Aseptic Dual Culture of the Aphid *Chaitophorus salijaponicus* with Different Tissues of *Salix* Hosts

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The apterous generation of the viviparous aphid *Chaitophorus salijaponicus* was propagated aseptically on *in vitro* cultures of host shoots for more than 34 passages and 17 months. On the best suited host (*Salix alba*) a single nymph yielded a colony of 126.3 ± 24.4 individuals within 4 weeks. Other tissues than normal host shoots (callus, root cultures, fasciated shoots) did not support aphid propagation. Aphid colony development accelerated the senescence of host shoots and severely inhibited the development of adventitious root systems. The aspects of insect surface sterility, aphid colony development and its influence on the host are discussed.

Introduction

For the study of plant-parasite relationships dual aseptic cultures of the parasites and tissues of their host plants offer many potential advantages:

- preservation and propagation of parasites under quarantine conditions (exclusion of all other organisms);
- complete control of the chemical and physical environment;
- possibility of addition of metabolic precursors, inhibitors, or stains through the culture medium;
- easy cloning of plants and, possibly, parasites.

Although these advantages have been exploited in studying many associations between viruses, bacteria, fungi, and nematodes and their respective host tissues (Ingram and Helgeson, 1980) the use of dual cultures to study the long-term interaction between plants and phytophagous insects has been restricted to very few examples (Askani and Beiderbeck, 1991; 1994; Raman and Beiderbeck, 1992). Whereas aseptic host tissues can be grown in vitro easily, the preparation of aseptic insects is often limited by the availability of life stages resistant to sterilizing treatments, e.g. eggs of the grape Phylloxera (Rilling, 1975) or larval stages firmly adhering to their substrate (Raman and Beiderbeck, 1992).

This paper describes the dual culture of the viviparous aphid *Chaitophorus salijaponicus* with different tissues from species of its host plant genus *Salix*. The build-up of colonies of this aphid and its influence on the growth of micropropagated shoots are studied.

Material and Methods

In Vitro Cultures of plant tissues

Shoot cultures

Shoot cultures were obtained from clones of *Salix alba* L., *S.fragilis L.* and from clone *6/87* of *S.viminalis* L.(Institut f. Schnellwachsende Baumarten, Hannoversch-Münden) using single-node explants from greenhouse-grown plants. They were surface-sterilized (Neuner and Beiderbeck, 1993) and cultivated on medium ACM (Ahuja, 1983) supplemented with (mg * L^{-1}): Ca-gluconate 650, MES 500, IBA 0.1, BAP 0.05, sucrose 20000, gelrite^R (Roth, Karlsruhe) 2500. In some experiments hormone concentrations were modified as indicated.

Abbreviations: ACM, aspen culture medium; BAP, benzyladenine; IBA, indole butyric acid; MES, 2(N-morpholino)ethane sulfonic acid; NAA, α-naphthaleneacetic acid.

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Culture of fasciated shoots

Pathologically deformed shoots of *S.alba* ("wirrzopf" cultures) were cultivated on hormone-free medium ACM (Ihrig and Beiderbeck, 1995).

Root cultures

Hairy roots of *S.alba* obtained after infection with *Agrobacterium rhizogenes* were grown on a modified ACM medium (Hauth and Beiderbeck, 1992).

Callus cultures

Callus of *S.alba*, originally obtained from a mite gall, was cultured on a medium ACM supplemented with (mg * l^{-1}): Ca-gluconate 650, MES 500, NAA 0.9, BAP 0.5, sucrose 20000, gelrite® 5000.

Shoot and callus cultures were performed in test tubes (2.5 cm ø) covered with Sigma caps, root cultures in Petri dishes (9 cm ø) sealed with Parafilm®, and wirrzopf cultures in baby food jars closed with Magenta® caps.

The media were adjusted to pH 5.5–5.7 and autoclaved for 20 min at 1.2 * 10^5Pa. Cultures were kept under the following conditions:

24°C, light-dark regime of 12:12h with 800–2400 lux of fluorescent light (Sylvania GTE cool white). Root cultures were grown in darkness.

At the time of inoculation with aphids all cultures were in a state of active growth.

Establishment of dual cultures

Isolation of aphids

Accidentally, plants of *S.alba* growing in the greenhouse were colonized by the aphid *Chaitophorus salijaponicus niger* MORDWILKO 1929. Under aseptic conditions (clean bench) but without prior surface sterilization 6 virgines were transfered to 3–6 weeks old shoot cultures of *S.alba* by means of an insect pin mounted on a needle holder. To prevent escape of the aphids the culture tubes were covered with Cellophan® (sterilized in 70% ethanol for at least 15 min) and kept under culture conditions. 1 culture showed no visible contamination with microorganisms, and offspring from this culture were used for further experimentation.

Handling of aphids

Under aseptic conditions and using insect pins virgines were taken from aphid colonies younger than 4 weeks and transfered to healthy fresh plant cultures. Culture vessels were closed with Cellophan® and kept under culture conditions.

Surface sterility of aphids

Aphids from dual cultures were pressed onto plates with a medium consisting of beef extract, peptone, agar (DIFCO), sucrose and yeast extract (SERVA) (Lippincott and Heberlein, 1965), incubated at 27°C for 7 days and occasionally up to 4 weeks and screened for microbial contamination.

Evaluations

During 4 weeks following inoculation the development of aphids or aphid colonies in dual cultures and the influence on host tissue growth was studied by

- measuring the body lengths and head and abdominal widths using an ocular scale with a dissecting microscope;
- determining colony size and time for development of colonies;
- determining fresh weight of plant material and chlorophyll content (Arnon, 1960).

Mean values and standard deviations are reported.

Results

Aphid colony development during standard culture

Our standard culture was started with a single micropropagated plant of S.*alba* and a single individuum of *C.salijaponicus* in its 1st larval stage (virgo). The subsequent aphid development included three larval stages (3.3 days, 3.1 days and 2.6 days) which were followed by the adult stage lasting about 21 days. Compared to free living specimens (Müller, 1986) the adults grown *in vitro* were rather small with a body length of 1.5 ± 0.10 mm, a head width of 0.37 ± 0.02 mm, and an abdominal width of 0.75 ± 0.04 mm.

Under test tube conditions a single adult produced a parthenogenetic offspring of 43 ± 7. Larvae borne shortly before the death of their mother were often deformed or dead. Occasionally two types of deviating individuals appeared: dwarfs

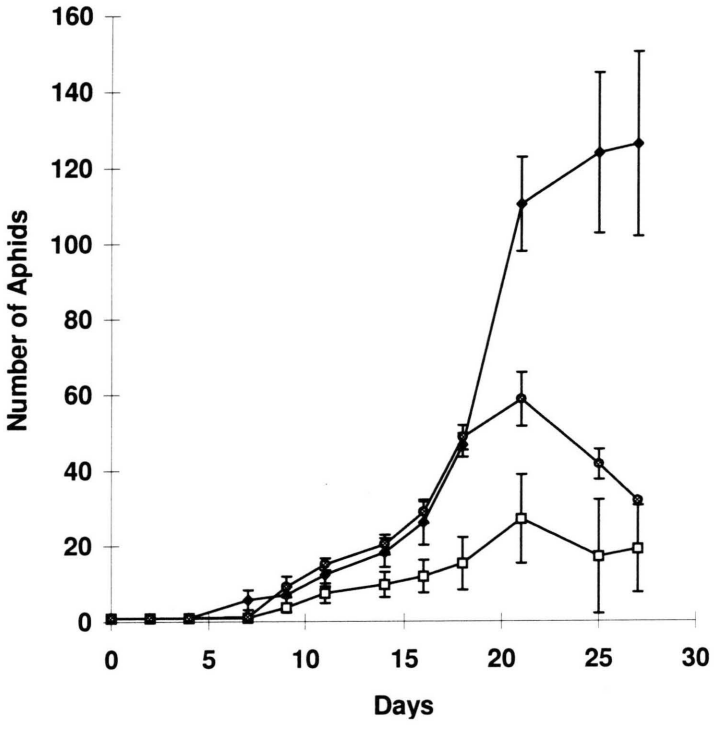

Fig. 1. The development of aphid colonies on shoots of different hosts inoculated with 1 aphid. ◆ *Salix alba;* □ *S.viminalis;* ☹ *S.fragilis.*

(body length less than 1 mm) which produced offspring of normal size and body proportions; and giants (body length more than 1.8 mm) which were unable to reproduce. Also, rarely, in cultures older than 4 weeks alate females were detected (frequency less than 10^{-3}).

Since during an extended culture period adults reaching an age of 2.3 days produced offspring by themselves colony size increased to 110.4 ± 12.5 individuals per test tube after 3 weeks and 126.3 ± 24.4 individuals per test tube after 4 weeks (Fig.1).

During this increase of the colony size the aphids spread from the inoculated organ (generally a leaf) to other leaves and, after 3 weeks, the shoot axis. After a colony size of 100 ± 20 individuals was attained larvae and adults started emigrating from the plant to the medium or the test tube surface.

On standard cultures *C.salijaponicus* was propagated continuously for more than 34 passages (2–4 weeks each) and more than 17 months.

The cultures produced seemed truly aseptic: In the beginning of the experiments the offspring of the starting culture was tested for surface contamination. From 16 aphids transfered to nutrient broth plates none produced any microbial colonies after incubation at 27°C for up to 4 weeks. And additional sterility tests during a period of 6 months did not reveal any contamination.

Aphid colony development in modified plant cultures

The standard culture was modified in different ways and aphid development studied:

Variation of phytohormone supply

Varying the BAP concentration from 0 to 0.25 mg * l^{-1} and the IBA concentration from 0 to 0.3 mg * l^{-1} did not affect duration of larval stages, adult body size, life expectancy or number of offspring. This may be a consequence of the rather narrow variation of hormone concentrations (Scheurer, 1976) which could not be extended without disturbing plant growth patterns *in vitro.*

Variation of the number of inoculated aphids

If *S.alba* plants were inoculated with 10 aphids instead of 1 a maximum colony size was obtained

Table I. The development of aphid colonies depending on inoculum size.

Day	Number of aphids	
0	1±0	10±0
5	1±0	10±0
10	13.4±3.2	98.0±17.3
15	25.0±2.5	110.0±23.1

much earlier, at day 15, after which colony size decreased by death of individuals (Table I).

Derooting of plants

From a number of plants (4–6 weeks old) the roots were cut off, the shoots transfered to fresh medium and inoculated with 10 aphids immediately. These rootless plants did not allow the development of large colonies: a maximum colony size of 39 ± 9.5 was obtained after 15 days which is less than half of that on rooted plants.

Inoculation of different tissues of *S.alba*

Aphids of different stages were inoculated onto hairy root, wirrzopf and callus cultures of the optimal host *S.alba*. On hairy roots (7 experiments) mortality was 46% after 3 days. Only on 2 plates offspring was produced and died within 5 days. On wirrzopf cultures (9 experiments) aphids did not survive 12 days and offspring died after 2 days. And on callus cultures (4 experiments) larvae of all stages and adults died within 8 days without producing offspring. Supposedly, a specific spectrum of allelochemicals responsible for successful interaction between aphids and their hosts is restricted to plant tissues in a specific stage of differentiation (Ananthakrishnan and Raman, 1993).

Inoculation of different *Salix* species

On *S.alba* shoots a single individuum generated a colony of 126.3 ± 24.4 within 4 weeks. Shoots of other *Salix* species were less effective hosts: On *S.viminalis* a maximum colony size of 27 ± 7.1 was obtained 3 weeks after inoculation and on *S.fragilis* a maximum colony size of 58.8 ± 15 (Fig.1).

Aphid influence on the development of S.alba plants

Senescence phenomena

At the time of inoculation with aphids the shoots had a length of 6.2 ± 1.6 cm which is about 2/3 of the final length attainable in the test tubes.

In spite of apical growth sooner or later these plants showed symptoms of senescence. Beginning at the shoot basis leaves started yellowing followed by leaf necrosis and/or abscission of lower leaves. These senescence processes were accelerated by colonization with aphids: Whereas on 100% of the control plants yellowing was observed after 20 days, on plants inoculated with aphids the same was observed 10 days after inoculation already. And 20 days after inoculation with 10 aphids all plants had heavy leaf necroses and some plants had died.

This acceleration of senescence processes is reflected by changes of the total chlorophyll content of the plants (Table II). Whereas in control plants the chlorophyll content increased to a maximum at day 20, in cultures inoculated with 1 aphid it remained constant and thereafter decreased more rapidly than in the control plants.

Table II. Chlorophyll content of control plants and plants inoculated with 1 aphid.

Day after inoculation	Chlorophyll content (mg * g FW^{-1}) Control plants	Plants with 1 aphid
0	0.59±0.09	–
5	0.69±0.07	0.62±0.15
10	0.83±0.1	0.61±0.08
20	0.96±0.04	0.62±0.07
25	0.83±0.13	0.47±0.08

Plant growth

By inoculation with 1 aphid the elongation growth of *S.alba* plants was not influenced significantly for up to 20 days. Later, growth of control plants continued whereas colonized plants stopped growing.

Because of considerable variation of in vitro plant growth a significant influence of aphid colonization on the fresh weight of rooted plants could not be detected.

Table III. Influence of aphid colonization on fresh weight of rootless shoots and on adventious root development of *S.alba*.

Day	Control plants			Plants with 10 aphids		
	mg Fresh weight	Roots per plant	Total root langth	mg Fresh weight	Roots per plant	Total root length
0	95.3±4.5	0.0±0	0,0 ±0	–	0.0±0	0.0±0
5	111.0±23.8	2.0±0	1.65±0.15	142.8±27.7	5.5±1.5	6.6±1.5
10	–	8.5±0.5	27.6 ±0.05	–	4.0±0	7.3±0.75
15	–	8.0±0	47.8 ±3.8	–	7.0±3.0	12.6±2.5
20	164.7±21.3	16.0±4.0	65.6 ±20.9	83.8±10.8	8.0±0	12.4±0.8

But on rootless shoots aphid colonization caused a heavy weight loss (Table III).

Adventitious root formation

Many (50–92%) shoots of *S.alba* started formation of adventitious roots 5 days after derooting. This process was strongly hampered by aphid colonization of the shoots. The final number of roots per plant and the elongation growth of the newly formed roots were reduced significantly (Table III).

Discussion

Establishment of dual cultures

Generally, the establishment of aseptic dual cultures consisting of insects and their plant hosts is impeded by the lack of insect stages resisting surface sterilization (see Introduction). Such stages are not available for viviparous aphids but there is increasing evidence that many aphid individuals are contaminated with microorganisms only weakly although they are handling carbohydrate-rich solutions (phloem sap, excretions): When individuals of *Dysaphis spp.* colonizing *Crataegus monogyna* were transfered to tissue culture media only 20% proved contaminated by microorganisms (experiments not reported). *Aphis illinoisensis* could be reared on micropropagated shoots of grape without prior sterilization and without contamination problems (Webb *et al.*, 1994). And in our experiments with *Chaitophorus salijaponicus* 1 out of 6 aphids proved noncontaminated and the other 5 were contaminated weakly. Therefore the presence of surface decontamination mechanisms is postulated, e.g. the deposition of antibiotic substances on aphid surfaces.

Propagation of Chaitophorus salijaponicus in dual culture

The parthenogenetic apterous phase of the life cycle of *C.salijaponicus* could be preserved on shoots of natural hosts under aseptic and controlled experimental conditions for more than 17 months. So, dual culture offers an excellent method of quarantine propagation of aphids where no synthetic diet is available.

Influence of Aphid colonization on host plant tissues

Under the experimental conditions the natural senescence of *S.alba* is accelerated by aphids presumably by the uptake of nutrients from the plant (see ref. in Thomas and Stoddard, 1980).

The most profound changes of plant growth were observed on rootless shoots where the aphid activities and the process of root initiation compete for nutrients supplied by shoots. This compares to observations with micropropagated *Veronica* plants colonized by *Myzus cerasi* (Askani and Beiderbeck, 1993).

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