



COST Action 0807

Working Group 2 - Insect vectors

Grapevine Yellows Vector Sampling and Monitoring Training School

Bernkastel-Kues, Germany 5th to 9th of July, 2010

Organizers: Michael Maixner, Phyllis Weintraub, Barbara Jarausch



Institute for Plant Protection
in Fruit Crops and Viticulture



Berlin Museum of Natural History



Johannes Gutenberg University, Mainz
Department of Ecology



AIPlanta - Institute for Plant Research



Gilat Research Center

Organizers

Julius Kühn-Institut, Federal Research Centre for Cultivated Plants
Institut for Plant Protection in Fruit Crops and Viticulture

www.jki.bund.de

Berlin Museum of Natural History

Leibniz Institute for Research on Evolution and Biodiversity at the Humboldt University Berlin

www.naturkundemuseum-berlin.de

Johannes Gutenberg-University Mainz

Department of Ecology - Population Biology

www.oekologie.biologie.uni-mainz.de/population_biology/popbiolframe.htm

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COST Action 0807: Integrated Management of Phytoplasma Epidemics in Different Crop Systems

Julius Kühn-Institute, Federal Research Centre for Cultivated Plants

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Verbandsgemeinde Bernkastel-Kues

Moselland Winzergenossenschaft

Organizing Committee

Dr. Michael Maixner (JKI)

Dunja Kröhner (JKI)

Christel Neuerburg (JKI)

Yvonne Kappel (JKI)

Dr. Barbara Jarausch (AIPlanta)

Dr. Phyllis Weintraub (Volcani Center)

Prof. Dr. Hannelore Hoch (Berlin Museum of Natural History)

Dr. habil. Jes Johannesen (Johannes Gutenberg-University Mainz)

Miriam Imo (Johannes Gutenberg-University Mainz)

Tentative program - Schedule may be changed due to weather conditions!
- Talks are indicated by a ●, other points are practical exercises

Sunday, July 4

Arrival of participants

Monday, July 5

- 9:00 Welcome, presentation of workshop program, technical information
- 9:30 Introduction of participants
- 10:00 ● Characterization of the sampling region (Maixner)
- 10:30 ● Auchenorrhyncha taxonomy and systematics - identification of major families (Hoch)
- 11:00 Coffee break
- 11:15 ● Biology and identification of Cixiids (Hoch)
- 12:00 ● Auchenorrhyncha phytoplasma vectors (Weintraub)
- 12:30 Lunch
- 13:30 Field work: Life sampling of *H. obsoletus* and other Cixiids from different host plants
- 16:30 Selection of interesting species and preparation for identification
or
Setup of transmission trials to plants;
- 18:00 End of session

- 19:00 Guided tour to the old part of Bernkastel

Tuesday, July 6

- 9:00 • Sampling strategies (Maixner)
 - Timing; trap types; handling of trapped insects
- 9:30 DNA-extraction from insects (optional)
 and
 Identification of field samples of the previous day
- 12:30 Lunch
- 13:30 Field work: - Sweep net and leaf-blower sampling of life insects
 - Sticky traps, emergence traps
- 16:30 DNA-extraction (continuation and finish)
 and
 Setup of transmission trials to plants
- 18:00 End of session

Wednesday, July 7

- 9:00 • Phytoplasma detection in GY-vectors (Maixner)
 - PCR detection and RFLP-typing; Test strategies: individual testing / batch tests
 - transmission trials
- 9:30 • Analysis of the genetic structure of *Hyalosthes obsoletus* populations
 (Johannesen / Imo)
- 10:15 Practical analysis of population genetic data (Johannesen/Imo)
 or
 Recording of vibrational signals from *H. obsoletus* (Hoch)
 and
 Setup of PCR-reactions for stolbur detection (optional)
- 13:00 Lunch
- 14:00 Field work: Collection of *Oncopsis alni* from *Alnus glutinosa*
- 16:00 Setup of transmission trials using artificial feeding medium
 and
 Gel electrophoresis of PCR-products
- 18:00 End of session
- 19:00 Visit of the 'Moselland' wine-growers cooperative

Thursday, July 8

- 9:00 Tuf-typing of stolbur isolates - digestion of pre-amplified PCR-products
- 9:45 • Influence of habitat structure on the occurrence of *H. obsoletus*
and the different epidemiological cycles of Bois noir (Maixner)
- 10:15 Practical analysis of population genetic data (Johannesen/Imo)
or
Recording of vibrational signals from *H. obsoletus* and/or *Oncopsis alni* (Hoch)
- 13:00 Lunch
- 14:00 Field work: Sampling of *H. obsoletus* at habitats of different suitability
- 17:00 Termination of transmission trials
- 18:00 End of session
- 20:00 Farewell barbecue

Friday, July 9

- 9:00 Gel electrophoresis of digested tuf-products
- 10:00 Documentation of results
- 11:00 Final discussion of results
- 12:30 End of workshop

A. Auchenorrhyncha identification

I. Auchenorrhyncha Taxonomy and Systematics – Identification of Major Families Biology and Identification of Cixiidae

Hannelore Hoch, Museum für Naturkunde, Berlin

With ca. 45,000 described species the Auchenorrhyncha are the largest hemimetabolous insect taxon. While the majority of species is tropical or subtropical, there are ca. 800 species in central Europe. Exclusively phytophagous, Auchenorrhyncha are characterized by their highly specialized sucking mouthparts, and known to feed on xylem, phloem or suck the content of plant cells. Correlated with this specialized diet are trophic interactions with endosymbiotic organisms (such as bacteria and fungi) and mutualistic relationships with other animals feeding on Auchenorrhyncha excretions (honey-dew), among them ants, lizards, moths, and snails. Concerning their host range, Auchenorrhyncha species display varying degrees of specialization, some being extremely polyphagous, while others are oligophagous to monophagous, with some species utilizing a single plant species. Consequently, Auchenorrhyncha play an important role in virtually all ecosystems, with some species causing serious problems on crops such as rice, corn, cotton, sugarcane, potato, and grapevine.

While Auchenorrhyncha are rather modest in regard to body size (less than 10 mm in most species), they are insect record-holders in regard to nymphal development (17 years), and sound-intensity (80-100 decibels at a distance of 18m).

The lecture will give an overview on the diversity and current systematics of the Auchenorrhyncha, introduce the major families and their morphological characteristics, as well as present several aspects of leaf- and planthopper behaviour (e.g., intraspecific communication, reproduction, defense), highlighted by several short video sequences. Special emphasis will be on the biology and identification of the Cixiidae.

II. Literature and keys for identification of European Auchenorrhyncha

The following books and keys for the identification of Auchenorrhyncha are available during the workshop:

Biedermann, R., Niedringhaus, R. (2004). Die Zikaden Deutschlands. Bestimmungstabellen für alle Arten. WAB-Fründ, Scheeßel: 1-409.

Giustina, W. della (1989). Homoptères Cicadellidae. Vol 3. Compléments aux ouvrages d'Henri Ribaut. Faune de France 73. INRA, Paris: 1-350.

Holzinger, W.E., Kammerlander, I., Nickel, H. (2003). The Auchenorrhyncha of Central Europe. Vol 1: Fulgoromorpha, Cicadomorpha excl. Cicadellidae. Brill, Leiden-Boston: 1- 673.

Ribaut, H. (1936): Homoptères Auchenorhynques I (Typhlocybidae). Faune de France 31. Lechevalier et Fils, Paris: 1-228

- Ribaut, H. (1952): Homoptères Auchenorrhynques II (Jassidae). Faune de France 57. Lechevalier et Fils, Paris: 1-474.
- Ossiannilsson, F. (1978). The Auchenorrhyncha (Homoptera) of Fennoscandia and Denmark. Part 1: Introduction, infraorder Fulgoromorpha. Fauna Entomologica Scandinavica Vol. 7, part 1. Scandinavian Science Press Ltd., Klampenborg: 1-222.
- Ossiannilsson, F. (1981). The Auchenorrhyncha (Homoptera) of Fennoscandia and Denmark. Part 1: The families Cicadidae, Cercopidae, Membracidae, and Cicadellidae (excl. Deltocephalinae). Fauna Entomologica Scandinavica Vol. 7, part 2. Scandinavian Science Press Ltd., Klampenborg: 223-593.
- Ossiannilsson, F. (1983). The Auchenorrhyncha (Homoptera) of Fennoscandia and Denmark. Part 3: The family Cicadellidae: Deltocephalinae, Catalogue, Literature and Index. Fauna Entomologica Scandinavica Vol. 7, part 1. Scandinavian Science Press Ltd., Klampenborg.: 594-978.

B. Collection and trapping of Grapevine Yellows vectors

I. Auchenorrhyncha Phytoplasma Vectors

Phyllis G. Weintraub, ARO, Volcani Center, Gilat Research Center

The Hemiptera is a large and diverse order of exopterygote insects, which occur throughout the world. The order is now divided into 3 suborders: Heteroptera (true bugs), Sternorrhyncha (scale insects, aphids, whiteflies and psyllids) and Auchenorrhyncha (leafhoppers, planthoppers, cicadas, treehoppers and spittlebugs). The single most successful suborder of phytoplasma vectors is the Auchenorrhyncha, of which only about 100 leafhopper and planthopper species transmit phytoplasmas.

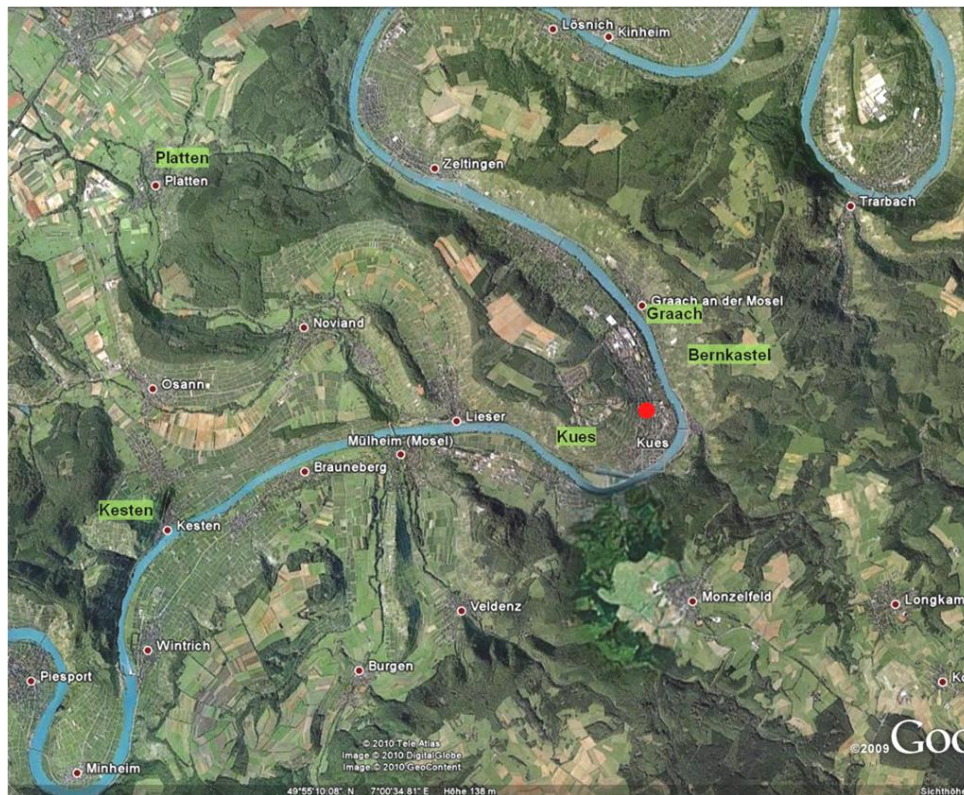
Hoppers possess several characteristics that make them efficient vectors of phytoplasmas: (a) they are hemimetabolous; thus, nymphs and adults feed similarly and are in the same physical location and often both immatures and adults can transmit phytoplasmas; (b) they feed specifically and selectively on certain plant tissues, which makes them efficient vectors of pathogens residing in those tissues; (c) they have a propagative and persistent relationship with phytoplasmas; (d) they have obligate symbiotic prokaryotes that are passed to the offspring by transovarial transmission, the same mechanisms that allow the transovarial transmission of phytoplasmas.

Phytoplasmas are acquired passively during feeding; the duration necessary to acquire a sufficient titer is the acquisition access period (AAP). The AAP can be as short as a few minutes but is generally measured in hours, and the longer the AAP, the greater the chance of acquisition. The time that elapses from initial acquisition to the ability to transmit the phytoplasmas is known as the latent period (LP) and is sometimes called the incubation period. During the LP the phytoplasmas move through and replicate in the competent vector's body; but eventually, to be transmitted, they must penetrate specific cells of the salivary glands. This LP can take from 10 days to a few months depending on the vector-phytoplasma relationship, temperature and other factors.

The interaction between insects and phytoplasmas is complex and variable. The complex sequence of events required for an insect to acquire and subsequently transmit phytoplasmas to plants suggests a high degree of fidelity between vector species and the phytoplasmas that they transmit. However, numerous phytoplasmas, such as AY and WX strains are transmitted by several different insect species. Vector–host plant interactions also play an important role in determining the spread of phytoplasmas. Polyphagous vectors have the potential to inoculate a wider range of plant species; whereas the monophagous *Scaphoideus titanus* is limited to grape.

II. Sampling area

The sampling area around Bernkastel-Kues is situated in the center of the Mosel wine region, one of the thirteen viticultural areas in Germany. Viticulture is stretched along the Mosel River and its tributaries Saar and Ruwer from the French and Luxemburg border in the southwest to Koblenz in the northeast where the Mosel meets the Rhine River. With approximately 8.500 ha the area is the fifth largest viticultural region in Germany. The most important cultivar is ‘Riesling’ with about 60 % of the vineyard surface. Vine growing in one of the northernmost areas (50th parallel) is restricted to favorable microclimatic conditions. The steep slopes of the river valley facing south and southwest provide best conditions for grape growing. The Mosel is one of the world’s largest areas of steep slope viticulture with around 40 % of the vineyards on slopes with an incline of more than 30 %. With an incline of 65° the ‘Bremmer Calmont’ is considered the world’s steepest vineyard. The average yearly temperature at Bernkastel-Kues is 10.5 °C with 690 mm rainfall. More than half of the rain falls during the vegetation period from April to September.



The sampling area around Bernkastel-Kues. The institute is marked by a red dot. Sampling sites are indicated by green labels.

The intense insolation on the steep slopes together with the predominant Devonian slate that retains the day's heat provide favorable conditions not only for grapevine but also for insects. Many xerothermic species e.g. the Bois noir vector *Hyalesthes obsoletus* were originally restricted to those habitats and are still most numerous there. Consequently, the steep slope viticultural areas of Mosel and the Middle-Rhine valleys are the sites where Bois noir traditionally occurs and reaches a high incidence.

Two types of grapevine yellows are present in the Mosel region. Their distribution and incidence within the area are closely correlated with the presence of the respective vectoring Auchenorrhyncha species. Bois noir (BN; called 'Schwarzholzkrankheit' or 'Vergilbungskrankheit' in German) is widespread and of high economic significance, while Palatinate Grapevine Yellows (PGY) is of minor importance and more or less restricted to single vines. Flavescence dorée is not present in Germany.

III. Vectors of grapevine yellows that are present in the area

There are two confirmed vector species of grapevine yellows present in the region, *Hyalesthes obsoletus* and *Oncopsis alni*. Two other phytoplasma vectors, *Reptalus panzeri* and *Macropsis fuscula* are also regularly found in the same environment. *H. obsoletus* (Cixiidae) transmits stolbur phytoplasmas (16SrXII-A) to various wild and cultivated herbaceous plants, but also to grapevine, where the pathogen causes BN. Another quite common Cixiid is *Reptalus panzeri*, a species that transmits stolbur to corn in the Balcan (Maize redness). Although stolbur has been occasionally detected in specimens of *R. panzeri* from the Mosel, this planthopper failed so far to inoculate test plants with the pathogen in transmission trials. Therefore, the species is not considered to be a vector of Bois noir in Germany. There is evidence that *R. panzeri* in central and southern Europe might be different (Hoch, pers. comm.).

Oncopsis alni (Macropsidae) transmits phytoplasmas of the elm yellows-group (16SrV). This leafhopper is strongly monophagous on *Alnus* (mainly *glutinosa*) and transmits Alder yellows phytoplasmas (AldY) to and from alder. A high proportion of alder trees of the region is infected with this pathogen. Very rarely single specimens of *O. alni* are found on grapevines growing close to alder trees. Inoculation of the vines with AldY causes PGY.

Macropsis fuscula is another Macropsid vector that transmits rubus stunt phytoplasma (16SrV) to *Rubus* spp. It is quite common and frequently found on sticky traps around vineyards and sometimes even inside on small *Rubus* stands. Although infected specimens are not rare, the rubus stunt phytoplasma was never detected in grapevine so far.

Neither Flavescence dorée nor its vector *Scaphoideus titanus*, are present in Germany.

Other Cixiids known or suspected to transmit phytoplasmas and/or γ -3 proteobacteria are occasionally found in the vineyards or the surrounding areas: *Cixius wagneri*, *Pentastiridius leporinus*, *Reptalus quinquecostatus*



Hyalesthes obsoletus (top left) and *Oncopsis alii* (top right) are vectors of grapevine yellows in Germany. *Reptalus panzeri* (bottom left) and *Macropsis fuscula* (bottom right) are vectors of stolbur and rubus stunt, respectively, but do not transmit grapevine yellows in Germany although they are present in the vineyard agroecosystem.

IV. Host plants and habitats

Hyalesthes obsoletus

Hyalesthes obsoletus is a polyphagous species that prefers herbaceous hosts. Since the plant-hopper hibernates in the nymphal stage, only perennial plant species may serve as host plants, although the adults can be found on a wide range of additional plant species (feeding hosts). The major host plants in Germany are *Convolvulus arvensis* (bindweed) and *Urtica dioica* (nettle). The former is the traditional host plant in Germany. It is widespread in the viticultural agro-ecosystem, both within vineyards and on uncultivated plots. Bindweed plants infected by stolbur show symptoms like stunting, upright growth, and occasionally also yellowing. However, where herbicides have been sprayed the symptoms cannot be clearly distinguished from herbicide damage.



Symptoms on Convolvulus arvensis. Left: Stand with healthy and symptomatic plants. Middle: Stolbur infected bindweed, upright growth, small leaves, stunted. Right: Bindweed with herbicide damage.

Urtica dioica has long been known as an important host in southern Europe (Italy), but only during the last decade *H. obsoletus* started to commonly colonize nettle in Germany, too. Populations on this host plant extended the range of the planthopper to additional wine growing regions and to sites of less favorable microclimatic conditions. Nettle is less frequently found within the vineyards where it usually grows in small stands between the vines or on the embankments of terraced vineyards. It is more often distributed along the vineyard borders, on fallowed plots or along ditches. Sparsely growing nettles at dry locations are preferred, while vigorously and dense growing stands on humid soil are usually not colonized by *H. obsoletus*. Infected plants of *U. dioica* do not show unambiguous symptoms.



*Nettle stands in a vineyard (left) and a fallow field (middle) that are colonized by *H. obsoletus*. Dense stands on humid soil (right) are no suitable habitats for the planthopper.*

Additional host plants of *H. obsoletus* that are of minor significance in the Mosel area are *Calystegia sepium* and perennial species of *Ranunculus*. Nymphs are occasionally found on the roots of *Artemisia vulgaris*, *Cirsium arvense*, *Medicago sativa*, *Senecio erucifolus*, and *Taraxacum officinale*, too. While infected *C. sepium* do not show clear symptoms, infected *Ranunculus* decline quickly and only healthy plants survive in the field.

Beside the true host plants on which *H. obsoletus* completes its life cycle, many other plants including annual species are used as feeding hosts by the adult planthoppers. Therefore, many of those plants are occasionally found infected by stolbur phytoplasma (e.g. *Solanum nigrum*), but they do not play a role in the epidemiology of stolbur phytoplasmas.



Additional host- and feeding plants of H. obsoletus. From left to right: Calystegia sepium; Ranunculus sp.; Artemisia vulgaris; Solanum nigrum (Stolbur infected plant is dark colored).

Regardless of the host plant species the distribution of *H. obsoletus* is extremely irregular on a small scale. While numerous vectors are found on some plants or plant stands, other plants growing nearby are not colonized at all. When host plants grow along the borders of vineyards there is often a gradient of both, *H. obsoletus* catches and diseased vines. The planthopper clearly prefers sparse vegetation, probably because of a better microclimate due to a more intense insolation of the soil.

Reptalus panzeri

Reptalus panzeri is also a polyphagous Cixiid planthopper. In the vineyard environment it is present on *Clematis vitalba* and various herbaceous plants such as *Urtica dioica*, *Ranunculus sp.*, and *Echium vulgare*. It occurs at about the same time as *H. obsoletus* but is not as widespread as this species. Stolbur has not been detected so far in *C. vitalba* or *E. vulgare*.



Host plants of R. panzeri: Stand of Clematis vitalba at a vineyard border (left), and Echium vulgare inside a vineyard (right).

Oncopsis alni

This leafhopper is closely associated with *Alnus glutinosa* and *Alnus incana*. Larvae of the univoltin species appear in May and adults are present from June to August. While immature stages feed on leaves, the adults usually sit on young branches and twigs. *O. alnus* (even infected individuals) can be found in low numbers on sticky traps in vineyards where alder trees are nearby.



Alnus glutinosa (left) is the host plant of *Oncopsis alni*: The picture in the middle shows a typical site where grapevine and alder grow in close vicinity and Palatinate GY can be expected. The lower twigs of older alder trees are best for sampling of *O. alni*.



While the larvae of *Oncopsis alni* feed on the underside of leaves of *Alnus glutinosa*, the adults are usually found on young twigs where they often sit in the branchings.

V. Trapping techniques

Different traps and sampling techniques can be applied to collect and monitor GY vectors according to the objectives of the studies. With most techniques it is difficult to estimate the population density but information about the occurrence of vectors, their host plant association, and the periods of their activity can be gathered

Sticky traps

Sticky traps can be used as chromotropic (yellow color) or transparent traps depending on the purpose of the study. Yellow traps are attractive for planthoppers and leafhoppers. They are applied for the monitoring of the adult flight activity or to check the occurrence of particular species. Transparent traps are useful e.g. if the movement of insects is studied without any attraction by the traps. For catching *Hyalesthes obsoletus* it is advisable to expose sticky traps close to or within stands of host plants rather than exposing them randomly. Traps within the weed layer are much more effective than those in the canopy of the vines. Sticky traps should be replaced at least every fortnight. During wet weather or if insects should be removed for subsequent DNA-extraction, the exposure time should not exceed one week. Insects can be removed from traps with a drop of benzene that dissolves the glue. It is also used for cleaning the specimens from glue residues. Before DNA-extraction the insects need to be dried thoroughly to remove all benzene.

Emergence traps

Emergence traps consist of a rigid frame of plastic or metal that is in tight contact to the soil. It is covered by a tent whose tip opens into a container with collecting liquid (ethanol 40%, water 30 %, glycerin 20 %, acetic acid 10%). All insects that emerge from soil crawl up to the light and fall into the liquid where they are preserved. The liquid should be removed every week. The traps allow the quantitative trapping of the emerging insects, for example in ecological or control studies, but the insects in the collecting liquid cannot be used for laboratory testing anymore. A major problem for the use of emergence traps is the small area they cover, considering the patchy distribution of many Cixiids in the soil.

Sweep net

The sweep net is the most effective sampling tool and a selective one, too. Only the interesting species are removed from the net with an exhaustor and all other insects can be released. A robust net with a straight rim should be used for the collection of Cixiids from low vegetation. Sweeping the vegetation for a defined period of time is a possibility to collect rough information about the abundance of vectors. Again, due to the clustered distribution of both, host plants and vectors, the validity of the data is limited. On the other hand, plant species can be swept selectively and data about host plant association can be gathered. Insects caught by sweep net are usually not damaged and can be used for laboratory studies including transmission trials. Caught insects can be kept for some hours in vials containing a leaf of a host plant. For longer periods, potted host plants with plastic cages are more suitable.

Vacuum insect collectors

Leaf blowers or D-VAC-collectors are suitable where Auchenorrhyncha are sampled from dense vegetation, trees and bushes, or from the soil. The machines are usually driven by a gasoline engine. Insects are sucked in a hose and collected in a mesh or nylon stocking before they pass the fan of the machine. In dry conditions and with not too intensive vacuum many vectors remain

alive and in a condition that allows to keep them e.g. in transmission experiments for a couple of days.



Yellow and transparent sticky traps exposed to monitor the flight of *H. obsoletus*. Emergence traps (right) allow the quantitative trapping of Cixiids that emerge from the soil as adults.



Collecting leafhoppers from the canopy with a leaf blower and from nettle using a sweep net.

VI. Determination of the time of emergence of adult *Hyaletthes obsoletus*

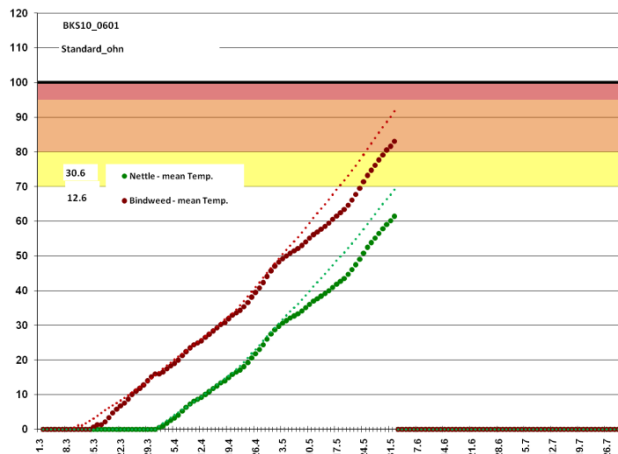
Hyaletthes obsoletus is a univoltine species. Like the larvae of many Cixiidae the immature stages live in the soil, where they feed on the roots of herbaceous plant hosts. Eggs are deposited on the root collar of host plants and the young larvae start to feed on roots in the upper 5 cm of the soil. In October they are already in a depth of 10-15 cm. The nymphs move down to 18-25 cm to hibernate and stay there until March. In April they move up again and are found from May to the emergence of adults in June in the upper 10 cm of the soil.

When the nymphs move back to the surface of the soil in spring they come under the influence of air temperature. The time they need to complete their life cycle depends on temperature. Therefore, the emergence of first adult vectors can be estimated by the calculation of degree-day sums. On the one hand this helps growers to cease weed control in good time to avoid the movement of adult vectors from their eradicated weed hosts to grapevine, and on the other hand it facilitates timing of monitoring and sampling activities. Traps should be exposed and sampling should start as soon as

around 90 % of the threshold degree-sum have been accumulated. The maximum flight activity can be expected within two to three weeks after the emergence of first adults.

Since host populations of *H. obsoletus* on *Convolvulus arvensis* (including those on *Calystegia sepium* and *Ranunculus spp.*) and *Urtica dioica* differ with respect to phenology different sets of parameters are required for the calculation:

Parameter	<i>H. obsoletus</i> populations on ...	
	Bindweed <i>C. arvensis</i>	Nettle <i>U. dioica</i>
Threshold temperature [°C]	5.8	5.0
Start of degree-day calculation	March 9	Apr 01
Required degree-days for flight	1053	1160



Sample output from June 1, 2010 of the degree day calculation for the start of the emergence of adult *H. obsoletus* at Bernkastel-Kues. For the bindweed populations (red), 83 % of the required degree-days are reached while the long term average is 92 % (small red dots). The corresponding figures for the nettle populations are 61 % and 69 %.



Immature stages of *H. obsoletus* live in the soil. Left: First instar larva. Middle; Nymphs are often found together in nests. The walls are lined with wax fibers. Right: Nymph feeding on a nettle root.

The calculation is based on daily mean air temperatures (2 m above ground). Unlike most degree-day based methods, all the degrees above 0 and not only those exceeding the threshold temperature are summed up if the threshold is reached; if not, 0 is added to the DD-sums (e.g: a daily average of 4.9 °C adds nothing to the degree-day sum, while a temperature of 7.8 adds 7.8 to the sum; see Maixner & Langer, 2006 for details). An Excel-Worksheet is available for calculation degree-days and graphic presentation. The average date of emergence of adult *H. obsoletus* at Bernkastel-Kues is June 5 for bindweed populations and June 18 for the planthoppers that live on nettle. The flight activity lasts for four to six weeks.

In the Bernkastel area agricultural weather stations are available. The data of the nearest weather station are used to calculate flight data of *H. obsoletus* at a particular location. A mobile datalogger can be used to record temperature data for special purposes or at locations where a weather station is not nearby. In this case, however, data have to be retrieved from the logger regularly using a mobile computer.



C. Estimation of the infection rate of vector populations

The methods described here for DNA extraction and PCR testing are those used in our lab and applied during the training school. There are many other procedures that work as well. Please refer to the original papers if you are interested in those.

I. Extraction of total DNA from individual insects

1. Place each leafhopper in a 1.5 ml plastic reaction tube, add 400 μ l (smaller insects like *H. obsoletus* and *O. alni*) to 600 μ l (larger insects like *Reptalus panzeri*) of extraction buffer, and grind the leafhopper using a micropestle.
2. Incubate in a thermomixer at 65°C for 20 min. and 1,300 min⁻¹
3. Add chloroform/isoamylalcohol (24:1 V/V) in the same volume as the extraction buffer (400-600 l), vortex.
4. Centrifuge at 6,000 rpm (ca. 3,200 g) for 5 min in a microfuge
5. Transfer upper aqueous phase (without the middle layer) to a new 1.5 ml reaction tube
6. Repeat steps 3 to 5
7. Add 400 (to 600) μ l ice-cold isopropanol and mix thoroughly, keep in -20°C for at least 20 min
8. Centrifuge at 4 °C for 6 min at 22,000 g in a precooled rotor
9. Discard the supernatant and wash pellet with 300 μ l of 70% ethanol
10. Centrifuge at 4 °C for 6 min at 22,000 g in a precooled rotor
11. Discard ethanol
12. Dry the pellet in a vacuum concentrator for approximately 5 min until it is transparent (Not too long!)
13. Dissolve the DNA pellet in 150 μ l TE buffer or ddH₂O.
14. Store the DNA at -20 °C

Extraction buffer (1000 ml)

20.0 g	CTAB	= 2 %
81.8 g	NaCl	= 1.4 M
20.0 ml	0.5 M EDTA	= 10 mM
15.8 g	Tris-HCl	= 100 mM

pH 8.0

II. Extraction of total DNA from grouped insects and group testing

1. Groups of up to 5 insects can be homogenized together in a 1.5 ml reaction tube. Larger groups are ground with the appropriate amount of buffer in a mortar.

No. of insects	1	3	5	10	15	20
Extraction buffer (µl)	400	600	800	1200	1500	2000

2. After homogenization transfer an aliquot of 400 µl to a new 1.5 ml tube and proceed as described above.

It is possible to detect a single infected individual of *H. obsoletus* in a batch of 20 grouped specimens.

Estimation of infection rates from group tests

Group testing is useful if large numbers of insects are tested and/or low rates of infection of vector populations are expected. Note that in the case of group-tests the proportion of positive tests does not represent the level of infestation of a population, since the test of a batch sample yields a positive result regardless of the proportion of positive individuals in the group. For example, it is possible to achieve a positive PCR result for groups of 20 *H. obsoletus* if only one specimen is infected. However, the population infection rate can be estimated e.g. with the method of Bhattacharyya et al (1979).

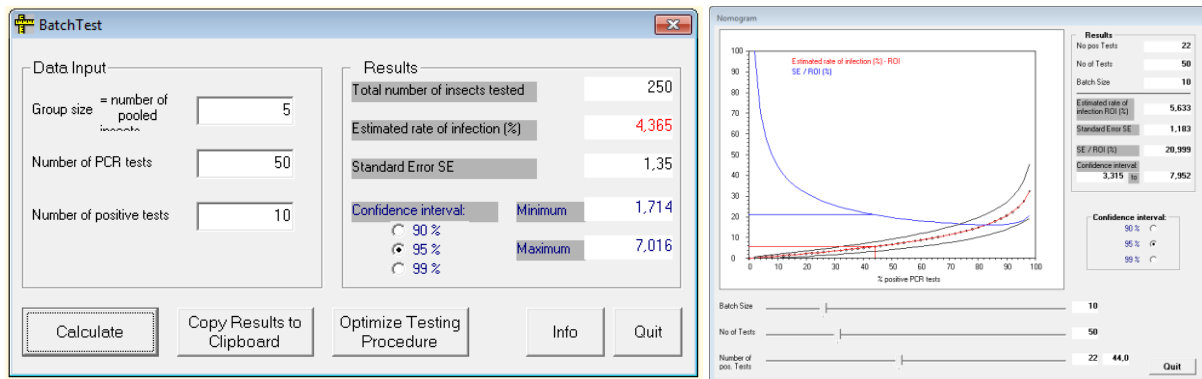
The number R of negative batches has the binomial distribution $b(n,p)$ with n as the total number of batches, each containing m insects, and p the probability that all m insects in a group are negative. Therefore, the field infection rate (FIR) of a tested population can be estimated by

$$\text{FIR} = 1 - (R / n)^{1/m}.$$

As an example, if 100 insects in 20 batches of 5 specimens are tested and five 5 tests are positive, the proportion of positive tests is 25 % but the estimated FIR is only 5.6 %:
 $m=5, n=20, R=(20-5)=15, \text{FIR} = 1 - (15 / 20)^{1/5} = 5.6 \%$. It is also possible to calculate the standard error and the confidence interval of FIR.

The tool “BatchTest” can be used to calculate FIR from the results of group tests. After input of group size, number of tests and number of positive tests it returns the estimated field infection rate, the standard error and the confidence interval.

FIR can only be calculated if $R>0$, that means not all tests are positive. Therefore, the lower the test number and the bigger the batch size, the lower is the maximum FIR to be estimated. For example, with 50 groups of 10 insects FIR can be estimated from 0 % to 32 %, while a batch size of 5 insects allows the estimation of FIR up to 54 %. Having a rough idea about the possible level of infection rate in a population is useful in order to choose an appropriate group size. A procedure included in BatchTest can help with this decision.



III. PCR detection of phytoplasmas in vectors

PCR mix for 20 reactions

50 µl	DNA Polymerase Reaction buffer	yellow microtube
	(10x incl. 15 mM MgCl ₂)	
50 µl	dNTP-Mix, 2 mM	blue microtube
50 µl	Primer 1: 5 µM	red microtube
50 µl	Primer 2: 5 µM	red microtube
250 µl	water	

Mix and then pipette the mixture into a tube that contains 2.5 µl (5 Units) DNA Polymerase

Mix thoroughly and prepare 20 reaction tubes with 23 µl each of reaction mix and 2 µl of extracted DNA

Centrifuge reaction tubes at 4000 rpm and put them in the thermocycler.

Routine detection of stolbur-phytoplasma (16SrXII-A)

Primers fStol/rStol (Maixner et al., 1995) are specific for 16SrXII-A. The specific amplification product has a size of approximately 570bp.

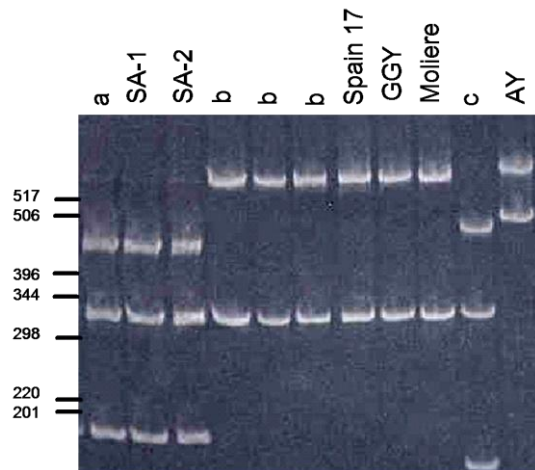
fStol: 5'-GCC ATC ATT AAG TTG GGG A-3';
rStol: 5'-AGA TGT GAC CTA TTT TGG TGG-3'

Amplification: 1 x 94 °C - 2'
 30 x 94 °C - 60"
 58 °C - 60"
 72 °C - 45"
 1 x 72 °C - 2'

Tuf-typing of stolbur phytoplasmas

A sequence of the *tuf* gene encoding the elongation factor Tu (Schneider et al., 1997) can be used to distinguish host specific strains of stolbur-phytoplasmas (Langer & Maixner, 2004). The *tuf*-type 'a' is associated with *Urtica dioica*, *tuf*-type 'b' is found in *Convolvulus arvensis* and *Calystegia sepium*, and *tuf*-type 'c' is only known from *C. sepium* so far.

The *tuf*-detection system is less sensitive than the fSTol/rStol system. It is therefore not used for routine detection of stolbur, and a nested-PCR protocol is used to amplify the *tufAY*-fragment. The primers used are from Schneider et al. (1997). In the first round the non-specific primers fTuf1/rTuf1 are used to amplify a fragment of approximately 1.100 bp. The primers fTufAy/rTufAy allow the specific amplification of a 940 bp fragment from phytoplasmas of the Aster-Yellows (16SrI) and stolbur (16SrXII) groups. Digestion of this fragment with the restriction enzyme *Hpa*II leads to the three different RFLP-profiles 'a', 'b', or 'c' from stolbur phytoplasmas associated with Bois noir (another 16SrXII-group phytoplasma [bindweed yellows] that is found occasionally in *C. arvensis*, too, yields a RFLP profile different from the three associated with BN).



Restriction profiles of the tufAY-fragment with HpaII. All strains except AY are stolbur. AY=aster yellows is also amplified with fTufAy/rTufAy, however the restriction profile is different from the stolbur strains.

First round PCR with primers fTuf1/rTuf1. The specific amplification product has a size of approximately 1.100 bp.

fTuf1: 5'-CAC ATT GAC CAC GGT AAA AC-3';

rTuf1: 5'-CCA CCT TCA CGA ATA GAG AAC-3'

Amplification: 1 x 94 °C - 2'
 35 x 94 °C - 30"
 45 °C - 30"
 72 °C - 60"
 1 x 72 °C - 2'

Dilute the product of the first round 1:100 (v:v) and use 2 µl as template for the second round PCR.

The second round PCR is done with primers fTufAy/rTufAy. The specific amplification product has a size of approximately 940 bp.

fTufAy: 5'-GCT AAA AGT AGA GCT TAT GA-3'

rTufAy: 5'-CGT TGT CAC CTG GCA TTA CC-3'

Amplification: 1 x 94 °C - 2'
 35 x 94 °C - 30"
 45 °C - 30"
 72 °C - 60"
 1 x 72 °C - 2'

Digestion of PCR-products:

Enzyme-mix for 25 reactions:

13 µl Restriction enzyme *Hpa*II (10 units/µl)
 50 µl Enzyme buffer
 50 µl water

Pipette 4.5 µl of the enzyme mix to 15 µl of fTufAY/rTufAY PCR-product. Centrifuge shortly. Incubate at 37 °C for at least 2 hours.

Use 'small fragment size' agarose for electrophoresis

Routine detection of alder yellows/Palatinat grapevine yellows phytoplasma in *Oncopsis alni* (16SrV-group)

Alder yellows is widespread in *Alnus glutinosa*. Around 80 % of the trees in the Mosel and Palatinat region are infected. The phytoplasma is transmitted to and from *Alnus glutinosa* by the Macropsid leafhopper *Oncopsis alni*. This species occasionally inoculates grapevine, too. The infected vines show a severe yellows disease called Palatinat grapevine yellows.

The primer pair fAY/rEY (Ahrens et al., 1994) allows the amplification of a 16S rRNA-gene fragment of approximately 300 bp specifically from EY-group phytoplasmas. To achieve specificity for elm yellows group phytoplasmas the protocol has to start with high annealing temperature

fAY: 5'-GCA CGT AAT GGT GGG CACTT-3'

rEY: 5'-CGA AGT TAA GCC ACT GCT TTC-3'

Amplification:

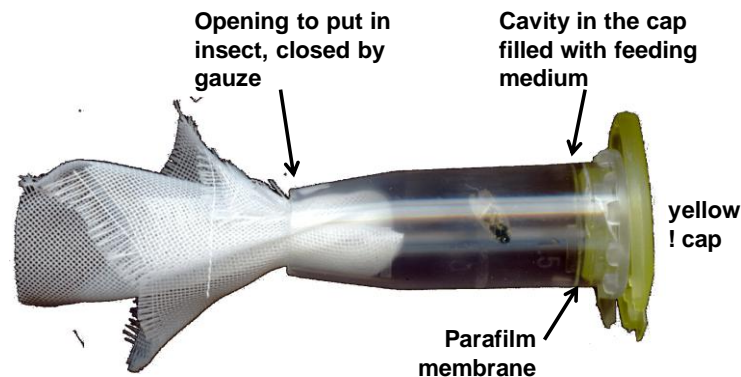
1 x	94 °C - 2'	2 x	94 °C - 60"
2 x	94 °C - 60"		63 °C - 60"
	65 °C - 60"		72 °C - 30 "
	72 °C - 30 "	25 x	94 °C - 60"
2 x	94 °C - 60"		58 °C - 60"
	64 °C - 60"		72 °C - 45"
	72 °C - 30 "	1 x	72 °C - 2'

IV. Experimental transmission of phytoplasmas

Since PCR is a highly sensitive detection method phytoplasmas ingested with plant sap by sucking insects can be detected even in non-vector species. Transmission tests are therefore indispensable to prove the vectoring ability of a suspected vector. While searching for a vector keep mind, that the most numerous species are not necessarily the most likely to transmit a disease. Transmission to an artificial feeding medium is a quick method to proof the principal ability of a species to transmit a particular phytoplasma. However, because transmission to a particular plant host is influenced by the feeding preferences of the insects, the unquestionable identification of a vector requires its inoculation of the plant species in focus, too.

a. Transmission to artificial feeding medium

The procedure according to Tanne et al. (2001) is used with few modifications. The cavity of a microcentrifuge cap is filled to the top with feeding medium and sealed with a parafilm membrane. The cap is then fit to a tube containing an individual insect. Tubes are placed horizontally on a table. Since light and yellow color are attractive for many Auchenorrhyncha a yellow cap is used which is oriented to a light source like window or a lamp. Most species sooner or later start to penetrate the membrane and begin feeding through it, thus injecting saliva to the medium. After the end of the defined transmission period the insects are removed and tested for phytoplasma infection subsequently. The medium is removed from the cap with a pipette and either frozen or immediately subjected to DNA extraction. A nested PCR procedure is appropriate for testing the medium for phytoplasmas. See Ge & Maixner (2003) for details.



Test preparation

- Add approx. 290 μ l of artificial medium to each yellow 1.5 ml Eppendorf tube cap with a Pasteur pipette
- Seal the cap using a piece of parafilm (stretch it as thin as possible).
- Remove the bottom end of a white 1.5ml Eppendorf tube and fit it to the cap containing artificial media.
- Put the insect inside the tube and block the tube end with a piece of gauze
- Place the tubes horizontally with the cap side facing the light. Keep them in room temperature

Feeding medium - 5% TE-Sucrose

Add 5 g sucrose to 100 ml routine TE buffer, autoclave (120 °C, 15 min) and store in aliquots at 4 °C.

Phytoplasma DNA extraction from feeding medium

- Detach the cap from the incubating tube and remove the sealing membrane. Fit another 1.5ml Eppendorf tube on it. (When necessary, Add relevant volume of TE buffer inside the cap to compensate the solution lost during feeding)
- Centrifuge for a few seconds to collect the artificial medium
- Centrifuge at 22.500g for 10 min, discard supernatant
- Add 10 µl of 0.5M NaOH and 20 µl TE-SDS
- Spin solution down to the bottom
- Incubate at 65 °C for 15 min
- Add 70 µl pre-chilled isopropanol and store the tube in –20 °C for at least 30 min
- Centrifuge at 22.500 g for 6 min at 4 °C, discard the supernatant.
- Wash pellet with 100 µl of 70% ethanol
- Centrifuge at 22.500 g for 3 min at 4 °C, discard the supernatant
- Vacuum dry the pellet for 5 min
- Resuspend the pellet with 20 µl TE buffer or water.

1% TE-SDS

Dissolve 15.76g Tris-HCl in 80ml H₂O, add 4ml 500mM EDTA Stock solution, 1g SDS, Adjust PH to 8.0 by add 12M NaOH, add H₂O to the end volume of 100ml.

Phytoplasma detection in medium by nested-PCR

A nested PCR is required to detect phytoplasmas from the feeding medium. If the group specific primers fStol/rStol or fAy/rEY or other sequences from the 16S gene are used for detection, the primers P1/P7 or other primers that allow to amplify the complete 16S-gene and the adjacent spacer region are suitable for the first round PCR (Schneider et al., 1995).

fP1: 5'-AAG AGT TTG ATC CTG GCT CAG GAT T-3'

rP7: 5'-CGT CCT TCA TCG GCT CTT-3'

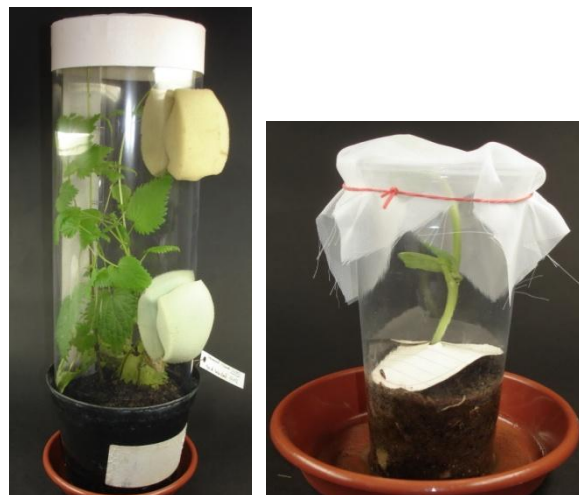
Amplification: : 1 x 94 °C - 2'
 30 x 94 °C - 60"
 52 °C - 60"
 72 °C - 60"
 1 x 72 °C - 2'

Dilute the product of the first round 1:100 (v:v) and use 2 µl as template for the second round PCR.

b. Experimental inoculation of host plants

Plants to be used for experimental transmission need to be definitely healthy. They should be grown from seed or, in the case of grapevine cuttings, need to be subjected to a hot water treatment (50 °C during 45 min; only suitable for dormant material) before rooting. For quick results, herbaceous plants like *Vicia faba* are suitable for many Auchenorrhyncha species and show symptoms if infected within four to six weeks. Inoculated grapevine cuttings usually require a period of dormancy and new growth to exhibit disease symptoms.

It is advisable to use cages with individual plants for the tests, either disposable ones in the case of small plants and short inoculation periods or larger cylinders of plastic or glass. Holes closed by removable foam plugs are useful for the manipulation of the insects in the cages. The top should be covered by gauze to allow ventilation. It is also advisable to cover the soil by foam layer that facilitates to find and remove dead insects. These should be removed at least every second day if they are intended for DNA extraction later on. Cages are kept at 20 °C and 16 h of light. An experimental inoculation period of 7 days is usually sufficient. If non-host plants of the vectors are used mortality is often high and the test period is usually shorter (e.g. with *H. obsoletus* and grapevine).



Usually, groups of insects are put together on individual test plants. Similar to group tests in PCR, it is possible to estimate individual transmission rates from the results of group transmission tests. The transmission rate represents the probability that a single insect of the population used will inoculate a plant. The method of Burrows (1987) is commonly used to estimate it:

n = total number of inoculated plant
t = number of infected plants
s = number of insects grouped on one plant
 p_{tr} = individual transmission rate

$$p_{tr} = 1 - \left[1 - \frac{t}{n + (s-1)/2s} \right]^{\frac{1}{s}}$$

If the proportion of infected vectors p_{inf} is known, the inoculation efficiency p_{ie} , that is the probability that a single infected vector inoculates a plant, can be calculated as $p_{ie} = p_{tr} / p_{inf}$.

If the transmission rate is already known, the optimum group size for a given number of test plants can be obtained from a table in the paper of Burrows (1987).

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Appendix

Participants

Acs	Zoltan	Hungary	acs.zoltan@fitolab.hu
Chireceanu	Constantina	Romania	cchireceanu@yahoo.com
Delic	Duska	Bosnia & Herzegovina	duskadelic@yahoo.com
Jrejiri	Fouad	Lebanon	fjrejiri@hotmail.com
Lolic	Biljana	Bosnia & Herzegovina	b_lolic@yahoo.com
Özgen	Inanc	Turkey	inancoz@hotmail.com
Sabate	Jordi	Spain	jordi.sabate@irta.cat
Strauss	Gudrun	Austria	gudi_strauss@yahoo.de
Torrini	Giulia	Italy	giulia.torrini@isza.it
Vellios	Evangelos	Greece	evellios@agr.uth.gr
Zwolinska	Agnieszka	Poland	a.zwolinska@iorpib.poznan.pl
Hoch	Hannelore	Germany	hannelore.hoch@mfn-berlin.de
Imo	Miriam	Germany	imo@uni-mainz.de
Jarausch	Barbara	Germany	barbara.jarausch@agrosience.rlp.de
Johannesen	Jes	Germany	jesjo@uni-mainz.de
Kappel	Yvonne	Germany	yvonne.kappel@jki.bund.de
Kröhner	Dunja	Germany	dunja.kroehner@jki.bund.de
Maixner	Michael	Germany	michael.maixner@jki.bund.de
Neuerburg	Christel	Germany	christel.neuerburg@jki.bund.de
Weintraub	Phyllis	Israel	phyllisw@volcani.agri.gov.il