Dual Culture of *Plasmopara viticola* and Grapevine and Its Application to Systemic Fungicide Evaluation

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ABSTRACT

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Successful dual cultures of *Plasmopara viticola* and grapevine (cvs. Cabernet Sauvignon and Cabernet Franc) were achieved by tissue culture techniques. A simple and rapid method of inoculation to produce large numbers of dual cultures for experiments is described. Following inoculation, sporangia were produced on leaves and stems after 5 days and leaf chlorosis appeared 7 days later. Viable sporangia have been maintained in culture for 12 wk, and regular subculture of grapevines and reinoculation with sporangia have maintained dual cultures for 9 mo. The dual cultures were used to demonstrate the systemic and curative properties of metalaxyl on grapevine downy mildew. Results suggested that the system may be used to screen fungicides or to compare these properties in various fungicides. The system could also be used to screen grape cultivars rapidly for resistance to *P. viticola*.

The downy mildew fungus *Plasmopara viticola* (Berk. et Curt. ex de Bary) Berl. et de Toni, an obligate parasite of grapevine (*Vitis vinifera* L.), is difficult to maintain for experiments as it not only requires the culture of the host but also the provision of conditions favorable for infection and sporulation. The advantage of using tissue culture techniques to grow and maintain this parasite was first realized by Morel (9), who successfully inoculated grapevine callus tissue cultures with aseptic sporangia of the fungus and established dual cultures. He was able to maintain dual cultures for extended periods by subculture and reinoculation and to obtain aseptic sporangia from these cultures for experiments.

Since Morel's (9) pioneering experiments, 22 dual cultures of other obligate parasites and their hosts have been established (2,6,7). However, these parasites, including *P. viticola,* have all been established with callus as their host, and there have been no reports of dual cultures of obligate parasites with tissue-cultured plants. Dual culture has been used as a simplified experimental system for the investigations of structure and physiology of host-parasite interaction of several obligate parasites and has provided a source of contaminant-free spores or mycelium for physiologic experiments (7). Although sporangia of *P. viticola* isolated from vine callus were used in fungicide tests (10,11), the use of dual cultures as a simplified system for fungicide testing has not been reported.

Considerable advances have now been made with clonal propagation of plants by tissue culture methods (14). Because successful clonal propagation of grapevine has recently been reported (3), the dual culture of *P. viticola* and tissue-cultured plants was attempted. This paper describes the establishment of the dual culture and its application as a simple experimental system to evaluate metalaxyl to control grapevine downy mildew.

MATERIALS AND METHODS

Grapevine cultivars Cabernet Sauvignon and Cabernet Franc were propagated by tissue culture methods (3). Following initiation of cultures in liquid medium, shoots were proliferated on 30 ml of solid medium in 250-ml polycarbonate containers (78 × 76 mm) with clear screw caps. Shoots excised from proliferating cultures were rooted in 28-ml polycarbonate tubes (76 × 25 mm) containing 10 ml of one-half strength Murashige and Skoog (15) basal medium gelled with 0.7% (w/v) Difco Bacto agar. All cultures were maintained at 23 ± 1 C under constant 3.0 klux illumination provided by cool white fluorescent light on a 16-hr photoperiod.

The isolate of *P. viticola* used was obtained from naturally infected leaves collected from a vineyard at Blackwood, a suburb of Adelaide, South Australia. A suspension of sporangia prepared by shaking sporulating leaf pieces with distilled water was used to inoculate Sultana (syn. Thompson Seedless) plants maintained in the glasshouse. The inoculum was applied to leaves with a hand sprayer; following inoculation, the leaves were enclosed in clear polyethylene bags for 48 hr. Infected leaves were detached 10–14 days later, and sections of leaf with "grease spot" symptoms were excised and surface-sterilized by dipping in 70% alcohol for 30 sec followed by a 5–10 min immersion in 0.5% sodium hypochlorite solution containing 0.01% Citowet wetting agent. The leaf sections were rinsed three times in sterile distilled water and then placed on moistened filter paper in sterile petri dishes, which were sealed by wrapping with Parafilm M. After 48 hr of incubation at 20 C in the dark, sporangia produced on leaf sections appeared free of contaminants and were used as a source of aseptic inoculum.

Table 1. Effect of metalaxyl on the production of *Plasmopara viticola* sporangia on cultivars Cabernet Franc and Cabernet Sauvignon

Treatment	Leaves (no.)	Leaves (no.) in each disease category[w]					
		0	1–25	26–50	51–75	76–100	
Cabernet Franc							
Control (water)	94	38	25	15	12	4	a[x]
Metalaxyl at 25 mg a.i./L[y]	66	19	25	13	9	0	a
Metalaxyl at 25 mg a.i./L[z]	93	66	22	2	3	0	b
Metalaxyl at 50 mg a.i./L[y]	39	18	18	3	0	0	c
Metalaxyl at 25 mg a.i./L, incorporated in agar	104	104	0	0	0	0	d
Cabernet Sauvignon							
Control (water)	26	9	6	1	6	4	a
Metalaxyl at 25 mg a.i./L[z]	22	15	7	0	0	0	b
Metalaxyl at 25 mg a.i./L, incorporated in agar	33	33	0	0	0	0	c

[w] 0 = no sporangia on leaves, 1–25 = 1–25% of leaf surface covered with sporangia, etc.
[x] Treatments with no letter in common are significantly different at the 5% probability level.
[y] Applied 4 days after inoculation.
[z] Applied 3 days after inoculation.

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Dual cultures were initiated by the modified inoculation technique described for grapevine callus (12). With the aid of a dissecting microscope and fine-pointed forceps, sporangia were removed from incubated leaf sections and transferred to droplets of sterile distilled water placed previously on the undersurface of leaves of in vitro plants. When a few dual cultures were established, sporulating leaves were used as a source of aseptic inoculum to infect large numbers of in vitro plants. This was done by removing plants from polycarbonate tubes, trimming their roots, and then dipping the shoots in a suspension of sporangia (2×10^3/ml) prepared 30 min before by shaking sporulating leaves in sterile distilled water. Plants were inoculated when they were at least 60 mm high and had produced five to 11 leaves. Following inoculations, the plants were immediately transplanted into 20 ml of fresh medium in larger (150 ml; 105×42 mm) polycarbonate tubes. All operations were carried out in a laminar flow cabinet. Dual cultures were incubated in identical light and temperature conditions as with tissue cultures. Observations were made regularly on the development of the fungus in dual cultures.

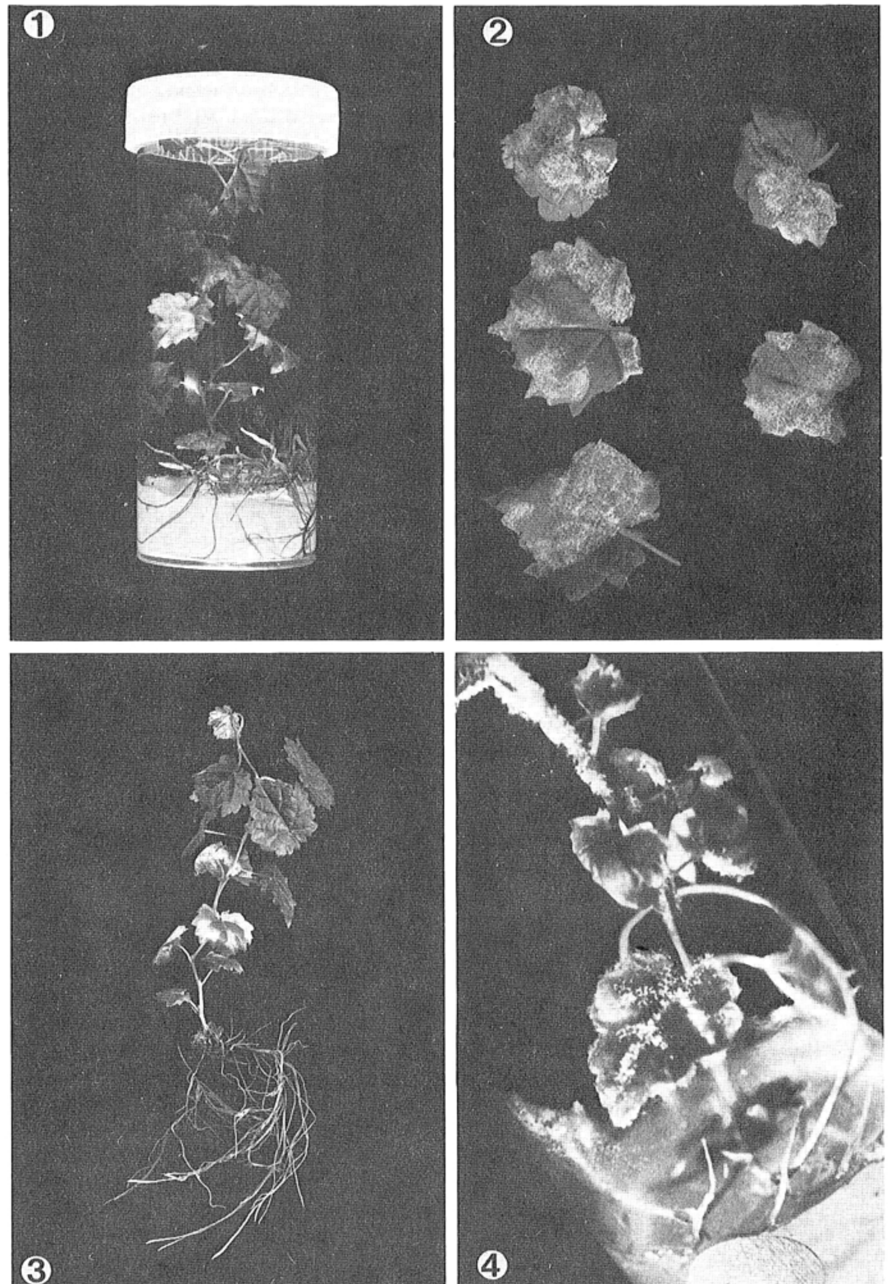
Metalaxyl [*N*-(2,6-dimethyl-phenyl)-*N*-(methoxyacetyl) alanine methylester] as Ridomil, 25% active ingredient (a.i.) wettable powder, was used to test the dual culture as a means of evaluating the systemic and curative properties of a fungicide. A sterile stock solution of metalaxyl at 1 g a.i./L was prepared by dissolving 2 g of Ridomil in 4 ml of acetone and making up to 500 ml with sterile distilled water. To test the curative properties of the fungicide, plants of Cabernet Sauvignon and Cabernet Franc were individually removed from tubes 3 or 4 days after inoculation and the shoots were immersed for 15–20 sec in a suspension of metalaxyl at either 25 or 50 mg a.i./L prepared by diluting stock fungicide solution with sterile distilled water. Control plants were dipped in sterile distilled water containing 0.04% acetone, a concentration similar to that used in the treatment with Ridomil at 50 mg/L. Plants were gently shaken and drained after dipping to prevent excess fungicide solution draining into the agar medium after they were replanted into tubes.

The systemic properties of the fungicide were evaluated by transplanting plants immediately after inoculation into 150-ml tubes containing 20 ml of culture medium to which metalaxyl at 25 mg a.i./L had been added. All plants were incubated until sporulation was evident in nil fungicide treatments, after which they were removed from tubes to determine the number of sporulating leaves and the area of sporulation (Table 1). Leaves were assessed by estimating the leaf area covered with sporangia and rating each leaf into one of the categories shown in Table 1. The data were analyzed using a log-linear model assuming the error distribution approximated the Poisson distribution. The analysis produces the "loglikelihood ratio" statistic, which is asymptotically χ^2 distributed (5). This was used to group different treatments according to whether treatments and disease rating were independent.

RESULTS AND DISCUSSION

Successful infections of tissue-cultured plants of grapevine cultivars Cabernet Sauvignon and Cabernet Franc were achieved by inoculation with aseptic sporangia of *P. viticola,* and dual cultures of the host and parasite were established (Figs. 1–4). The initiation of dual cultures by placing sporangia directly into drops of water on the undersurface of leaves was tedious and resulted in low numbers of infected leaves. The subsequent technique of dipping grapevine shoots into a suspension of sporangia was more successful; more than 60% of the leaves became infected, and the fungus sporulated on not less than 40% of these leaves on their lower surfaces (Table 1). This relatively simple technique facilitated the inoculation of large numbers of plants and gave consistent results, allowing it subsequently to be used in fungicide experiments.

In both grapevine cultivars, the first sign of infection was sporulation that appeared 5 days after inoculation. This incubation period is similar to that reported on grapevines by others (8).



Figs. 1–4. Dual culture of Cabernet Franc grapevine and *P. viticola* 4 wk after inoculation. **(1)** An infected plant in culture. **(2)** Sporulation on the undersurface of leaves. **(3)** Chlorotic leaf symptoms occurring with continued healthy shoot growth. **(4)** Sporulation on the upper leaf surface and stem.

Most sporulation occurred on the undersurface of the leaves (Fig. 2), but some also formed on stems and on the upper leaf surface (Fig. 4). Sporulation on the upper leaf surface is rare on grapevines in the field. Its occurrence in tissue-cultured grapevines may be caused either by changes in the thickness of the epidermis (enabling the conidiophores to penetrate directly through the upper leaf surface) or by the increase in numbers of stomata (through which sporulation occurs on the upper leaf surface). Although neither of these aspects was investigated, it is known that tissue-cultured plants have juvenile leaves (4,13), and the epidermal thickness and stomatal number could vary considerably from adult leaves. The sporangia produced in dual cultures were normal but were borne on relatively long conidiophores (500–1,300 μm), in contrast with those (300–500 μm) reported for *P. viticola* in the field (1).

Chlorotic leaf symptoms in dual cultures appeared 7 days after the production of sporangia. The production of sporangia 1–2 days before the symptoms were visible is apparently not uncommon in other situations where conditions favor fungus sporulation. It appeared that conditions in dual culture favored the host physiology and resulted in a long delay in symptom expression. The leaf symptoms observed in dual cultures (Fig. 3) were typical of those described for infected grapevines (1). After a few weeks in culture, most of the infected and sporulating leaves were alive; in some plants, however, chlorotic leaves and infected stems became necrotic. Following inoculation, no plants were killed, and infected plants continued to grow from shoot tips and axillary buds (Fig. 3). No sporulation occurred on the new growth, which suggests that infections had not become systemic and also that secondary infection did not occur on the plants.

Dual cultures were maintained for up to 12 wk before subculturing was necessary. This differs from dual cultures with grapevine callus tissue, which required subculturing every 4–6 wk (9,12). Our studies showed that the viability of sporangia produced within 3 wk after inoculation was greater than 80%. Although the viability of older sporangia was not measured, sporangia obtained from 12-wk-old cultures were infective when used as inoculum. With regular subculture of the grapevine and reinoculation with the fungus, dual cultures have been maintained for more than 9 mo.

Both grapevine cultivars in dual culture with *P. viticola* were used successfully to evaluate the systemic and curative properties of metalaxyl. When inoculated plants were incubated for 3–4 days before the shoots were dipped in metalaxyl at 25 or 50 mg a.i./L, there was a considerable reduction in the numbers of leaves bearing sporangia and in the area of sporulation when compared with the untreated inoculated plants (Table 1). Complete inhibition of sporulation did not occur, possibly because of the low rates of fungicide used and also the time of application—in this case, 1 and 2 days before sporangia appeared. No sporangia were produced on inoculated plants growing in medium containing metalaxyl at 25 mg a.i./L (Table 1).

The results have confirmed the curative (17) and systemic properties of metalaxyl (16). They show that the dual culture technique may also be a useful method to screen fungicides or to compare the curative and systemic properties of different fungicides for the control of grapevine downy mildew and other obligate parasites. The propagation of grapevines by the tissue culture technique described is rapid, and abnormal genetic conditions were not detected in plantlets (3). Hence, the dual culture system could also be used as a means of rapidly screening grapevine cultivars for resistance to *P. viticola.*

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