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In vitro propagation of *Dactylosphaera vitifolii* SHIMER (Homoptera: Phylloxeridae) on shoot and root cultures of a *Vitis* hybrid

by

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In vitro-Vermehrung von *Dactylosphaera vitifolii* SHIMER (Homoptera: Phylloxeridae) an Sproß- und Wurzelkulturen einer *Vitis*-Hybride

Zusammenfassung: Methoden zur langfristigen Mikrovermehrung (Sproßspitzenkultur) und zur Kultur von transformierten Wurzeln (*Agrobacterium rhizogenes*) einer *Vitis*-Hybride wurden entwickelt. Das Wachstum dieser Organkulturen wurde charakterisiert. Nach Inokulation mit Eiern der Reblaus lief auf diesen Kulturen der parthenogenetische Lebenszyklus der Reblaus *in vitro* ab.

Bei 23,6 °C reagierten mehr als 80 % der Sproßkulturen innerhalb von 2 Wochen nach der Inokulation mit der Bildung von Beutelgallen an jungen Blattspreiten, Schwellungen der Petioli und ausnahmsweise der jungen Sprosse, und etwa gleichzeitig legten die entwickelten Tiere neue Eier ab. Die beiden Vorgänge der Gallbildung und der Reblausvermehrung konnten kontinuierlich über 2,5 Jahre fortgeführt werden, indem alle 1—3 Wochen Reblauseier aseptisch auf neue mikrovermehrte Sprosse übertragen wurden.

Mehr als 90 % der Wurzelkulturen beherbergten innerhalb 1 Woche nach der Inokulation Larven an den jungen Wurzelabschnitten und reagierten dort mit Krümmungen und Verdickungen. Nach ca. 2 Wochen waren neue Eier abgelegt und die Zahl der Tiere und Eier nahm bis zur 5. Woche stark zu.

Damit erlauben die beiden Verfahren der Dualkultur die Bildung von Blatt- und Wurzelgallen und die Reblausvermehrung unter Ausschluß weiterer Organismen.

Key words: hybrid, tissue culture, shoot, hairy root, growth, phylloxera, dual culture, gall, propagation, method.

Introduction

There are many potential advantages of using dual *in vitro* cultures of plant tissue or organ and phytophagous insects to study insect-plant relationships:

— preservation and maintenance of a pest under quarantine conditions;
— control of the chemical and physical environment;
— control of inoculum density;
— addition of metabolic precursors, inhibitors or stains to the host or the parasite through the medium.

Although these advantages have been exploited in studying many associations between viruses, bacteria or fungi and their respective host-plant tissues (INGRAM and HELGESON 1980), the use of *in vitro* plant systems to study their long-term interactions with phytophagous invertebrates has been restricted to nematodes (ZUCKERMAN *et al.* 1971; SAVKA *et al.* 1990). Dual cultures of plant tissues and plant feeding insects have been started without lasting success (MOTT *et al.* 1978; NAPPEN 1981; CROUGHAN and QUISENBERRY 1989; COOK *et al.* 1990). However, in pioneering studies grape phylloxera has been propagated temporarily on callus raised from gall tissue (PELET *et al.* 1960) or callus and root forming explants of *Vitis* (RILLING and RADLER 1960; RILLING 1975). In

this paper, we report the long-term propagation of grapevine phylloxera under aseptic and controlled environmental conditions using micropropagated shoot cultures and hairy-root cultures of the *Vitis* host.

Material and methods

Plants

Cuttings of a 'Weißer Amerikaner' vine, probably a hybrid between *V. vinifera* L. and *V. labrusca* L. (det. Dr. EIBACH, Geilweilerhof, pers. communication) were obtained from a local winegrower at Heidelberg, rooted in pots and kept in a greenhouse (ca. 60 % RH, a 14 : 10 h light regime with natural dayligth, in winter supplemented by light from Osram HWLS bulbs, 500 W, 20—35 °C depending on weather conditions).

Micropropagation of plants

Green shoots with about 12 nodes were defoliated and divided into 1 to 2-cm single-node cuttings. These were surface-sterilized by consecutive treatments with 1 % $NaO\overset{\cdot}{C}l$ + 0.03 % Tween 20 for 12 min; 70 % ethanol for 10 s; 3 washes with sterile water for 5 min each. While using this protocol, on average 3.9 ± 5.4 % of the explants were contaminated.

3 segments were placed horizontally in Petri dishes (9 cm ∅) with the bud above the surface of the medium. MS medium containing 30 g/l sucrose, 12 g/l agar (Difco) and 1 mg/l BAP served as propagation medium (MURASHIGE and SKOOG 1962; CHEE and POOL 1985). The plates were incubated at 23.6 °C and 14 : 10 h photoperiod using fluorescent white light of 1300 lux.

For subcultures, 5 shoots of 1—2 cm length sprouting from the vegetative axillary buds of single-node explants were excised and subcultured for 8 weeks on 20 ml propagation medium using 9-cm ∅ Petri dishes. 4 weeks after the isolation of single-node explants and 8 weeks after transfer of the newly developed axillary shoots to propagation medium, the development of the cultures was evaluated.

Rooting

After 8 weeks on propagation medium, shoots with 3—4 nodes were transferred to 200-ml baby food jars closed with Magenta® caps and containing 20 ml of rooting medium. This medium consisted of the minerals and vitamins of BLAICH's medium (HOOS and BLAICH 1988), 30 g/l sucrose, 12 g/l agar, 0.01 mg/l NES and 0.03 mg/l BAP. The cultures were incubated as before under fluorescent white light of 3100 lux.

For long-term routine propagation, every 7 d shoot tips consisting of the apical bud and two adjacent nodes were excised from micropropagated plants (ca. 6 weeks old) and cultured under rooting conditions (see before). Within 2—3 weeks, from decapitated shoots 1—3 quiescent buds sprouted, which could be used again to obtain additional shoot tip cultures.

Induction and culture of transformed hairy roots (RIKER *et al.* 1930)

Agrobacterium rhizogenes strain 15834 (American Type Culture Collection) was cultured in NYS medium (LIPPINCOTT and HEBERLEIN 1965) at 25 °C for 2 d. A hypodermic syringe filled with these bacteria was used for the wounding and inoculation of

internodes from *Vitis* plants grown *in vitro* (see above) 6 cm in height. After inoculation, the plants were incubated as before under fluorescent white light of 2800 lux.

2—3 weeks after inoculation, roots of 1—2 cm length developed at the inoculation sites. They were excised and grown in Petri dishes (5 cm Ø) containing 7.5 ml of a rooting medium. This medium consisted of half-concentrated macro- and microelements of the medium used by HOOS and BLAICH (1988), vitamins of medium B5 (GAMBORG *et al.* 1968) and additionally (mg/l): $FeSO_4$ — 27.8; Na_2EDTA — 37.3; Ca-pantothenate — 0.8; riboflavine — 0.015; sucrose — 60,000; agar (Difco) — 12,000; pH 5.8. The Petri dishes were incubated at 27 °C, under a light regime of 12 : 12 h with natural light of 5—50 lux. After 4 weeks of culture, the root showing the most abundant growth (almost 10-fold length, with several ramifications) was divided into segments of 2 cm length each with an apex and the segments were transferred to fresh rooting medium, covered with filter paper (Schleicher and Schüll, 595) to prevent penetration of the roots into the agar layer.

Every 3 weeks, subcultures from these roots were made as described. No indication of bacterial contamination (*A. rhizogenes*) has ever been recognized during the experiments.

Culture of normal roots

Non-transformed roots were obtained from 6—8 weeks old micropropagated plants and cultured on the same medium and under the same conditions as hairy roots.

Propagation of grape phylloxera on plant tissues

Plants of 'Weißer Amerikaner' were grown in the greenhouse in insect-proof cages. Gall-bearing leaves were fixed to young shoot axes and subsequently galls developed on the host plants.

For sterilization of phylloxera eggs, a procedure of RILLING (1975) was modified: Using a magnifying glass (8 ×) in a laminar flow cabinet, the orange egg masses bulging out of gall openings were harvested by means of a needle and placed in a funnel lined with filter paper moistened with an aqueous solution of 1 % NaOCl. After 200—300 eggs had been collected in this way, they were rinsed for 5 min with 1 % NaOCl containing 0.03 % Tween 20 as a wetting agent. Care was taken that the egg masses were spread during the rinsing procedure. Subsequently, the eggs were washed with sterile water for 15 min, thereby accumulating them at the base of the funnel. The filter paper carrying the eggs was placed in a Petri dish and flattened for transfer of the eggs to the host plant organs using a fine needle.

10—20 eggs each were placed on unfolded leaves (about 0.5 cm) of 3.5—4.5 weeks old *in vitro* plants or close to the tips of cultured roots recently transferred onto fresh medium. Egg clusters obtained from *in vitro* plants were wiped onto a piece of moist linen cloth. After drying the surplus water in the air current of the laminar flow cabinet, a distinct number of eggs were transferred gently to new cultures.

Results

A. *In vitro* growth of shoots

1. Propagation of shoots

In 5 experiments, 12 single-node explants each were placed onto the propagation medium. 4 weeks later, axillary buds sprouted from 83.2 % (± 12.3 %) of the explants.

On average, 3.2 (± 1.0) shoots/explant had been formed and 61.9 % (± 6.5 %) of these shoots had a length between 1 and 2 cm and consisted of 3—4 nodes.

In 3 experiments, 25 newly formed shoots each were severed and subcultured on the propagation medium. After 8 weeks, 9.5 (± 1.0) shoots/explant had developed. 61.0 % (± 4.8 %) of these shoots were between 1 and 2 cm in length, consisting of 3—4 nodes.

Fig.1

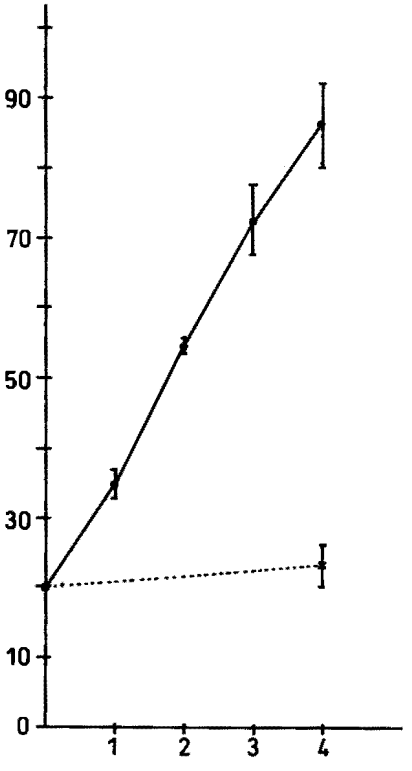

Fig.2

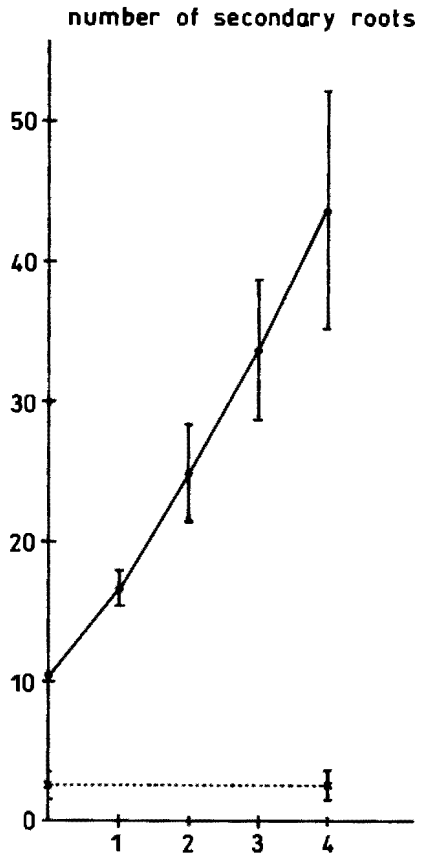

Fig. 1: Length of the main roots from transformed and non-transformed root explants after different culture periods.

Fig. 2: Number of secondary roots/explant on transformed and non-transformed root explants after different culture periods. ●——● = transformed, ×·····× = non-transformed explants. Bars indicate standard deviation.

Abb.1: Länge der Hauptwurzeln von Explantaten transformierter und nicht-transformierter Wurzeln in Abhängigkeit von der Kulturdauer.

Abb. 2: Zahl der Sekundärwurzeln an Explantaten transformierter und nicht-transformierter Wurzeln in Abhängigkeit von der Kulturdauer. ●——● = transformierte, ×·····× = nicht-transformierte Explantate. Balkenlänge = Standardabweichung.

2. Rooting

In 4 experiments, 25 shoots each were transferred to the rooting medium. Within 3 weeks, 88.5 % (± 8.4 %) had formed roots. After 6 weeks, each of the rooted plantlets was 4.5—7.5 cm high and consisted of 8—12 nodes. For routine propagation, shoot tips of these rooted plants were transferred to rooting medium (200 explants). Within 3 weeks, 93.7 % (± 7.9 %) had formed roots, after 5 weeks the new plants were 4.5—7.5 cm high, showing 6—10 nodes.

B. *In vitro* growth of isolated roots

1. Growth of the main root

Segments of 10 transformed roots (5 replicates) and 10 non-transformed roots (3 replicates) were placed onto rooting medium and the root development was studied. While the transformed roots grew 4-fold in length within 4 weeks, the non-transformed roots showed only traces of elongation at best (Fig. 1). In each case, the white root explants turned brown within the 4 weeks of culturing. With non-transformed roots this browning reaction occurred all over the whole explant, including the zone of apical meristem (root tip). With the transformed roots the browning reaction started at a distance of 30—40 mm proximally to the root tip.

After 4 weeks, the epidermis of the brown parts of transformed roots showed spontaneous fissures, exposing the loosely packed cortical cells and sometimes giving rise to calli. At these fissures roots easily broke into pieces, interfering with the handling of the roots, and made further recording of root lengths impossible. However, in spite of the appearance of fissures, the transformed roots continued growing (compare Fig. 3).

2. Number of secondary roots

Root explants of 20 mm length used in these experiments consisted of the main root and several secondary roots. An explant from transformed roots bore an average of 10.3 (± 2.1) secondary roots versus 2.6 (±1.1) secondary roots originating from a non-transformed root.

During the subsequent culture period, the transformed roots continuously produced additional secondary roots leading to a 4-fold rise within 4 weeks. On the contrary, the non-transformed roots produced no additional secondary roots during the 4 weeks following explantation (Fig. 2).

3. Elongation of secondary roots

At the time of explantation, the longest secondary roots had a length of 5 (± 3) mm in the case of transformed and non-transformed roots as well. After 4 weeks of culture, the secondary roots from non-transformed explants had never increased in length, whereas those from transformed roots had reached an average length of 53 (± 1) mm.

4. Formation of tertiary roots

On non-transformed roots no tertiary roots could be observed, whereas on transformed ones tertiary roots began to grow 3 weeks after explantation on the longest secondary roots.

5. Fresh weight increase of root systems

The changes of fresh weight of whole root systems derived from transformed and non-transformed root explants differed greatly (Fig. 3). The fresh weight of trans-

formed root systems increased more or less constantly for 6 weeks and subsequently growth slowed down. After 8 weeks, the fresh weight had increased 29-fold. On the contrary, no fresh weight increase was observed with non-transformed root systems.

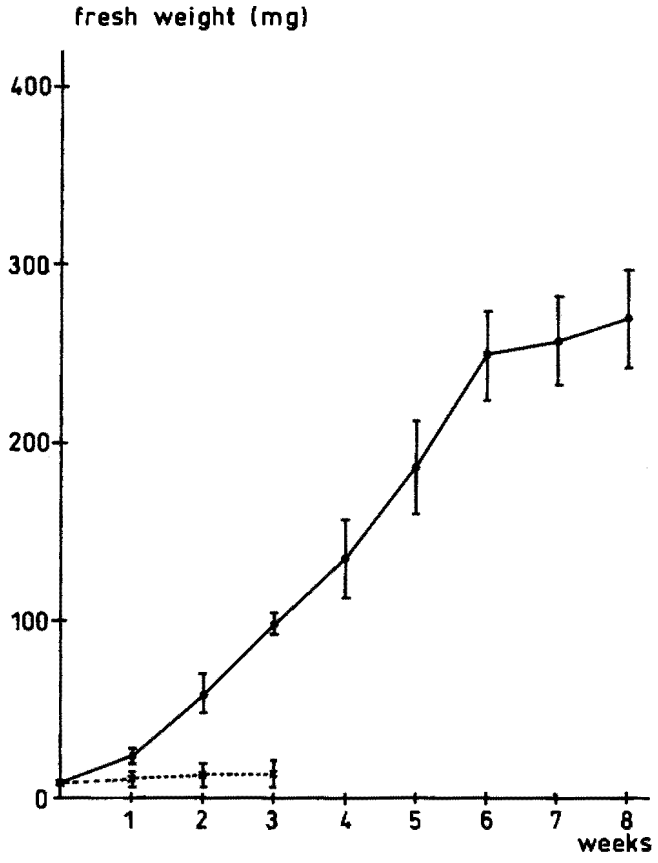

Fig. 3: Fresh weight/explant of transformed (●——●) and non-transformed (×·····×) root systems after different culture periods. Bars indicate standard deviation.

Frischgewicht transformierter (●——●) und nicht-transformierter (×·····×) Wurzelsysteme in Abhängigkeit von der Kulturdauer. Balkenlänge = Standardabweichung.

C. Dual cultures of grape phylloxera with *in vitro* propagated plants

When *in vitro* plants were inoculated with eggs, the percentage of contaminated isolates depended solely on the pretreatment of the eggs: eggs harvested from healthy greenhouse-grown galls and surface sterilized yielded 3.3 % (± 3.3 %) contaminated plants (3 experiments with 10 plants each), whereas eggs harvested from fungus-infected galls yielded 83 % contaminated plants.

Leaf gall formation *in vitro* was visible on 70.4 % (± 16.3 %) of the plants 10—14 d after inoculation. On the upper leaf surfaces, yellow-green spots with a central gall

Table 1

Development of grape phylloxera and gall formation on shoot cultures of *Vitis* (with standard deviations)

Entwicklung der Reblaus und Gallbildung an Sproßkulturen von *Vitis* (mit Standardabweichungen)

Weeks after inoculation	Galled leaves/plant	Galls/leaf	% Galls on		
			Lamina	Petiolus	Shoot
2	1.4±0.6	4.6±4.2	97.2±4.7	0	2.6±4.5
6	2.8±0.9	6.6±5.1	89.5±5.7	3.8±2.6	2.6±1.4

opening could be seen against the dark-green background of the laminae and on the lower sides of 2.5—3 cm long leaves pouch galls of 3—5 mm ∅ were evident. Inside the galls about 2 weeks after inoculation, phylloxera started to oviposit and soon yellow egg masses became visible at the gall openings. These eggs were used for aseptic inoculation of additional plants omitting surface sterilization of eggs. On 82.6 % (± 13.0 %) of these second 'generation' of inoculated plants, new leaf galls formed 10—14 d after inoculation (Table 1) with egg masses protruding through the gall openings. These dual culture cycles have been repeated until now for 2.5 years (about 110 times in overlapping experiments) without any significant changes in gall development and egg production. Depending on the age of the galls, 20—80 eggs could be harvested from a single gall, leaving some wasted eggs behind.

Gall formation was induced on leaves of about 0.5 cm length, still folded around the shoot apex. Fully unfolded leaves (more than 1 cm length) never formed galls. Galls developed on shoots (Table 1) consisted of curved, red-coloured swellings.

When culturing inoculated *in vitro* shoots over extended periods, gall formation increased considerably (Table 1), each new gall yielding additional eggs and aphids; also petioli became swollen and curved. Exceptionally root galls were formed on those roots protruding over the agar surface.

On *in vitro* shoots oviposition continued until about 10 weeks after inoculation. Thereafter, plants were heavily damaged: the terminal buds became brown and dried out, the shoot axes lost mechanical stability, the petioles curved downwards with laminae turned yellow. The root system became brown even when not colonized by phylloxera. Control plants devoid of phylloxera showed similar symptoms of decay only 6 weeks later.

D. Dual culture of grape phylloxera with *in vitro* propagated hairy roots

Root segments (2—3 cm) including an apical meristem were transferred to fresh rooting medium covered with filter paper and immediately inoculated with 10—20 eggs. 1 week after inoculation, 96.6 % (± 4.7 %) of the roots harboured an average of 7.3 (± 4.7) living larvae/root (Table 2). These were preferentially settling close to the root tips, inducing curved swellings (nodosities). A few larvae occurred more proximally on the roots and on the callus tissue which had formed at fissures of the root cortex. The proximal parts of the roots responded with thickening, whereas no alterations were observed in the calli. At this time and later, part of the larvae died because they were trapped in the liquid film between root and filterpaper.

During the following period of culture, part of the larvae developed into adults which in turn laid eggs. Consequently the number of phylloxera (larvae and adults) and

Table 2

Development and propagation of grape phylloxera on hairy roots of *Vitis* (with standard deviations)

Entwicklung und Vermehrung von Rebläusen an transformierten Wurzeln von *Vitis* (mit Standardabweichungen)

Weeks after inoculation	% Roots with adults	Number of phylloxera/root		
		Total	Adults	Eggs
1	0	7.3 ± 4.7	0	0
2	27.4 ± 3.6	5.4 ± 4.2	2.1 ± 0.9	20.8 ± 10.4
3	95.5 ± 6.7	10.5 ± 13.0	2.6 ± 2.1	26.0 ± 5.9
4	91.6 ± 6.9	13.2 ± 16.4	4.0 ± 3.4	32.6 ± 11.0
5	93.3 ± 9.4	21.7 ± 18.4	5.0 ± 3.8	54.2 ± 18.7

eggs/root increased to about 26 and 54, respectively (Table 1), until the 5th week after inoculation. Further dual growth of phylloxera and roots was observed up to the 8th week but quantitative evaluation was no longer possible because of the high numbers of insects and secondary and tertiary roots.

Conclusions

Shoot culture

For the initiation of shoot cultures single-node segments of greenhouse-grown plants were explanted. Young shoots growing from such explants were micropropagated on a cytokinin containing medium and their rate of propagation and elongation was comparable to that of the hybrid 'Remaily Seedless' (CHEE and POOL 1985). The shoots obtained were easily rooted by transfer to rooting medium, without any further manipulations like low salt content of the medium and paper bridges on liquid medium as reported by HARRIS and STEVENSON (1982). Having obtained a certain number of plantlets, the transfer to a cytokinin containing medium was dispensable since the side shoots, developing after decapitation of the rooted plantlets, provided sufficient resources for continuous propagation.

Root culture

Since non-transformed roots did not elongate or ramify on the many media tested in preliminary experiments, roots resulting from transformation of *Vitis* shoots by *Agrobacterium rhizogenes* (MUGNIER 1988) were used which yielded reasonable elongation, fresh weight increase and — most important for dual culture studies — abundant ramification. Growth of these roots was more or less restricted to the surface by culturing them on agar plates covered with filter paper.

Dual cultures

On shoot cultures as well as on hairy root cultures phylloxera were kept aseptically starting with surface-sterilized eggs. The aphids induced leaf and root galls of typical appearance, completed their parthenogenetic life cycle from egg to adult and propagated. As in field and greenhouse plants (STERLING 1952; ANDERS 1960), only young plant organs and tissues like root tips, folded leaves and young petioles were competent

to gall formation. In addition, tender parts of *in vitro* grown shoots also produced galls. In previous experiments, isolated roots devoid of adhering shoot parts (BOUQUET 1983; GRANETT *et al.* 1983) were shown to respond to colonization by grape phylloxera with gall formation. Now, using hairy roots as host organs, the continuous observation of the insect behaviour and of plant responses has become possible. On hairy roots, the development from egg to adult takes about 2 weeks, which is characteristic of the interaction between roots of susceptible plants and phylloxera (GRANETT *et al.* 1983).

On shoots, the continuous microscopic observation of the insects and the galls is technically impossible but, due to the lack of moisture stress on the eggs, propagation of phylloxera was considerably increased; thus shoot cultures are well suited for long-term propagation and storage under quarantine conditions.

This work establishes that the two forms of dual culture are appropriate for various experimental purposes. Compared to experiments under greenhouse conditions, dual *in vitro* cultures of grape and phylloxera offer several advantages envisaged in the introduction.

Summary

Using a *Vitis* hybrid, methods of long-term micropropagation of shoots and culture of hairy roots (transformed by *Agrobacterium rhizogenes*) were developed. The growth of these organ cultures was characterized. The cultures were used as feeding substrates for grapevine phylloxera. Starting with eggs, at 23.6 °C the parthenogenetic life cycle of the aphid proceeded *in vitro*.

Within 2 weeks after inoculation, more than 80 % of the shoot cultures responded with the formation of galls on young leaf blades and swellings on petioles, and, exceptionally, young shoots. At about the same time new eggs were deposited. Gall formation and propagation of phylloxera could be perpetuated for 2.5 years by aseptical transfer of eggs to freshly micropropagated shoots every 1—3 weeks.

Within 1 week after inoculation, more than 90 % of the younger parts of root cultures harboured larvae and responded with curvatures and thickenings. After 2 weeks, phylloxera oviposited and during the following weeks the number of eggs and animals increased considerably.

Thus, both forms of dual culture enable leaf and root gall formation and propagation of phylloxera excluding further organisms.

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