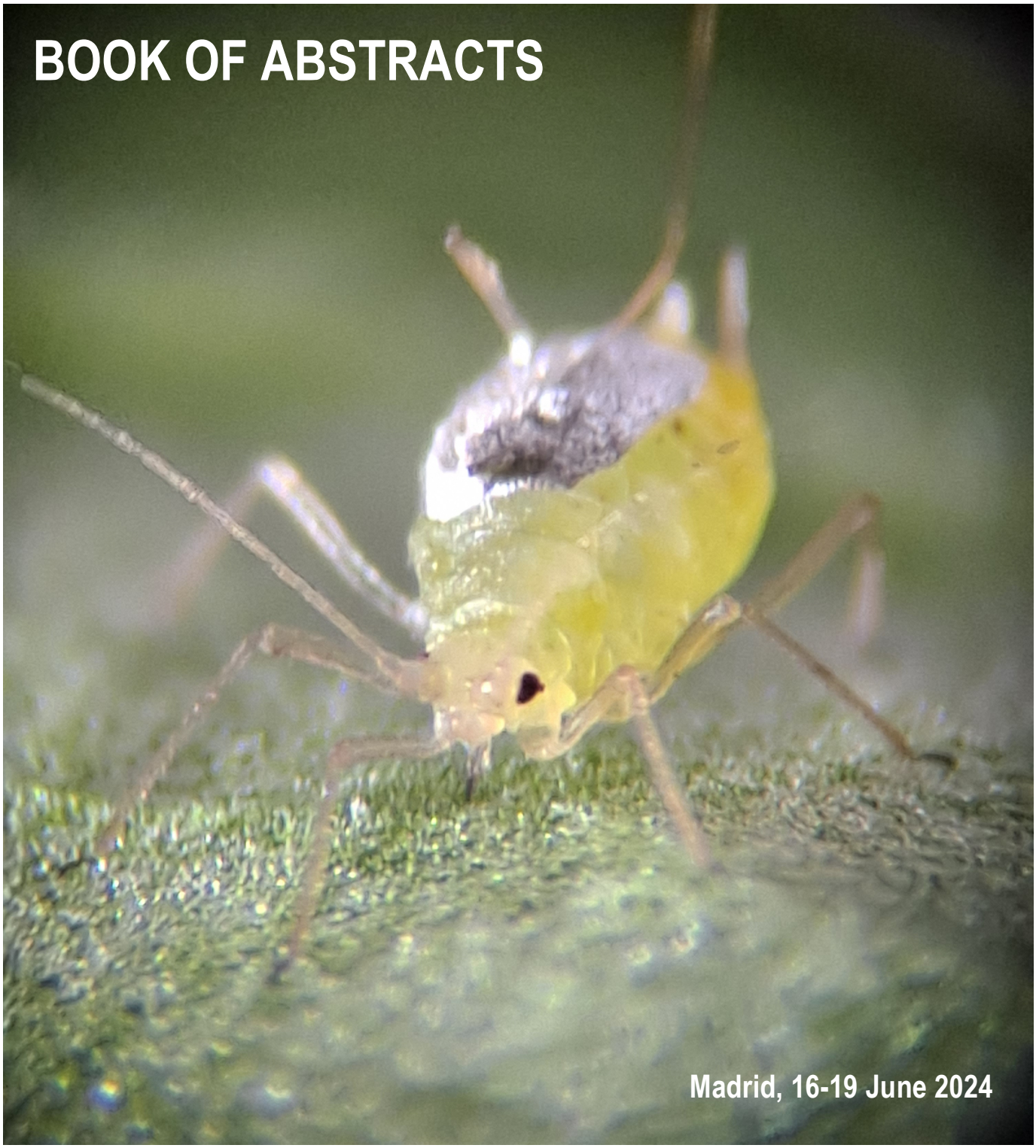
1st European EPG Workshop

BOOK OF ABSTRACTS



Madrid, 16-19 June 2024

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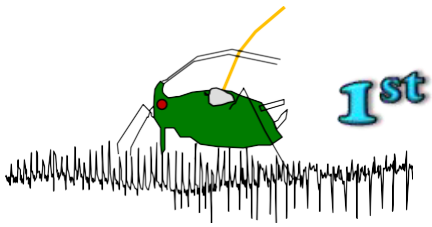

1st European EPG Workshop

Madrid, 16-19 June 2024

Program

Venue: ICA-CSIC (16-19 June 2024). Madrid, Spain

Day 0: 16 June
18:30h-20:00h Welcome Reception & Registration (at ICA-CSIC, Calle de Serrano, 115 b, Chamartín, 28006 Madrid)

Day 1: 17 June
8:45-9:00 Registration (ICA-CSIC)

9:00-10:00 Keynote presentation: Introduction to EPG monitoring of insect behavior. History, background & electronics

Keynote Speaker: Prof. W.F. Tjallingii

10:00-10:30: EPG standard waveforms for different insects (waveform library): Phloem feeders
Speaker: Prof. Alberto Fereres

10:30-11:00: Coffee break & Poster Session

11:00-11:30: EPG standard waveforms for different insects (waveform library): Xylem feeders Speaker: Dr. Clara Lago

11:30- 12:00: Non-standard EPG waveforms of aphids and waveform features due to device adjustments
Speaker: Prof. W.F. Tjallingii

12:00-12:30. Participant talks

12:00-12:15 Insights from EPG with whiteflies: Position-fixation techniques and tethering
Speaker: Lize Braat
12:15-12:30 EPG as a tool to understand plant/arthropod interaction for crop protection
Speaker: Juan Manuel Alba Cano

12:30-14:00 Lunch & Poster Session

14:00-14:30 Wiring, setting up and calibration of EPG equipment and computerized data acquisition in EPG recording
Speaker: Dr. Elisa Garzo

14:30-16:00 Afternoon Hands-on

16:00-16:30 Coffee break & Poster

16:30- 18:00 Afternoon Hands-on (cont.)

Day 2: 18 June

09:00 -9:30 EPG analysis & data processing for phloem feeders
Speaker: Dr. Elisa Garzo

09:30-10:00 EPG analysis, data processing and workbooks for xylem feeders
Speaker: Dr. Daniele Cornara

10:00-10:30 Keynote talk. An interactive Platform for efficient automatic analysis of EPG signal of piercing sucking insects combining changepoint detection and Machine learning
Keynote Speaker: Prof. Bernard Reynaud

10:30-11:00: Coffee break & Poster Session

11:00-11:30 Guidelines for experimental design, data analysis and interpretation
Speaker: Dr. Aranzazu Moreno

11:30-12:00: Behavioural response to climate change
Speaker: Dr. Piotr Trebicki

12:00-12:45 Participants talks

12:00-12:15 Speed and directionality of electrical signal propagation in plants in response to physical stress
Speaker: Claudio C. Ramírez
12:15-12:30 Identification of plant virus proteins responsible for the manipulation of host phenotype and vector behavior
Speaker: Quentin Chesnais
12:30- 12:45 Identification of plant cues involved in *Rhopalosiphum padi*'s host alternation
Speaker: Rituparna Ghosh

12:45-14:00 Lunch & Poster Session

14:00-16:00 Afternoon Hands-on

16:00-16:30 Coffee break & Poster

16:30- 18:00 Afternoon Hands-on (cont.)

Day 3: 19 June

9:00-9:45 Applications of EPG technique: Transmission of plant pathogens by insect vectors
Speaker: Prof. Alberto Fereres

9:45-10:30: Keynote talk. Applications of EPG technique: Host plant resistance and understanding mode of action of chemical compounds using EPG
Keynote Speaker: Dr. Beata Gabrys

10:30-11:00: Coffee break & Poster Session

11:00-11:30: EPG in combination with additional techniques: cryofixation, CT scan and confocal, stylectomy, video-tracking recording, artificial diets, honeydew clock
Speaker: Dr. Jaime Jiménez

11:30-12:00: Participants talks

11:30-11:45 Cellulose synthase gene expression analysis of *Solanum lycopersicum* exposed to combined effects of drought and TYLCV virus
Speaker: Samra Mirzayeva
11:45-12:00 Aphids as bioelectrodes to measure changes in plant membrane potential in parallel with physiological changes as a result of pathogen infection
Speaker: Torsten Will

12:00-12:30: Questions and Discussion

12:30-14:00 Lunch & posters

14:00-16:00 Afternoon 'Hands-on'. EPG experiments coupled with video-tracking recording, cryofixation, artificial diets, honeydew clock

16:00-16:30 Coffee break & Poster Session

16:30- 18:00 Afternoon Hands-on (cont.)

20:00h: Closing dinner (Restaurante Tragantia, Calle del Príncipe de Vergara, 210, Chamartín, 28002 Madrid)

NOTE: Participants should bring their own laptop to the workshop and download and install the EPG installation software in advance (at: https://epg.csic.es/downloads)

Posters

Alteration of aphid feeding behavior in sugar beet genotypes results in decreased beet yellows virus transmission
Anabella Heintz, Quentin Chesnais, Camille Gutierez, Alessandra Maia-Grondard, Michael Stange, Sandra Otte, Martin Drucker, Véronique Brault

Does SBR - a new disease of sugar beet - affects the feeding behavior of *Pentastiridius leporinus*?
Brita Kais, J. Koehler, B. Czarnobai De Jorge, Anna Markheiser, J. Gross

Measuring principles in EPGs: Circuit properties and Ohm's law relationships
Freddy Tjallingii

Cold treatment of infected plum fruits reduces the transmission of the PPV by aphids
Claudio C. Ramírez, Angélica González-González, Jessica Devia-Parra, Isabel Ramírez-Abarca, Mónica Madariaga

Exploring diurnal rhythms in aphid feeding behavior through the lens of the Electrical Penetration Graph technique
Daniel Kunk

How do endosymbionts modulate plant virus transmission by aphid vectors?
Patricia Sanches, Consuelo De Moraes, Mark Mescher

1st European EPG Workshop

AFTERNOON Hands-On PROGRAM

Day 1: Beginners Program

EPG recording (data acquisition)

DEMO EPG set up:
- Equipment parts
- Noise and Faraday cage
- Amplifier test*

Aphid, whitefly, spittlebug & stink bug wiring
- Making an insect electrode
- Proper electrode attachment
- Insect and plant mounting*

Making a first EPG recording
- Stylet+-d acquisition software*
- Start: name, comments & adjustments
- Starting*: 1) Giga ON
  2) 'insects ON'
- Optimal voltage adjustment*

Day 2: Stylet+ software practice

File conversion
- Automatic and comment lines
- Manual conversion (separate conversion)

Analysis software (stylet+a)
- Use of day 1 data files or sample files
- Moving, zooming and .ppt signal pictures
- Exporting signal samples
- Making a 'Book of reference signals'

Retrieving EPG data
- Cursor use and data grid
- Saving the data grid
- Frequency & smooth tools

- DATA PROCESSING SOFTWARE:
- Excel Workbooks for calculation of EPG variables (3 programs)
- Automatic EPG waveform recognition (2 Programs)

Day 3: Demonstration: *other techniques combined with EPG*

- Honeydew clock for collecting droplets of plant sap
- Cryofixation of aphid stylets
- Stylectomy of aphid stylets
- Video-tracking recording of spittlebug and aphid feeding
- EPG recording in artificial diets

Bring in any questions or problems

* Program items that can be extended for advanced users:

- Special plant voltage adjustments
- Life establishing of R- and emf-components
- Event marks in unused channels

Introduction to EPG monitoring of insect behavior. History, background & electronics

Freddy Tjallingii[1]

[1] EPG Systems, Dillenburg, Wageningen, The Netherlands.

The basic idea for making a plant and a piercing sucking insect part of an electrical circuit was introduced by McLean & Kinsey (1964) and is now called the 'AC-system'. Later this was changed to a DC-system (Tjallingii, 1978), which expanded the recorded biological activities during insect-plant interactions. EPG signals reflect mechanical stylet movements, ingestion from and saliva excretions into cells and specific plant tissues. Such activities, generally triggered by plant chemicals or physical properties, often appeared to play a crucial role in the acquisition and inoculation of plant pathogens. All activities occurring during plant penetration by the insect that changes the electrical properties in primary circuit contribute to the EPG signal. The two main electrical properties that have an impact on the signal are: 1) voltages generated during insect activities, called electromotive force (emf) components, and 2) voltage changes due to electrical resistance fluctuations that modulate the circuit voltages, called R-components. A minor role is played by capacities in the primary circuit. Relations between EPG signal waveforms and their biological causes will be discussed.

EPG standard waveforms for different insects (waveform library): Phloem feeders

Alberto Fereres[1]

[1] Instituto de Ciencias Agrarias – Consejo Superior de Investigaciones Científicas (ICA-CSIC). Calle de Serrano 115 dpdo, 28006 Madrid, Spain.

Most of the EPG waveforms for phloem feeders were first characterized for aphids and then extended to other sternorrynchas such as whiteflies. EPG waveforms are characterized by their amplitude, frequency and voltage level (positive or negative).

Basically, the most common DC-EPG waveforms that have been described for aphids are: A: high amplitude and frequency waveform occurring after the first stylet contact with the epidermis; B, low frequency (0.2-0.3 Hz) superimposed a base waveform of 4-5 Hz correlated with the excretion of gelling saliva droplets used by aphids to form the salivary sheath; C, a complex waveform representing intercellular stylet pathway before reaching the vascular bundle; PD or potential drop, embedded during the stylet pathway phase (waveform C) correlated with a brief (5-10s) intracellular stylet puncture of a living cell; E1, occurs at the intracellular level, negative voltage with positive pulses of 2-3 Hz correlated with salivation activities in the phloem sieve elements: E2, intracellular waveform with negative pulses (0.5-4 Hz) associated to sap ingestion from phloem sieve elements; F, a waveform of high frequency (11-19 Hz) at the extracellular level representing penetration difficulties or derailed stylet mechanics: G, an extracellular waveform of 4-9 Hz associated with active xylem ingestion from xylem vessels (dead cells).

Other phloem feeders such as psyllids may exhibit other characteristic waveforms such as waveform D, which represents the first phloem contact just before E1 waveform that has a very specific morphology (square crested waves at 4-8 Hz and a second flat phase with positive spikes at regular intervals with repetition rate of about 2 Hz and a somewhat decreasing voltage).

EPG standard waveforms for different insects (waveform library): Xylem feeders

Clara Lago[1]

[1] Institute of Agricultural Sciences – Spanish Research Council (ICA-CSIC). Calle de Serrano 115 dpdo, 28006 Madrid, Spain.

Xylem-feeders are hemipteran insects that exclusively belong to the suborder Cicadomorpha, Superfamilies Cercopoidea (spittlebugs and froghoppers) and Cicadoidea (cicadas), and to the family Cicadellidae, subfamily Cicadellinae (sharpshooters). Most species of these xylem fluid-feeders pose significant threats to agriculture, as they transmit the well-known bacterial plant pathogenic bacterium, *Xylella fastidiosa* (Wells).

These insects have evolved to feed on xylem-sap, which is one of the hardest-to-extract components due to high tension and poor in nutrients. Central to their feeding behaviour is the cibarium, an efficient muscular pump located between the stylets and the esophagus. The cibarium marks the transition point to the foregut or alimentary canal. The food canal in the stylets are connected to the cibarium through a narrow channel called the the precibarium, lined with chemosensory papillae separated in two by the precibarial valve. Besides, these insects tend to be polyphagous, broadening their nutritional options. The insertion of the stylets into the host plant tissues and uptake of xylem-sap are critical steps in host-plant discrimination. *Xylella fastidiosa* relies on the completion of these steps by its insect vectors to escape the host plant and infect healthy plants, thus surviving and evolving. Feeding behavior thus plays a crucial role in the tripartite interaction among vector, host, and bacterium. Recent studies employing DC Electrical Penetration Graphs (EPGs) have standardized waveform patterns for xylem feeders, identifying and describing different waveform patterns: (i) np, non-probing; (ii) C, pathway; (iii) Xc, xylem contact; (iv) Xi, xylem ingestion (frequency > 0.1 Hz); (v) LF, low-frequency xylem ingestion (frequency < 0.1 Hz); (vi) N, non-pathway interruption of xylem activity; (vii) R, resting; (viii) Xe, behaviour putatively associated with *X. fastidiosa* inoculation; (ix) W, stylets withdrawal and (x) Esc, escaping of the individual from the host plant. In this presentation, we will describe and compare the feeding behavior of xylem-sap feeding insects.

Non-standard EPG waveforms of aphids and waveform features due to device adjustments

Freddy Tjallingii[1]

[1] EPG Systems, Dillenburg, Wageningen, The Netherlands.

Anomalous waveforms during the classified waveforms – mainly pathway - will briefly be reviewed. Moreover, the waveform features as modified by the circuit voltage level V will be focused. Several of these waveforms occur during (stylet) pathway phase: A, B, C, as part of waveform C; phloem-pd, pseudo-pd, and pd-B; and E1e, although most underlying insect activities are still unknown.

A major unpredictable voltage is caused by the two electrode potentials. These are originated by the two metal-electrolyte interfaces Pe1 and Pe2: the soil-plant electrode and the insect-insect electrode interface that represent a galvanic cell property in the primary measuring circuit. The voltage level V is the result of the Pe1&2 and can be controlled by using adjustments of the voltage supply (Vs or 'Plant voltage' buttons) in the DC-EPG device. The (sum of the) electrode potentials will be shown in EPG recordings from a special experiment.

The voltage level V and sign changes the features of most waveforms due to the fact that its level is modulated by the insect-plant resistances changes. In general the emf- and R-components in every waveform are intertwined. Waveform E2 will be used as an example to show these effects.

Insights from EPG with whiteflies: position-fixation techniques and tethering

Lize Braat[1]

[1] Plant Breeding, Wageningen University & Research, P.O. Box 384, 6700 AJ, Wageningen, The Netherlands.

The sweet potato whitefly, *Bemisia tabaci*, is a globally destructive pest found in both greenhouses and field settings. Due to increasing concerns about insecticide impacts on the environment and human health, alongside rising insect resistance, there's a growing demand for environmentally friendly alternatives like resistance breeding. Despite this need, only a few studies have examined adult whitefly feeding behavior in detail, using electronical penetration graph (EPG) recording techniques. At Wageningen University's Department of Plant Breeding, whitefly resistance in plants is assessed by evaluating adult survival, oviposition, and population building. The incorporation of EPG techniques would offer a valuable means to directly observe whitefly feeding behavior, thereby elucidating factors in resistant plants linked to decreased survival rates. However, the system that was in place, adapted to aphids, did not allow for effectively studying adult whiteflies. Therefore, a vacuum system, adapted from aphid tethering techniques, was created to facilitate the whitefly tethering process for EPG recordings. Then, trials were performed with various diameter sizes of platinum and gold wires of varying diameter (25 µm, 12 µm, and smaller with the help of solvents) to tether adult whiteflies. The acquired EPG data was utilized to assess the success of tethering whiteflies. In future trials, the efficacy of employing 2.5 µm diameter Wollaston process platinum wires with the vacuum system will be explored, aiming to advance whitefly research and integrate the EPG technique to elucidate whitefly resistance mechanisms in plants.

EPG as a tool to understand plant/arthropod interaction for crop protection

Juan Manuel Alba[1], Enrique Moriones[1], Marta Montserrat[1]

[1] IHSM La Mayora-UMA-CSIC: Instituto de Hortofruticultura Subtropical y Mediterránea "La Mayora", Universidad de Málaga, Consejo Superior de Investigaciones Científicas, Málaga, Spain.

Plants are constantly threatened by a plethora of biotic stresses that can severely affect their survival and reproduction, such as herbivores, microorganisms and viruses causing diseases (parasites, hereafter). As a response, plants are forced to develop mechanisms to resist the attack from plant parasites, and these mechanisms of resistance become a selection pressure for plant parasites to develop new strategies to cope with plant defences. This arm-race drives the intimate interaction between plants and consumers and usually its effects are transmitted up to the food web. One of the main research cores at the Institute for Subtropical and Mediterranean Horticulture is dedicated to apply fundamental knowledge for crop protection, i.e. understanding pest formation to develop efficient techniques and sustainable strategies for crop protection, as an alternative to the use of pesticides. Understanding the feeding behavior of herbivorous arthropods is essential to comprehend several of the plant resistant mechanisms that are approached in our institute. The aim of this presentation is to discuss the potential use of Electro Penetration Graphs as a tool to develop better strategies for crop protection.

Acknowledgement: This presentation is supported by the project QUAL21 012IHSM.

Wiring, setting up and calibration of EPG equipment and computerized data acquisition in EPG recording

Elisa Garzo[1]

[1] Instituto de Ciencias Agrarias – Consejo Superior de Investigaciones Científicas (ICA-CSIC). Calle de Serrano 115 dpdo, 28006 Madrid, Spain.

The Electrical Penetration Graph Technique (EPG) is the most powerful tool for studying the feeding behavior of pierce-sucking insects such as hemipterans, as well as thrips. Here, we will provide the basics on how to set-up and calibrate DC-EPG equipments. Giga-8dd device and its predecessors (Giga-8d, Giga-8 and Giga4) have been developed by EPG Systems (Wageningen, The Netherlands) to record EPG signals generated when the insect inserts its stylets into the plant tissue. The EPG device consists of a main control box and 4 or 8 separate EPG probe units for each channel. The insect is attached with a gold wire (12 or 18.5 µm diameter), which is glued to a thicker copper wire (2 cm length) using water-based silver paint. Subsequently, the insect electrode is carefully inserted into the input connector of each channel's EPG probe, while the plant electrode is inserted into the soil of the plant container. The circuit is closed when the insect inserts its mouthparts into the plant. To minimize external interference, the measuring EPG probe is mounted inside a Faraday cage. The output signals to the computer are digitized by an internal AD converter before being transmitted to the computer. To optimize signal quality, each EPG recording and insect (channel) should be individually adjusted. Finally, EPG signals are acquired and analyzed the specific software Stylet + on the Windows platform. Instructions on how to calibrate and acquire EPG signals will be explained.

EPG analysis & data processing for phloem feeders

Elisa Garzo[1]

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The Electrical Penetration Graph Technique (EPG) is a useful tool but very time consuming mainly because the large amount of data generated. Proper identification of EPG waveforms as well as the calculation of EPG variables is often tricky and time-consuming. Consequently, several software programs have been developed for automatic calculation of EPG variables. Here we will present a new user-friendly Excel Workbook that uses a standardized list of EPG variables and follows expert guidelines for calculating them. The program developed in Visual Basic for Applications (VBA) is a step up from the existing software and allows easy data analysis and interpretation. It also includes a novel option for dealing with the common problem of 'truncated' - waveforms artificially terminated by the end of recording. The only requirement to run the program is Microsoft® Excel software running under a PC environment. The EPG Workbook provides researchers with a reliable and standardized tool for automatic calculation of up to 127 EPG variables for phloem-sap-sucking insects.

EPG analysis, data processing and workbooks for xylem feeders

Daniele Cornara[1]

[1] University of Bari Aldo Moro, DiSSPA, Via Amendola 165/A, 70126 Bari (Italy).

Xylem-feeding is apparently the only requirement making an insect a competent vector of the bacterium *Xylella fastidiosa*, nowadays considered one of the most feared threats to agriculture and landscape in Europe. Electrical Penetration Graph (EPG)-assisted transmission experiments are the workhorse to elucidate the bacterium transmission dynamics and how biotic and abiotic factors influence vector-plant interaction and pathogen epidemiology.
Excluding the pioneering work of Crane in the '70s, EPG studies on xylem-sap feeders are relatively recent compared to other insects as aphids, and the biological meaning of most of the identified waveforms remains elusive. Additionally, scientists willing to use the EPG to explore the xylem feeders-plant interaction are faced with several challenges spanning from the way insects should be handled for EPG-experiments, to how EPG recs should be marked and statistically analyzed, to how the statistical results should be "biologically" interpreted. For example, EPG-connected xylem feeders frequently generate aberrant patterns that make marking and interpretation of the recordings far from being trivial. Furthermore, the use of relatively thin gold wires to avoid excessive constrains during the recording is counterbalanced by the wire being too fragile in case the insects attempt to escape from the plant, resulting in early termination of the recs and consequent problems in data analysis.
Here I describe the current standard practices on how to handle xylem feeders EPG-recordings, starting from basic rules and hints on how to plan experiments and carry out the recs, how to mark the files using a workbook devised purposedly for xylem feeders, and possible ways to statistically analyze the data.

An interactive Platform for efficient automatic analysis of EPG signal of piercing sucking insects combining changepoint detection and Machine learning

Reynaud B[1]., Turpin P., Morin R., Molinaro F., Nibouche S.

[1] Cirad, UMR PVBMT, Saint-Pierre, La Réunion, France,

EPG systems (AC or DC) are used to study the behaviour of piercing - sucking insects. Voltage changes are amplified and acquired over several hours and on several channels simultaneously. The digital files are then analysed manually using dedicated software (Stylet®, Windaq ®), which is time-consuming. We first proposed a software for acquisition and automated classification of EPG recording, but the classification rate needed to be improved (EPG-Soft 2002, Reynaud *et al.* 2004). Since then, A2EPG software was developed by Adasme-Carreno and al. (2015), which automatically recognises most aphid waveforms (except E2 and F) but still requires manual expertise. To enhance EPG-Soft, we have experimented various segmentation techniques for the EPG signal, as well as diverse deep learning methods for its post-segmentation classification. The combination of this two techniques (segmentation and classification) with a careful selection of specific physical variables has significantly improved the accuracy of automatic signal classification, thus providing optimized performance in the classification process. The construction and the validation of the different methods were carried out by training the two different machine learning models (SVM, RDMF) on three sets of signal datasets from three insects with distinct feeding behaviour: an aphid (*Melanaphis sacchari* on sugarcane), a delphacid (*Peregrinus maidis* on maize) and a leafhopper (*Cicadulina mbila* on maize .To evaluate the performance of each insect model, various statistics such as the confusion matrix and the kappa coefficient were used. By using robust classification on each dataset, we obtained correct classification ranging from 83% to 100% for the seven main waveforms (Np, B, G, F, pd, E1, E2). Kappa (K) statistic was calculated on the comparison of manual versus automatic classification of 8 hours EPG recordings (n=20). Kappa which measures reliability varies between 1 (total agreement) to 0 (total disagreement). The mean Kappa for EPG recordings: *M. sachari* (n=8) *P. maidis* (n=7) and *C. mbila* (n=5) were 0.75, 0.51 and 0.66 respectively. We performed the automatic analyses again on EPG recordings of *M. sacchari* on susceptible and resistant sugarcane varieties made previously manually analysed (Fartek et al.,2012). The automatic analysis obtained the same significant ranking of the varieties for the total durations of waveform phases. The methods used for automatic classification appear to be fairly robust and generic for piercing-sucking insects, provided that the library is properly appraised, standardised and enriched. We will publish this new « EPG Soft » version as a "toolkit" for visualization, expertise and processing EPG signals with a user-friendly interface, grouped in a R Package open source.

Guidelines for experimental design, data analysis and interpretation

Aranzazu Moreno[1]

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When conducting experiments using the EPG technique, there are numerous considerations to take into account to minimize potential effects on the calculation of EPG variables and their interpretation. Therefore, various aspects need to be considered both before conducting the recordings and during them, as well as once they have been completed, during their analysis. The first step to consider is the experimental design, which should align with the objective pursued in our study: the experimental unit, standardizing insects and plants, the number of repetitions, and other factors. Then, pre-recording and recording aspects must be considered: insect handling and wiring, recording duration, and the timing of recording initiation could significantly influence the results of our experiment. Finally, the method used for calculating the EPG variables and their subsequent analysis and interpretation are crucial for drawing accurate conclusions. The numerous variables to be considered in an EPG experiment to derive biological conclusions will be discussed.

Behavioural response to climate change

Piotr Trębicki[1]

[1]Applied BioSicences, Macquarie University, Sydney, Australia.

The changing climate is exerting a significant impact on ecosystems worldwide. Moving forward, these changes are predicted to have profound effects on food production and food security, not only through direct impacts but also through changes in outbreaks of pests and diseases. Insect vectors cause damage through feeding and colonization of the plant but also through transmission of plant pathogens. Climate change and subsequent changes in plant physiology will affect vectors' distribution, frequency, and behaviour. Hence, understanding how plants can cope with pests and diseases and how ferocious pests will be is critical for maintaining or increasing current food production. Among others, water availability, temperature, and CO_2 concentrations are considered as important factors that will affect interactions between plants, pests, and pathogens. The electrical penetration graph is a powerful tool that enables us to explore those intriguing interactions. By creating a future environment and exposing plants, insects, and pathogens, through electrical penetration graph studies, we can describe the interactions and explore opportunities that will enable us to stay a step ahead. Here, I will explore how climate change will affect behavioural responses, how the electrical penetration graph can be implemented, and the opportunities and challenges that lie ahead.

Speed and directionality of electrical signal propagation in plants in response to physical stress

Francisco Ibacache-Carrión[1], Erwan Michard[1] and Claudio C. Ramírez[1]

[1] Instituto de Ciencias Biológicas, Universidad de Talca, 1 Poniente 1141, Talca, Chile.

Electric signals in plants are of great interest to plant biologists because they provide a means for plants to transmit information from damaged areas to healthy ones. It is understood that these signals can propagate through the phloem, but there is limited knowledge regarding the characteristics of this transmission. One unresolved question is whether the velocity of signal propagation (VPS) through the phloem differs between leaves. Specifically, it is not clear whether there are variations in the propagation speed of electrical signals between leaves in the acropetal (from base to tip) and basipetal (from tip to base) directions within the plant. To address this question, experiments were conducted using Vicia faba L. plants, the pea aphid and the electrical penetration graph (EPG) technique, which involves using phloem-feeding insects as bio-electrodes to observe electrical signals. Three experiments were conducted to evaluate the directionality of VPS. In the first experiment, an aphid was placed as an electrode on a leaf located in the central region of the plant, and then a leaf located immediately above was subjected to mechanical damage, allowing for the recording of basipetal propagation. In a second experiment, the damage was inflicted on a leaf located in the lower position, enabling the recording of acropetal propagation. After establishing the values of basipetal and acropetal VPS, a third experiment was designed to simultaneously measure propagation speeds in both directions. The results of these experiments consistently showed a higher propagation speed in the acropetal direction compared to the basipetal direction. This finding could contribute to a deeper understanding of how plants coordinate their responses to damage across different parts of the plant.

Acknowledgement: Fondecyt Regular Grant #1230406 (ANID-Chile).

Identification of plant virus proteins responsible for the manipulation of host phenotype and vector behaviour

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Host and vector manipulation by plant viruses is an evolutionary concept describing the effects induced by viruses on host-plant phenotype and vector behavior, in ways that favor their transmission. To date, the effects of viral infections on host-plant/insect vector interactions have been well documented, but the underlying molecular mechanisms are largely unknown. We are studying cauliflower mosaic virus (CaMV, *Caulimoviridae*) and turnip yellows virus (TuYV, *Solemoviridae*), two viruses transmitted by the green peach aphid (*Myzus persicae*) with different modes of transmission (semi-persistent non-circulative and persistent circulative, respectively), and therefore subjected to very different selection pressures. We have characterized the manipulative effects of these viruses and identified two CaMV proteins and one TuYV protein responsible for altering different sub-phases of the aphid probing or feeding behavior on infected plants. These virus-specific behavioral alterations (e.g., increased number of intracellular penetrations on CaMV-infected plants, longer phloem sap ingestion on TuYV-infected plants…) appear well adapted to the specific transmission mode of each virus and could have important repercussions on virus acquisition by the vector. Transcriptomic and metabolomic analyses of the infected plants revealed a high number of deregulated genes and changes in metabolic pathways (such as those involved in the biosynthesis of ethylene, glucosinolate, and jasmonic acid) that could be responsible for the aphid behavioral alterations observed. To get closer to a comprehensive understanding of this tripartite molecular dialogue, functional validation work is currently underway. Finding the genes and metabolic pathways that viruses manipulate in their host plants might help design strategies to stop virus spread by selecting, for example, plants that are less susceptible to virus manipulation.

Identification of plant cues involved in *Rhopalosiphum padi*'s host alternation

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Plant biochemistry plays a crucial role in insects' host location and selection. In this study, we tested the hypothesis that specific plant cues are sensed by the insects and triggered host plant alternation, i.e., the obligate migration between plants of unrelated families. This was tested in the aphid *Rhopalosiphum padi*, which alternates between two hosts annually, with *Prunus padus* as the exclusive primary or winter host in Europe and grasses as secondary hosts, which include several economically important cereals such as wheat, oats, barley, and wild grasses. *R. padi* produces several morphs (parthenogenetic and sexual) across its life cycle to withstand different hosts. In the spring, wingless parthenogenetic female produces winged parthenogenetic females (emigrants) which migrate to the secondary hosts. This host alternation occurs in two steps, emigrant production on the primary host and emigrant migration from the primary to the secondary host.

Plant biochemistry changes with the leaf maturation. In this study, changes in phloem composition with leaf (*P. padus*) age, were extensively studied to find whether they are perceived by *R. padi* as cues to trigger emigrant production. To define the critical period (a time window of leaf maturity within which drastic changes in phloem composition occur which might be perceived by *R. padi* as emigrant production cues, several parameters (length, chlorophyll content, penetrativeness by EPG, and phloem metabolite composition) are being measured. Several phloem collection methods (centrifugation, EDTA-exudation, and stylectomy) were compared, and based on that, phloem samples were collected. Detailed metabolite profiles were generated. Using this information, a two-week period of leaf-aging was identified as the critical period. Indeed, insect assays confirmed that changes in this critical period are sufficient to change morph. Notably, amino acid concentrations decreased drastically within this period making the diet nutritionally poor which might lead to emigrant production. Complementation assays using artificial diets are in progress to identify the key plant metabolites.

Emigrants feed on *P. padus* for a couple of days until they get repelled by it and attracted by the secondary hosts. This is the second step of host alternation. The role of plant volatile organic compounds (VOCs) in insect repulsion and attraction (push-pull) are shown using different plant-insect systems. Whether *R. padi* emigrants are sensitive to volatile cues of its primary and secondary hosts is being studied. Plant headspace VOCs were characterized with leaf-maturity and their role is being tested in olfactometric assays and electroantennography. Together, this study explores the role of plant compounds in aphids' host-switching. Finally, by identifying the chemical basis of host migration of agriculturally hazardous pests like aphids, this study can impact sustainable pest management by identifying novel bioactive compounds.

Applications of EPG technique: Transmission of plant pathogens by insect vectors

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EPG has been a very valuable tool to understand the transmission of plant pathogens by their insect vectors. EPG-assisted transmission experiments have been conducted in the past to correlate the acquisition/inoculation of plant pathogens with specific probing or feeding activities within the plant tissues. The acquisition process of a plant pathogen can be monitored in real time by placing an insect vector on an infected source plant and then interrupting the probe when a given waveform is observed. Similarly, the inoculation process can be monitored when a probe is interrupted when a specific waveform is observed while a viruliferous insect probes on a healthy test plant. Transmission of several plant pathogens have been associated to specific EPG waveforms and have helped to elucidate specific transmission mechanisms. For example, non-persistent virus transmission has been associated to salivation-ingestion activities of aphids during their first intracellular stylet punctures. The inoculation of semi-persistently transmitted phloem restricted viruses by aphids has been associated to brief intracellular stylet penetrations in phloem sieve elements or companion cells (or phloem pds). More recently, a specific waveform named Xe representing egestion of fluids contained in the foregut into xylem vessels, has been associated to the inoculation of *Xylella fastidiosa* to grapevine by sharpshooters.

Applications of EPG technique: Host plant resistance and understanding mode of action of chemical compounds using EPG

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The electrical penetration graph technique (EPG) visualizes the movements of aphid mouthparts' stylets in individual plant tissues. Various activities associated with the aphid stylets penetration in plant tissues are collectively termed aphid probing behavior. During probing, aphids collect samples of sap, first - from peripheral plant tissues and, second – from sieve elements, the basic aphid food source. At each phase of probing, allelochemicals detected in these sap samples may alter aphid behavior. Depending on whether an allelochemical is a phagostimulant or a phagodeterrent, aphids may continue probing and/or feeding or withdraw the stylets. Therefore, the parameters describing aphid behavior during probing are good indicators of plant suitability or the interference in probing by chemical or physical factors characteristically present in individual plant tissues. At present, aphid control depends mainly on the use of neurotoxic insecticides. Due to the repeated applications, many aphid species have developed resistance to several aphicides. The use of resistant plant species or cultivars and/or targeted chemicals that would repel aphids or deter their settling on plants is one of the most promising approaches. Therefore, the understanding and modification of aphid behavior during the pre-ingestive and ingestive phases of probing are crucial in designing alternative methods of aphid control.

Following the biopesticide-related approach to aphid control, we present the results of our multi-year research on aphid responses to plant resistance and aphid probing behavior-modifying activity of several natural and structurally modified allelochemicals. We show that using aphids as sensors it is possible to demonstrate indirectly the localization of natural deterrent factors and that the exogenously applied xenobiotics may penetrate the plant cuticle and epidermis and pass into deeper tissue layers. We also show that the transcuticular application of certain allelochemicals may cause disturbances in plant recognition and acceptance, which may finally reduce aphid infestation.

EPG in combination with additional techniques: cryofixation, CT scan and confocal, stylectomy, video-tracking recording, artificial diets, honeydew clock

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The Electrical Penetration Graph (EPG) technique has been largely used to study the feeding behavior of pierce-sucking insects to understand insect-host plant relationships and the transmission of vector-borne plant pathogens. However, EPG can be also applied for a wide range of approaches when combined with additional techniques. One of the most prolific combinations has been the correlation of EPG waveform patterns produced by insects with the subsequent identification of the specific punctured plant cells using microscopic studies. For this purpose, EPG recordings were terminated by cryofixation using liquid nitrogen and further study of the stylet's tip position by confocal laser-scanning microscopy (CLSM) or terminus of the salivary sheath by microcomputed tomography (CT-scan). By using this combination, the occurrence of previously uncharacterized EPG patterns produced by insects could be correlated with the specific insect activity. Moreover, specific biological activities during specific EPG waveforms can be determined combining EPGs and video-tracking recording. In case of spittlebugs, observation of the cibarial muscles activity through the tender and translucent nymphal cuticle was correlated with the corresponding waveform produced. EPGs can be also combined with the unique stylectomy technique for collection of phloem sap exudates from stylets severed by radiofrequency microcautery (RFM) and further collected into a microcapillary tube. Through these techniques, the presence of target compounds in the phloem sap of the host plant can be determined. Moreover, sap ingestion can indirectly be determined by quantification of honeydew excretions. This can be achieved by collecting honeydew droplets using a honeydew clock, consisting on a rotating paper strip attached to a clockwork, thus collecting the honeydew droplets. Besides studying the feeding behavior of insects on their host plants, EPG can be also used to monitor the feeding behavior on artificial diet devices. This is of special importance to check for successful feeding and acquisition of the target solute in the diet. The combination of EPGs with all these additional techniques will be further discussed.

Cellulose synthase gene expression analysis of *Solanum lycopersicum* exposed to combined effects of drought and TYLCV virus

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Drought is becoming a vital agricultural problem with climate warming. Extended drought also influences plant-pathogen interactions. Tomato yellow leaf curl virus (TYLCV) is one of the most dangerous quarantine viruses infecting ~30 different kinds of plants. Here, we investigated the combined stress, abiotic (drought) and biotic (TYLCV) stress, to study possessing the ability to protect tomato host plants against severe conditions. The Money Maker (MK, UK) seeds were germinated in insect-free growth chamber at 26/20^{0}C, day/night and a 16-h light/8-h dark cycle with 60-70% relative humidity. At 3-4 true leaf stages, tomatoes were used as recipient plants for the single-leaflet grafting method. As inoculum source, TYLCV infected tomato plants were used for grafting inoculation. TYLCV was respectively confirmed by duplex PCR using specific primer triplets *AV632*, *AV950*, and *AC1048* before grafting inoculation. Besides, 4 *cellulose synthase gene* families, which are based on the gene expression analysis, play a role in drought and virus resistance, *CesA*/*Csl genes (Csl-H1, Ces-A2, Csl-D3,2, Csl-D3,1*) were analyzed by the *qRT-PCR* method. As a result of molecular studies, it was found that *CesA*/*Csl genes* genes were significantly up-regulated in TYLCV-infected tomatoes. *Csl-H1* gene demonstrated 6 fold-change rise on 1st day and approximately 36-fold increase on 25th. Combined stress applied samples manifested the highest gene expression in the *Ces-A2* gene. Interestingly, the dual stressed tomatoes, which showed a 4 fold-change upregulation in the *Csl-H1* gene on day 1, manifested a 7.5 fold-change, downregulation on day 25. *Csl-D3,2* and *Ces-A2* genes showed no significant increase or decrease in gene expression level on day 25 compared to the 1st-day results. The *CesA*/*Csl genes*, biosynthesis of which occurs in the MK cultivar, is involved in the defense against TYLCV infection.

Aphids as bioelectrodes to measure changes in plant membrane potential in parallel with physiological changes as a result of pathogen infection

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Barley yellow dwarf virus (BYDV) infects various members of the family Poaceae, including cereals such as wheat and barley. In addition to the typical symptoms such as yellowing and dwarfing, it also causes changes in the leaf anatomy, as has been shown for barley. This can be seen, among other things, in a smaller diameter of the individual sieve elements. Based on the observations that BYDV infection leads to the long-term death of individual sieve elements, we suspect an early influence of the infection on the membrane potential of the sieve elements. To test this hypothesis, we used aphids of the species *Rhopalosiphum padi* as bioelectrodes using the electrical penetration graph technique in order to measure the relative membrane potential of the sieve elements and neighboring cells. The relative membrane potential determined by EPG is used to calculate index values, which have the relative membrane potential of the sieve elements as a reference point. The observations show that the membrane potential of the sieve elements in leaves of infected barley is weakened. Measurements of the mass flow inside sieve tubes show that it is reduced in BYDV infected plants, which is probably related to the changes in membrane potential. The study shows that aphids can not only be used for observations of action potentials, but their use as bioelectrodes can also provide important insights into plant physiological changes as a consequence of pathogen infection.

Alteration of aphid feeding behavior in sugar beet genotypes results in decreased beet yellows virus transmission

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Since 2018, neonicotinoid seed treatment for sugar beets has been banned. As a result, proliferation of aphids potentially carrying yellowing viruses led to significant yield losses up to 60%. In the lab, we applied electrical penetration graph technique (EPG) to identify antixenosis resistance in sugar beet accessions that could limit virus transmission by aphids.

The feeding behavior (by EPG) of aphids (*Myzus persicae*) on non-infected plants was studied on 14 genotypes and one control cultivar. We selected 13 parameters known to be important for virus transmission to discriminate the 15 genotypes. Based on the EPG results, genotypes M and 11 were identified has being well accepted by aphids, while genotypes 24 and 26 seemed to be poorly accepted by aphids, which could lead to a decrease in virus transmission.

To address whether the differences observed in the aphid feeding behavior could be correlated to virus transmission efficiency, aphid transmission tests of beet yellow virus (BYV) were conducted. BYV, acquired from the control cultivar, could be inoculated at a high rate to the four selected genotypes. In contrast, transmission of BYV to control cultivar after acquisition of BYV from genotypes 24 and 26 was significantly reduced (18-31%) compared to acquisition from genotypes M and 11 (84-89%) showing the ability of these genotypes to reduce virus acquisition.

In a second step, we tested whether BYV infection would alter aphid feeding by recording their feeding behavior on the four infected genotypes. In parallel, the plant metabolomic profiles were analysed in infected and non-infected plants. Metabolomic analysis highlighted a potential role of flavonoids in the aphid feeding responses, with only a minor impact of BYV infection. Only genotype 24 was affected by BYV infection with modifications of flavonoids levels in plants and a decrease of aphid's total probing time on plants. These modifications could explain the reduced acquisition and then transmission of BYV by aphids.

Our analyses showed that aphid feeding behavior recording by EPG on healthy plants is suitable to identify genotypes in which aphid's behavior is altered. This aphid trait could be correlated to the transmission of BYV by *M. persicae*.

Does SBR - a new disease of sugar beet - affects the feeding behavior of *Pentastiridius leporinus*?

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The "Syndrome Basses Richesses" (SBR) is a bacterial disease of sugar beet (*Beta vulgaris*), which reduces sugar content in the beet and causes significant yield losses. The causative agent of the SBR is the γ-proteobacterium *Candidatus* Arsenophonus phytopathogenicus (CAp), which is located in the phloem sap of the host plant and transmitted by the feeding activity of the planthopper *Pentastiridius leporinus* (Hemiptera: Cixiidae). Direct control of the pathogen and its vector is not yet possible and up to now, there are no resistant beet varieties available. A critical barrier to SBR containment and control is the insufficient knowledge of the multitrophic interaction between pathogen, host plant and vector. One aim of our research is the elucidation of the host finding and host acceptance behavior of this planthopper.

It is quite unclear whether the vector *P. leporinus* benefits from a sugar beet infection with CAp and is therefore preferably found in the cultivated area. Comparative studies of the phloem sap of CAp-infected sugar beet revealed significantly lower levels of monosaccharides and an increased concentration of six amino acids in infected plants. Three of these amino acids are essential for insects, so the phloem sap of infected beets could be a better food source. Whether this difference is reflected in the feeding behavior of *P. leporinus* is currently being investigated by using electropenetrography (EPG). 128 recordings are currently being analyzed to determine the different feeding behavior of *P. leporinus* on infected and healthy plants.

Measuring principles in EPGs: Circuit properties and Ohm's law relationships

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Voltage sources and resistances in the primary EPG circuit are dissected and their role in determining waveform features is discussed. EPG signals include electromotive force (emf)- and R-components; caused by fluctuating generated voltage (emf) and resistance (R) properties in the measuring (or primary) circuit. Electrode interfaces between the metal of the insect and soil (plant) electrode and their connected 'electrolyte'; the soil and the insect, respectively, make a galvanic cell (battery) of the circuit. Their contribution to the signal depends on Ohm's law relationships in the primary circuit. The EPG signal is represented by the input voltage Vi across the input resistor Ri, and Vi is the voltage between the measuring point (M) and ground, connected the two input terminals of the head stage amplifier. The signal voltage Vi is a fraction of the circuit voltage V. Similarly, the emf-component is the measured fraction Viemf of the original generated Vemf. The measured Viemf/Vemf fraction is the emf-component sensitivity (emf sensitivity), which is equal to Vi/V. Thus Vi/V and Viemf/Vemf can be used both to express the emf-component sensitivity. The Vi/V sensitivity is determined by the insect resistance as well (insect resistance as such cannot be measured; therefore we use "resistance between the 2 electrodes", Rbe). The emf-component sensitivity can plotted against the input resistance in the primary circuit. For every constant Rbe the emf-component sensitivity Vi/V will follow a sigmoid (s-) curve. The sensitivity increases with Ri.

Unlike the emf sensitivity the R-sensitivity cannot be expressed for a single Rbe (insect resistance). The R-component sensitivity depends on Rbe fluctuation range (ΔRbe) between two Rbe values, represented by the low and high value of the ΔRbe range. This is represented by the ΔVi/V range between the two Rbe sigmoid curves in the Vi/V-Ri plot. The Vi dependence of the value and sign of V is discussed in talk 1.

Cold treatment of infected plum fruits reduces the transmission of the PPV by aphids

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The Sharka disease or plum pox, which is caused by Plum pox virus (PPV), it is one of the most serious diseases in plants of the genus Prunus. This non-persistent virus is acquired by aphids (Hemiptera: Aphididae) from infected plants when they probe and penetrate the surface of infected plant tissues with their stylets and eventually inoculate healthy plant tissues. In stone fruit trees, fruits can be a virus source for aphids and thus contribute to the dissemination of PPV. Here we study whether subjecting PPV-infected plum fruits to a period of low temperatures (15 days under 0°C) reduces the transmission to healthy leaves. By using the EPG technique, we found that the transmission of PPV by aphids from infected fruits that were subjected to a period of low temperature was much lower than the transmission from infected fruits not subjected to that period of low temperature. This finding suggests that the likelihood of PPV transmission mediated by aphids from export fruits that have been subjected to a cooling period to resident plants in the destination locations is low.

Acknowledgement: Grant #PYT-2019-0091 FIA-Chile: "Desarrollo de estrategias de mitigación de la enfermedad de Sharka".

Exploring diurnal rhythms in aphid feeding behavior through the lens of the Electrical Penetration Graph technique

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While plants are known to undergo significant daily changes in metabolism and thereby their phloem content, it is unclear how phloem feeders, specifically aphids, adapt their feeding behavior in response to these daily oscillations. This study investigated the diurnal feeding behavior of the bird cherry-oat aphid (*Rhopalosiphum padi* L.) on wheat using the Electrical Penetration Graph (EPG) technique. Aphids were monitored at set intervals over a 24-hour period under both long-day (LD) and short-day (SD) conditions. A subset of aphids was also observed continuously for 24 hours. Our findings suggest that aphid feeding behavior remains largely consistent throughout the day, with only minor variations in behavior observed. Notably, extended salivation periods were observed during the nighttime in the short-day groups. These findings challenge the assumption of significant diurnal variation in aphid feeding behavior. However, the suitability of EPG as a method for detecting subtle behavioral differences over extended periods will be discussed, raising questions about the sensitivity and validity of this technique in long-duration behavioral studies. This research contributes to our understanding of rhythms present in aphid behavior and the methodological considerations in studying insect feeding patterns.

How do endosymbionts modulate plant virus transmission by aphid vectors?

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Endosymbiotic bacteria modulate aphid traits relevant to the transmission of plant viruses, including via strong interactive that appear conducive to disease transmission. We recently reported that the transmission of pea enation mosaic virus (PEMV) to fava beans (*Vicia faba* L.) was highest when wingless pea aphids (*Acyrthosiphon pisum*) harbored the facultative endosymbiont *Hamiltonella defensa*. However, the mechanisms leading to such phenotypes remain unknown, as well as whether endosymbionts also modulate virus transmission by winged (alate) aphids. To address this knowledge gap, we explored the effects of different endosymbiont sets on pre- and post-transmission steps relevant to PEMV transmission by both wingless and winged pea aphids. In a series of laboratory assays using qPCR, we analyzed the titers of virus acquisition and inoculation by aphid vectors, as well as the expression levels of molecules that might influence post-virus transmission steps, such as aphid salivary proteins and host plant defenses induced upon aphid feeding. Our results show that PEMV transmission is modulated by endosymbionts in wingless but not winged aphids. Additionally, we found that virus transmission by aphids harboring only the obligate symbiont is higher in winged than in wingless individuals, but this pattern is reversed when aphids also harbor the facultative endosymbiont *H. defensa*. Such differential patterns were also consistently observed in transmission-relevant traits, including titers of PEMV inoculation, levels of aphid salivary proteins, and induced host plant defenses, indicating that endosymbionts mediate post-acquisition and inoculation steps of virus transmission. Altogether, these findings highlight a potentially finely-tuned interplay between vector adaptations and disease ecology and the interface between microbial ecological interactions and disease transmission. Further studies addressing the effects on aphid feeding patterns might provide a more holistic understanding of the mechanistic impacts on vector-virus interactions.

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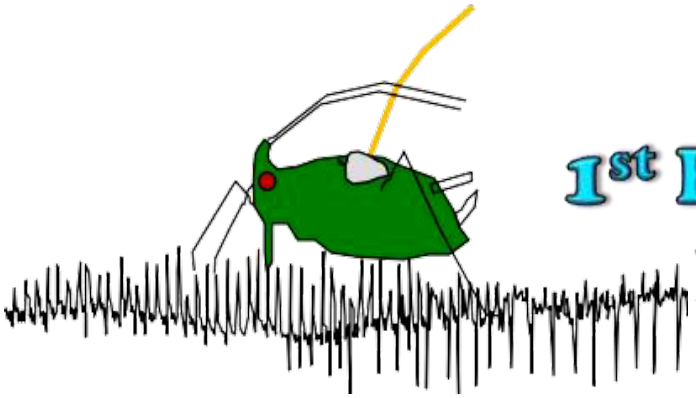

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