Development of an *in vitro* Dual Culture System for Grapevine and *Xiphinema index* as a Tool for Virus Transmission

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Grapevine fanleaf virus (GFLV) is a nepovirus that is transmitted to grapevines by the ectoparasitic nematode *Xiphinema index*. GFLV causes severe losses in yield and quality in viticulture worldwide. Presently, laborious and time-consuming field trials or greenhouse tests are necessary for screening putative GFLV resistance in new grape genotypes developed in breeding programmes. We developed an *in vitro* dual culture system for grapevines and nematode vectors that requires less time and space than inoculation experiments done in the greenhouse. Virus infection of *in vitro* grapevines was investigated using immunocapture-reverse transcriptase-polymerase chain reaction (IC-RT-PCR) analysis. The development of root galls induced by feeding nematodes on *in vitro* grapevines was also analysed. Virus infection in grapevines in the dual culture with viruliferous nematodes was detected six weeks post-inoculation. Root galls were always absent from parasitised *in vitro* grapevines with detectable virus infection, whereas they developed on some parasitised, but virus-negative tested grapevines. Therefore, root galls cannot be used as a reliable indicator for parasitism and virus transmission.

INTRODUCTION

Grapevine fanleaf disease, caused by various nepoviruses, is responsible for severe losses in viticulture worldwide. The most economically important is grapevine fanleaf virus (GFLV), which is transmitted to grapevines by the soil-dwelling ectoparasitic vector nematode *Xiphinema index* (Longidoridae) (Andret-Link *et al*., 2004b; Hewitt *et al*., 1958). Natural resistance against grapevine fanleaf virus that could be used in breeding programmes has not been identified in any *Vitis* species. Consequently, several research groups focus their efforts on the introduction of virus resistance into rootstocks and scion varieties using a transgenic approach. This approach will require a simple and fast screening system for the evaluation of putative virus-resistant grapevines. However, some *Vitis* species reveal tolerance of *X. index*. This could reduce the rate of inoculation, but grapevines remain susceptible to the virus (Andret-Link *et al*., 2004a).

Mechanical standard inoculation procedures are not applicable in grapevine, and biolistic virus delivery and infection via electroporation methods are difficult (Valat *et al*., 2003a; Valat *et al*., 2003b). Green grafting or micrografting are effective to infect grapevines (Lahogue and Boulard, 1996; Lahogue *et al*., 1995), but due to the high virus load that is delivered into the vascular system, this method seems to be inappropriate to evaluate virus resistance established at the cellular level, which is the place of virus replication (Lahogue and Boulard, 1996; Staudt, 1997; Valat *et al*., 2003a). Virus transmission using the vector nematode mirrors natural conditions and, therefore, this inoculation method will lead to the most reliable assessment of virus inoculation. However, testing candidate grapevines in field trials over several years is laborious, expensive and time consuming (Vigne *et al*., 2004; Walker and Wolpert, 1994). Furthermore, although nematode-mediated virus transmission under greenhouse conditions is possible, it is inconvenient for screening large numbers of candidate grapevines within a short time (Valat *et al*., 2003a).

The aim of the present work was to develop an *in vitro* dual culture system for virus transmission via *Xiphinema index* that will need few resources in terms of space and time. Parameters required to establish the *in vitro* dual culture were investigated, as well as their effect on nematode survival. Furthermore, the frequency of nematode-mediated virus infection and root gall development in *in vitro* grapevines was also investigated.

MATERIAL AND METHODS

Establishment of a *Xiphinema index* population

Xiphinema index was reared under greenhouse conditions on *Ficus carica*, which provided a permanent source of nematodes that could also be used as an aviruliferous control population. Viruliferous nematodes were obtained from exposure on GFLV-infected *Vitis* varieties (Herold, Portugieser, Müller-Thurgau and Sylvaner). The plants were grown in sand (grain size 0.2 to 0.5 mm), which was heat sterilised at 200°C for 8 h, and was placed in 3 L pots. The field capacity of the sand substrate was adjusted to approximately 60%. Field capacity at 100% is the

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amount of liquid held by the soil after excess water has drained due to gravitational force. The content of liquid was calculated by weighing and subtracting the dry weight of the substrate. Based on the weight of substrate and liquid, a percentage less than 100% of field capacity was calculated and adjusted.

In vitro culture of grapevine

In vitro grapevines were established from cuttings obtained from plants grown under greenhouse conditions. Young shoots were harvested, cleaned with ethanol (70%), cut into one-node segments and surface sterilised with CaOCl (7%) for 20 min. Cuttings were rinsed three times with sterile water and placed into culture tubes containing $^1/_2$ MS salts and vitamins (Murashige and Skoog, 1962), supplemented with 2.0% sucrose and 0.3% gelan gum without growth regulators. Standard growth conditions for *in vitro* grapevines were 24°C and a 16/8 h light cycle (60 µmol m^{-2} s^{-1}). These conditions enabled the growth of explants (5BB, 125AA, Binova, SO4) with a multiplication rate of five cuttings obtained from one plantlet within six weeks.

In vitro dual culture of *Xiphinema index* and grapevine

For the *in vitro* dual culture, sand (140 g, grain diameter 0.2 to 0.5 mm) was used as substrate. The sand was placed in $^1/_2$ L WECK jars, which were closed with a lid and a felt ring for better ventilation. The jars containing sand were autoclaved, dried and $^1/_2$ MS medium without gelan gum was added to the substrate to obtain a field capacity of 100%. To adjust the field capacity to 100%, 37 mL of liquid was added to 140 g of sand. The medium with or without sucrose was adjusted to a pH of 5.8 or 7.0 respectively. Two grapevine cuttings were cultivated in each jar under standard growth conditions. *Xiphinema index* was added to the *in vitro* cultures after three weeks culture initiation, when the roots were approximately 2 cm long and the shoot length had reached 2 to 4 cm. During the pre-incubation period, the field capacity of the sand substrate reached a value of about 60% due to evaporation. For dual culture initiation, nematodes of the greenhouse stocks were washed out, collected on a sieve with a mesh diameter of 40 µm, and adult individuals were handpicked using a bristle. Subsequently, nematodes were rinsed several times with sterile water to reduce the contamination of the dual culture during incubation. An additional disinfection treatment of the nematodes before inoculation, as described by Bavaresco and Walker (1994), was not carried out because of the reported negative effects on *X. index* survival and feeding behaviour. Desiccation of the nematodes on the substrate surface was prevented by placing the nematodes directly on the roots with the help of a sterile spatula. On the outside, the bottom of the jar was covered with tinfoil to avoid irritation of the nematodes by light influx. For the analysis, the plants were uprooted and the nematodes were washed out and collected for extraction.

Virus detection in grapevine and nematodes

Immunocapture (IC) reverse-transcriptase (RT)-PCR was used as a highly sensitive and reproducible system for virus detection in the grapevines, based on the description of Wetzel *et al*. (1992; 2002) and Valat *et al*. (2003b). For the IC step, samples of grapevine leaves and roots were homogenised separately in grapevine extraction buffer (Bioreba, Reinach, Switzerland) and chilled on ice. Microplates (Nunc) were coated (15 mM Na_2CO_3, 33 mM $NaHCO_3$, pH 9.6) with GFLV-specific antibody (1:1000, Bioreba, Reinach, Switzerland), incubated at 37°C for 4 h and rinsed three times with washing buffer (137 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 5% Tween, pH 7.4). Samples (150 µL) were loaded on coated microplates and incubated at 4°C overnight. After rinsing the microplates three times with washing buffer, viral RNA was released from the virus particles by vortexing with 20 µL TritonX100 (10%, 70°C) for a few seconds.

Different protocols are described for RNA extraction from nematodes (Esmenjaud *et al*., 1994; Van der Wilk *et al*., 1994; Demangeat *et al*., 2004). However, for the extraction of total RNA from *X. index* we followed the protocol of Van der Wilk *et al*. (1994), which provides RNA applicable for RT-PCR assays within a short time. Nematodes were ground with glass beads by vortexing in 150 µL DEPC-treated water for 5 min. After extraction with chloroform, the RNA was precipitated with 2.7 vol ethanol and 0.1 vol sodium acetate (3 M, pH 5.2) and dissolved in 10 µL DEPC-treated water. Two µL of the obtained samples were analysed using the one-step RT-PCR kit Superscript II RT/Platinum Taq (Invitrogen, Karlsruhe, Germany), following the manufacturer's instructions. Species-specific primers (10 mM) targeting the internal transcribed spacer (ITS) region of *Xiphinema index* were used for RT-PCR to check the quality of the extraction and to verify the species (Wang *et al*., 2003, Hübschen, unpublished). The thermal scheme for one-step RT-PCR was as follows: reverse transcription at 50°C (30 min), initial denaturation at 94°C (5 min), 40 cycles of denaturation at 94°C (1 min), annealing at 57°C (45 sec), extension at 68°C (2 min), and a final elongation step at 68°C (10 min). The *Xiphinema index*-specific primers yield a PCR product of 340 bp.

The presence of virus in grapevines and nematodes was checked by GFLV-specific RT-PCR. Three µL of RNA samples were used for amplification with one-step RT-PCR, with the following thermal cycling scheme: reverse transcription at 50°C (30 min), initial denaturation at 95°C (5 min), 40 cycles of denaturation at 95°C (30 sec), annealing at 55°C (30 sec), extension at 68°C (45 sec), and a final elongation step at 68°C (7 min). The GFLV-specific primers (10 mM, for 5´-TAC CGA CTG GGA CGA ACA CAT TGG-3´, rev 5´-AGA TTC ACG CCT TGG TTC CTC CTG-3´) target a conserved region of the viral movement protein (MP) and yield an amplicon of 298 bp. PCR products were separated by electrophoresis in an ethidium bromide-containing agarose gel (1.0%) and were visualised with UV-light illumination.

RESULTS

Development of the dual culture system *in vitro*

For the cultivation of *Xiphinema index* and grapevine plants in an *in vitro* dual culture system, the substrate for culturing *X. index* in the greenhouse and *in vitro* conditions for grapevines were combined. For each experiment, four to six one-node cuttings per rootstock (two per jar) were cultured. Experiments were repeated twice. The different rootstocks showed similar growth and, therefore, they were not further discriminated in the presented results. As no plant developed using LS medium (Linsmaier and Skoog, 1965) in initial experiments, only $^1/_2$ MS medium was used for the further studies. The results of the preliminary experiments with grapevines grown *in vitro* on sterilised sand as substrate under different conditions are summarised in Table 1.

Root development and shoot growth were improved by adding 2% sucrose to the $^1/_2$ MS medium. Furthermore, a medium at pH 5.8 yielded a more extended root system compared to a medium at pH 7.0. The roots of plants grown in the *in vitro* dual culture system showed root hairs, a pale brownish cortex, and were less fragile compared to roots grown in gelan gum-solidified medium in *in vitro* standard culture. The root system formed in the sand substrate appeared similar to the roots of grapevines grown in pots under greenhouse conditions.

A three-week period of pre-culture of the *in vitro* cuttings provided the plants with well-developed roots suitable for inoculation with *X. index*. The incubation time following the inoculation with 20 nematodes per jar was six weeks. Nematode survival rates of approximately 75% were found in sucrose-free jars, whereas nematodes were hardly able to survive in the dual cultures established with sucrose-containing medium (Table 1). There were only slight differences in the survival rate of nematodes between the medium at pH 5.8 and at 7.0 respectively, but covering the outside of the jar bottom with tinfoil increased the survival rate (Table 1). In contrast to the results obtained by Sultan and Howard (1991), we found little effect of water content of the substrate in the initial experiments as long as the water content was within the range of 40 to 70% field capacity. To establish suitable conditions for plant development and the survival of the nematodes, jars with sucrose-free $^1/_2$ MS medium (pH 5.8) with tinfoil covering the jar bottoms were selected for further inoculation experiments.

Inoculation experiments

With reference to Staudt (1997), who suggested 20 plants per experiment for evaluation in greenhouse tests, we used this number of plants in our *in vitro* dual culture experiments. In each of the two experiments, 20 rootstock plantlets were inoculated with 20 nematodes per jar after three weeks pre-culture. After additional incubation of six weeks, a total of 37 of the plants could be analysed. The roots of 14 plants (38%) tested positive for GFLV using IC-RT-PCR. Six of the plants with infected roots also revealed systemic infection of the shoot. Plants of all four test rootstocks were affected and no difference in susceptibility to virus infection or frequency of parasitism by the nematodes was found. The survival rate of the nematodes was 89% in these experiments. Nematodes were collected from each jar and pooled after the experiment, and total RNA was extracted. Before inoculation, the species and stage of development of individual *X. index* were verified by microscopy. The species was also confirmed through RT-PCR of total RNA extracts with *X. index*-specific primers (Figure 1A). The RT-PCR assay with GFLV-specific primers revealed the presence of viral RNA in the extracts from the nematodes (Figure 1B).

Root galls were rarely found in the dual culture and did not appear before three weeks post-inoculation (Figure 2A). The *in vitro* galls were smaller and less conspicuous than the root galls from heavily-infested greenhouse cultures (Figure 2B), which revealed swollen tissue that often was accompanied by necrosis with small dark brown or even black spots.

Systemically, GFLV-infected grapevines were first found three weeks post-inoculation by investigation of leaf samples. However, the incubation period of the experiments lasted six weeks. Selected examples of inoculated grapevines analysed through IC-RT-PCR six weeks post-inoculation are presented in Figure 3. Roots and leaves of grapevine 1 (G1: R1, L1) indicate systemic infection, whereas in grapevine 2 (G2: R2, L1) virus infection was only detectable in the roots. Grapevine 3 (G3: R3, L3) is shown as an example of a parasitised but non-infected plant. It is important to note that the roots of the GFLV-positive grapevines G1 and G2 reveal no gall formation. Grapevine G3 showed root galls, although virus infection could not be detected in this plant. Overall, root galls were never visible after a six-week incubation period on GFLV grapevines that had tested positive, but galls developed on the roots of three GFLV grapevines that had tested negative.

The presence of root galls in only those *in vitro* grapevines that had tested negative for GFLV after inoculation could be due to the feeding of aviruliferous nematodes. However, aviruliferous nematodes were rarely found in the stocks reared on GFLV-infected grapevines in the greenhouse. Aviruliferous individuals occur when attached virus particles are lost during the moult stage (Taylor and Robertson, 1970). Before becoming viruliferous again, the nematode needs to feed on a virus-infected plant. Alternatively, an explanation for the *in vitro* grapevines with root galls that tested negative for GFLV could be that the gall devel-

TABLE 1

The effect of various parameters influencing grapevine development and the survival of vector nematodes in in vitro dual culture.

Tested plants (n)	Culture media $^1/_2$ MS[2]	Cuttings with roots[3] (%)	Cuttings with shoots[3] (%)	Inoculated X. *index*[4] (n)	Surviving X. *index*[5] (%)
14	pH 5.8 / 10g/L sucrose	88	94	140	5
16	pH 5.8	75	81	160	51
14	pH 7.0 / 10g/L sucrose	94	94	140	2
14	pH 7.0	69	94	140	36
12	pH 5.8 / 10g/L sucrose[1]	75	67	120	0
16	pH 5.8[1]	75	25	160	75
12	pH 7.0 / 10g/L sucrose[1]	75	92	120	3
12	pH 7.0[1]	42	33	120	73

Two plants per jar; [1] bottom of jar was covered with tinfoil; [2] field capacity for pre-culture was adjusted to 100%;
[3] growth of plants estimated three weeks after pre-culture; [4] inoculation with 20 individuals per jar;
[5] estimated six weeks post-inoculation.

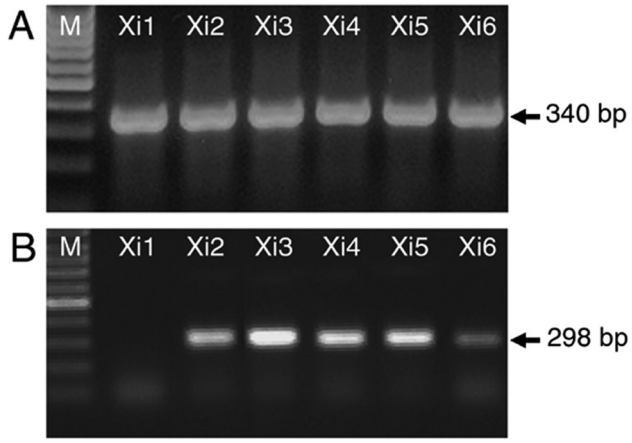

FIGURE 1

Identification of *Xiphinema index* and GFLV by RT-PCR of RNA extracts from pooled samples of 10 individuals each. (A) Detection of *X. index*, (B) detection of GFLV; Xi 1: *X. index* population from ficus (GFLV-free); Xi 2 to 6: different *X. index* populations reared on GFLV-infected grapevines; M: GeneRuler 100 bp DNA-ladder, Fermentas.

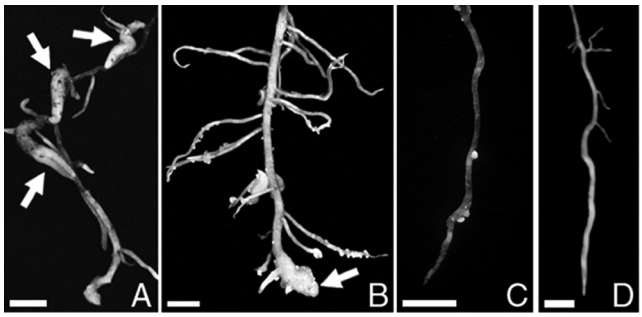

FIGURE 2

Grapevine roots with galls induced by parasitising *Xiphinema index*. (A) Root from *in vitro* sand culture showing gall development three weeks post inoculation (arrows). (B) Root from greenhouse culture with intensive gall formation, several months post inoculation (arrow). Healthy grapevine roots from *in vitro* (C) and greenhouse plants (D). Scale: 5 mm.

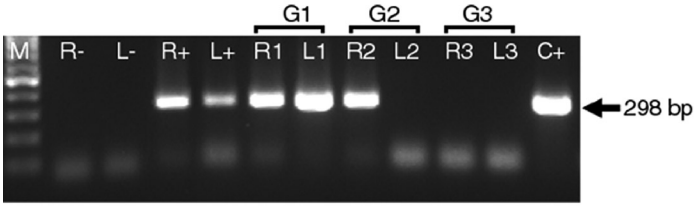

FIGURE 3

Virus detection by IC-RT-PCR. Non-infected roots (R-) and leaves (L-) as negative controls, and roots (R+) and leaves (L+) of a GFLV-infected grapevine as positive controls. Root and leaf samples (R1/L1 to R3/L3) from grapevine plants (G1 to G3) grown in the *in vitro* dual culture system for six weeks. Grapevine G3 developed root galls, but gall development was absent on the roots of grapevines G1 and G2. GFLV-specific amplification product of 298 bp. C+: cDNA of GFLV. M: GeneRuler 100 bp DNA ladder, Fermentas.

opment of the parasitised roots might decrease the expansion and spreading of the virus due to compartmentalisation of the affected plant tissue. This could cause a delay or even inhibition of the infection in the roots of *in vitro* grapevines. However, the fact that the *in vitro* grapevine plants revealing virus infection after the inoculation did not develop root galls due to the parasitism of nematodes presently cannot be explained, because the trigger that is responsible for gall development is still unknown.

DISCUSSION

The establishment of an *in vitro* dual culture system for the inoculation of grapevine plants with GFLV using the natural transmission path has been described. The advantage of *in vitro* dual culture compared to infection experiments in greenhouses or in nematode-infested field trails is the shortening of the incubation time to approximately six weeks before detection of the established virus infection in the plant. In addition to the three weeks pre-culture, the entire inoculation experiment lasted only nine weeks. As reported by Staudt (1997), inoculation experiments on greenhouse grapevines last several months. Valat *et al.* (2003a) reported an overall nine-month period to complete virus transmission and infection under greenhouse conditions.

Due to the use of $^1/_2$ L jars for the *in vitro* dual culture, the infection experiments need much less space than the experiments performed with containers in greenhouse experiments. Furthermore, the *in vitro* inoculation experiments were performed in a growth chamber under controlled environmental conditions, which are more stable than greenhouse conditions.

The small number of viruliferous nematodes per test (20 nematodes two *in vitro* plants) in the *in vitro* dual culture system allows higher numbers of infection experiments compared to greenhouse trials. Bouquet (1981) used 50 to 300 individuals per kg of soil, while Valat *et al*. (2003a) performed infection experiments in 25 m^3 containers containing 100 to 150 viruliferous *X. index* per kg of soil. Similarly to greenhouse and field experiments, the infection rate of grapevines in the *in vitro* dual culture system may depend on the activity and the feeding behaviour of the nematodes. In our nematode stock population in the greenhouse, we found variability in propagation and feeding characteristics, but could not identify any influencing parameters.

Although the infection rate of about 38% seems to be low in the inoculation experiments with the *in vitro* dual culture, it should be noted that the incubation time was only six weeks. Lahogue *et al*. (1995) reported an infection rate of 51% after an incubation period of six months for virus inoculation by grafting. Considering this, the infection rate obtained with the *in vitro* dual culture appears absolutely competitive. Compared to micro- or green grafting of candidate genotypes onto infected grapevine rootstocks, this dual culture system simulates a natural infection process and theoretically enables a better evaluation and interpretation of resistance. Grafting methods can lead to a high load of virus particles in the candidate plant via the vascular system, making it difficult to efficiently inhibit the replication of the virus by a resistance mechanism at the cellular level. Lahogue and Boulard (1996) reported the limited reproducibility of the green grafting technique and concluded that green grafting was inappropriate for the identification of virus-resistant grapevine cultivars due to high inoculum pressure. Comparative experiments

with different infection systems (e.g. green grafting, mechanical inoculation, bombardment) using transgenic, putative virus resistant (Jardak-Jamoussi *et al*., 2003; Reustle *et al*., 2003) and susceptible grapevines are in progress.

Furthermore, this study shows that the development of galls on the parasitised roots of *in vitro* grapevines is not a reliable indicator for nematode feeding and virus transmission. The development of root galls is obviously not always initiated by feeding nematodes on the roots of *in vitro* grapevines, because the *in vitro* grapevines that revealed GFLV infection after inoculation with nematodes did not develop root galls. It is recommended than an analysis with RT-PCR be performed to investigate if plants are infected with the virus after using the dual culture for inoculation.

CONCLUSIONS

The described reduction in space and time for virus inoculation with the *in vitro* dual culture system enables the testing and evaluation of large numbers of putative virus-resistant grapevines. New candidate plants developed and cultured *in vitro* can be tested directly without any adaptation to greenhouse conditions for inoculation experiments. Using the *in vitro* dual culture, only plants susceptible to GFLV should become infected after the feeding of viruliferous nematodes on the roots. Plant lines with a reliable resistance should theoretically not become infected with GFLV by nematodes. Candidate grapevines revealing GFLV infection after six weeks post-inoculation can be recognised and sorted out in an early stage of selection. Apart from this, uninfected plants of the same line with the identical genetic background should be evaluated with particular caution. These plants also might not have reliable virus resistance.

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