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Review Article

Scientific Opinion: Improving the Definition of Grape Phylloxera Biotypes and Standardizing Biotype Screening Protocols

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Abstract: Grape phylloxera biotypes are defined by their specific performance on, or preference for, a particular host (e.g. feeding on a particular rootstock). Numerous studies have phenotyped phylloxera, particularly in regard to their performance on various hosts but the results are difficult to compare because of the lack of a homogenous nomenclature and a standardized protocol for phenotyping. In an effort to improve communication within the scientific community, we offer a simplification of the phylloxera biotype classification to allow clear data interpretation and effective communication. We also introduce the standard techniques employed for phylloxera phenotyping and discuss their advantages and disadvantages.

Introduction

The term biotype was first applied to grape phylloxera last century (Prinz 1937). However grape phylloxera (*Daktulosphaira vitifoliae* Fitch) biotypes did not become well known until after

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the outbreak of a previously unknown strain of phylloxera capable of feeding and developing, thus producing tuberosities on the *Vitis vinifera* x *V. rupestris* rootstock hybrid (AXR#1) in the late 1980s (Granett et al. 1983). This biotype, commonly known as Biotype B, caused substantial economic losses in California. In general when referring to biotypes a specific performance on, or preference for, a particular host as measured by survival, development and individual life stages resulting in growth of populations or individual insects of phylloxera is implied in phylloxera literature. Although host suitability and intrinsic performances of insects are not synonymous (Singer, 1986); the term is often attributed to the aggressiveness of the phylloxera in question (King and Rilling 1991, Corrie et al. 1997, Anonymous 2014) . The term "biotype" has been used for strains of insect pests that vary in their response to hosts (Claridge and Den Hollander 1983), and the variants are often classified with numbers or letters. The genetic background of the variants is often not clearly defined, and the phenotypic variation could be based on allelic genotypes, or at the level of an individual or population levels (Sandström and Pettersson 1994, Downie 2010).

Over the last 30 years, numerous studies have phenotyped phylloxera, particularly in regard to their performance on various hosts and by assessing insect survival, development and growth. The physiological response by the hosts in the form of gall production as nodosities and tuberosities on roots or galls on leaves has been also used (Powell et al. 2013). The results are sometimes difficult to interpret and compare because of the lack of a homogenous nomenclature and a standardized protocol for phenotyping. In an effort to improve communication among phylloxera researchers, we offer this simplification of the phylloxera biotype classification, which should facilitate easier interpretation and comparison of results.

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Phylloxera Phenotypes

Aggressiveness and virulence are terms that have been used inconsistently in the literature when phylloxera biotypes and strains are described in terms of their performance (e.g. their rate of development and reproduction) on different hosts and in different environments, or in terms of the damage they cause to hosts (e.g. Granett et al. 2001b, Herbert et al. 2010). Furthermore, the term "biotype" is sometimes used for mixed populations of similarly performing strains, rather than for a specific genotype. In addition, aggressiveness is often measured in terms of life table parameters, which essentially assess the insect's developmental characteristics, rather than its impact on the host plant (Granett et al. 1985). Generally, the phylloxera development response is strongly dependent on the environment (host, abiotic factors, etc.). Despite known genetic and physiological differences in grape phylloxera populations, comparative studies of grape phylloxera morphology have so far yielded no evidence that strains can be distinguished based on morphological characteristics (Forneck and Huber 2009). The development of defined protocols for phylloxera phenotyping is needed to further promote research into the development of biomarkers associated with differences in phylloxera aggressiveness.

Phylloxera Genotypes

Over the past 30 years, the existence of different phylloxera strains has become more apparent because of differences in their performance on a range of *Vitis* genotypes. New strains can evolve, such as biotype B (Granett et al. 1985), which overcame the partially resistant rootstock genotype AXR#1 (Sullivan 1996). A wide-range of phylloxera strains has been reported in Europe (Song and Granett 1990, Forneck et al. 2001b, Yvon and Peros 2003), Australasia (King and Rilling 1985, Corrie et al. 1997, Corrie et al. 2002, Umina et al. 2007), Canada (Stevenson 1970), South Africa

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(De Klerk 1979), USA (Williams and Shambaugh 1988, De Benedictis and Granett 1992) and China (Du et al. 2008). It is not possible to accurately characterize the distribution of all strains in different countries without a standard genetic basis and set of protocols for strain identification, standardized phenotyping protocols and without a link between phenotype (in terms of performance and effects on the host) and genotype. However, it is likely that two Australian strains G1 and G4 (subsequently defined as superclones (Umina et al. 2007)), both with accurate genetic descriptions, do represent strains that have not been found in any other grape-growing country (Powell et al. 2013).

In order to better define phylloxera genotypes, a standardized genotypic protocol was proposed by the ISHS Phylloxera Work Group in 2014 which defines and names phylloxera genotypes suitable for comparative studies (Forneck et al. submitted); and an open database for phylloxera genotypes („PHYLLI“) https://www.dnw.boku.ac.at/wob/international-phylloxera-genotype-database/ is now available.

An updated concept of “biotypes” and aggressivity in grape phylloxera

Currently phylloxera biotypes are named according to their performance on and damage to (nodosity, tuberosity) a particular *Vitis* host for field clones, or for lineages characterized by their phenotype. Superclones are strains that constitute 40–60 % of a population in a region (Vorburger et al. 2003), and have higher fitness and damage levels on a general set of host plants (Powell et al. 2013).

Proposed Biotype classification

It is strongly suggested that phenotyping of phylloxera strains include both the life table parameters of the phylloxera strain under study and the evaluation of host plant responses, whether root feeding

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induces either nodosities (organoid galls on root tips) or tuberosities (galled tissue on mature roots) or both, or intermediate galls on mature roots (also called pseudotuberosities) (Powell and Korosi 2014), or root necrosis. Host plant response by leaf feeding strains can be evaluated as to whether leaf feeding induces a complete gall, or incomplete gall (with reproducing phylloxera) or incomplete/partial galls (without reproducing phylloxera), or leaf necrosis. Any feeding and growth of phylloxera requires a gall providing nutritive tissue and resources to allow the insect to develop to the adult stage and subsequently reproduce. We suggest that the term biotype be used as a category designating shared phenotypic traits. In practice, and with viticultural relevance (i.e. effective management), phylloxera strains that are equal in their ability to establish and develop on a particular host plant consequently belong to the same biotype group (see below). The level of aggressivity is measured by the rate of life-stage development and subsequent population increase on either type of gall and can vary among phylloxera strains within the biotype group.

A biotype classification based on a review of existing literature is proposed below with parameters for classification based on phylloxera and host plant interactions (Table 1).

Biotype A group: strains showing superior performance on nodosities and tuberosities on *V. vinifera* roots and limited performance on nodosities on rootstock roots derived from crosses between American *Vitis* species (Granett et al. 1985, King & Rilling 1985).

Biotype B group: strains showing superior performance on nodosities and tuberosities on the roots of rootstocks derived from American *Vitis* species crossed with *V. vinifera* (e.g. AXR#1) and limited performance on nodosities on the roots of rootstocks derived from crosses between American species (Granett et al. 1985).

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Biotype C group: strains showing superior performance on nodosities and pseudotuberosities on the roots of rootstocks derived from American *Vitis* species (e.g. T5C, 101-14 Mgt, C3309) and reduced ability to establish on *V. vinifera* roots (King and Rilling 1985, Forneck et al. 2001a, Kocsis et al. 2002).

Biotype D group: strains originating on leaves of susceptible American *Vitis* species and rootstocks derived from American *Vitis* species with reduced ability to establish nodosities on the roots of rootstock or nodosities and tuberosities on *V. vinifera* roots (Downie et al. 2000, Kellow et al. 2002, Vidart et al. 2013).

Biotype E group: strains showing superior performance of nodosities and tuberosities on susceptible *V. vinifera* roots, on nodosities and pesudotuberosities on the roots of some rootstocks derived from crosses between American *Vitis* species, and on rootstocks roots derived from crosses between American *Vitis* species and *V. vinifera* (Powell and Krstic. 2015, Trethowan and Powell 2007).

Biotype F group: strains showing superior performance on both leaves and roots of *V. vinifera* and reduced performance on rootstock roots (MA Walker, pers. comm.).

Biotype G group: strains showing superior performance on nodosities and leaves of rootstocks derived from American *Vitis* species (e.g. T5C, 101-14 Mgt, C3309) and superior performance on leaves of *V. vinifera* but reduced ability to establish nodosities and tuberosities on roots of *V. vinifera.* (Forneck et al. 2016).

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Current worldwide phylloxera biotype status

In Europe, the majority of phylloxera strains screened to date belong to the Biotype C group, with occasional occurrences of Biotype A and Biotype F (e.g. Forneck et al. 2001b, Kocsis et al. 2002, Powell et al. 2013, A. Forneck, personal communication, 2015). However, very few strains have been characterized, and more information is needed. In Australia the most widely distributed strains identified belong to the Biotype A group, including the two existing superclones, G1 and G4, although among the strains tested some fit in the Biotype B, D or E group (Umina et al. 2007, KS. Powell, personal communication, 2015). The geographical dominance of the Biotype A group in Australia may be the result of growing mainly non-grafted *V. vinifera* in this country. In California strains of Biotypes A, B, C and D have been found with shifting ratios over time (e.g. Granett et al. 2001). In Uruguay, Brazil and Peru Biotype A and F group strains have been found (Bao et al. 2015).

Although potential damage and genetic diversity of phylloxera strains is high, no convincing evidence has been provided for a single specific phylloxera strain being responsible for *Vitis* decline. The two superclones that singularly infest and kill own-rooted *V. vinifera* vines relatively rapidly in Australia are an exception to this observation. It is likely that such specific interactions are not known in other viticulture regions of the world because of the generally high diversity of strains that exist in vineyards and even on single vines, inconsistent procedures for identifying aggressivity and respective damage potential, and limited or reduced awareness that phylloxera is a potential cause of vineyard decline.

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Proposed phylloxera biotyping protocols

A range of rearing methods have been used to study interactions between grape phylloxera and *Vitis* hosts including excised roots (Granett et al. 1985; 1987, De Benedictis and Granett 1993, De Benedictis et al. 1996, Makee et al. 2004), *in vitro* propagation (Pelet et al. 1960, Askani and Beiderbeck 1991, Forneck et al. 1996, Grzegorczyk and Walker 1998, Kellow et al. 2002), whole plants in plastic tubes (Yvon and Leclant 2000, Forneck et al. 2001a), whole potted plants (Boubals 1966, Ramming 2010, Herbert et al. 2010, Pavloušek 2012) and field grown grapevines (Boubals 1966, Porten and Huber 2003, Trethowan et al. 2007). Each technique has technical advantages and disadvantages and may not provide equal or comparable results for biotyping. Because of the economical importance of phylloxera root-feeding biotypes we focus on presenting and discussion bioassays for root-feeding biotypes. Bioassays for leaf-feeding phylloxera (whole plant assays: aseptic dual culture, potted, caged under controled environmental conditions or on caged or clipped leaves of field grownd plants) exist but are not described in detail here.

Here we propose standard procedures for biotype maintenance and screening of root-feeding phylloxera according to scientific standards aimed at reproducibility and feasibility. As there are several ways in which biotype screening can be conducted, and because the phylloxera-host plant interactions may differ depending on the protocol used, a generic standard for each bioassay type is described.

Excised root bioassay

The excised root bioassay has been widely used as a rootstock screening system for several decades. It has the advantage of allowing comparative studies of phylloxera life-stage development, but the disadvantage of using excised plant material which may affect secondary metabolite response and

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hence the interaction between phylloxera and its host. The standard we suggest is based on a modified method of Granett et al. (1983) as follows:

Excise *V. vinifera* lignified roots from mature plants and cut into 70-80 mm long pieces, with a root diameter of 10-15mm. Dip roots in Ridomil™ fungicide at the rate of 7.3 g per 3 L water to deter fungal attack and then rinse three times in sterile water to remove excess fungicide residues. Air dry root pieces aseptically in a laminar flow cabinet under UV light. Place dried root pieces on a filter paper lined 90×25 mm petri dish. Wrap both ends of the roots in cotton wool and moisten daily with sterile distilled water. Place twenty phylloxera eggs sourced from a single clonal lineage on each root piece with a fine soft paintbrush. Use a minimum of ten replicates of each rootstock/phylloxera biotype combination. Seal Petri dishes with Vitifilm™ or similar clingwrap to prevent phylloxera escaping and keep in dark at constant temperature (growth room set at 25±2°C) for eight weeks. Record phylloxera survival and development weekly. Ensure removal of fungal infestation by judicious use of 70% ethanol on fungal hyphae at regular weekly intervals, taking care to avoid contact with phylloxera as ethanol can reduce phylloxera survival.

In vitro **bioassay**

Aseptic dual culture systems allow fine-tuned host-parasite interaction studies and detailed analysis of non-compatible or compatible interactions. They can also be used to produce plant tissue suitable for further gene expression, microscopic or metabolomic studies. The system has also been used to study phylloxera biotypes, since all *Vitis* species root well *in vitro* and provide optimized host conditions for both root- and leaf-feeding phylloxera. The system can be run under quarantine conditions all year round. The disadvantages are the time, costs and the potential contamination risk

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of the cultures. The standard we suggest is based on the method used by Forneck et al. (1996) as follows:

Micropropagated green cuttings were pre-rooted in ½ X Murashige & Skoog medium and transferred into culture vessels partially filled with medium, poured with a sloped surface so that about half of the vessels base is covered. After 14d of growing the vessels are returned to an upright orientation to promote root growth towards the media-free side of the vessel. Between 10-50 phylloxera eggs (depending on the size of the vessel) are surface sterilized and spread over the roots with an autoclaved fine paintbrush or pipette. Phylloxera first instars will start feeding immediately after hatching and galls form 1-2 dpi (days post inoculation). Environmental conditions for the aseptic dual culture should be set at between of 22-25°C to reduce condensation in the vessel. If this cannot be achieved consider using an autoclaved cloth to absorb condensation water. Dual aseptic culture has been successful in a range of vessels from petri dishes to 2L jars. For purposes of biotyping, phylloxera development can be recorded weekly and stages and galls can be marked with a pen to track molting and survival. This system also allows leaf galling phylloxera to be observed in real-time, since galls rarely close entirely under *in vitro* conditions.

In planta **bioassay**

An *in planta* bioassay system is a whole plant system with no induced effects caused by root excision and is performed within cages to prevent phylloxera spread and migration. The bioassay has been introduced in several variations of which we suggest either the “bottle- system” (Forneck et al. 2001b) or the “potted trial with root enclosures” (Korosi et al. 2007). In order to have comparable data we suggest calibrating the phylloxera population data and plant responses (e.g.

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nodosities, pseudotuberosities and tuberosities) in relation to the root biomass, which is dependent on the pot/vessel chosen.

The advantage with the potted trial is that 'root enclosures' allow damage and phylloxera abundance to be assessed at the end of the trial with limited sample sorting. The advantages of the bottle method is include the ability to observe root development/damage without disturbance, limited space requirements and the ability to easily insect-proof the system to limit migration. The methods we recommend are a modified version of Korosi et al (2007) and Forneck et al. (2001b) respectively, and running the assay at 25±2°C and 16hr photoperiod.

Potted bioassay with root enclosures

Twenty phylloxera eggs are placed on a single lignified root piece (1.5-4.0 mm diameter) on the root system of the potted vine and wrapped in 50 μm mesh with Tanglefoot™ insect trap applied around the enclosure to prevent phylloxera escape. Repot the infested vines and place in a mesh bag (50 μm mesh) tied at the trunk of the vine and sealed with Tanglefoot™ to prevent phylloxera cross contamination between pots. Keep plants under controlled conditions for eight weeks. At harvest carefully wash root pieces and collect the washing and examine for phylloxera life stages and assess root damage by counting tuberosities, pseudotuberosities and nodosities (Korosi et al. 2003).

Bottle bioassay

Bottles are constructed from plastic soda bottles and fitted with an insect proof silk-screen baffle to allow airflow and humidity control. No drainage holes are required. The soil consists of a 3:2 mix of peat moss and soil mix (1:1:2 sand/loam/fir bark). Rooted green or dormant cuttings are planted in 1000 ml of soil, irrigated with 200 ml of water. Twenty eggs are placed on a moistened filter paper, which is then rolled into a tube and inserted deep into the soil. The first interaction can be observed

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after 3 dpi. Keep plants under controlled conditions for two generations of phylloxera (eight weeks). Collect data for each generation on the visible root area adjacent to the bottles surface. Expand data collection after harvesting to the entire root system to collect data on nodosities and tuberosities (Forneck et al. 2001a).

In field bioassay

In field bioassays are best used where single biotypes exist although in some instances when testing rootstocks they can be used where mixed biotypes occur (Trethowan and Powell 2007). There are two methods of biotype assessment, root assessment and emergence trap assessment and they can be used either singularly or in combination. If conducted throughout the season root assessment allows monitoring of all phylloxera life stages whilst emergence traps allow monitoring of phylloxera dispersive stages first instar and adult alates. Bioassays can either be conducted in areas in which a natural infestation of phylloxera is present or if feasible in a quarantined area where field inoculation is allowed.

In field root bioassay

Phylloxera abundance on grapevine roots is best quantified, over one to three successive seasons, in early- to mid-summer when phylloxera life stage activity is at its peak and all life-stages may be present. Assessing relative phylloxera abundance on roots can be conducted in a destructive or a non destructive manner. Using the destructive method root samples (2–8 g dry weight), including both lignified and non-lignified roots, are excised from the sample vine (10-20 sample vines are recommended per treatment). The excised root can then either be examined directly or washed carefully and examined, under a dissecting microscope, to record the number of each phylloxera life-stage and the number of nodosities, pseudotuberosities and tuberosities. Samples should be

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collected at 3-week intervals. The roots are then oven-dried at 70°C for 48 h and weighed to quantify root dry weight and the insect/root ratio determined (Powell et al. 2003)

In field emergence trap bioassay

Phylloxera abundance is best monitored in early- to mid-summer when phylloxera activity is at its peak using plastic emergence traps over one to three successive seasons. These traps consist of 3-5 liter translucent plastic bowls, inverted and placed at a distance of 10 cm from the sample vine trunk (10-20 sample vines are recommended per treatment). Traps should be secured adjacent to vine trunks with 3 metal tent pegs (Powell et al. 2000). Traps are rinsed with tap water prior to placement resulting in a film of condensate, which effectively traps emerging phylloxera dispersive life-stages. Trapped insects are collected at 3-week intervals post-placement by removing the pegs, inverting and washing with 70% ethanol into plastic vials. Trap samples are then examined using a low power binocular microscope. Emergent phylloxera life-stages, consisting of first instar nymphs and winged alates are recorded.

Conclusion

When choosing a bioassay for biotyping root-feeding phylloxera one should consider that all bioassays presented except the excised root bioassay, promote nodosity, tuberosity and pseudotuberosity based feeding with a whole plant response; whereas the excised root bioassay only allows testing of strains feeding on tuberosities and pseudotuberosities. We suggest using the excised root bioassay in combination with any lab-based bioassay to biotype phylloxera. Biotyping within the field is occassionally done using destructive or non-destructive techniques with root bioassays and/or emergence trap bioassays. Screening of field samples strains for their performance

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on leaves, requires a bioassay with whole plant response and is mostly done in the bottle bioassay but could also potentially be conducted in the field. Although field conditions are difficult to control (eg. climatic and edaphic), field bioassays can also be used to study the impact of phylloxera biotypes on whole plant performance and grape yield. For standardization purposes and to ensure precise experimental screening conditions, we strongly suggest that phylloxera are selected from single founder lineages and a range of insect growth and development parameters (survival rate, fecundity, instar development time) are assessed. Ideally standard biotypes should be co-screened as standards for comparison.

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Table 1 Biotype differentiation according to feeding sites and feeding organ (galled tissue) and insect development (Capital letter indicates superior insect development in relation to compared host plants, small letters indicate lower/limited insect development, **-** indicates neither gall or insect development, or **?** no information available). Letters attributed to **t**uberosities, **n**odosities, **p**seudotuberosities, leaf **g**alls)

Biotype	Feeding tissue	*Vitis vinifera*			Rootstocks (*V. vin*. x American *Vitis* species			Rootstocks (American *Vitis* species)			Reference
A	Root	T	N	-	t	n	-	-	n	-	Granett et al. 1985, King & Rilling 1985.
	Leaves	-			G			G			
B	Root	t	n		T	N		-	n	-	Granett et al. 1985.
	Leaves	-			-			-			
C	Root	-	n	-	T	N	P	-	N	P	King and Rilling 1985, Forneck et al. 2001a, Kocsis et al. 2002
	Leaves	-			G			G			
D	Root	t	n	-	-	N	-	-	N	-	Kellow et al. 2002. Corrie and Hoffmann 2004.
	Leaves	-			?			G			
E	Root	T	N	-	T	N	?	T	N	P	Powell and Krstic 2015.
	Leaves	-			-			-			
F	Root	T	N	-	?	?	?	?	n	?	M.A. Walker pers. communication
	Leaves	G			?			G			
G	Root	?	?	?	?	N	?	-	N	?	Forneck et al. 2016
	Leaves	G			G			G			