient method of inoculation of apple viruses to apple seedlings

Yamagishi, Noriko, Sasaki, Shintarou, Yoshikawa, Nobuyuki
Plant Pathology Laboratory,Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan

Virus inoculation to original plants is an important steps in research, for example, to satisfy Koch's postulates, to test resistance to viruses in a breeding program, and to analyze gene function by virus vectors etc. However, it is generally difficult to inoculate viruses to woody fruit trees like apple, and an efficient inoculation method has not been developed thus far. In this study, we showed that a biolistic inoculation of total RNAs from infected tissues resulted in a high infection rate in apple seedlings. Total RNAs were extracted from *Chenopodium quinoaleaves* infected with Apple latent spherical virus (ALSV) or Apple chlorotic leaf spot virus (ACLSV). The RNAs were biolistically inoculated to the cotyledons of apple seedlings using a Helios Gene Gun System (20~30 ug RNA per plant). The inoculated seedlings were grown in a growth chamber at 25 C and then analyzed by Northern blot hybridization two weeks after inoculation. The results indicated that 61 out of 66 plants (92%) inoculated with ALSV and 6 out of 7 plants (86%) inoculated with ACLSV were found to be infected with each virus. Thus, the biolistic inoculation of total RNA from infected tissues to apple seedlings is an efficient inoculation method of apple viruses and the method can be applied to other virus-fruit tree combinations.

Nucleotide analysis of pome fruit virus isolates detected in apple and pear samples from Italy and India

Ferretti, L.[1], Hallan, V.[2], Ram, R.[2], Rana, T.[2], Dhir, S.[2], Negi, A.[2], Thockchom, T.[2], Zaidi, A.A.[2], Barba, M.[1]
[1]C.R.A. - Centro di Ricerca per la Patologia Vegetale, Via C. G. Bertero, 22, 00156 Rome, Italy
[2]Virology Lab, Institute of Himalayan Bioresource Technology,. Palampur-176061, HP, India

In the frame of a joint research project between Italy and India field surveys were done in different pear and apple growing areas of North of India and Central and Southern Italy. Samples were collected from plants belonging to common and local varieties and molecularly analyzed for the detection of the main pome fruit viruses (*Apple stem pitting virus*, *Apple stem groving virus*, *Apple chlorotic leaf spot virus*, *Apple mosaic virus*) by using harmonized diagnostic protocols. In order to evaluate phylogenetic relationship among isolates of different geographical origin positive samples of each virus were sequenced and the obtained sequences were compared with those retrieved in gene bank. The sequence homology were evaluated and a phylogenetic tree was built.

Detection of pear vein yellows disease caused by Apple stem pitting foveavirus (ASPV) in Hatay province of Turkey

Sertkaya, Gülsen
Department of Plant Protection, Faculty of Agriculture, University of Mustafa Kemal,
31034 Antakya/Hatay-Turkey
Contact: gsertkaya@mku.edu.tr

Apple stem virus (ASPV, genus Foveavirus) cause *Pear vein yellows disease* (PVYD) in pear (*Pyrus communis*). The purpose of this study was to investigate the relation of ASPV with PVYD in pear trees are exhibited severe leaf symptoms in Hatay province of Turkey. A survey was carried out in 9 pear orchards and 3 nurseries to investigate PVYD and to inspect the symptoms associated with the disease on pear in Hatay. A few number of quince trees planted in the inspected pear orchards were also detected for the disease. Leaf symptoms consist of yellow vein banding, reddening and flecking along veins were observed during the summer till winter (dropping leaves) on pear. Symptoms of PVYD were not found on quince trees. ApMV and ACLSV which are important viruses of pome fruits were also investigated on pear orchards and quince (*Pyrus cydonia*) trees in pear orchards. The shoot and leaf samples were taken randomly from inspected trees in orchards and seedlings in nurseries in July for ApMV, ACLSV and for (ASPV) in September in 2008. A total of 47 samples from local pear cultivars (Mustafa Bey and Ankara cvs.) and 6 from quince trees (unknown cultivar) were collected. All samples were detected for presence of ApMV and ACLSV by ELISA. ApMV, ACLSV infections were not found in detected samples. Fifteen samples randomly selected from 25 symptomatic and all of 5 asymptomatic pear samples were also tested for ASPV by biological indexing. Sap extracts were mechanically inoculated on herbaceous test plants. Symptoms including vein clearing and leaf necrosis were observed on *G. globulosa* and *N. occidentalis* test plants. Twelve samples collected from symptomatic and asymptomatic pear samples were found to be infected with the disease by sap transmission tests. ASPV found present in 60,0% of the tested samples (12/20). This preliminary study demonstrated that a high rate of ASPV infections were presence on local pear cvs. in the province.

Determination of the effects of APPLE stem grooving virus on some commertial apple cultivars

Birisik, Nevzat[1], Baloglu, Saadettin[2]
[1]Adana Plant Protection Research Institute. Department of Phytopathology. 01321 Yuregir, Adana.
[2]Plant Protection Department of Agriculture Faculty. University of Çukurova. 1235 Balcalı, Adana
Contact: nevzatbir@yahoo.com

The studies to determine the effects of *Apple stem grooving capillovirus* (ASGV) on external and physical characteristic of some commercial apple cultivars were carried out in Adana Plant Protection Research Institute's screen house facility in Turkey during 2006-2008. The selected cultivars for this aim were Jerseymac, Fuji, Golden Delicious, Summerred, Granny Smith, Vista Bella, Galaxy Gala and Starking. The selection of the cultivars was based on their common use by growers in the country. All cultivars were grafted on M9 rootstock and potted in screen house. Turkish io-50 ASGV isolate, which had been obtained from previous works from an Anna apple tree, was used for inoculation by chip budding, and the success of inoculation was confirmed by DAS-ELISA. The trial was evaluated two years after inoculation, based on six external and two physical parameters of the inoculated trees. The results demonstrated that ASGV has no statistically important effects on length of tree, number of the branches, average and total length of the branches, and leaf dry matter. However, ASGV decreased the trunk diameter about 18%, and the woody dry matter in a statistically significant rate, whereas the angle of the branches from the trunk increased in an average about 22% by ASGV infection. The cultivars reacted differently to the virus inoculation and stem grooving symptoms were observed on some tested cultivars.

Virus diseases of pomes fruit trees in Belarus

Kukharchyk, Natalia, Kastrickaya, Manana
Institute For Fruit Growing, Kovaleva Street 2, Samokhvalovichy, Minsk region, 223013 Republic of Belarus
Contact: Kychnataly@tut.by

Virus diseases diagnostics of fruit crops were begun in 1998 in Belarus. Before that time, only fragmentary researches of apple-tree viruses using woody test were carried out. The purpose of our work was monitoring of contamination by viruses and production of certified varieties and vegetative propagated rootstocks of *Malus*, *Pyrus* and *Cydonia*. Tests on woody indicators in the field and in the glasshouse and serological tests (DAS-ELISA) were used for virus disease detection. Currently apple trees are infected mainly by *Apple chlorotic leaf spot trichovirus* (ACLSV, 59,32 %), however, data about ACLSV strongly differ for various plantings, varieties and rootstocks. So, the virus presented in 66,38 % of varieties and rootstocks propagation material while in fructifying plantings it was revealed in 54,75 % of the samples. *Apple stem grooving virus* was detected in propagation material (21,74 %) and in fructifying plantings (8,89 %). This virus infects completely many old varieties, cultivated for a long time. Apple mosaic ilarvirus was detected only in 3,50 % of samples from apple trees. *Apple stem pitting virus, Apple proliferation phytoplasma*, virus-like diseases and viroids weren't observed using woody tests during 4 years. ACLSV was identified on the average in 59,09 % of *Pyrus* varieties and rootstocks propagation planting and in *Cydonia* rootstocks planting. Fructifying plantings were infected by this virus very differently – numbers of varieties were free from the virus (new cultivars), another had 38 – 100% infected plants. According to our researches, plants with a negative test result for all pathogens listed in Belarussian Certification scheme, were transferred to the nuclear stock collection (16 apple varieties, 7 pear varieties, 4 rootstock forms).

Viruses of pome fruits in Bosnia and Herzegovina

Lolić, B.[1], Matić, S.[2], Hassan, M.[3], Đurić, G.[4], Di Serio, F.[5], Myrta, A.[6]
[1]Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (BA), Italy and Faculty of Agriculture, Stepe Stepanovića 75, 78000 Banja Luka, Bosnia and Herzegovina
[2]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale, Sezione di Bari, Via Amendola 165/A, 70126 Bari, Ital
[3]Plant Pathology Division, Faculty of Agriculture, Fayoum University, Fayoum, Egypt
[4]Faculty of Agriculture, Stepe Stepanovića 75, 78000 Banja Luka, Bosnia and Herzegovina
[5]Istituto di Virologia Vegetale del CNR Via Amendola 165 /A, 70126-Bari, Italy
[6]Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (BA), Italy.
New address: Certis Europe, Via A. Guaragna, 3 21047 Saronno (VA) Italy
Contact: f.diserio@ba.ivv.cnr.it

During autumn 2005 and summer 2006, field surveys were carried out to assess the sanitary status of pome fruit trees in Bosnia and Herzegovina. Inspections were done in the main pome fruit growing areas including 10 orchards, 2 nurseries and one varietal collection. A total of 65 apple and 50 pear cultivars were tested by biological indexing for the presence of *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem pitting virus* (ASPV), *Apple stem grooving virus* (ASGV) and *Apple mosaic virus* (ApMV). The average infection level was 81%. Both species showed similar infection rate (83% for apple and 78% for pear). The most frequent viruses of apple were ACLSV (72%) and ASPV (69%), whereas for pear ASGV (69%) and ACLSV (64%). The same number of samples were additionally tested by ELISA, but biological indexing showed as more reliable than ELISA for virus detection. Multiplex RT-PCR results of 20 randomly selected apple cultivars were in line with biological indexing. Results of our surveys report for the first time the presence of ACLSV, ASPV, ASGV and ApMV on pome fruits in Bosnia and Herzegovina.

Detection and Identification of Apple Stem Pitting and Apple Stem GroovingAffecting Apple and Pear in Egypt

Sahar, A.Y.[1], Moawed, S.M.[2], Shalaby, A.A.[1]
[1]Plant Pathology Res. Ins. Giza Egypt
[2]General Admenestration of seed test and Certification
Contact: saharyoussef@link.net

Apple stem pitting virus (ASPV) and *Apple stem grooving virus* (ASGV) are important apple, pear and other fruit crops viruses. They infect many commercial apple and pear cultivars and occur either individually or mixed infections causing yield loses. Young green bud and/or base of petiole, were collected from naturally infected apple and pear trees from different location in Egypt. Both viruses were detected frequently in apple and pear samples. A total of 420 trees from 9 different orchards were tested; 13% ASPV-infected and 17% ASGVinfected trees were recorded. Mixed infection with ASPV and ASGV was recorded in 4.% of the trees. Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) has been used as a routine method for the detection of ASPV and ASGV and for screening of virus-free materials generated from elimination programs. However, this method involves more steps and thus time consuming. Total RNA was isolated from 100 mg fresh affected apple and pear leaf tissue using Qiagen RNeasy plant mini-kit (Qiagen, Crawley, UK), according to the manufacturer's instructions. The one step-RT-PCR method was performed using ASPV and ASGV - specific primer for each virus. A 316 bp fragment for ASPV and 524bp fragment for ASGV were amplified and indicated the presence of ASPV and ASGV in affected apple and pear. Southern blot hybridization of the amplified products to digoxigenin (DIG)-labeled cDNA probe for ASPV and ASGV were used to confirm the detection results No product was detected in amplified extracts from uninfected apple and pear samples. The detection of ASPV and ASGV by one step-RT-PCR assay were successful and appear useful for testing pome fruit germplasm in quarantine or budwood certification programs.

Current Status of Apple Mosaic Virus in Turkey

Ertunc, Filiz[1], Sokmen, Miray A.[2], Sezer, Arzu[3], Canik, Digdem[1]
[1]Ankara University, Faculty of Agriculture, Department of Plant Protection,Ankara
[2]19 Mayıs University, Department of Plant Protection,Samsun
[3]Hazelnut Research Institute;Giresun
Contact: ertunc@agri.ankara.edu.tr

Apple mosaic virus is one of the most important virus infection of apple and hazelnut production in Turkey. It distributes especially with pollens and infected plant material especially with scions. The patogen is present almost all of the hazelnut orchards placed on Black Sea coast , besides in apple production the ApMV was present only on stunted Granny Smith plantations. The other common and local varieties seem to be resistant to the patogen to some extent. The presence of patogen first was confirmed by DAS-ELISA tests and RT-PCR. Rna extraction was succeded in apple tissues but it failed by hazelnut tissues. Hazelnut specimens were subjected to paasage to *Phaseolus vulgaris* (bean) plants and then,RNA will be extracted from bean and will be subjected to RTPCR.

First survey of pome fruit viruses in Morocco

Afechtal, M., Milano, R., Djelouah, K., D'Onghia, A.M.
Istituto Agronomico Mediterraneo, Bari, Italy (IAMB)

A preliminary assessment of the presence of pome fruit viruses in Morocco was carried out. Twenty orchards and nurseries were surveyed in the regions of Midelt, Meknès and Azilal. A total of 100 samples (apples and pears) were collected and tested. Biological indexing was made in a climatized greenhouse using the following indicators: *Mallus pumila* 'Spy 227' 'Radiant', 'R 12740 7A' and *Pyrus communis* 'LA/62'. All samples were also tested by ELISA for the presence of *Apple chlorotic leaf spot virus* (ACLSV), *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV) and *Apple mosaic virus* (ApMV). The prevailing viruses of apple were ACLSV (71%) and ASPV (58%), whereas ASGV was found in 12 tested trees. The same viruses were present, but less frequently, in pear: ACLSV (61), *Pear Vein Yellows Virus* (PVYV) (25%) and ASGV (18%). Only four apple trees were found to be infected by ApMV. Additional RT-PCR testing confirmed the high incidence of ACLSV and ASPV. This was the first report of pome fruit viruses in Morocco, indicating the high infections rate worsened by the recent report of the presence of fire blight (*Erwinia amylovora*) in the country. Moreover, a total

of 168 apples and 81 pears were sampled and tested for pome fruit viroids *Apple scar skin viroid* (ASSVd), *Apple dimple fruit viroid* (ADFVd) and *Pear blister canker viroid* (PBCVd) by tissue printing hybridization. No viroids were detected.

The evaluation of presence and the symptomology of viruses in commertial quince orchards in Turkey

Birisik, Nevzat[1], Baloglu, Saadettin[2]
[1]Adana Plant Protection Research Institute. Department of Phytopathology, 01321 Yuregir, Adana.
[2]plant Protection Department of Agriculture Faculty. University of Çukurova, 1235 Balcalı, Adana
Contact: nevzatbir@yahoo.com

Turkey is the biggest quince (*Cydonia oblonga*) producer country in the world with its production about 120.000 tons/year. Virus diseases *Apple stem pitting foveavirus* (ASPV), Apple *stem grooving capillovirus* (ASGV), *Apple mosaic ilarvirus* (ApMV) and *Apple chlorotic leaf spot trichovius* are known as viral pathogens that can affect quality and quantity of the quince production. This study was carried out in Mediterranean region of Turkey between 2006 and 2008. The study was based on the survey activity, the symptomatological observations and the detection of viruses by DAS-ELISA and/or RT-PCR techniques. During the survey activity, 33 commercial orchards in five different counties were visited and 115 samples were collected and examined. Laboratory results showed that 17.39% of the samples were infected by either single or mixed infection of any tested viruses. Single infection of ASPV, ACLSV and ASGV were found 12,17%, 5,21% and 2,60% whereas mixed infections of ASPV+ASGV, ASPV+ACLSV, and ASPV+ASGV+ACLSV were 2,60%, 3,47% and 1,73% respectively. However ApMV was not found in any tested samples. Infected trees were marked and observed monthly during the whole vegetation period for two years. The observed symptoms were evaluated in accordance with the laboratory results. During the study; leaf mosaics, leaf deformation, fruit malformation, gummy fruit, dwarfing of the tree, bud-union abnormalities and trunk deformations were observed.

Incidence of Ilarviruses in Latvian Fruit Orchards

Pupola, Neda, Kale, Anna, Jundzis, Maris, Morocko-Bicevska, Inga
Latvia State Institute of Fruit-Growing, Graudu Street 1, Dobele
Contact: neda.pupola@lvai.lv

Apple mosaic virus (ApMV), Prunus necrotic ringspot virus (PNRSV) and Prune dwarf virus (PDV) are most notable Ilarviruses that affect fruit trees. ApMV is named after the disease it causes in apple, the first host in which it was described. Many plant species of *Rosacea* family are susceptible to ApMV, including *Prunus domestica* and *Pyrus communis*. PNRSV was earlier classified as isolate of ApMV but now it is reclassified as separate virus. PNRSV is distributed worldwide, infecting *Prunus* spp. and causing necrotic spots and shot holes in leaves. PDV causes chlorotic and necrotic spots on leaves and stunting of trees. It frequently occurs in mixed infections with other *Ilarviruses* – PNRSV and ApMV. In order to study the incidence of *Ilarviruses* in orchards the samples from apple, pear and plum trees of different varieties were collected from commercial gardens during spring 2007 and 2008. Polyclonal antibodies were used for DAS ELISA test for large scale screening. Totally 890 samples from apple, 252 samples from pear and 655 samples from plum were tested. Preliminary results obtained with ELISA test showed that all tested *Ilarviruses* were present in orchards. With ApMV were infected 1% of apple samples and 2 % of plum samples, but not any pear samples gave positive result. PNRSV was detected in 14% and PDV in 12% of plum samples. Mixed infections of *Ilarviruses* were observed in 4% of tested plum. Frequently observed mixed infection was PNRSV with PDV which appeared in 2% of tested plum samples. The obtained data of ApMV incidence in apple and pear trees with ELISA were compared with RT-PCR results. The RT-PCR results showed that the ApMV incidence in apple orchards is 22%, but in pear orchards 20%. ApMV sequences from different apple, pear and plum isolates are going to be compared for further studies.

Detection of a divergent variant of Plum bark necrosis and stem pitting associated virus (PBNSPaV) in Prunus domestica with peach red marbling disease symptoms

Marais, Armelle[1], Faure, Chantal[1], Gentit, Pascal[2], Candresse, Thierry[1]
[1]UMR GD2P, IBVM, Université Bordeaux2, BP81, 33883 Villenave d'Ornon Cedex, France

[2]Ctifl, Centre de Lanxade, 24130 La Force, France
Contact: amarais@bordeaux.inra.fr

In order to study the etiology of the peach red marbling disease (PRMa), GF305 peach plants were inoculated by grafting with various sources of PRMa. Double stranded RNAs (dsRNA) were extracted from symptomatic leaves and submitted to random amplification with highly degenerated primers. DsRNAs of high molecular weight were detected in one PRMa source from a Prunus domestica. After cloning and sequencing, seven clones were found to contain viral sequences displaying homology with different regions of the genome of Plum bark necrosis and stem pitting associated virus (PBNSPaV), a member of the *Ampelovirus* genus in the family *Closteroviridae*. The nucleotide identity levels observed ranged between 84% (625 bp fragment in the HSP90 gene and a 203 bp fragment in the HSP70 gene) and 87% (320 bp fragment in the helicase domain of ORF1a and 475 bp fragment in the major CP gene). At the amino acid level, amino acid sequence identity levels in the different regions are borderline with the 10% divergence species demarcation criteria in the *Ampelovirus* genus. Based on these preliminary observations, the molecular characterization of the PRMa-virus was pursued by sequencing the gaps between the different regions already available to yield a continuous ca. 8kb partial genomic sequence. The comparison of this sequence with that of PBNSPaV will allow the clarification of the taxonomic position of this agent. In parallel, the association of this virus with the PRMa disease will be evaluated by looking for this virus in various PRMa sources.

Biological characterization of Apricot latent virus (ApLV)

Grimová, Lenka[1], Rysánek, Pavel[1], Zouhar, Miloslav[1], Bazzoni, Alessandra[2], Minafra, Angelantonio[2], Palmisano, Francesco[2], Savino, Vito[2]
[1]Department of Plant Protection, Czech University of Life sciences Prague, Str. Kamýcká 129, Prague 6 Suchdol, 165 21, Czech Republic
[2]Dipartimento di Protezione delle Piante e Microbiologia Applicata, Universitá degli Studi and Instituto di Virologia Vegetale CNR, Sezione di Bari, Via Amendola 165/A, 70126 Bari, Italy
Contact: lgrimova@seznam.cz

Apricot latent virus (ApLV, ALV) was the first described in Moldova on symptomless apricot (*Prunus armeniaca*) cv. Silistra introduced there from Bulgaria in 1993. Based on the complete coat protein gene sequence, this novel virus was classified in the *Foveavirus* genus. The virus naturally infects apricot trees with no apparent symptoms. ApLV, however, causes yellow spot symptoms on leaves of graft-inoculated peach seedlings. Considering that, up to now, little information on biological properties of ApLV was available, the present study was focused on the identification of new potential woody hosts, further on the study of the associated symptomatology and the complete virus biology. The results of our findings are reported herein. Virus-free plants consisted of peach, apricot, cherry and plum varieties were infected by graft inoculation in 2004. Chip buds from ApLV infected peach seedlings (Apr-47, Palestinian isolate) were used in this study. The 200 bp ApLVspecific cDNA fragment was obtained from all 33 tested Prunus cultivars. Due to these results, the ApLV woody host range was extended by new varieties of *P. persica*, *P. avium*, *P. armeniaca* and for the first by japanese and european plum cultivars. Clear cut symptoms associated to ApLV were exhibited by the peach varieties and by one apricot cultivar. Tracking the virus in the different stone fruit species were carried out during the growing season, from these results the best period for reliable diagnosis of ApLV was suggested. Finally, ApLV occurence in the different plant organs, such as flower, fruit, leave and bark tissues, was determined.

Occurrence of Little cherry virus-1 on *Prunus* ssp. in Baden-Württemberg

Schroeder, Manfred, Petruschke, Michael
Landwirtschaftliches Technologiezentrum Augustenberg, Reinsburgstr. 107, D-70197 Stuttgart

A survey on Little cherry virus-1 (LChV-1) has been performed at three different sites between 2004 to 2006 in the State of Baden-Württemberg, including a commercial growing site, a state nursery and a garden for evaluation of trueness of variety. Ten Varieties of *Prunus avium* and a single one of Prunus serrulata were proved partly or totally infested. None of the infested trees has shown distinctive disease symptoms. In addition, testing of different varieties and types of certified rootstocks gave only negative results. The patched distribution pattern of trees of various infestation longevities within both the commercial nursery and the variety trueness evaluation garden can hardly justify the involvement of insect vectors in LChV-1 transmission in the field. At a scattered orchard of Baden-Württemberg LChV-

1 was detected on a local variety of sweet cherry and a wild tree of *Prunus avium*. Despite the occurrence of virus disease symptoms on the local variety, no relationship can be drawn to LChV-1. Further testing proved the presence of apple mosaic virus in addition, which is known to induce the observed symptoms. Studies on the shoots of some infested cherry trees being used for scion propagation showed an uniform dispersal of LChV-1 in all over the tree. Recent studies are conducted to verify the responses of young trees of the variety ‚Regina' to an artificially infestation with either LChV-1 or LChV-2. First year results indicate that - in complete contrast to LChV-2 - there are no adverse effects of LChV-1 on the single fruit weight, fruit yield, fruit size nor on circumference.

Transmission of Little cherry virus -1 (LChV1) by Cuscuta europea to herbaceous host plants

Jelkmann, Wilhelm, Hergenhahn, Felix, Berwarth, Constanze
Julius Kühn Institute, Institute for Plant Protection in Fruit Crops and Viticulture, Schwabenheimer Str. 101, D-69221 Dossenheim, Germany

Cuscuta europea as vector for transmission of *Little cherry virus*-1 (LChV-1) to *Nicotiana occidentalis* '37B'. Little cherry disease has been associated with two different long flexuous filamentous viruses of the family Closteroviridae. *Litte cherry virus*-1 (LChV-1) is an unassigned member in the family while *Little cherry virus*-2 (LChV-2) has been assigned to the genus Ampelovirus. Both viruses have been characterized on molecular level. The viruses can be found both alone and in mixed infections. The disease is distributed worldwide in ornamental and sweet cherry and has a great impact on fruit quality of infected trees. Symptoms produced by infected trees consist of small angular and pointed fruit that do not fully ripen and are imperfectly colored. Fruit have reduced sweetness and are unsuitable for consumption. There is evidence that some strains of LChV-1 on sensitive cultivars are either latent or symptoms are less severe compared to those caused by LChV-2. The disease is readily graft transmissible to cherry. There is no known vector associated with LChV-1, however, LChV-2 is transmitted by the apple mealybug (*Phenacoccus aceris*). Both viruses can be detected by RT-PCR. In order to identify alternative hosts different *Cuscuta* species were investigated in transmission trials. LChV-1 and -2 were graft inoculated onto Prunus avium F12 rootstocks and parasited by *Cuscuta europea*. *N. occidentalis* '37B' served as receptor host plant and could be infected systemically with LChV-1. Virus detection from *Cuscuta* and *N. occidentalis* tissue was done by RT-PCR. Virus transmission was not successful for LChV-2. Propagation of LChV-1 by mechanical transmission on *N. occidentalis* failed, however, the virus was serially transferred by grafting.

First report of Little cherry virus 1 in cherry in Turkey

Ulubas Serce, Cigdem, Gazel, Mona, Caglayan, Kadriye
Mustafa Kemal University, Plant Protection Department, Hatay/Turkey
Contact: culubas@gmail.com

Little cherry disease (LChD) is a serious virus disease of sweet (*Prunus avium*), sour (*P. cerasus*) cherry and several ornamental cherry trees. One Ampelovirus species, *Little cherry virus* 2 (LChV-2) and one unassigned species in the Closteroviridae, *Little cherry virus* 1 (LChV-1) have been associated with LChD. Symptoms produced by the infected trees consists of small, pale colored fruits with reduced sweetness and the interveinal areas of upper leaf surface turn red-violet or become bronze colored, while the midrib and the main veins retain their green color. The trees in an orchard located in Osmaniye, Turkey had bronze leaves on the upper shoots in July-August of 2007-2008 and no fruit set. In October 2008, the trees had out of season flowers with pink petals and bronze sepals. Flowers from seven cherry trees cv Napoleon and shoots from one rootstock cherry tree cv Mahaleb were collected from the orchard. Total nucleic acids from these samples were extracted as described Foissac et al. (2001) and used as template for reverse transcription. PCR were performed using primer sets specific for LCV-1 or LCV-2 (Rott and Jelkmann, 2001). Wheras all samples were negative for LCV-2, one cherry cv Napoleon and mahaleb samples gave a 419 bp fragment of LCV-1. The PCR product of mahaleb sample was sequenced and sequence analysis showed 89 % nucleotide identity to GenBank Accession Nos. Y10237 and X93351. To our knowledge, this is the first report of LChV-1 in Turkey.

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Susceptibility of a new range of apricot cultivars to apple mosaic ilarvirus

Gentit, Pascal, Ramat, Charlotte
Ctifl, Centre de Lanxade BP21 24130 La Force – France

Seven apricot cultivars were examined for their susceptibility to *Apple mosaic virus* (ApMV) in greenhouse during one year. Observations were carried out in order to determine if it is possible to use this test for selection of new cultivar regarding their suceptibilty to some viruses before planting them in a region infected by a determine virus. All the cultivars were sensitive to ApMV with different level of symptoms.

Serological Identification for some Important Viruses on Stone Fruits in Saudi Arabia

Alhudaib, Khalid
PO Box 55009 Alhassa 31982 Saudi Arabia
Contact: alhudaib@hotmail.com

To assess stone fruit viruses in spring 2007, field surveys were carried out in the stone fruit growing area (Al Juof - North of Saudi Arabia). Several virus symptoms including green mottle, vein clearing, necrotic spots, chlorosis and/or discoloration and symptoms less were collected and tested for the presence of *Plum pox virus* (PPV), *Prune dwarf virus* (PDV) and *Prunus necrotic ringspot virus* (PNRSV) using double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA). Total of 67 leaf samples (38 peach and 29 apricot) were tested. Result showed that 28 out of 67 leaf samples were infected with one and/or more viruses. The most common viruses were (PNRSV) with rate of 12/67, followed by PDV 9/67 and PPV 7/67. Detecting of mixed infection of PPV+PDV+PNRSV were 3 samples, PPV+PDV also were 3 samples and PDV+PNRSV were 2 samples. Further investigations are in need for other commercial orchards and nurseries. The serological result demonstrates that this is the first report of the detection of PPV, PDV and PNRSV in Saudi Arabia.

Keywords: ELISA, PPV, PDV, PNRSV, virus, detection, Saudi Arabia Abstract:

First occurence of Cherry virus A (CVA) in Czech Republic

Grimová, Lenka, Zouhar, Miloslav, Rysánek, Pavel, Drabesová, Jana
Department of Crop Protection, Czech University of Life Sciences Prague, Kamycká 129,
Praha 6 - Suchdol, Czech Republic
Contact: lgrimova@seznam.cz

Cherry virus A (CVA), a recently described member of the genus *Capillovirus*, was the first reported in *Prunus cerasus* in Germany. The virus does not appear to cause any obvious symptoms in the plants, but when combined with other viruses it may effect the severity of symptoms, or it may have some influence on graft incompatibility in susceptible combinations of scion and rootstock. The CVA is widely distributed in Europe and North America. In a survey for the assessment of the sanitary status of sour and sweet cherry crops in in the Czech Republic, leaf samples from 200 trees were collected in the Research and Breeding Institute of Pomology Holovousy Ltd. in 2008. Positive amplification in reverse transcription polymerase chain reaction (RTPCR), with one set of specific primers, was used to detect virus. Sequenced analyses of the RT-PCR products identified *Cherry virus A*. This is the first record of the occurence of CVA in the Czech Republic. This work provides the starting point for research on the occurence of the virus in planting material.

Occurrence of Prunus necrotic ringspot virus and Prune dwarf virus in sweet cherries in locality Veleherad (South Moravia, Velehrad)

Navrátil, Milan, Safarova, Dana
Department of Cell Biology and Genetics, Faculty of Science, Palacky University in Olomouc, Slechtitelu 11, 783 71 Olomouc, Czech Republic
Contact: milan.navratil@upol.cz

Old extensive orchard of wild cherry (*Prunus avium* L.) located in Velehrad (South Moravia, Czech Republic) was examined for *Prunus necrotic ringspot virus* and *Prune dwarf virus* during two years. DAS-ELISA detection kits (Bioreba AG) were used to detect both viruses according instructions of manufacturer. Occurrence of both viruses was confirmed. The problems of sampling and detection are discussed.

Identification of ilarviruses in almond and cherry fruit trees using nested PCR assays

Maliogka, Varvara I.[1], Charou, Anastasia[1], Efthimiou, Konstantinos[1], Katsiani, Asimina T.[1], Chatzivassiliou, Elisavet K.[2], Katis, Nikolaos I.[1]
[1]Plant Pathology Lab, School of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Greece
[2]Democritus University of Thrace, Department of Agricultural Development, 193 Pantazidou St., 68200 N. Orestiada, Greece

Stone fruits are known to be susceptible to ilarvirus infections. A nested PCR has been recently developed for the generic detection of *Ilarviruses* amplifying a 371 bp RdRp fragment. Using this method a survey was conducted on a number of almond and cherry trees and revealed high rates of *Ilarvirus*-related infections. For further identification of the viral agents involved in these infections the nested PCR step of the generic assay was modified to specifically detect *Prune dwarf virus* (PDV), *Prunus necrotic ringspot virus* (PNRSV) and *Apple mosaic virus* (ApMV). For the detection of each virus specific downstream primers were designed from conserved RdRp regions and used in respective nested PCR assays. The application of the same thermocycling profile allowed all amplifications to run in parallel. Ilarvirus isolates from different hosts were used for the evaluation of the detection range of the assays. A total of 265 almond and 196 cherry samples were collected from different districts of Greece. In almond trees the incidence of PNRSV and PDV was 41% and 21.5%, respectively. Both viruses were detected, though in lower rates (10%), in wild almonds. In cherry orchards the opposite was observed with PDV (56.6%) being the prevalent virus followed by PNRSV (19.4%). Mixed infections with both viruses were also encountered in approximately 10 and 17% of cherry and almond trees, respectively. ApMV was not detected in any of the samples tested. This is the first extensive survey conducted in Greece in order to monitor the distribution of these viruses using molecular assays.

Molecular characterization of the 3' part of the genome of divergent Cherry virus A isolates and development of a polyvalent CVA-specific PCR detection test

Svanella-Dumas, Laurence, Marais, Armelle, Candresse, Thierry
UMR GD2P, IBVM, Université Bordeaux2, BP81, 33883 Villenave d'Ornon Cedex, France
Contact: amarais@bordeaux.inra.fr

Previous studies on *Cherry virus A* (CVA) diversity based on the sequence of an internal fragment of the RdRp (the PDO fragment) have shown the existence of five phylogenetic groups, with up to 19% genetic divergence (Marais et al., 2008). Moreover, the prevalence of CVA in asymptomatic cherry trees, including certified material, was found to be very high (around 80%). Remarkably, outside of the major phylogenetic cluster, available detection assays based on PCR or on molecular hybridization failed to detect all the tested CVA isolates. In order to develop a more polyvalent test allowing the detection of all CVA isolates, the 3' half of the genome of representative members of each CVA cluster was sequenced. The comparison of the obtained sequences allowed the design of three primer pairs that were evaluated in RT-PCR assays. The two first primer pairs differed from each other by the location of the reverse primer; they are located in the region of overlap between the RdRp and the movement protein genes and allow the amplification of cDNA fragments of respectively 302-bp or 443-bp. The third couple of primers permit the amplification of an approximately 340-bp fragment comprising the 3' end of the CP gene and a part of the 3' UTR. In a two step RT-PCR assay, the three primer pairs allowed the detection of all the CVA isolates tested, including members of the divergent cluster corresponding to the CVA isolates from non-cherry hosts. (Marais et al. (2008). Acta Horticulturae, 781, 37-45)

Effects Associated with Graft-transmissible Agents Found in the Peach Variety 'Ta Tao 5'

Gibson, Philip[1], Reighard, Gregory[2], Marini, Diana[3], Scott, Simon[4]
[1]Gwinnett Technical College, 5150 Sugarloaf Parkway, Lawrenceville, GA, 30043 USA
[2]Department of Horticulture, Clemson University, Clemson, SC 29634
[3]P.O. Box 78, Gral. San Martin (5570) Mendoza, Argentina
[4]Department of Entomology, Soils and Plant Sciences, Clemson University, Clemson, SC 29634
Contact: PGibson@gwinnetttech.edu

The peach variety 'Ta Tao 5' is host to at least three graft-transmissilble agents. Extraction and characterization of these agents indicates that they are variants of *Peach latent mosaic viroid* (PLMVd), *Apple chlorotic leaf spot virus* (ACLSV) and an uncharacterized *Foveavirus* referred to as *Asian prunus virus* (APV). Each of these agents is used separately, in defined combinations and in concert in a field

study designed to identify their graft-transmissibility and consequent contribution to phenological changes in the peach varieties 'Springprince' and 'Juneprince.' Field data record significant changes in bloom date, vegetative growth and fruiting in both varieties tested. Further, such phenological variations associated with 'Ta Tao 5' differ significantly from artificial combinations of inoculants. The use of 'Ta Tao 5' as an inoculant source to manipulate growth and development of peach trees is unique when compared with other sources.

Assessment of the main stone fruits viruses and viroids in Algeria

Meziani, S.[1], Rouag, N.[2], Milano, R.[1], Kheddam, M.[3], Djelouah, K.1
[1]Istituto Agronomico Mediterraneo, Bari, Italy (IAMB)
[2]UFA, Faculté des Sciences, Département d'Agronomie, Setif
3 Centre National de Contrôle et de Certification des Semences et Plants (CNCC)
Contact: djelouah@iamb.it

In order to improve the sanitary status of the propagating material of stone fruits, a field survey was conducted to assess the main viruses and viroids affecting stone fruits in selected growing areas and their distribution according on the collected material by using serological and molecular detection methods. Serological assays were carried out to detect: *Plum pox virus* (PPV), *Prunus necrotic ring spot virus* (PNRSV), *Prune dwarf virus* (PDV), *Apple mosaic virus* (ApMV) and *Apple chlorotic leaf spot virus* (ACLSV). Moreover, tissue-print hybridization was performed to detect *Peach latent mosaic viroid* (PLMVd) and Hop stunt viroid (HSVd). Among nearly 2000 trees tested, no PPV infection was detected, while 14% of them reacted positively to at least one virus. The highest infection rate (17%) was reported in both nurseries and commercial orchards. PNRSV was the most detected virus (9%), followed by ApMV (3%) and PDV (1.5%). Cherry was the most infected species (20%). As for viroids, a high infection rate was recorded for PLMVd (9%)and HSVd (5%); the highest infection rate was reported in mother blocks and varietal collections.

Surveying Viruses on Ornamental Trees and Shrubs in Hungarian Botanical Gardens

Nemeth, Maria[1], Nyerges, Klara[2], Hangyal, Rozalia[2], Kosa, Geza[3]
[1]Central Agricultural Office, Budaőrsi út 141-145., 1118 Budapest, Hungary
[2]Agricultural Office of County Fejér, Plant Protection and Soil Conservation Directorate, Orszag út 23., 2481 Velence, Hungary
[3]Institute of Ecology and Botany, Hungarian Academy of Sciences Akotmány u. 2-4., 2163 Vácrátót, Hungary

In Hungary the occurrence of *Plum pox virus* (PPV) in ornamental and wild *Prunus* sp. has been surveyed since 2002. In 2005 this program was extended to other viruses occurring on woody plants kept in botanical gardens and collection. So far the following species were found to be infected with different viruses (*Prune dwarf virus* PDV, *Prunus necrotic ringspot virus* PNRSV, *Apple mosaic virus* ApMV, *Cherry leafroll virus* CLRV): *Prunus yedoensis* 'Moerheimii', *P. serrulata* 'Tai Haku', *P. serrulata* 'Pink Perfection', *P. serrulata* 'Ychio', *P. spinosa* 'Purpurea', *P. spinosa* 'Plena', *P. mume* L., *Lonicera sataliensis*, *Lonicera kaukazika*. Biological indexing and serology (ELISA) were used to detect the viruses.

Survey for PPV and PNRSV in nurseries and orchards in the northwest region of Iran

Sokhandan Bashir, Nemat
Crop protection Dept., University of Tabirz, Iran 51664
Contact: sokhandan@tabrizu.ac.ir

In survery for *Plum Pox virus* (PPV) and *Prunus Necrotic Ringspot Virus* (PNRSV), 313 samples with the following symptoms were collected from nurseries and orchards in the northwest part of Iran. Chlorotic ring spot, necrosis and vein yellowing on apricot; mosaic and midrib crinkling on peach, calico leaf on almond; chlorotic ringspot in sour cherry; vein yellow, leaf distortion, mosaic and small fruit on cherry were evident. Samples suspected to be infected by PPV were inoculated on *Nicotiana* spp., pea, *Chenopodium quinoa*, beans, *Gomphrena globosa* and broad bean plants. Samples suspected to have infection with PNRSV were inoculated on cucumber plant. Infections with the viruses were revealed in the collected samples and the inoculated plants were by DAS-ELISA and/or DASI-ELISA by the use of respective antibodies. PPV- related symptoms developed on the inoculated plants: chlorotic leaf spot on *N. benthamiana* and beans and purple spots on *G. globosa* leaves. On cucumber plants, dwarfing and

chlorotic yellow spots occurred. Also, dsRNA was extracted from PPV-infected plants and subjected to IC-RT-PCR which ended up in amplification of the expected 243 bp fragment. Thus, PPV was detected with ELISA and IC-RT-PCR; PNRSV by ELISA and glass inoculations.

Health status of the pome- and stone fruit planting material imported to serbia

Paunović, Svetlana, Jevremović, Darko
Kralja Petra I 9, Čačak, Serbia
Contact: institut-cacak@eunet.rs

The paper summarizes the results of the analysis of the pome- and stone fruits planting material intended to be imported to Serbia on the presence of quarantine and economically important viruses and phytoplasmas during 2004- 2009. Examinations have been done on the officially inspected samples according to the phytosanitary law regulations of the Republic of Serbia. A total of 325 samples have been analyzed: 89 rootstock samples (*Malus domestica*, *Pyrus communis*, *Cydonia oblonga*, *Prunus cerasifera*, *P. persica*, *P. armeniaca*, *P. avium* and *P. mahaleb*); 215 samples of apple, pear, plum, peach and nectarine, apricot, sweet and sour cherry varieties; and 21 samples of seed (*P. cerasifera*, *P. persica*, *P. armeniaca*, *P. avium*, *P. mahaleb* and *P. amygdalus*). On the presence of viruses samples were analyzed by ELISA test, while the PCR test was performed for the detection of phytoplasmas. Depending on the fruit species, type of the sample, vegetation season, category of the planting material and country of origin laboratory tests were performed on the adequately viruses (*Plum pox virus*, *Prune dwarf virus*, *Prunus necrotic ringspot virus*, *Cherry leaf roll virus*, *Arabis mosaic virus*, *Strawberry latent ringspot virus*, *Raspberry ringspot virus*, *Tobacco black ring virus*, *Tomato ringspot virus, Apple mosaic virus*, *Apple stem pitting virus*, *Apple stem grooving virus* and *Apple chlorotic leafspot virus*). Four apple samples were tested on the presence of '*Candidatus* Phytoplasma mali' and 5 pear samples on the presence of '*Candidatus* Phytoplasma pyri'. Plant viruses were detected in 5 samples (1.54%). One rootstock sample *Prunus avium*, originating from Hungary, was found to be infected with Prune dwarf virus. Two plum samples, also from Hungary, were infected with *Plum pox virus*. *Apple mosaic virus* was found in one apple sample from Belgium, and one apple sample from Italy was infected with *Apple chlorotic leafspot virus*. No phytoplasmas were found in tested samples.

Investigations on the phytosanitary status of the main stone fruits nurseries and mother plants in Albania

Musa, A.[1], Merkuri, J.[2], Milano, R.[1], Djelouah, K.[1]
[1]Istituto Agronomico Mediterraneo, Bari, Italy (IAMB)
[2]Plant Protection Institute, Durres, Albania

To assess the virus and viroids infections of the most important stone fruits in Albania, surveys were carried out in nurseries, mother plots and commercial orchards in the main fruit trees-growing areas. The presence of virus and viroids was assessed by visual inspections and laboratory tests. During field surveys, more than 5,000 trees were individually inspected for symptoms expression. A total of 749 were tested, showing to be highly infected (27%) by one or more viruses; in particular, Sharka infection was detected in all the selected areas and in plants of different origin (nurseries 29%, mother plants 14% and orchard 12%). *Prunus necrotic leaf spot virus* (PNRSV) and *Apple chlorotic leaf spot virus* (ACLSV) infections were frequent in peach and plum, while *Prunus dwarf virus* (PDV) was more frequent in cherry. Regarding viroids, 740 samples were tested for *Peach latent mosaic viroid* (PLMVd); the infection rate was, as for viruses, quite high (23%), particularly on peach (60% of tested samples). This study highlighted the quite alarming situation, especially due to the presence of PPV infection in nurseries; urgent measures should be taken to avoid a serious crisis and deterioration of fruit trees industry in Albania.

Detection of systemic pathogens in tissue culture

Liefting, Lia, Shiller, Jason, Harper, Scott, Ward, Lisa, Clover, Gerard
Plant Health and Environment Laboratory, MAF Biosecurity New Zealand, P.O. Box 2095,
Auckland 1140, New Zealand
Contact: cloverg@maf.govt.nz

Access to genetic material is crucial to enable plant-based industries to remain competitive, for example by introducing traits such as disease resistance, improved yield or taste. New genetic material can be

moved internationally in a variety of forms such as nursery stock, seed or pollen. However, clonal material such as cuttings or tissue culture must be traded if specific genotypes are required. Tissue culture is of lesser biosecurity risk than other forms of nursery stock and generally has fewer phytosanitary requirements. However, bacteria and viruses may infect tissue culture without showing obvious disease symptoms. Testing is often required to ensure disease freedom but there is uncertainty as to whether plants must first be deflasked. There is concern that detection efficacy might be compromised if the pathogen concentration were smaller in tissue-cultured plants than in deflasked plants. However, allowing plants to remain in tissue culture throughout quarantine would be beneficial because international trade would be faster and cheaper. Research has been initiated to investigate whether two important and commonly regulated pathogens, *Plum pox virus and Xylella fastidiosa* can be detected reliably in tissue culture plants of *Prunus and Vitis*, respectively.

Investigation on rose mosaic disease of rose in Hatay-Turkey

Sertkaya, Gülşen
Department of Plant Protection, Faculty of Agriculture, University of Mustafa Kemal, 31034 Antakya/Hatay-Turkey
Contact: gsertkaya@mku.edu.tr

Several viruses including *Prunus necrotic ringspot virus* (PNRSV) and *Apple mosaic virus* (ApMV) are associated with rose mosaic disease (RMD). Field inspections were carried out during the years of 2008 and 2009. Characteristic symptoms include chlorotic line patterns (zigzag pattern), vein-banding and mottles in leaves were observed during spring. Symptoms were also evident during summer on leaves produced until early summer. Flower abnormalities as phyllody were also exhibited during autumn. Distortion and reduction in flower size and early leaf drop have been observed on symptomtomatic plants in winter period. Leaf samples taken from 15 rose plants include different cvs. neighboring stone fruit orchards tested by mechanical inoculation tests on herbaceous plants and enzyme-linked immunosorbent assay (DAS-ELISA) for presence of *Prunus necrotic ringspot ilarvirus* (PNRSV), *Apple mosaic ilarvirus* (ApMV) and *Arabis mosaic nepovirus* (ArMV) which are the viruses related to RMD. *Catharanthus roseus*, *Chenopodium amaranthicolor*, *C. quinoa*, *Cucumis sativus*, *Nicotiana clevelandii*, *Phaseolus vulgaris*, *Vigna unguiculata* test plants were incubated after mechanical inoculation for symptom appearance at a temperature range between 25±2°C in an insect-proof room. Symptoms include chlorotic local lesions, systemic necrosis, stunting and yellow mottling were began to appear on *C. quinoa* and *Cucumis sativus* 2-3 weeks after sap inoculation. Serological tests of test plants are in progress. According to preliminary results of investigations on symptomatic rose plants, the causal agent of RMD is PNRSV. The rose plants exhibited symptoms in home gardens are going to re-tested for the viruses in spring period of 2009 by ELISA.

The development of resistance to cucumber mosaic virus using intrabodies specific for the viral replicase

Matic, Slavica, Burrone, Oscar, Thompson, Jeremy R., Tepfer, Mark
Plant Virology Group, ICGEB Biosafety Outstation, Via Piovega 23, 31056 CaTron di Roncade, Italy

Recombinant antibodies expressed in plants have been used recently with success in conferring resistance against plant viruses without the perceived biosafety risks associated with pathogen-derived resistance. We applied 'Intracellular Antibody Capture Technology' (IACT) for the development of resistance to *Cucumber mosaic virus* (CMV), one of the plant viruses with the widest host range, including both herbaceous and woody plants. Applying the IACT method, a single-chain variable antibody fragment (scFv) library was used against three proteins derived from the RNA-dependent RNA polymerase (RdRp) of CMV subgroup I strain I17F with the aim of blocking their function and thus preventing viral infection in planta. The proteins analyzed were - 'Full-length' (839 aa) consisting of the complete 2a gene, 'Motifs' (132 aa) covering conserved motifs (IV-VII) and 'GDD' (22 aa) centered on the GDD conserved motif (VII) complex of CMV. The scFv library (4 x 104 colonies with 95% diversity) was screened for positive interactions by using a yeast two-hybrid system. Of the three RdRp proteins tested the 'Full-length' and 'Motifs' proteins interacted with 96 and 25 library prey constructs, while the 'GDD' protein caused transactivation and was discarded from further analyses. Those scFvs that have tested positive in back-transformations will now be analysed for interaction with the 2a viral protein in vivo using a pPVX expression vector, and mammalian system. After which stably tobacco

transformed with positively interacting scFvs will be produced and screened for the degree and breadth of resistance. This work provides a model system for the development of resistance to other plant viruses.

Keywords: yeast two-hybrid system, scFv library, viral proteins, screening, transformation

Agro-Ecological Incidence Of Pepper Veinal Mottle Virus, Genus Potyvirus, Family Potyviridae, On Cultivated Pepper (Capsicum AnnuumL.) in Nigeria.

Fajinmi, A.A.
Crop Protection Department (COLPLANT)
Contact: ayofaji@yahoo.com

The agro-ecological distribution of *Pepper veinal mottle virus* (PVMV) (Family Potyviridae, genus Potyvirus) and its disease incidence and severity were observed on cultivated pepper between year 2002 to 2005 in six agro-ecological zones in Nigeria, comprising the major pepper producing areas of the humid forest, derived savanna, southern Guinea savanna, mid-altitude, northern Guinea savanna and Sudan savanna the virus was isolated and its physical properties determined. PVMV was confirmed to be present in cultivated pepper fields showing characteristics PVMV disease symptoms in the six agro-ecological zones surveyed but with significant difference in disease incidence and severity within the agroecological zones. Out of the three thousand suspected viral infected pepper leaves sample collected from the fields in the six agro-ecological zones, 88% were confirmed to be PVMV positive through the serological test while 12% were found to be negative. The incidences of PVMV diseases were observed to be high in the derived savanna and the humid forest compared with the other agro-ecological zones. The percentage PVMV disease incidence ranged between 39.14% with 34.48% severity in the Sudan savanna to 50.12% incidence and 43.85% severity in the derived savanna zone.

Towards generation of an infectious full- length cDNA clone of Apple stem pitting virus

Arntjen, Anja, Jelkmann, Wilhelm
Julius Kühn Institute, Institute for Plant Protection in Fruit Crops and Viticulture, Schwabenheimer Str. 101, 69221 Dossenheim, Germany
Contact: anja.arntjen@jki.bund.de

Our aim is the generation of an infectious full - length cDNA ASPV clone which will be used in studies on disease development and symptom expression. Two strategies for the generation of an infectious full - length clone of ASPV are being followed. Initially a ligation strategy has been developed by subdividing the genome of the ASPV isolate PA 66 into six fragments. During amplification and digestion of the fragments high sequence variability occurred, which required determination of the whole genome. The sequence of the new genotype PB 66 has only 80% sequence identity with the original isolate PA 66. The ligation strategy has been modified and the sequence was subdivided into three fragments, which will be ligated into the Plasmid 1657 containing the 35S promoter. The second strategy is based on a full - length PCR of the ASPV genome to circumvent the variability of the virus RNA. It is based on the higher conservation of the 3' and 5' ends of the sequence of RNA viruses, because of their importance for virus infectivity. To amplify the 9,3kb PCR fragment it was necessary to adapt the PCR protocol and to test different polymerases. The obtained full - length fragment will also be inserted into the Plasmid 1657.

Molecular Characterization and full length Genome Sequencing of Citrus Yellow Mosaic virus associated with Rangpurlime cultivar

Singh, J.[1], Baranwal, V.K.[2]
[1]Sardar Vallabh Bhai Patel University of Agriculture and Technology, Meerut, U.P, India
[2]Advanced center for plant Virology, Division of plant Pathlogy, I.A.R.I, New Delhi, 12, India
Contact: jeets_80@rediffmail.com

Citrus yellow mosaic virus (CMBV) is a badna virus reported from India. It causes graft transmissible severe mosaic disease in Rangpurlime. There are three viral diseases viz. Tristeza, Indian citrus ringspot. The genome of the virus associated with sweet orange has been sequenced and characterized in USA in 2001 (Huang and Hartung, 2001). As this was the only report available on genome characterization of CMBV, the study was undertaken to sequence and characterize full genome of CMBV associated with Rangpurlime from Tirupathi (Andhra Pradesh). The full length genome of *Citrus yellow mosaic virus* associated with Rangpur lime consists of 7522 nucleotides with (G+C) content 43.75% and comprises of

six ORFs. ORF3b is the largest ORF and consists of putative cysteine-rich region (CX2CX11CX2CX4CX2C) and cysteine-rich, zinc finger-like RNA binding domain (CXCX2CX4HX4C). ORF3b also contains domains homologous to those of aspartic protease, reverse transcriptase and RNase H which are highly conserved among all plant pararetroviruses. ORF3A contains domain homologous to those of movement protein. The intergenic region of 724 nucleotides between ORF6 and ORF1 consists of putative promoter elements. ORF1 has the plant cytosolic t-RNA methionine binding site and the numbering of nucleotides of CMBV genome starts from here. Genome of CMBVRL consists of unique EcoRV, XbaI and NcoI restriction sites. The total molecular weight of genome is 4564.6 kDa.

Assessment of molecular diversity in the polymerase gene of several Citrus tristeza virus isolates in northern and southern Iran

Sadeghi, M.S.[1], Afsharifar, A.R.[2], Niazi, A.[2], Izadpanah, K.[2]
[1]Plant Virology Research Center, College of Agriculture, Shiraz Univ. Shiraz, Iran
[2]Plant Virology Research Center, College of Agriculture, Shiraz University, Shiraz, Iran
Contact: izadpana@shirazu.ac.ir

Citrus tristeza virus (CTV) contains a monopartite, single stranded RNA genome organized into 12 open reading frames (ORFs). CTV isolates show a considerable degree of sequence diversity in the 5'-half of the genome. This virus is widely distributed in all citrus growing regions of northern and southern Iran. In this study genetic diversity of 16 Iranian CTV isolates based on sequence of the 5'- terminal part was determind using two overlapping primer pairs from ORF1. Total RNA was extracted from Fars (6 isolates), Boushehr (4 isolates) and Mazandaran (6 isolates), reverse-transcribed and amplified. CN487/CN489 primer pair spanning nucleotides 697 to 1105 of the CTV genome produced the expected 409 bp amplicon for all isolates. A phylogenetic analysis based on the nucleotide sequences of the CN487/CN489 amplified product and published sequences clustered the Iranian CTV isolates into two groups. While on the basis of the CN488/CN491 amplified product, these isolates formed four groups. The results showed that while various isolates fall into different clusters, each cluster includes both Fars, Boushehr and Mazandaran isolates. The results of multiple alignment of the nucleotide sequences of these 16 isolates showed a higher genetic variability between nt. 1082 and 1484 than between nt. 697 and 1105 of the CTV genome. Furthermore, based on the sequences corresponding to nucleotides 1082 to 1484, the Iranian CTV isolates showed most similarity to those from California (SY568, 98%) and Japan (NuagA, 98%). However the percentage identity on the basis of the other genomic region (nt. 697-1105) was 91-98% to the Japanese isolate (NuagA). Noticeably, the close relationship of the Iranian CTV isolates to the Californian and Japanese isolates, have already been shown in several studies that have been conducted on the CP gene analysis. This may reply the origin of the Iranian CTV isolates that possibly have been derived from the Californian and Japanese isolates, through importation of infected planting material in 1964 and 1969 respectively.

An international effort to study the diversity of Plum pox virus.

Glasa, M.1, Shar,C.O.2
[1]Institute of Virology, Bratislava, Slovakia
[2]consortium
Contact: Miroslav.Glasa@savba.sk

The understanding of the geographic dynamics and the genetic evolution of *Plum pox virus* (PPV) populations is crucial for an efficient management and control of the disease. Intensive research of the PPV variability in the last years has allowed the identification of 2 new groups, i.e. Rec and W, showing that present knowledge of the PPV genetic diversity may still be completed by further discoveries. Moreover, occurrence of several divergent isolates, found within common strains, extends our vision of the intra-group variability and has the potential to limit the accuracy or efficiency of typing methods. An international effort supported by the European FP7 SharCo project has therefore been initiated with the aim to provide a realistic view on the current diversity of PPV worldwide, by the large-scale analysis of complete and/or partial (P3-6K1, CP) genome sequences for a large number of natural field isolates. The obtained sequence data accompanied by standardized information (country/region of origin, natural host, symptoms….) for the analysed isolates will be integrated in a webqueryable reference database. A

centralised lyophilised collection of PPV isolates is being simultaneously developed, together with a living collection of epidemiologically or molecularly interesting isolates.

Preliminary results on resistance to PPV-M in *Prunus persica* (L.) Batsch

Casati, Paola, Bassi, Daniele, Spadone, Paola, Bianco, Piero A.
Dipartimento di Produzione Vegetale, Università degli Studi, via Celoria 2, 20133, Milano. Italy
Contact: piero.bianco@unimi.it

Plum Pox virus (PPV) is the causal agent of Sharka disease, one of the most dangerous disease of the stone fruits and in particular for peach. PPV is present in several areas in the world including North America and Asia. In Europe, where Sharka was firstly reported and where is still spreading, PPV is infecting both fruit trees and wild plant species. In these cases, the application of quarantine measures is time consuming, expensive and not effective, also when virus-free material is employed for the plantation of new orchards. A fast spread of the strain PPV-M recently occurred in several areas in Europe ad in particular in Italy, where the disease is causing severe losses on peach crop, threatening the nursery industry as well. Where Sharka is endemic the only sustainable strategy is the employment of resistant varieties. In this work the results of the assays conducted in the winter of 2007-2008 on 20 peach [*Prunus persica* (L.) Batsch] accessions inoculated with PPV-M strain in greenhouses and periodically checked for symptom expression, are presented. Serological and molecular tests (RT-PCR) were individually carried out on all the plants in order to verify the presence of the virus. 'GF305' seedlings were the healthy control. Moreover, 'GF305' plants, graft-inoculated with PPV-M inoculum, were also included as positive control. Four peach accessions were found asymptomatic and virus free while the remaining accessions were found tolerant either (2) or susceptible (14). Putatively *Myzus persicae* resistant 'PI 914559' and 'S 6699' peach accessions were found healthy after 24 hours of exposure to PPV-M infected *Myzus persica*, while the control 'GF305' seedlings was found positive to ELISA test just after one hour of exposure to the infected aphids.

Reaction of PPV infected scions of European plum genotypes grafted onto rootstocks with hypersensitivity resistance to PPV

Mühlberger, Louisa, Neumüller, Michael, Treutter, Dieter
Dürnast 2, D-85354 Freising
Contact: muehlberger@wzw.tum.de

Via latently infected plant material, *Plum pox virus* (PPV) spreads over short and long distances throughout countries where PPV host plants are cultivated. At present, the only way to circumvent this way of virus dissemination in *Prunus domestica* is the use of genotypes with hypersensitivity resistance to PPV. It is well known, that scions of genotypes with a high hypersensitivity index grafted onto PPV infected rootstocks die off after a short period of growth. Vice versa, PPV infected buds grafted onto branches of hypersensitive trees are rejected by the hypersensitive genotype. So the use of hypersensitive genotypes could prevent the spread of PPV infected trees from the nursery. The present study deals with the use of genotypes with hypersensitivity resistance as rootstocks for genotypes sensitive to PPV. As rootstocks interspecific hybrids between *P. domestica* and *P. spinosa* and between *P. domestica* and *P. cerasifera*, respectively, were used. In spring 2008, each 200 plants of four rootstocks with hypersensitivity resistance were grafted with buds taken from trees of PPV infected plum genotypes. Most of the shoots growing from the grafted buds stopped growth after a short period and died off. The length of the scion shoot prior to their death varied between 0.3 and 50 cm. Some buds were rejected by the rootstock so that they did not start growing. 53 out of 800 plants grew until the autumn and were kept under dormancy conditions for three months. In spring 2009, 51 % of the remaining plants died off, some after a short growing period and others without shooting. 25 out of 26 plants showed no PPV symptoms, PPV could not be detected by DAS-ELISA. There was only one plant still growing in April 2009 which was proofed to be infected by PPV. The results indicate that the use of hypersensitive rootstocks might be a powerful strategy to avoid the short and long distance transport of PPV via infected plant material.

The inheritance of the hypersensitive resistance of European Plum (*Prunus domestica* L.) against the Plum pox virus

Lichtenegger, Ludwig[1], Neumüller, Michael[2], Treutter, Dieter[3]
[1]ludwig.lichtenegger@wzw,.tum.de
[2]neumueller@wzw.tum.de
[3]dieter.treutter@wzw.tum.de
Contact: ludwig.lichtenegger@wzw.tum.de

According to our present knowledge, the use of the hypersensitivity resistance is the most promising mechanism to control the economic effect of Sharka disease in European plum (*Prunus domestica* L.). The inheritance of the resistance trait was investigated on progenies of crossing combinations with at least one hypersensitive cultivar. 47 different crossing combinations with about 1000 seedlings have been proved for sharka resistance. These combinations resulted from crossings between hypersensitive and sensitive genotypes, hypersensitive and hypersensitive ones and between hypersensitive genotypes and quantitatively resistant ones. The progenies were tested for resistance by using a double grafting method with a virus infected interstem in the green house. The percentage of hypersensitive seedlings in crossing combinations with two hypersensitive parents is significantly higher than in combinations with only one hypersensitive parent. In crossing combinations with one or two hypersensitive parents the percentage of seedlings in hypersensitive classes 1 and 2 is equal. The results of the inheritance of hypersensitive resistance obtained so far confirm former results and help for a better understanding of the inheritance of the resistant trait. The selected genotypes provide a base for further selection and breeding of PPV resistant European plum cultivars.

The spatial distribution of Plum pox virus (PPV) in the leaves of European plum cultivars with different degrees of PPV resistance

Paskaš, K., Neumüller, M., Treutter, M.D.
Technische Universität München, Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt, Fachgebiet Obstbau, Dürnast 2, D-85354 Freising email:paskas@wzw.tum.de,
Contact: fax: ++49 81 61 71 53 85

There are two components of virus resistance in plants: (1) The reduction of the virus titer within the plant tissue and (2) the inhibition of the systemic spread of the virus within the plant. Both or only one of these components can get effective in resistant cultivars. In order to determine the degree of PPV resistance in 250 cultivars and selections of Europen plum (*Prunus domestica*), the spread of PPV in the leaf blades was observed. Cultivars to be tested were chip budded on myrobalane seedling rootstocks infected with PPV-D isolate. Plants were grown in an insectproof greenhouse. In June, leaves were taken, immediately put into fixations soluton, embedded into resin and cut into 5 µm sections. PPV was localised within the leaf tissue using the Immunogold Silver Staining Method applied after sectioning. Observations were made with the light microscope. Different patterns of PPV spread within the leaf blade of plum cultivars could be described. Along with the determination of the virus titer within the leaves the determination of the spatial distribution of PPV could provide additional information for the reliable characterisation of the PPV resistance of PPV host genotypes.

The investigation of Plum-Pox-Virus infections in some peach and apricot cultivars and rootstock

Trandafirescu, Marioara, Indreias, Alexandra
Research Station for Fruit Growing Constanta,
Contact: mtrandafirescu@yahoo.com

The stone fruit tree species can be infected by a lot of viruses which are not visible symptoms, such as *Plum-Pox virus* has became one of the most frequently and the most grave disease in Europe for all species of *Prunus genus*. That is way, to introduce in cultivation as well as to obtain of peach and apricot cultivars with tolerance and genetic resistance to *Plum-Pox-virus* have reprezented one of the important obiective of the research programme at the Research Station for Fruit Growing *Constanta*. This paper presents the data regarding the susceptibility of some peach and apricot cultivars and rootstock confronted by the natural infection with local *Plum-Pox virus* strains. The material taken into study was represented by the peach and apricot cultivars and rootstock with constitute the national collection from Research Station for Fruit Growing *Constanta*. The *Plum-Pox-virus* detection has been made using biological and serological method. In the biologic test were used GF 305 seedlings for peach and Luizet cultivar for apricot. The observations pursued to detect typical symptoms of the *Plum-Pox virus* attack, both on indicator leaves. It is remarquable that on the one hand there was large differences of manifestations of the *Plum-pox virus* symptoms among the species (peach and apricot). An the other hand, there was hight variability regarding the manifestation intensity of the attack among the cultivars and hibrids.

Susceptibility of different prunus rootstocks to natural Plum pox virus (PPV-D) infection in Spain

Vidal, E., Moreno, A., Bertolini, E., Capote, N., Gil, M., Martínez, M.C., Gorris, M.T., Collado, C., Cambra, M.
Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias (IVIA), Carretera Moncada-Náquera km 5, 46113 Moncada, Valencia, Spain.
Contact: mcambra@ivia.es

The use of less susceptible rootstocks to natural *Plum pox virus* (PPV) infection is one of possible strategies to reduce viral incidence in nursery blocks. The main Prunus rootstocks used in the Spanish stone fruit industry were evaluated during two consecutive vegetative periods (2006-2007) in two different areas in Valencia (Spain) with different PPV inoculum pressure: Llíria (high) and Carlet (low). The tested rootstock species were: Nemaguard, *Prunus marianna* GF8-1, Adesoto 101, Cadaman, Myrobolan 29C and Garnem. The Llíria plot was analyzed in three occasions (Fall 2006, Spring 2007 and Spring 2008), the Carlet plot was analyzed in two occasions (Fall 2006 and 2007) by DASI-ELISA (5BIVIA, Durviz, kit). The virus incidence in Llíria was 59,4% in Spring 2008. The most susceptible

rootstocks were: Adesoto 101 (96,9%) and Mariana GF8-1 (96,1%) and none plant of *Cadaman* and Garnem rootstocks were infected. In Carlet, virus incidence was lower (0,3% in Fall 2007) and only Adesoto 101 (1,4%) and Nemaguard (0,6 %) rootstocks were infected. Aphid species were monitored by Moericke yellow water traps sited in both localities from May 2006 until October 2007. May resulted the month with more abundant caught aphids. Cumulative numbers of aphid species were similar in both plots: 5,575 (Llíria) and 5,265 (Carlet). Aphid species landing on the crop were estimated by the sticky shoot method during a complete year. The main PPV-vector aphid species that landed on the grown rootstocks in Llíria and Carlet were *Aphis spiraecola* (56.4% and 56.8%) and *A. gossypii* (4.1% and 12.7%), respectively.

Evaluation of transgenic *Prunus domestica* L., clone C5 resistance to Plum pox virus

Kumar Kundu, Jiban, Jarosova, Jana, Polak, Jaroslav
VURV, Drnovska 507, 16106 Prague
Contact: j.jarosova@vurv.cz

Plum pox virus (PPV) is one of the most devastating diseases of *Prunus* species. Since few sources of resistance to PPV have been identified, transgene-based resistance offers a complementary approach to developing PPV-resistant stone fruit cultivars. C5, a transgenic clone of *Prunus domestica* L., containing the PPV coat protein (CP) gene, has been described as highly resistant to PPV in greenhouse tests, displaying characteristics typical of post-transcriptional gene silencing (PTGS). Moreover, C5 trees exposed to natural aphid vectors in the field remained uninfected after 4 years while susceptible transgenic and untransformed trees developed severe symptoms within the first year. In our study, a high and permanent infection pressure of PPV-Rec was provided by bud grafting of inoculum in the field trial of clone C5 conducted in the Czech Republic, in which PPV-infected and healthy control trees were used. Moreover, trees with combined inoculations by PPV, ACLSV and PDV were also used in the trial. The presence of the viruses throughout the tree tissues, the relative titre of the viruses and symptoms on C5 trees have been monitored over the years. The resistance stability of C5 clones under permanent infection pressure is discussed.

Evaluation of different peach genotypes for resistance to PPV-M

Pasquini, Graziella[1], Ferretti, Luca[1], Gentili, Andrea[1], Verde, Ignazio[2], Micali, Sabrina[2], Barba, Marina[1]
[1]CRA-PAV Centro di ricerca per la Patologia Vegetale Via C.G. Bertero, 22 00156 Roma
[2]CRA-FRU Centro di Ricerca per la Frutticoltura Via Fioranello, 52 00134 Roma
Contact: marina.barba@entecra.it

A total of 122 peach seedlings, obtained in different cross breeding events using *Prunus* genotypes considered tolerant or resistant to *Plum pox virus* (PPV), were evaluated for the response to PPV–M infection. Tested selections were grafted onto GF305 peach seedlings and successively infected with a PPV-M isolate. For each selection three plants were inoculated and an healthy one was maintained as negative control. Symptoms evaluation was performed according to a detailed scale as reported by Decroocq et al., (2005). The response of infected plants to the challenge of PPV inoculum was evaluated either on the selections or the GF305. Different diagnostic approaches (ELISA, RT-PCR and TaqMan real time RT-PCR) were used for virus detection. Plant response was classified into five groups as suggested by Faggioli et al., 1999, according to the symptoms and the virus detection. After two years of trials a percentage of 82.8% (101/122) of the thesis resulted sensitive, showing symptoms, with different severity, both on the selection and GF305; the 4.1% (5/122) did not show symptoms on the selection, but gave a heavy symptomatology on the rootstock, whereas no symptoms were observed in the remaining thesis (16/122). Among the 21 asymptomatic selections, 15 resulted negative when assayed both in ELISA and RT-PCR, but all of them were positive when assayed with the more sensitive TaqMan real time RT-PCR (Olmos et al., 2005), that revealed the presence of a very low virus titer. On the basis of these results, 7 selections from 'Maria Aurelia' x SD45 F1 hybrid (*Prunus persica* x *Prunus davidiana*) and 8 selections obtained from different cross breeding between commercial peach cultivars and weeping peaches (S2678) could be considered 'highly tolerant' or resistant and must be submitted to further investigations.

Molecular characterization of some new Canadian isolates of Plum pox virus

James, Delano[1], Varga, Aniko[1], Glasa, Miroslav[2], Thompson, Dan[1]

[1]Sidney Laboratory, CFIA, 8801 East Saanich Road, Sidney, BC, V8L 1H3
[2]Institute of Virology, SLovak Academy of Sciences, Dubravska 9, 84505 Bratislava, Slovakia
Contact: Delano.James@inspection.gc.ca

Plum pox virus (PPV) was first detected in Canada in 2000. Since then an intensive survey program has be undertaken to determine the distribution of the virus with the goal of eradicating the virus from Canada. A number of Canadian isolates have been characterized and only isolates of PPV strain D have been found in commercial orchards. This research was conducted in part to: a) determine the relationship of PPV D isolates found in commercial orchards with PPV D isolates found in homeowner or residential properties; and b) analyze unusual isolates to confirm strain and/or determine identity. A total of 5 homeowner isolates were obtained for analysis. Four isolates were confirmed as strain D isolates, and formed 3 distinct clades. One of these isolates (H- 0170) grouped with the Canadian Subgroup II, 2 isolates (H-4688, H-4880) grouped with the Canadian Subgroup I, and the fourth isolate (H-4782) formed a separate and distinct clade. The fifth homeowner isolate was strain typed as a member of the strain PPV Rec.

Key words: *Pepper veinal mottle virus*, Incidence, Severity, Pepper, Agroecological Zones.

Biolistic transfection of plants by infectious cDNA clones of Plum pox virus

Subr, Zdeno[1], Nagyova, Alzbeta[2], Glasa, Miroslav[1]
[1]Institute of Virology SAS, Bratislava, Slovakia
[2]Comenius University, Faculty of Sciences, Bratislava, Slovakia
Contact: virusubr@savba.sk

Plant biolistic transfection by two *Plum pox virus* (PPV) infectious cDNA clones (strains PPV-M and PPV-D) using the gene gun apparatus PDS 1000-He (Biorad) was optimized. *Nicotiana benthamiana* plants were germinated by five on Petri dishes (diameter 6 cm) with MS growth medium. In the age of four weeks (5 – 6 leaf stage, total leaf surface about 1.5 cm2 per plant) the plants were subjected to biolistic transfection and three days later they were transplanted into common soil substrate. The plant survival after transplantation was about 70 %, the transfection efficiency was over 80 % (compared to 6 % efficiency reached by mechanical plant inoculation). The plants showed typical PPV symptoms two weeks post transfection (leaf distortions and mosaic). The virus presence was confirmed by immunoblotting, RT-PCR, as well as by successful transmission by sap to healthy plants and subsequent virus purification. The cotransfection of *N. benthamiana* plants by PPV-M and PPV-D led to mixed infection with prevalent PPV-D.

In vivo thermotherapy and in vitro chemotherapy of plums, apricots and peaches artificially infected with PPV-D and PPV-M strains.

Hauptmanova, Alena, Polak, Jaroslav
VÚRV v.v.i., Drnovska 507, 16106, Prague
Contact: hauptmanova@vurv.cz

Plum cultivars Čačanská lepotica and Švestka domácí, apricot cvs. Leskora and Velkopavlovická, peach cvs. Redhaven and Earliglo artificially infected with PPV-D and PPV-M were treated by in vivo thermotherapy at 37°C. A successful treatment was recorded in cases of plum cv. Čačanská lepotica and apricot cvs. Leskora and Velkopavlovická. Plum cv. Čačanská lepotica and apricot cv. Velkopavlovická were PPV-D free, apricot cv. Leskora was PPV-M free seven and nine months after finishing the in vivo thermotherapy. However, both of the peach cultivars remained PPV infected after the treatment. Furthermore, five peach trees died during the treatment. In vitro cultures of plum cv. Bluefree and apricot cv. Hanita infected with *Plum pox virus* (PPV) were used for the virus elimination by chemotherapy. Low ribavirin concentrations of 5 and 10 mg.l-1 in MS medium were applied in the treatment. PPV was completely eliminated by ribavirin in concentration of 5 mg.l- 1 in plum cv. Bluefree within twenty weeks, and in apricot cv. Hanita in twelve weeks of the application. The presence of PPV was not proved by RT-PCR. Clones of plum cv. Bluefree and apricot cv. Hanita were re-tested by RT-PCR one year after the termination of the ribavirin treatment and negative results confirmed the elimination of PPV. PPV free clones rooted in modified MS medium by Paunovic (2007) during six weeks.

Distribution of Plum pox virus strain in natural sources in the Czech Republic.

Polak, Jaroslav, Kominek, Petr

VÚRV v.v.i., Drnovska 507, Prague, 16106, Czech Republic
Contact: polak@vurv.cz

In the Czech Republic, the distribution of *Plum pox virus* (PPV) has been monitored for the last 15 years. Also, individual strains of PPV have been monitored since the end of the 20th century. PPV-M was typed in natural sources of plum, myrobalan and blackthorn from 1999 to 2004. PPV-M was detected in 5, 88% of investigated plum trees; 7, 41% of myrobalan trees and 4, 0% of blackthorn shrubs, respectively. Distribution of PPV-D, PPV-M and PPV-Rec was investigated in 2005-2008. 52-94 samples of plum, myrobalan and blackthorn were tested in individual years. PPV was detected by DAS-ELISA with specific polyclonal antibodies; PPV-M by DASI-ELISA with specific monoclonal antibodies; PPV-D, PPV-M and PPV-Rec were detected by RT-PCR. The presence of PPV-D varied from 94, 7% to 100%, the presence of PPV-M from 0, 0% to 3, 2% and the presence of PPV-REC from 0, 0% to 2, 1% during 2005-2008. More than 95% of natural sources of PPV were infected with PPV-D and less than 2, 5% of natural sources of PPV were infected with PPV-M or PPV-Rec. The presence of PPV-C and PPV-ElAmar was not proved in plum, myrobalan and blackthorn trees infected with PPV.

Typing and distribution of Plum pox virus isolates in Romania

Zagrai, Ioan[1], Zagrai, Luminita[1], Kelemen, Beatrice[2], Petricele, Ioana[3], Pamfil, Doru[3], Popescu, Octavian[2], Preda, Silvia[4], Briciu, Alexandru[3]
[1]Fruit Research and Development Station Bistrita, 3, Drumul Dumitrei Nou Street, 420127, Bistrita, Romania
[2]Babes Bolyai University, Faculty of Biology and Geology, 42, Treboniu Laurian Street, Cluj-Napoca, Romania
[3]University of Agricultural Science and Veterinary Medicine, 3, Manastur Street, Cluj-Napoca, Romania
[4]Fruit Research and Development Station Valcea, 464, Calea lui Traian Street, Ramnicu Valcea, Romania
Contact: izagrai@yahoo.com

Plum pox or Sharka, caused by *Plum pox virus* (PPV) is considered as the most destructive disease of plum. Although PPV is widespread in all plum growing areas from Romania and causes serious yield losses, little is known about the variability of its isolates at country level. For this reason, a large-scale study was performed with the aim to get a picture of the prevalence and distribution of PPV strains in plum. During three years surveys, 200 PPV isolates collected from 23 different plum orchards from Transylvania, Moldavia and Muntenia areas were investigated. DAS-ELISA and IC/-RT-PCR were used for PPV detection. PPV strains were serologically determined by TAS-ELISA using PPVD and PPV-M specific monoclonal antibodies. Molecular strain typing was done by RTPCR targeting three genomic regions corresponding to (Cter)CP, (Cter)NIb/(Nter)CP and CI. RFLP analysis was used to distinguish D and M strains, based on RsaI polymorphism located in (Cter)CP. All PCR products targeting (Cter)CP and 13 PCR products spanning the (Cter)NIb/(Nter)CP were sequenced. The typing of PPV isolates revealed that PPV-D is the prevalent strain in all the three areas. The higher incidence of PPV-D was noticed in Moldova (84%) and the higher rate of PPV-Rec was recorded in Transylvania (18%). The mixed infections (D+Rec) was more frequent in Muntenia (24 %).Overall results provided that in Romania the predominant strain is PPV-D (73%), follow with a much lower frequency by PPV-Rec (14%). Mixed infections (PPV-D+PPV-Rec), which might generate additional variation by recombination, are also frequent (13%).

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Cloning and sequencing of a mild naturally induced PPV isolate

Vassilakos, Nikon
Benaki Phytopathological Institute, 8 S. Delta str., Kifissia, Attica,
Contact: c.varveri@bpi.gr

A mild PPV isolate (PPV-B2) was naturally induced after low temperature treatment of *Nicotiana benthamiana* plants infected with the local isolate PPV-DGR. Compared to the mother isolate, PPV-B2 is not aphid transmissible, replicates less efficiently in *N. benthamiana* and *N. clevelandii* and causes no symptoms on the last mentioned experimental host. Both isolates were cloned, sequenced and 14 amino acid substitutions were determined between them as follows: two in P1, two in HC-Pro, two in P3, one in

CI, two in 6K2, four in NIa and one in NIb. Three nucleotide substitutions were observed in the untranslated 5' and 3' terminal regions. In addition, PPV-D-GR, which does not succeed in invading *Prunus* spp. host plants, differed from the reference PPV-D (X16415) isolate with 32 substitutions scattered among P1, HC-Pro, P3, NIa/VPg and CP proteins. The biological impact of the above substitutions is under investigation.

Assessment of the genetic structure of Plum pox virus (PPV) in Serbia

Jevremović, Darko1, Paunović, Svetlana1, Dallot, Sylvie2
[1]Fruit Research Institute, Kralja Petra I 9, Čačak, Serbia
[2]INRA-UMR BGPI- TA A54/K- Campus international de Baillarguet, 34398 Montpellier cedex 5,, France
Contact: darkoj@tfc.kg.ac.rs

Numerous studies have reported the widespread occurrence of the main strains of PPV (PPV-M, -D and -Rec) in Central and Eastern Europe these last years (Matic et al., 2006, Dallot et al., 2008, Gadiou et al., 2008, Zagrai et al., 2008). In Serbia, Sharka disease is known to occur since the mid-1930's and the coexistence of all three PPV-M, -D and –Rec strains has been reported (Jevremovic et al., 2008). Moreover, it has been hypothesised that PPV-Rec could originate from ex-Yugoslavia (Glasa et al., 2005). The objective of this present study was to assess the genetic structure of the PPV populations in this country and to identify potential factors determining such a structure. 185 peach, apricot and plum trees were sampled in 53 orchards located in 27 distinct sites during a large survey undertaken in the main stone fruit growing regions of the country in 2005 and 2006. All samples were used directly for PPV diagnostic, strain typing and sequencing. PPV diagnostic and strain typing was performed by IC-RT-PCR using classical already published primers targeting the CIP, CterNIb-NterCP and CterCP coding regions. The genetic diversity of the PPV populations was assessed by sequencing a 427 bp PCR fragment located in the CterNib-Nter CP coding region for 67 isolates originating from the three main Prunus species and from the different sampling sites. Moreover, 19 isolates were further sequenced on both entire CP and partial P3-6K1 coding regions. The prevalence, host and geographical distributions of the three PPV strains were evaluated and evidence of genetic differentiation within each PPV strain was further investigated. The results of these analyses will be presented.

Preliminary studies on the use of the Cascade Rolling Circle Amplification technique for Plum pox virus detection

Hadersdorfer, Johannes[1], Neumüller, Michael[1], Fischer, Thilo[2], Treutter, Dieter[1]
[1]Technische Universität München; Unit of Fruit Science; Dürnast 2; D-85354 Freising-Weihenstephan
[2]Ludwig-Maximilians-Universität München; Chair for Biochemistry and Plant Physiology; Großhadener Str. 2-4; D-82152 Planegg-Martinsried
Contact: johannes.hadersdorfer@wzw.tum.de

Isothermal techniques for the amplification of nucleic acids have emerged in the last years. In contrast to the Polymerase chain reaction (PCR), the most prevalent method to amplify DNA in vitro, the reactions can be run at constant temperatures. Specificity and sensitivity are at least as high as by using PCR and the methods are less time consuming. Therefore, the isothermal amplification of nucleic acids provides also a powerful tool for the detection of *Plum pox virus* (PPV), the causal agent of the Sharka disease. The cascade rolling circle amplification (CRCA), first described by Thomas et al. (1999), is based on the rolling circle mechanism a lot of viruses use to replicate their genome multiplicatively. It is advanced by the amplification of the released strand to achieve exponential accumulation of DNA. Circular Probes, also called Padlock probes (PLP), which arise from the ligation of the terminal region of DNA probes upon side by side hybridization to the target serve as template (Nilsson et al. 1994). For detecting PPV by CRCA the RNA was extracted and reverse transcribed to cDNA using a PPV specific primer. Several PLPs varying in length and sequence of the complementary region to the cDNA were designed and tested. Furthermore, different pairs of primers for the subsequent amplification were developed. For specific ligation Ampligase and T4 DNA Ligase were tested. In CRCA, two polymerases with strong strand displacement activity were compared: Phi29 DNA Polymerase and Bst DNA Polymerase. These enzymes differ in the optimal reaction temperature. Ligation as well as amplification do occur, but there is high background amplification also in negative and no template controls. Discrimination is possible after restriction digestion is carried out. As proven by sequencing of reaction products non-specific

signals are a result of primer polymerization. Current work focuses on the reduction of the background amplification and improvement of the sensitivity.

Sampling and analysis of symptomless plants for Plum pox virus detection in nurseries

Vidal, E., Bertolini, E., Moreno, A., Martínez, C., Gorris, M.T., Capote, N., Gil, M., Cambra, M.
Centro de Protección Vegetal y Biotecnología. Instituto Valenciano de Investigaciones Agrarias (IVIA). Carretera Moncada-Náquera km 5. 46113, Moncada, Valencia, Spain.
Contact: mcambra@ivia.es

Conventional sampling and extract preparation methods for ELISA must be modified to reduce contamination risks when highly sensitive molecular amplification methods are used. The modifications include: hand collection of samples, special disruption procedures, preparation of extracts into plastic bags, composite samples, etc. Different methods are proposed for large scale testing by both serological and molecular methods as well as the results of comparisons between visual inspections and laboratory analysis, are discussed. In symptomless plants, 3-4 mature leaves should be hand collected from the basal part of one-year-old shoots of nursery plants. The basal part of the leaves including the peduncle should be used for extract preparation. For many years PPV positive detection was associated to the presence of symptoms: visual inspection of symptoms and DASI-ELISA analysis for PPV were coincidental in 82% of the nursery plants, however in 8% of symptomless plants PPV was detected. PPV detection in composite samples (using 3 leaves or 3 dormant buds/plant) was higher by real-time PCR than by ELISA, especially during dormant period. PPV detection using 5 complete spurs or dards per mother plant in winter showed 6.1% and 11.8% post-test probability of disease on trees that tested negative by spot real-time RT-PCR and DASI-ELISA analysis, respectively. The analysis of 4,224 *Prunus* nursery plants by spot real-time RT-PCR and DASIELISA (using 5B-IVIA) showed 93.6% of coincidental results. All this data should contribute to improve protocols for PPV detection in nurseries.

Survey on plum pox virus in Norway

Blystad, Dag-Ragnar[1], Spetz, Carl[1], Haugslien, Sissel[1], Ørstad, Kari[1], Munthe, Tor[1], Knudsen, Randi[2]
[1]Norwegian Institute for Agricultural and Environmental Research
[2]Norwegian Food Safety Authority
Contact: dag-ragnar.blystad@bioforsk.no

In 1998 *Plum pox virus* (PPV) was detected for the first time in Norway. Virus symptoms were observed on several trees in a collection of plum cultivars at Njøs Research Station in the Sogn og Fjordane County in West Norway. The Norwegian Food Safety Authority and the Norwegian Crop Research Institute immediately started surveying other variety collections around the country, nuclear stock material and orchards in all important plum-growing areas. Since 1998 we have surveyed the main part of the commercial plum orchards in Norway. About 75 000 individual trees have been tested. About 1 % of the trees have been found infected by PPV. Only the PPV-D strain has been found. It is suspected that the main infection source was infected plums or apricots imported to Njøs around 1970 or earlier. In most plum orchards in Norway, the spread of PPV by aphids is relatively slow. Therefore, we expect to be able to eradicate PPV from commercial plum orchards in the near future.

The eradication work is continuing.

The presence of Peach latent mosaic viroid (PLMVd) in Greece

Boubourakas, I.N.[1] Faggioli, F.[2], Luigi, M.[2], Barba, M.[2], Fukuta, S.[3], Kyriakopoulou, P.E.[1]
[1]Agricultural University of Athens, Department of Plant Production Science, Plant Pathology Laboratory, 11855 Athens, Greece
[2]CRA – Centro di Ricerca per la Patologia Vegetale, Via C.G. Bertero, 22 – 00156 Rome, Italy
[3]Biotechnology Group, Aichi Agricultural Research Center, 1-1 Sagamine, Yazako, Nagakute, 480-1193 Aichi, Japan

Peach latent mosaic viroid (PLMVd) is the causal agent of the economically important peach latent mosaic disease, which is responsible for reduction of tree vigor and fruit quality. In Greece, PLMVd has been reported for the first time in pear and wild pear, in 2001 and in 2004 in apricot. On the other hand, in Greece, there is no information on the presence of this viroid in its main host, peach and its germplasm. In this study the presence of PLMVd in peach and peach germplasm (nursery stock) was examined thoroughly, as well as in other *Prunus* species, and in pome fruit species. Leaf samples were collected from orchards in Pella and Imathia prefectures of Macedonia, and in Magnesia and Argolida prefectures of Thessaly and Peloponnesus, respectively. The presence of the viroid was ascertained by RT-PCR assays, slot-blot hybridization, nucleotide sequencing and RT-LAMP assays. RT-LAMP assays were used for fist time internationally for the detection of PLMVd. Peach (48/53), plum (11/24) apricot (4/15) and cherry (2/15) tree samples were found infected with PLMVd. It was interesting to see that more than 50% (108/214) of peach germplasm examined (nursery stock) was found infected. The viroid was also detected in pear, wild pear and quince samples. Our positive results were obtained by using three different assays as well as nucleotide sequence analysis and were performed in three different labs in three countries.

Pospiviroidae viroids in naturally infected stone and pome fruits in Greece

Kaponi, Maria S.[1], Faggioli, Francesco[2], Luigi, Marta[2], Barba, Marina[2], Kyriakopoulou, Panayota E.[1]
[1]Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece
[2]CRA-PAV, Centro di Ricerca per la Patologia Vegetale, 00156 Rome, Italy
Contact: mkaponi@yahoo.gr

Viroid research on pome and stone fruit trees in Greece is important, as it seems that such viroids are wide spread in the country and may cause serious diseases. Our research dealt with three Pospiviroidae species infecting pome and stone fruits, namely *Apple scar skin viroid* (ASSVd), *Hop stunt viroid* (HSVd) and *Pear blister canker viroid* (PBCVd). Tissue-print hybridization, reverse transcription-polymerase chain reaction (RT-PCR), cloning and sequencing techniques were successfully used for the detection and identification of these viroids on a large number of pome and stone fruit tree samples from various areas of Greece (Peloponnessos, Macedonia, Thessaly, Attica and Crete). The 47 complete viroid sequences obtained (25 ASSVd, 11 PBCVd and 11 HSVd) were submitted to the GenBank. Our results showed the presence of ASSVd in apple, pear, wild apple (*Malus sylvestris*),wild pear (*Pyrus amygdaliformis*) and sweet cherry; HSVd in apricot, peach, sweet cherry, apple and wild apple; and PBCVd in pear, wild pear, quince, apple and wild apple. This research confirmed previous findings of infection of Hellenic apple, pear and wild pear with ASSVd (Kyriakopoulou, P.E., and Hadidi, A.1998, Boubourakas et al. 2006), and pear, wild pear and quince with PBCVd (Kyriakopoulou et al., 2001, Boubourakas et al., 2006). Our findings also revealed for the first time the natural and mixed infection of apple and wild apple with ASSVd, HSVd and PBCVd, of apple and pear with ASSVd and PBCVd, and of apple with ASSVd and HSVd, as well as the natural infection of Hellenic sweet cherry and peach with HSVd. Finally, to our knowledge, this is the first published report of detecting HSVd in infected apple and wild apple and ASSVd in sweet cherry.

Detection by Tissue Printing Hybridization of Pome Fruit Viroids in the Mediterranean Basin: Incidence and Biodiversity

Di Serio, F.[1], Afechtal, M.[2], Attard, D.[3], Choueiri, E.[4], Gumus, M.[5], Lolic, B.[2], Matic, S.[6], Navarro, B.[1], Myrta, A.[7]
[1]Istituto di Virologia Vegetale del CNR, Via Amendola 165/A, 70126 Bari, Italy
[2]Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (BA), Italy
[3]Ministry for Rural Affairs and the Environment, plant biotechnology center, Lija, Malta
[4]Department of Plant Protection, Lebanese Agricultural Research Institute, Tal Amara, P.O. Box 287, Zahlé, Lebanon
[5]Ege University, Department of Plant Protection, Faculty of Agriculture, 35100 Bornova, Izmir, Turkey
[6]Dipartimento di Protezione delle Piante e Microbiologia Applicata, University of Bari, 70126 Bari, Italy
[7]Certis Europe, Via A. Guaragna 3, 21047 Saronno (VA) Italy
Contact: f.diserio@ba.ivv.cnr.it

Available data on the incidence and biodiversity of *Pome fruit viroids* in Mediterranean basin are limited. Before starting a research survey to fill this gap, a tissue-printing hydridization (TPH) method to detect *Apple scar skin viroid* (ASSVd), *Pear blister canker viroid* (PBCVd) and *Apple dimple fruit viroid* (ADFVd) has been developed, validated and used in large-scale indexing of *Pome fruit viroids* in Bosnia and Herzegovina, Malta, Lebanon and Turkey. A total of about 1,200 trees have been tested. Positive results obtained by TPH were confirmed by at least one additional detection method (RT-PCR and/or Northernblot hybridization). PBCVd was detected in 18%, 12% and 8% of the tested pear trees in Bosnia and Herzegovina, Malta and Turkey, respectively, showing a wider diffusion of this viroid than expected. Interestingly, in all these countries several ancient native cultivars tested positive to PBCVd infection. In contrast, ASSVd was never detected and ADFVd was only detected in symptomatic trees (cv. Starking Delicious) in Lebanon, confirming a restricted spread of these viroids in Mediterranean basin. Full-length cDNA clones of PBCVd and ADFVd of the different geographic origin were molecularly characterized, with several new polymorphic positions in the genome of both viroids being identified. In the frame of this research, PBCVd in Bosnia and Herzegovina (1), Malta (2) and Turkey (unpublished), and ADFVd in Lebanon (4) were first recorded, indicating that TPH is a useful technique for exploring *Pome fruit viroid* spread.

First report and molecular analysis of Apple scar skin viroid in sweet cherry

Kaponi, Maria S.[1], Faggioli, Francesco[2], Luigi, Marta[2], Barba, Marina[2], Kyriakopoulou, Panayota E.[1]
[1]Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece
[2]CRA-PAV, Centro di Ricerca per la Patologia Vegetale, 00156 Rome, Italy
Contact: mkaponi@yahoo.gr

Apple scar skin viroid (ASSVd) is a serious pathogen of pome fruits. Recently, it has been reported in Chinese apricot and Chinese peach (Zhao and Niu 2006, 2008). In the context of our research on fruit tree viroids in Greece, ASSVd was initially detected in a sweet cherry tree cv *Tragana Edessis* (Rupka) from Florina (Macedonia) by RT-PCR and this finding was confirmed by direct sequencing. This tree is located at the edge of a newly established apple orchard, along with other sweet cherry and wild cherry (*Prunus avium*) trees. In order to verify this interesting finding, we examined 4 sweet cherry trees, 2 wild cherry trees and their neighbouring apple trees for ASSVd in the orchard. The examination was done by imprint hybridization using an ASSVd-specific DIG-labelled probe at stringent hybridization conditions and by RT-PCR using two different ASSVd specific primer pairs. We obtained ASSVd-positive results for all 6 cherry trees. No ASSVd was detected in the apple trees of the orchard. Purified ASSVdpositive RT-PCR products from the cherries were directly sequenced or cloned into the pGEM-T vector and then sequenced. ASSVd sequences were obtained from 5 trees. These sequences are 327-340 nucleotides long and share 97-99% identity with ASSVd isolates from Indian (Asian) apples. These results are similar to our data for other ASSVd variants from pome fruit trees in Greece. The cherry ASSVd sequences differ from the prototype isolate of ASSVd (Hashimoto and Koganezawa 1987) at 18-29 sites. There are 15 nucleotide changes common to all Hellenic ASSVd variants, i.e. from pome fruit trees and sweet cherry around Greece. There are no cherry-specific nucleotide changes in the ASSVd sequences obtained. To our knowledge, this is the first published report of detecting ASSVd in naturally infected cherry, including its molecular analysis.

Screening for fruit tree viroids in Lithuania

Abraitiene, Asta[1], Abraitis, Rokas[1], Staniene, Grazina[2], Hammond, Rosemarie W.[3], Owens, Robert A.[3]
[1]Eukaryote Genetic Engineering Laboratory, Institute of Biotechnology, V. Graiciuno 8, Vilnius LT-02241, Lithuania
[2]Plant Biotechnology Laboratory, Institute of Horticulture, Babtai, Kaunas district, LT-54333, Lithuania
[3]Molecular Plant Pathology Laboratory, USDA-Agricultural Research Service, Beltsville, MD 20705, USA
Contact: abraitiene@ibt.lt

Viroids are the smallest plant pathogens known so far, consisting of a small circular RNA which does not code for proteins and is infectious and able to replicate on a variety of host plant species. Viroids are classified into two families, the *Avsunviroidae*, that replicate in the chloroplasts of the infected cells and the Pospiviroidae, that replicate in the nucleus. *Avsunviroidae* family members invade mostly woody plant species. The members of Pospiviroidae invade mostly herbaceous plant species and some fruit trees of *Prunus* and *Malus genera*. A large number of fruit trees in the Lithuanian orchards were also found to be infected by the phytoplasmas. This work was aimed at testing fruit trees for the presence viroid infection. This is the first study aimed at searching for viroid infection in the fruit trees in Lithuania. Mature fruit trees of *Prunus* and *Malus genera* were first screened for viroid infection based on exposure of visual symptoms in 2007. The apple, cherry and apricot trees were tested in the Southern, Central, and Northern parts of Lithuania. Leaf samples from trees exposing decline, defoliation, leaf and branch proliferation, leaf wilting and smaller size, bark damage and changes in leaf color were analyzed using R(eturn) PAGE and RT-PCR assays. The obtained results showed that phytoplasmas are much more common in plants exposing decline and abnormal morphological traits in the trees of *Prunus* and *Malus* species than viroids.

Molecular characterization of hellenic variants of Apple scar skin viroid and Pear blister canker viroid in pome fruit trees

Kaponi, Maria S.[1], Faggioli, Francesco[2], Luigi, Marta[2], Barba, Marina[2], Kyriakopoulou, Panayota E.[1]
[1]Agricultural University of Athens, Iera Odos 75, 11855 Athens, Greece
[2]CRA-PAV, Centro di Ricerca per la Patologia Vegetale, 00156 Rome, Italy
Contact: pek@aua.gr

Apple scar skin viroid (ASSVd) and *Pear blister canker viroid* (PBCVd) are members of the genus *Apscaviroid* (family Pospiviroidae). In order to study the nucleotide sequence and secondary structure of Hellenic isolates of these viroids, a large number of naturally infected fruit trees were initially tested with imprint hybridization. Total RNA extracts from hybridization-positive samples were reverse-transcribed and amplified by polymerase chain reaction using 2 different specific primer pairs for each viroid. Purified RT-PCR products were directly sequenced or cloned into the pGEM-T vector and then sequenced. ASSVd variants from 2 apples, 3 wild apples (*Malus sylvestris*) and 2 pears are 330 nucleotides long. They differ from the prototype isolate of ASSVd at 3-14 sites, and 3 nucleotide changes are identical among all Hellenic variants. Most of the changes occur in the variable region of ASSVd (nucleotides 100-180). Hellenic ASSVd variants share significant homology (97-99%) with ASSVd isolates from Asian apples. Three variants, deriving from different hosts and areas, are identical to each other (wild apple and apple from Pella [Macedonia] and pear from Achaia [Peloponnessos]). PBCVd variants from 4 apples, 1 wild apple, 3 pears and 1 quince are 314-315 nucleotides long. There are 6-35 nucleotide changes between all Hellenic variants and the prototype PBCVd isolate. Nineteen changes are identical among the majority of the Hellenic variants, regardless of origin, and 28-35 changes occur in apple and wild apple PBCVd sequences. These differences are not located in a specific region of PBCVd. In addition, most Hellenic PBCVd variants are 91-98% homologous to Australian PBCVd isolates from pear, quince and nashi pear (*Pyrus pyrifolia*) and Bosnian pear isolates. The ASSVd and PBCVd Hellenic sequences can form rod-like secondary structures. This is the first detailed molecular study of ASSVd and PBCVd in Hellenic pome fruit orchards and wild pome fruit trees.

Identification of Peach latent mosaic viroid and hop stunt viroid in different peach cultivars showing dapple fruit, fruit yellow mosaic and cracked sutures symptoms

Luigi, Marta[1], Faggioli, Francesco[1], Barba, Marina[1], Giunchedi, Luciano[2]
[1]CRA-Centro di Ricerca per la Patologia Vegetale, 00156 Rome, Italy
[2]Dipartimento di Scienze e Tecnologie Agroambientali - Università di Bologna
Contact: francesco.faggioli@entecra.it

From the early 1990s, a fruit peach syndrome characterized mainly by small discoloured spots (dapple fruit) and/or yellow areas on skin (yellow mosaic), cracked suture and deformations occurs in most commercial orchards in the Emilia Romagna region (central-north part of Italy). In the past, *Peach latent mosaic viroid* (PLMVd) and occasionally *Hop stunt viroid* (HSVd) have been detected in trees with symptomatic fruits. In order to ascertain the presence and the frequency of these two viroids, fruit samples have been collected from five different cultivars: 'Royal Glory', 'Crimson Lady', 'Grenat', 'Diamond Princess' and 'Laura'. Dapple fruit symptoms affected all cultivars, whereas 'Grenat' samples also showed evident yellow mosaic and fruit deformation and 'Royal Glory' severe cracked sutures. The results obtained showed a large diffusion of the two viroids, mainly in mixed infection; more specifically PLMVd was found in 100% of 'Royal Glory', 'Diamond Princess' and 'Laura' samples, in 90% of 'Grenat' samples and in 70% of 'Crimson Lady' samples; HSVd affected 100% of 'Crimson Lady' and 'Laura' samples, 90% of 'Royal Glory' samples and 30% of 'Grenat' samples, whereas it was not found in the 'Diamond Princess' samples. The role that the viroids could play in the expression of the symptoms in the fruit peach samples has been complicated by the high number of samples infected by both viroids (60%); in any case, PLMVd was confirmed to be strictly associated with the yellow mosaic, cracked suture and fruit deformation symptoms. The aetiological origin of the dapple fruit disease, however, seem to be more complicated, since in the 'Diamond Princess' only PLMVd has been found to be associated with the symptoms, whereas in all other cultivars the presence of HSVd in high percentages could have influenced the symptoms expression.

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Real-Time Reverse Transcription PCR assay for Peach latent mosaic viroid detection

Grimová, Lenka, Kochanová, Michaela, Rysánek, Pavel
Department of Plant Protection, Czech University of Life Sciences Prague, Kamýcká 129, Praha 6 Suchdol
Contact: lgrimova@seznam.cz

Prunus spp. is affected by a couple of viroids, *Peach latent mosaic viroid* (PLMVd) and *Hop stunt viroid* (HSVd). In this study we focused on PLMVd, which is classified in the *Avsunviroidae* family and is widely distributed in peach germoplasm of Europe, Asia, Australia, North and South America. Peach latent mosaic disease is economically important because it affects fruit quality, reduces the lifespan of the trees, and causes peach trees to be more susceptible to other biotic or abiotic stresses. Reverse Transcription Real Time PCR for PLMVd detection was carried out. In our case, TaqMan probe was chosen due to its high specifity of reaction. Primers and the probe were designed by software Beacon Designer 7.0 and the reaction was carried out by Real-Time machine Opticon 2 (Software Opticon Monitor 3). PCR products were used as standarts in number of 108 – 10 16 copies. The method assay is accurate and reliably detects PLMVd in bark, leaf, fruit, flower blossom and pollen grain tissues of trees during the whole growing season. Furthermore, it is well adapted for the routine detection of PLMVd, because it eliminates any risk of contamination and it obviates the need for post- PCR processing steps. This system may replace the commonly used diagnostic techniques to detect this viroid.

Assessment of susceptibility to European stone fruit yellows phytoplasma of new plum variety and of five rootstock/plum variety combinations

Landi, Fabio[1], Prandini, Andrea[1], Paltrinieri, Samanta[1], Missere, Daniele[2], Bertaccini, Assunta[1]
[1]Alma Mater Studiorum' University of Bologna, Dista, Plant Pathology, viale Fanin 42 -40127 Bologna, Italy
[2]Centro Ricerche Produzioni Vegetali, Cesena, Italy
Contact: ipwg2007@gmail.com

During 2003-2008 a research was carried out to assess the plum susceptibility to infection by European stone fruit yellows (ESFY, '*Candidatus* Phytoplasma prunorum') phytoplasmas. Two different trials were carried out to verify susceptibility of new variety/cultivar compared to some currently available rootstock/scion combinations. Half of the new variety belonged to Japanese and the other half to European plum groups respectively and were evaluated in more than one plot each with four plants grafted into Myrabolan 29C. The orchard was located in a ESFY severely infected area of Northern Italy: yearly monitoring by visual inspection and PCR/RFLP identification of phytoplasmas allowed to verify an increasing phytoplasma presence in both symptomatic and asymptomatic plants. After five years 8 selections from Japanese plums showed ESFY symptoms or pathogen presence in 50% of the plants, and 9 selections showed 20% of infection. Only 9 selections showed absence of both symptoms and pathogen, although European selections/cultivars were not symptomatic plants belonging to 6 cultivars were positive to phytoplasma presence. In a parallel experiment 5 rootstocks were grafted with 3 Japanese variety: the majority of the combinations showed phytoplasma symptoms and presence starting from the first year after plantation; however two of the rootstocks appeared to induce a delay in symptoms appearance indicating that rootstock could probably induce some resistance to ESFY in Japanese plum but only for a some years after plantation.

Molecular identification of phytoplasmas associated with diseases of *Prunus* sp. at the Canadian Clonal Genbank

Michelutti, Roberto, Wang, Liping, Arocha, Yaima, Lalonde, Josh
Agriculture and Agri-Food Canada, Canadian Clonal Genebank, GPCRC, 2585 County Rd. 20, Harrow, Ontario, N0R 1G0
Contact: micheluttir@agr.gc.ca

Surveys for phytoplasma were conducted at the Canadian Clonal Genebank during 2008. Symptoms of leaf reddening and shot holes followed by tissue necrosis, were observed in *Prunus* sp., including apricot, peach and nectarine. Total DNA was extracted from leaf samples collected and used as a template in a nested polymerase chain reaction (PCR) with phytoplasma universal primers P1/P7-R16F2N/R16R2 that target the conserved 16S ribosomal RNA (rRNA). PCR products were subjected to RFLP analysis for partial characterization. Nested PCR products were purified, cloned and sequenced. Ribosomal sequences were compared to those of reference in GenBank and phylogeny was carried out to determine the relationships among phytoplasmas identified.

Detection and distribution of European stone fruit yellows in apricot cv. Bergeron and epidemiological studies in the province of Trento (Italy)

Poggi Pollini, Carlo[1], Forno, Flavia[2], Franchini, Sergio[3], Gobber, Marino[3], Lanzoni, Chiara[1], Mattedi, Luisa[2], Miorelli, Paolo[3], Profaizer, Davide[3], Ratti, Claudio[1]
[1]DISTA, Alma mater studiorum, Bologna, Italy
[2]Istituto Agrario S. Michele all'Adige (IASMA), Trento, Italy
[3]IASMA, CAT, Trento, Italy
Contact: carlo.poggipollini@unibo.it

The aim was to investigate the field performance of "Bergeron" on "Wavit" in 4 experimental fields in the province of Trento (Italy), where European Stone Fruit Yellows (ESFY) caused by "*Candidatus* Phytoplasma prunorum" has been constantly spreading since 2000. This included visual inspections for typical symptoms (early bud-break during dormancy and premature leaf-roll) and a highly sensitive real-time PCR assay; 25% of the propagation material was checked with this method and found to be healthy, before planting in 2005. The epidemiology of the disease was also studied by focusing: the presence of the vector Cacopsylla pruni (Scopoli) on conifers; the detection of "Ca. Phytoplasma prunorum" in psyllid eggs and the trasmission efficiency at different stages. This was done by exposing apricot trees in 2 locations, during 2 periods from January to July, to the overlapping presence in the orchards of the

reimmigrants and the new generation of *C. pruni*. The results obtained demonstrated that "Bergeron"seems to be highly susceptible to ESFY: typical bud-break was rarely observed, but up to 20-30% of the plants showed premature leaf-roll, fruit deformation and dieback. As regards the vector: *C. pruni* was caught only once on Picea abies during winter; "Ca.Phytoplasma prunorum" was found in 4 eggs samples from 4 locations; the preliminary results on the exposed trees confirmed that the reimmigrants could be the most efficient vectors at least on apricot. This research was supported by the Provincia Autonoma di Trento.

PCR/RFLP based method for molecular characterization of *'Candidatus* Phytoplasma prunorum' strains using aceF gene

Martini, Marta[1], Ferrini, Francesca[1], Danet, Jean-Luc[2], Ermacora, Paolo[1], Sertkaya, Gülşen[3], Delić, Duska[4], Loi, Nazia[1], Foissac, Xavier[2], Carraro, Luigi[1]
[1]Department of Biology and Plant Protection, University of Udine, Italy
[2]UMR GDPP, INRA and Universite Bordeaux 2, Villevave d'Ornon, France
[3]University of Mustafa Kemal, Faculty of Agriculture, Department of Plant Protection, Antakya, Turkey
[4]University of Banja Luka, Faculty of Agriculture, Banja Luka, Bosnia and Herzegovina
Contact: marta.martini@uniud.it

New molecular typing tools for phytoplasmas belonging to the 16SrX phytoplasma group have been recently developed based on non-ribosomal genes aceF, pnp, imp, and SecY. In the present work we chose to perform a PCR-RFLP method based on aceF. In fact, this genetic marker showed high variability among strains of 16SrX group; moreover it allowed to differentiate French hypovirulent '*Candidatus* Phytoplasma (Ca. P.) prunorum' strains from the virulent ones. Stone fruit samples were mostly collected in North-east Italy; few samples from Turkey and Bosnia & Herzegovina were also included in the work to explore variability. French hypovirulent and virulent strains were used as reference strains. Part of Italian samples was not field collected and they became infected by *Cacopsylla pruni* in controlled conditions. Sequencing of aceF gene was performed on part of the samples tested, and based on the alignment few restriction enzymes were selected for 'Ca. P. prunorum' strain differentiation. Nested-PCR was performed using previously developed primers on all samples, and RFLP analyses were carried out with HaeIII and Tsp509I enzymes. HaeIII enzyme permitted to split some of the Turkish and Bosnian samples from the others, including all the Italian and French samples. On the other hand, Tsp509I enzyme allowed differentiation within the Italian and French strains. Combining the results obtained with the two restriction enzymes it was possible to distinguish among the 'Ca. P. prunorum' strains investigated in this study, 4 different RFLP subgroup (indicated with AceFA, -B, -C and –D). In North-east Italy, where a large number of samples were processed, we can affirm that the strains belonging to AceF-A and B subgroups were the predominant and the mixed infection of the two strains was also quite common. Distinction between Italian hypovirulent and virulent strains is still under investigation.

Establishment of a quantitative real-time PCR assay for the specific quantification of *Candidatus* Phytoplasma prunorum in plants and insects

Jarausch, Wolfgang, Fuchs, Annette, Jarausch, Barbara
AlPlanta-IPR, RLP AgroScience, Breitenweg 71, 67435 Neustadt
Contact: wolfgang.jarausch@agroscience.rlp.de

A real-time PCR assay for the quantification of *Candidatus* Phytoplasma prunorum has been established which combines the specificity of detection with a low cost method of quantitative PCR. The assay uses the specific primers ECA1/ECA2 with a SYBR Green I protocol. A gene fragment of Ca. P. prunorum with the target of the primers has been cloned and is used as standard for quantification by the standard curve method. The assay has been successfully applied to measure the concentration of Ca. P. prunorum in insects as well as in different kinds of plant samples.

16SrI-B Phytoplasma Infections in Plum and in Sour Cherry in Lithuania

Valiunas, Deividas[1], Jomantiene, Rasa[1], Staniulis, Juozas[1], Davis, Robert E.[2]
[1]Phytovirus Laboratory, Institute of Botany, Zaliuju ezeru 49, Vilnius LT-08406, Lithuania;
[2]Molecular Plant Pathology Laboratory, USDA-Agricultural Research Service, Beltsville, MD 20705, USA
Contact: deividas_valiunas@yahoo.com

Recently, in the late summer of 2008, phytoplasma-related diseases of plum (*Prunus domestica* L.) and sour cherry (*Cerasus vulgaris* Mill., syn. *Prunus cerasus* L.) were detected in Lithuania. The plum exhibited symptoms of witches'-broom, shoot proliferation and abnormally small leaves . The diseased cherry developed symptoms related with unusual unseasonal time of flowering, some proliferation, dropping of leaves, and general decline of the tree. Molecular investigation by polymerase chain reaction, restriction fragment length polymorphism and sequence analysis of 16S rDNA, revealed phytoplasmas belonging to group 16SrI ('*Candidatus* Phytoplasma asteris' group), subgroup B. The insect-vector is unknown. The most important phytoplasmas in stone fruit plants worldwide are related to 16Sr-X, 16SrV, 16SrI, and 16SrIII phytoplasma groups. Previously obtained data and the results of this investigation indicate that the 16SrI-B subgroup of phytoplasmas is the most prevalent phytoplasma infection in both grass and woody plants in Lithuania. The subgroup 16SrI-B phytoplasma was identified in plums for the first time.

Evaluation of the susceptibility of pear and plum-trees varieties and rootstocks to Candidatus Phytoplasma prunorum by means of realtime PCR

Torres, E.*., Laviña, A., Sabaté, J., Bech, J., Batlle; A.
Laboratori de Sanitat Vegetal. DARP. Generalitat de Catalunya. 08040 Barcelona
Contact: ester.torres@gencat.cat

The diseases produced for phytoplasmas are difficult to control due to the fact that there are no available direct measures of control. One of the means to avoid the damages produced by these diseases is to have resistant or tolerant plant material. For the diseases produced for phytoplasmas has been observed that the minor expression of symptoms is owed often to the lack of reinfections and therefore to a minor concentration of the population of phytoplasmas. This fact is associated also in the difficulty of detecting the phytoplasma in the tolerant varieties though they are infected. For the *Prunus* species important differences of susceptibility to European stone fruit yellows (ESFY) phytoplasma have been mentioned, the apricot, Japanese plum and the peach trees are more susceptible than the *Prunus cerasifera* (Myrabolan) and that the *Prunus domestica* genotypes. Likewise in the monthly detection of the *Candidatus* Phytplasma pyri in infected trees of two varieties of pear (cv Blanquilla and cv Bartlett), the phytoplasma was detected with only PCR in trees of the variety Bartlett, whereas in the variety Blanquilla the accomplishment of the nested PCR was necessary. This was also related to the presence of symptoms that were much more evident in the variety Bartlett. The purpose of this work is to apply the real-time PCR to quantify the phytoplasma concentration in pear and plum trees previously infected by Ca. P. pyri and Ca. P. prunorum respectively. A selection of different pear and plum varieties and rootstocks were analyzed in order to establish the relation between the presence of symptoms and the estimated phytoplasma concentration. Samples were amplified both by nested-PCR with universal and specific primers and by Real time PCR.

Individuation of *Candidatus* Phytoplasma prunorum type a and type b in *Cacopsilla pruni* individuals

Ferretti, Luca[1], Gentili, Andrea[2], Poggi-Pollini, Carlo[3], Ermacora, Paolo[4], Pasquini, Graziella[5]
[1]CRA Centro di Ricerca per la Patologia Vegetale Roma
[2]CRA Centro di Ricerca per la Patologia Vegetale, Via C. G. Bertero, 22 Roma
[3]Dipartimento di Scienze e Tecnologie Agroambientali (DISTA), Università di Bologna
[4]Dipartimento di Biologia e Protezione delle Piante, Università di Udine
[5]CRA Centro di Ricerca per la Patologia Vegetale, Roma
Contact: luca.ferretti@entecra.it

Recent investigations on molecular characterization of the '*Candidatus* Phytoplasma prunorum' (16SrX-B subgroup), causal agent of the European Stone Fruit Yellows (ESFY) syndrome, on the non ribosomal tuf gene allowed to individuate two groups of isolates, named 'type a' and 'type b', with a distinct geographical distribution in Italian stone fruit growing areas (Ferretti et al., 2007, 2008). Considering the role of *Cacopsylla pruni* (Scopoli) in the epidemiological cycle of the disease, the presence of the two groups of isolates has been investigated also in infected individuals of the psillid, sampled in different Italian areas. Both types has been identified in *C. pruni* specimens captured on apricot, plum and wild *Prunus* species.

Phytoplasma manipulates psyllid vector behaviour by altering host plant odour

Gross, Jürgen, Mayer, Christoph J.
Julius Kühn Institute, Institute for Plant Protection in Fruit Crops and Viticulture, Schwabenheimer Str. 101, 69221 Dossenheim, Germany

Apple proliferation is one of the major plant diseases affecting apple growing in Europe. This disease is caused by *Candidatus* Phytoplasma mali (Bacteria: Mollicutes), which colonizes the phloem of infected trees. The main symptoms of this disease are dwarf sized fruits and the proliferation of axillary buds (witches brooms). The phytoplasma is vectored by the jumping plant louse *Cacopsylla picta* (Hemiptera: Psyllidae). Complex multitrophic interactions between *Malus domestica*, *C. picta*, and *Ca*. P. mali were investigated in laboratory and field. Results from Y-tube olfactometer trials showed that immature adults of *C. picta* differentiated between the odour of healthy and infected apple trees and preferred the odour of infected trees. GC-MS analysis of the headspaces collected from healthy and infected apple trees revealed the induction of the sesquiterpene ß-caryophyllene in infected trees. Y-tube olfactometer trials revealed ß-caryophyllene to be an attractant for *C. picta*. In conclusion, the pathogen manipulated its vector indirectly by inducing an allomone in the plant, which increased its attractiveness. Finally, the use of this compound for trapping the vector was proved in field experiment.

Detection and identification of phytoplasmas in pear trees in the Czech Republic

Franova, Jana[1], Pribylova, Jaroslava[1], Cermakova, Helena[1], Sucha, Jana[2], Paprstein, Frantisek[2]
[1]Biology Centre v.v.i., Institute of Plant Molecular Biology, Academy of Sciences, Branisovska 31, Ceske Budejovice
[2]Research and Breeding Institute of Pomology Holovousy Ltd., Horice
Contact: jana@umbr.cas.cz

Survey on the occurrence of phytoplasma diseases in pear trees was conducted in the Czech Republic over the years 2006 to 2008. A total of 160 pear trees with symptoms of premature leaf reddening, yellowing, dieback of branches and also asymptomatic trees were tested for the presence of phytoplasmas. Nested PCR procedures were employed with different sets of primers to amplify phytoplasma 16Sr DNA plus spacer region. Among the plants tested, 36 trees were positive in nested PCR analyses. However, by RFLP analyses of 16Sr DNAs (R16F2n/R2 nested PCR products) it was possible to identify phytoplasmas in 28 of the studied trees. '*Candidatus* Phytoplasma pyri' (ribosomal subgroup 16SrX-C, pear decline - PD), '*Candidatus* Phytoplasma asteris' (16SrI-C, clover phyllody – CPh), and '*Candidatus* Phytoplasma mali' (16SrX-A, apple proliferation – AP) were identified in 11, 7 and 2 pear trees, respectively. A mixed infection of PD with CPh (3 trees), PD with '*Candidatus* Phytoplasma solani' (16SrXII-A, stolbur – STOL) (1 tree), PD, AP and CPh (1 tree), AP with CPh (2 trees) and AP together with phytoplasmas belonging to ribosomal group 16SrI were identified after digestion with MseI, TruI, SspI, RsaI and HhaI endonucleases. Sequence analysis of selected samples and its comparison with the data available in the GenBank confirmed the phytoplasma presence and its

previous identification by RFLP. The identification of stolbur and apple proliferation represents the first report of such an occurrence of these phytoplasmas in pear trees.

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Seasonal variations of pear decline

Gazel, Mona[1], Ulubas Serce, Cigdem[1], Kadriye, Caglayan[1], Ozturk, Harun[2]
[1]Mustafa Kemal University, Plant Protection Department, Hatay/Turkey
[2]Ministry of Agriculture and Rural Affairs, Agriculture Directorate of Bursa Province, Bursa, Turkey.
Contact: monagazel@hotmail.com

Pear decline disease (*Candidatus* Phytoplasma pyri, PD) was monitored in pear trees cv Deveci in two orchards in Bursa, Turkey. PD infections on twenty pear trees in each orchard were previously determined by molecularly. Fluctuations of PD were determined throughout a year monthly. The tests were performed from roots, shoots, leaf midribs, fruits and flowers of the trees depend on the season. Samples were analyzed with PCR using P1/P7 and fU5/rU3 universal primer pairs. Nested PCR products were digested with RsaI restriction enzyme and digested products revealed the same profile of PD control. RFLP results were supported by sequencing of three selected PD isolates. The results revealed that the detection rate of PD had different averages according to the sampling tissue and the period. The flower tissues were sampled in March and the infection rate of PD was 75%. The fruit tissues only sampled in September and the infection rate of PD was 100%. Root, shoot and leaf samples were collected longer period of the year, but the rate of PD was comparatively less than flower and fruits. The infection rate in roots, shoots and leaves was found as 18, 19 and 10%, respectively. The present result have revealed that the best period to detect PD infection in pear trees was between November to March for root samples. April, October, November and December were the best time for leaves and PD could be detected in shoot samples whole year around except July and August.

Effect of pear decline for Turkish pear production

Ulubas Serce, Cigdem[1], Gazel, Mona[1], Caglayan, Kadriye[1], Ozgen, Mustafa[2]
[1]Mustafa Kemal University, Plant Protection Department, Hatay/Turkey
[2]Gaziosmanpaşa University, Agriculture Faculty, Horticulture Dept., Taşlıçiftlik, Tokat, TURKEY
Contact: culubas@gmail.com

Pear decline is an important threat for Turkish pear production. In this study, we attempt to compare several pomological characteristics, total phenolic content and total antioxidant capacities in *Candidatus* Phytoplasma pyri infected and noninfected 'Deveci' pear from Bursa, Turkey. The samples were taken in October 2008 on harvesting maturity from four infected and noninfected trees based on pear decline symptoms. Presence of *Candidatus* P. pyri was later confirmed by nested PCR tests. The result indicated that infection significantly reduced fruit size, width, length; and increased pH, color values of a, b and hue. Abortive and healthy seed numbers and weights, soluble solids and acidity did not change significantly. Similarly, the infection did not affect the flesh color. To investigate a possible differential response on skin and flesh of fruits, total phenolic (TP) and total antioxidant capacity (TAC) skin and flesh tissues analyzed separately. The results indicated that, as expected, infected skin tissue had higher total phenolic and total antioxidant capacity for both methods analyzed (TEAC and FRAP). Skin TP content increased from 806 to 923 µg gallic acid equivalent (GAE)/g fresh weight (fw) while flesh TP content increased from 195 to 249 µg GAE/g fw. TAC also found to be enhanced on infected fruits. On average, noninfected trees had 32.4 and 28.3 µmol TE/g fw for TEAC and FRAP, respectively. Infection increased these averages to 35.4 and 32.3 µmol TE/g fw tabulating 18 and 12% increase in flesh tissue. Similarly, the TEAC and FRAP averages increased from 4.0 to 5.8 and 3.3 to 4.9 µmol TE/g fw, respectively.

Identification of phytoplasmas associated with apple trees showing shoots proliferation and leaves deformation

Mirosława, Cieślińska
Research Institute of Pomology and Floriculture, Pomologiczna 18, 96-100 Skierniewice, Poland
Contact: mcieslin@insad.pl

Five apple trees showing proliferation of shoots and deformation of leaves were tested to detect the possible presence of phytoplasmas. Total DNA was extracted from shoots using DNeasy Plant Mini Kit (Qiagen GmbH) and was subjected to a nested PCR for amplification of phytoplasma 16S ribosomal (r) DNA. Universal primers P1/P7 followed by universal primers R16F2n/R16R2 as well as specific primers: R16(I)F1/R16(I)R1 for Aster yellows group, AY (16SrI) and fAT/rAS for Apple proliferation group, AP (16SrX) were used in a nested PCR. All phytoplasma rDNA fragments were amplified by nested PCR with R16F2n/R16R2. Using fAT/rAS AP-specific primers in nested PCR, products of expected size were obtained for DNA fragments extracted from four apple trees but not from 'Evelina' tree. Phytoplasma rDNA fragment isolated from this apple tree was amplified by nested PCR with R16(I)F1/R16(I)R1 AY-specific primers but not with fAT/rAS AP-specific primers. Restriction fragment length polymorphism (RFLP) of R16F2n/R16R2 primed nested PCR products was performed using endonucleases RsaI, BfaI, AluI, HpaII to identify phytoplasmas. Restriction profile of nested PCR products from four apple trees was identical to each other, to the control AP-infected apple and patterns published previously and indicated that they were infected by a phytoplasma classified in 16SrX group as the species *Candidatus* Phytoplasma mali. Samples from 'Evelina' apple trees produced a restriction profile identical to AY-infected strawberry – now classify as *Candidatus* Phytoplasma asteris. Nested PCR products primed with R16F2n/R16R2 were purified using QIAquick® Gel Extraction Kit (Qiagen, GmbH), sequenced and analyzed using Lasergene (DNASTAR) computer program. Sequence analysis of 16Sr DNA fragments of phytoplasmas isolated from apple trees confirmed the PCR-RFLP results.

Diagnostics of fruit tree phytoplasmas - Importance of latent infections

Mehle, Natasa[1], Ambrozic Turk, Barbara[2], Brzin, Jernej[1], Nikolic, Petra[1], Dermastia, Marina[1], Boben, Jana[1,3], Ravnikar, Maja1[1]
[1]National Institute of Biology, Department of Biotechnology and Systems Biology, Vecna pot 111, 1000 Ljubljana, Slovenia,
[2]Agriculture Institute of Slovenia, Hacquetova 17, 1000 Ljubljana, Slovenia, 3present adress: Institute of Microbiology and Immunology, Zaloska 4, 1000 Ljubljana, Slovenia
[3]present adress: Institute of Microbiology and Immunology, Zaloska 4, 1000 Ljubljana, Slovenia
Contact: natasa.mehle@nib.si

In the period 2000-2008 more than 1300 fruit trees from different regions of Slovenia were tested for the quarantine phytoplasma: Apple proliferation (AP), Pear decline (PD), and European stone fruit yellows (ESFY). The majority of the samples were collected within systematic official surveys, conducted in order to assess the presence of these phytoplasma diseases in Slovenia in production and mother plant orchards. Samples were taken from trees with and without expressed symptoms. DNA was extracted from the roots (latent infection) or shoots (trees with symptoms). The presence of phytoplasmas were checked using nested PCR, RFLP and real time PCR test (Hren et al., 2007). AP, PD and ESFY were confirmed as being present in several areas in Slovenia where fruit trees are cultivated. AP was found not only in apple, but also in stone fruit trees such as cherry, apricot and plum (Mehle et al., 2007). We observed that especially young trees didn't show typical symptoms (Lešnik et al., 2007) and some infected fruit trees were symptomless. Some latent infections were detected only by using high sensitive diagnostic methods, such as real time PCR, but one year later the infection was confirmed also by using less sensitive methods. Infected trees without symptoms represent a hidden source of infection, so an early detection of infected trees and their removal is almost equally important as intensive vector control, especially in newly established plantations in fruit tree growing areas with high disease occurrence.

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Differential host DNA methylation might be the cause of phytoplasma elimination upon the treatment with auxins

Jezic, Marin[1], Leljak-Levanić, Dunja[2], Ludwig-Müller, Jutta[3], Mladinić, Marin[2], Katić, Marija[1], Ćurković Perica, Mirna[1]
[1]University of Zagreb, Faculty of Science, Division of Biology, Department of Botany, Marulicev trg 9a, 10000 Zagreb, Croatia
[2]University of Zagreb, Faculty of Science, Division of Biology, Department of Molecular Biology, Horvatovac 102a, 10000 Zagreb, Croatia
[3]Institut für Botanik, Technische Universität Dresden, Zellescher Weg 20b, 01062 Dresden, Germany

Contact: mjezic@botanic.hr

Phytoplasmas from the class Mollicutes are wall-less, pleomorphic endocellular plant pathogenic bacteria that live in the phloem of their plant hosts. Phytoplasmas have reduced genomes of 530 – 1130 kb. Therefore, they are highly dependent on the intake of the nutrients form their hosts, cannot be grown on artificial media and cannot be inoculated mechanically on healthy plants to produce infection. It has been shown that a transfer of in vitro grown phytoplasma-infected *Catharanthus roseus* plantlets from medium supplemented with cytokinine, 6- benzylaminopurine (BA) to the one supplemented with auxins, indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) can induce remission of symptoms and even permanent elimination of '*Candidatus* Phytoplasma asteris' reference strain HYDB. To elucidate the possible mechanism of phytoplasma elimination from *C. roseus* shoots caused by IBA-treatment, we measured and compared endogenous auxins' levels and general methylation levels in healthy periwinkles, periwinkles infected with different '*Candidatus* Phytoplasma' species and phytoplasma-recovered periwinkles. Healthy shoots did not respond, or responded very weakly to exogenously added auxin, maintaining their phenotype, nominal levels of methylation and hormone concentrations. Phytoplasma-infection caused a change in endogenous levels of auxins in infected periwinkle shoots infected with different '*Candidatus* Phytoplasma' species, but general methylation levels were not statistically different from healthy plants except in the case of 'Ca. P. asteris', which was the phytoplasma strain eliminated from tissues when periwinkles were transferred to IBA containing medium. Therefore, low level of host genome methylation caused by 'Ca. P. asteris' infection of periwinkles, statistically elevated after IBA-treatment, might be the cause of this phytoplasma elimination.

Current status of European stone fruit yellows phytoplasma in Bosnia and Herzegovina

Delic, Duska[1], Mehle, Natasa[2], Lolic, Biljana[3], Ravnikar, Maja[2], Duric, Gordana[3]
[1]University of Banjaluka, Faculty of Agriculture
[2]National Institute of Biology, Department of Biotechnology and Systems biology, Ljubljana, Slovenia
[3]University of Banjaluka, Faculty of Agriculture, , Bosnia and Herzegovina
Contact: duskadelic@yahoo.com

Stone fruits from commercial as well as abandoned orchards were evaluated for ESFY presence during 2004-2007 years, monitoring western and southern districts of Bosnia and Herzegovina. In first survey conducted in period of 2004 till 2005 ESFY were identified on peach and apricot plants in both surveyed districts. During 2007 new survey were done and samples were taken from symptomatic and symptomless plants of peach (*Prunus persica*), apricot (Prunus armeniaca), plum (*Prunus domestica*), Japanese plum (*Prunus salicina*), myrobolan (*Prunus cerasifera*) and cherry (*Prunus avium*). Samples were analyzed using real-time PCR and nested PCR approaches. Concerning previous results, presence of ESFY phytoplasma was additionally identified in Japanese plum and myrobolan as two new phytoplasma hosts in Bosnia and Herzegovina.

Almond witches - broom phytoplasma (*Candidatus* Phytoplasma phoenicium) a real threat to almond, peach and nectarine

Abou-Jawdah, Y.[1], Abou -Fakhr, Efat[1], Hana, Sobh[1], Molino Lova, Marina[1], Vercesi, Annamaria[2], Piero Attilio, Bianco[1]
[1]Agricultural Sciences Department, Faculty of Agricultural and Food Sciences, American University of Beirut, P.O. Box 11-0236/ AUB, Riad El Solh, Beirut 1107 2020, Lebanon
[2]Di.Pro.Ve. sez. Patologia Vegetale, University of Milan, via Celoria 2, 20133 Milano, Italy
Contact: piero.bianco@unimi.it

Almond witches'- broom phytoplasma ('*Candidatus* Phytoplasma phoenicium') was recently reported in association with a devastating disease that killed over a hundred thousand almond (*Prunus dulcis*) trees in Lebanon and in Iran. This phytoplasma belongs to the pigeon pea witches' broom group (16SrIX) which was reported for the first time on stone fruits. The vector has not yet been identified, even though some preliminary data were gathered at our laboratory. However, more recently over a hundred peach and nectarine trees showed symptom of early flowering, rosetting and fruit abortion followed by shoot proliferation, chlorotic smaller leaves that gave the tree a bushy appearance. Most infected trees did not set any fruits. DNA sequencing demonstrated that this isolate is very closely related to 'Ca. Phytoplasma phoenicium'. In one field, the workers stated that in 2007 they observed only a limited number of trees with such symptoms, but that in 2008 approximately 90 trees were diseased. Farmers responded rapidly by eradicating symptomatic trees and in 2009 only few cases were observed and quickly eradicated. The rapid spread of Almond witches' broom over large geographical areas in Lebanon and Iran and the susceptibility of peaches and nectarines to this disease calls for immediate and coordinated regional and international action to establish a stricter local and international quarantine measures in order to eradicate or prevent the further spread of this emergent devastating stone fruit disease.

Results of patch- grafting of tissue infected by *'Candidatus* phytoplasma pyri' on pear and by *'Candidatus* phytoplasma prunorum' on apricot

Pastore, M.[1], Cardone, A.[2], Catucci, L.[2], Del Vaglio, M.[1], Gervasi, F.[1], Scognamiglio, G.[1], Bertaccini, A.[3]
[1]Unità di Ricerca per la Frutticoltura di Caserta C.R.A. Via Torrino, 3- 81100 Caserta, Italy;
[2]Centro di Ricerca e Sperimentazione in Agricoltura Basile Caramia, Via Cisternino, 281, Locorotondo - 70010 Bari;
[3]DiSTA, Patologia Vegetale Alma Mater Studiorum- University of Bologna, Viale Fanin, 4 – 4027 Bologna.
Contact: Pastore2000@inwind.it

Because the pear varieties 'Conference', 'Comice' and 'William', grafted in open field on different rootstocks resulted susceptible to *'Candidatus* phytoplasma pyri', transmitted by *Cacopsylla pyri*, and, in the same orchard, sixty-eight apricot varieties, all grafted on Prunus cerasifera, resulted differently susceptible to *'Candidatus* phytoplasma prunorum' , we planned a work program with the aim to discover a source of immunity among seven new combination of pear varieties/rootstock and six new combination apricot varieties/rootstock exposed to the contact with the associated phytoplasma micro-organisms, by patch grafting of infected tissues on their phloem. We used also the pear combination 'Comice' on *Pyrus* communis and the apricot combination 'Palummella' on *Prunus* cerasifera already resulted, in open field, both susceptible to the associated phytoplasma, transmitted by the specific vectors. For these experiments 776 health young plants, cultivated in pots, under insect proof greenhouses, were utilized. The molecular analyses, using PCR/RFLP technology, to verify pathogen presence were carried out on leaves collected in each vegetative season since 2003 to 2008. The new pear combination resulted susceptible to 'Ca. phytoplasma pyri' is 'William' on *Pyrus betulaefolia* but the evidence that either the pear combination 'Comice' on *Pyrus* communis or the apricot combination 'Palummella' on *Prunus cerasifera* resulted in the past susceptible were resulted, in these experiments, negative to molecular test does not permit us to declare immune all the other new combinations tested. So we can only conclude that this kind of grafting is not efficient in transmitting these two pathogen to young plants, cultivated in pots. We will continue to test other kinds of grafting techniques on these young plants in pots to understand if the different reaction to these two pathogens can be studied or not under green-house conditions.

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Evaluation of different detection methods of virus and phytoplasmas for a pear and apple certification program

Laviña, A*., Sabaté, J., Batlle, A.
Dpt. Protecció Vegetal, Institut de Recerca i Tecnologia Agroalimentàries (IRTA).
Contact: amparo.lavina@irta.cat

The virus *Apple stem grooving virus* (ASGV), *Apple stem pitting virus* (ASPV) and *Apple chlorotic leaf spot virus* (ACLSV), and the phytoplasmas *Candidatus* Phytoplasma pyri and Ca.P.mali cause significant diseases in Malus and *Pyrus* species. The sensibility of the molecular detection methods of these virus and phytoplasmas has been reported for years, nevertheless it is still recommended to confirm their absence by biological indexing. In this work the presence of this virus by means of RT-PCR and of Ca. P. pyri and Ca.P.mali by PCR is indicated, in trees obtained from certified plant material. Likewise, the detection of these viruses has been done in positive controls of different transmissible diseases like, Ring mosaic, Red mottle, and Russet wart. In all of them ASPV, ASGV and/or ACLSV have been identified, indicating that these are responsible for the disease on their own or in synergism with other viruses or pathogenic organisms. Different methods of extraction, primers and seasons have been tested. The best primers for the detection of ASPV and ASGV by RT-PCR are those used by Massart et al. (2008). With those primers it is possible to detect the two viruses simultaneously both in purified RNA or in crude extracts and also in the extractions done from phloem in winter and from young outbreaks in spring. For phytoplasma detection the best results were obtained with PCR using purified DNA and fO1/rO1 primers. The results obtained with other non ribosomal primers and with the PCR-dot blot method are also presented. The detection of ASPV, ASGV and ACLSV by RT-PCR and of the phytoplasmas Ca.P.Pyri and Ca.P.mali by PCR can be used year-round for the specific detection of these pathogens directly in their woody hosts.

Molecular detection of pear decline phytoplasma in pear trees and their biochemical responses

Canik, Didem[1], Dikilitas, Murat[2], Ertunc, Filiz[1]
[1]Department of Plant Protection, Faculty of Agriculture, Ankara University, Diskapi-Ankara/Turkey
[2]Department of Plant Protection, Faculty of Agriculture, Harran University, Osmanbey-Sanliurfa/Turkey

This study was carried out to determine the presence of phytoplasma diseases in pear fruit trees and their biochemical responses in the experimental research garden of Ankara University. Phytoplasmas were detected and characterized by PCR-RFLP analysis. PCR analysis was performed using the primer pairs of P1/P7 and fO1/rO1 to identify the phytoplasmas involved in the diseases. The infected trees, with symptoms or without symptoms, were found positive with the pear decline phytoplasma. Their biochemical responses such as PPO (Polyphenol oxidase), MDA (malondialdehyde), chlorophyll, carotenoids and anthocyanin contents were varied among the cultivars (Duchesse d'Angouleme, Beurre Hardy, Abbe Fetel, Coscia and Deveci) tested. However, no significant differences were observed in proline contents between the cultivars regardless of the presence of symptoms. The cultivars Duchesse d'Angouleme and Beurre Hardy were found more resistant to the effect of disease. The results suggest that the PD phytoplasma, detected in all infected trees, plays a crucial role on the biochemical metabolisms of the diseased plants. Therefore, the resistance of pear trees infected with PD phytoplasma could be easily checked by using biochemical markers.

Key words: Pear, phytoplasma, PCR-RFLP, proline, PPO, MDA.

Variation among *Apple chlorotic leaf spot virus* isolates affecting fruit tree cultivars and ornamental rosaceous hosts

Sobolev, I.[1], Dombrovsky, A.[1], Candresse, T.[2], Svanella-Dumas, L.[2], Spiegel, S.[1]
[1]Dept of Plant Pathology, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel; [2]UMR GDPP, INRA et Université Bordeaux 2, Centre INRA de Bordeaux, Villenave d'Ornon, France

Apple chlorotic leaf spot virus (ACLSV), the type member of the genus *Trichovirus*, is among the pathogens targeted in certification programs aimed at producing virus-tested propagation material. ACLSV exists worldwide in numerous strains (isolates) in a wide range of rosaceous hosts. ACLSV isolates from pome and stone fruit cultivars have been studied extensively. In the present study, RT-PCR performed with primers targeting the ACLSV coat protein region, was applied for isolates from natural,

poorly studied, ornamental hosts - dwarf flowering almond (*Prunus glandulosa*), medlar (*Mespilus germanica*) and hawthorn (*Crataegus* spp.). Alignment of the nucleotide sequences of virus isolates derived from these hosts showed sequence variability among clones of isolate Dfa1114 from dwarf flowering almond, Med from medlar and Haw from hawthorn but not Dfa1292. These results support previous suggestions that a mixture of ACLSV variants could be present in one tree. Most of the isolates studied by us have the combination of amino acid pairs described recently as crucial for infectivity (Yaegashi et al. 2007). Most isolates show the pairs also observed in isolate B6 but Haw shows the A4-like amino acids. However, variants Haw.6 and Dfa1114.5 have additional modifications at some of these positions. The effectiveness of these modifications needs to be further studied. A phylogenetic tree prepared with the above isolates and a large number of ACLSV sequences available in Genbank demonstrated that none of the isolates included in this study are closely related to previously analyzed isolates. The two Dfa isolates are related despite significant divergence (90% homology). Both isolates belong to a poorly resolved cluster, which contains mainly *Maloidae* isolates, among which is B6. Isolate Haw belongs to another, more resolved group, which contains, among others, the A4 isolate and an isolate from Hawthorn characterized in Greece (N. Katis, personal communication).

Application of recombinant antibodie fragments for supression of a viral disease caused by Tomato yellow leaf curl virus

Safarnejad, Mohammad Reza
Department of Microbial Biotechnology and Biosafety, Agricultural Biotechnology Research Institute, Karaj, Iran
Contact: safarnejad@abrii.ac.ir

Tomato yellow leaf curl virus (TYLCV) is a complex of geminivirus species prevalent in the tropics and sub-tropics, which causes severe diseases in economically important crops such as tomato. Conventional strategies for disease management have shown little success and new approaches based on genetic engineering need to be considered. We generated two single-chain variable fragment antibodies (scFv-ScRep1 and scFv-ScRep2) that bound strongly to continuous epitopes within the TYLCV replication-associated protein (Rep). The TYLCV-Ir C1 gene (encoding Rep) was expressed as glutathione-Stransferase (GST) and maltose-binding protein (MBP) fusions. Purified MBP-Rep was used to immunize mice allowing the construction of a scFv phage display library. Two specific recombinant antibody fragments (scFv-ScRep1 and scFv- ScRep2) were obtained through panning of naïve and pre-immunized phage display libraries. Immunoassays showed that scFv-ScRep1 recognized an Nterminal epitope of Rep, whereas scFv-ScRep2 recognized a more central epitope. Coding regions of these scFv fragments were sub-cloned into plant expression vector and expressed in plant, both as stand-alone and as N-terminal GFP fusions. Initial results indicated that both scFvs and both fusions accumulated to detectable level in the cytosol and nucleus of plant cells. Transgenic plants challenged with TYLCV-Ir showed that the scFv-ScRep1 but more so the fusion proteins were able to suppress TYLCV-Ir replication. These results show that expression of a scFv-ScRep1-GFP fusion protein can attenuate viral DNA replication and prevent the development of disease symptoms. The present article describes the first successful application of recombinant antibodymediated resistance approach against a plant DNA virus.

List of Autors

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J

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V

W

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