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Semi-selective media for the isolation of *Phaeomoniella chlamydospora* from soil and vine wood

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Summary. Two semi-selective culture media, F10S (PDA + folpet 10 ppm + streptomycin sulphate 1 g l^{-1}) and RB150S (PDA + rose bengal 150 ppm + streptomycin sulphate 1 g l^{-1}), were developed for the isolation of the phytopathogenic fungus *Phaeomoniella chlamydospora* from soil samples and vine tissues. The media were selected so that they would allow proper growth of the pathogen and would partially inhibit eleven other common fungal genera. Eight antifungal agents were tested: Folpan (a.i. folpet), Captazel (a.i. captan), Benlate (a.i. benomyl), Chipco (a.i. iprodione), Switch (a.i. cyprodinil + fludioxonil), rose bengal, and the bactericidal antibiotic streptomycin sulphate at several doses. Recovery of *Pa. chlamydospora* from wood samples was 40% better on RB150S and 50% better on F10S than on PDA, while the contaminants were reduced by 42% with RB150S, and by 48% with F10S. Pathogen reisolation from artificially contaminated soil samples was improved with F10S, while RB150S facilitated pathogen detection in samples containing moderate amounts of *Rhizopus, Penicillium, Alternaria* or *Trichoderma* or in soils heavily contaminated with bacteria. F10S and RB150S improved the isolation of *Pa. chlamydospora* from wood and soil and can be used as alternatives to current culture media.

Key words: rose bengal, esca, folpet, Petri disease, selective isolation.

Introduction

Phaeomoniella chlamydospora (W. Gams, Crous, M. J. Wingf. & L. Mugnai) Crous & W. Gams is a major causal agent of Petri disease (Mugnai *et al*., 1999), which causes decline and dieback of young grapevines, and it is also involved in esca. This fungus can be detected by a molecular technique, the amplification of Internal Transcribed Spacer (ITS) fragments of ribosomal DNA (rDNA), using specific primers. But even though a rapid method for its detection is available, its isolation and cultivation in a culture medium is still indispensable to study its biology and to acquire a better understanding of the disease aetiology and the inoculum sources.

Various modes of dispersal have been suggested for this fungus, including by colonization of pruning wounds (Larignon and Dubos, 2000), through vine propagation material (Ridgway et al., 2002; Halleen et al., 2003; Fourie and Halleen, 2004), and by per-

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sistence in the soil (Rooney et al., 2001; Whiteman et al., 2002; Damn and Fourie, 2005). Vine tissues and soil must therefore be considered potential sources of inoculum, although so far there have not been any reports of positive isolation from naturally infested soil. The possible inoculum density in the soil is therefore unknown, but the usual concentration of soil fungi is about 105 cfu g-1 (Singleton et al., 1992). The isolation of Pa. chlamydospora from vine tissue and soil in common synthetic media is particularly difficult due to the co-presence of fast growing micro-organisms. Therefore, it is important to formulate semi-selective media.

A semi-selective isolation medium is usually based on the exclusion of undesired micro-organisms, thus permitting the preferential growth of the desired fungus. For this purpose, in this study we examined the response of three isolates of *Pa. chlamydospora* and eleven other fungi frequently found as contaminant microflora or as pathogens involved in esca or Petri disease, on twenty combinations of eight chemicals with fungistatic and/or antibiotic effects at different doses, with the ultimate aim of improving the isolation of *Pa. chlamydospora* from the soil and from vine wood.

Materials and methods

Fungal isolates

The three isolates of *Pa. chlamydospora* used in this study (221.0, 1.44 and 7.01) had been isolated by our group from diseased vines collected in central Spain between 2001 and 2005 and were identified by morphological and molecular methods. Eleven fungal isolates (*Alternaria alternata, Fusarium oxysporum, Epicoccum nigrum, Rhizopus stolonifer, Coniothyrium* sp., *Botryosphaeria obtusa, Cylindrocarpon macrodidymum, Trichoderma* sp., *Penicillium italicum, Fomitiporia mediterranea* and *Phaeoacremonium aleophilum*) obtained during the isolation of *Pa. chlamydospora* from naturally infected vines were tested as accompanying micro-organisms.

Culture media

The basal medium potato-dextrose-agar (PDA, Difco, Detroit, Michigan, USA) and PDA with eight antifungal and antibacterial agents were tested: Folpan (a.i. folpet 80%, Aragro, Madrid, Spain); Captazel (a.i. captan 50%, Syngenta Agro, Madrid, Spain); Benlate (a.i. benomyl 50%, Agrex, Villafranca Padovana, Padova, Italy); Chipco (a.i. iprodione 50% p/p, Bayer CropScience, Alcacer, Valencia, Spain); Switch (a.i. cyprodinil 37.5% + fludioxonil 25%, Syngenta); rose bengal (Sigma-Aldrich, Madrid, Spain) and the antibiotic streptomycin sulphate (Duchefa Biochemie, Haarlem, The Netherlands) at different concentrations. Captan was tested at 1 and 10 ppm and benomyl at 1 and 5 ppm. Some of the media were also supplemented with streptomycin sulphate (1 g l^{-1}). Final formulations are shown in Table 1. Rose bengal was added to the medium before autoclaving. Plates with rose bengal were protected from light by wrapping in aluminium foil to avoid photo-oxidation, which increases the toxicity of this agent (Edwards and Seddon, 2001). The other additives were added after the medium had been autoclaved and cooled down to 45–50°C.

In a first series of experiments, the effect of the twenty media on *Pa. chlamydospora* was evaluated. In a second step, the growth and spore germination of the associated mycoflora were tested using those formulations which had been found to be less toxic to *Pa. chlamydospora*, or which had a low inhibitory effect considered acceptable for a semi-selective medium.

Mycelial growth

Mycelial growth of the *Pa. chlamydospora* isolates was evaluated on the various media (Table 1). Plugs of agar, 5 mm in diameter, were removed from the edge of four-week-old colonies actively growing on PDA, and inoculated in 85-mm diameter Petri dishes containing 15 ml of medium per dish. Dishes were incubated at 25°C in darkness. Radial growth of the colonies was recorded at 2-day intervals for two months by measuring two perpendicular diameters. Mycelial growth of any associated fungi was measured daily for 20 days, or until the mycelium of the fungus reached the edge of the dish. Mean daily growth rates were calculated and compared with the control on unamended PDA. The experiment was performed three times.

Sporulation

Three plugs of agar, 5 mm in diameter, were removed from the actively growing edge of a 4-week-old colony grown in each medium-isolate combination, placed in 10 ml plastic tubes with 5

ml of sterile distilled water, and vortexed for five seconds at 250 rpm (Whiting *et al*., 2001). The final concentration of the conidial suspension was determined by counting the spores with a haemocytometer. Three repetitions per treatment were used and the experiment was repeated twice.

Spore germination

Spores were harvested from the media by adding 10 ml of sterile distilled water to each Petri dish and gently rubbing the colony surface with a sterile scalpel. The suspensions were filtered through a sterile Whatman 113V filter (30 μm) and their concentration adjusted to 10^4 spores per ml. Dishes with 10 ml of water agar (WA, Panreac Química, Barcelona, Spain) and PDA amended with the fungicidal and bactericidal compounds were inoculated by adding 200 µl of the suspension, which was spread with a sterile glass rod. After 72 hours of incubation at 25ºC in darkness, spore viability was assessed by counting the germinated spores out of 100 observed per dish. A conidium was considered to have germinated if the germ tube was at least one-half the length of the spore as described by Dhingra and Sinclair (1995). Plates with unamended WA and PDA were used as controls. The experiment was performed four times.

Isolation from vine wood and soil samples

Media that permitted suitable growth of *Pa. chlamydospora* (F10S and RB150S) were also tested for *Pa. chlamydospora* isolation from wood and soil samples.

Fourteen vine-wood samples (one per vine) were collected from esca-symptomatic vines. Segments of the wood with its bark (2 cm^3) were surface-sterilized for 1 min in 70% ethanol and for 1 min in 2% NaOCl and rinsed in sterile distilled water. The segments were then sectioned with a sterile scalpel and wood pieces (5×1×1 mm) from the inside were plated onto the different media (five pieces per dish). Dishes were incubated at 25ºC in darkness and inspected for twenty days. Colonies of *Pa. chlamydospora* and other micro-organisms were recorded. The *Pa. chlamydospora* isolation rate was calculated as the number of fragments rendering positive isolation divided by the total number of fragments plated. In the same way, the contamination rate was calculated as the number of contaminated fragments divided by the total number of fragments. The isolation and contamination rates were calculated as the means of the 14 samples (5 replicates per sample).

To detect and isolate *Pa. chlamydospora* from natural soils, two sets of experiments were performed. First, five 800-g soil samples from each of two vineyards in the Madrid region were collected at a depth of 5–10 cm around symptomatic vines. Each sample contained soil from 4–5 points around the vine. *Pa. chlamydospora* was not detected by species-specific PCR (Pch1-Pch2 primers from Tegli *et al*., 2000) in any of these samples. Spore suspensions of *Pa. chlamydospora* were obtained as described for the spore germination tests and the concentration was adjusted to 10^4 conidia per ml. Soils were inoculated with 1 ml of spore suspension per gram of soil, and gently mixed by hand. One gram each of every soil + suspension mixture was placed in 250 ml flasks containing 150 ml sterile distilled water. The soil suspensions were mixed in a rotating shaker for 30 min at 150 rpm (De Cal *et al.,* 1996). Two hundred μl of each suspension was placed on each dish of each medium (3 dishes per medium) and spread with a sterile glass rod. Dishes were incubated at 25ºC in darkness and the number of colonies of *Pa. chlamydospora* and other micro-organisms was recorded after 20 days. Reisolations were considered positive when uncontaminated separated colonies of *Pa. chlamydospora* were obtained despite the presence of some contaminants in the same dish. The isolation rate was calculated for each of the 10 soils as the percentage of dishes with a colony presence.

In the second experiment, three soil samples were similarly collected from 3 vineyards in the Madrid region. The spore suspension was a mixture of five *Pa. chlamydospora* isolates (Pch9, Pch223, 9.27, 221.0 and 9.43) at 10^2 conidia per ml. The soils were inoculated with 1 ml of the *Pa. chlamydospora* suspension per dish (85-mm diameter plastic Petri dishes with 20 g of soil each, 18 dishes per soil) and were incubated at 10ºC in darkness for 6 months. To determine *Pa. chlamydospora* and contaminant recovery, 0.2 g soil per dish was suspended in 0.4 ml sterile distilled water, vortexed for 60 sec, diluted to 1/100, and 20 μl per dish was placed on each medium tested. This dilution allowed colony counting without excessively favouring the target pathogen over less concentrated contaminants. Dishes were incubated at 25ºC in darkness and the number of

colonies of *Pa. chlamydospora*, bacteria and saprobic fungi was recorded after 20 days. Isolation and contamination rates were calculated as the means of the number of colony-forming units per dish observed in the 3 soils (54 replicates).

Statistical analysis

Mycelial growth data were subjected to GLM repeat measures ANOVA. Two variables, *Pa. chlamydospora* isolate and formulation, were considered grouping factors (between variables), and time was introduced as the repeated factor (within variables). Within-subject effects were analysed with multiple paired t-tests using the Bonferroni adjustment, while between-subject effects were estimated using Tukey's post-hoc comparison test.

One-way ANOVA was used to compare the mycelial growth rates of the accompanying microflora and sporulation, while the GLM procedure was used for spore germination analysis. Means were separated using Tukey's multiple range and the Duncan tests. All statistical analyses used SPSS software version 14.0.

Results

Mycelial growth of *Pa. chlamydospora*

The three *Pa. chlamydospora* isolates showed a very similar response to the different formulations ($F=2.60$, $P=0.12$). The between-subjects statistical test revealed significant effects ($F=751.94$, $P<0.001$) for the variable formulation. The greatest daily myc-

Table 1. Denomination and formulation of the culture media that allowed colony growth, and the growth parameters of *Pa. chlamydospora* analysed: mycelial growth rate, sporulation and conidial germination (after 72 h). Means followed by different letters differ significantly at $P=0.05$ by Tukey's test.

Medium denomination	Medium formulation[a]	Mycelial growth[b] (mm day^{-1})	Sporulation[c] (conidia ml^{-1})	Conidial germination (%)
PDA/WA	PDA/WA	1.1±0.4 b	6.1×10^6 d	99.8±0.3 a
S	SS 1 g l^{-1}	0.7±0.1 e	5.9×10^6 d	19.4±1.3 h
F0.5	Folpet 0.5 ppm	1.1±0.0 b	5.9×10^6 d	31.8±0.3 f
F1	Folpet 1 ppm	1.1±0.0 b	ND	ND
F10	Folpet 10 ppm	1.1±0.1 a	6.5×10^6 c	66.3±1.6 b
F0.5S	Folpet 0.5 ppm + SS 1 g l^{-1}	0.7±0.1 e	5.9×10^6 d	27.0±1.5 g
F10S	Folpet 10 ppm + SS 1 g l^{-1}	0.8±0.1 d	6.9×10^6 b	50.7±3.0 c
F1S0.5	Folpet 1 ppm + SS 0.5 g l^{-1}	0.9±0.1 c	7.7×10^6 b	36.7±0.7 e
RB10	RB 10 ppm	0.9±0.1 c	5.7×10^6 d	6.7±1.0 j
RB 50	RB 50 ppm	0. 5±0.1 f	ND	ND
RB150	RB 150 ppm	0.5±0.0 f	7.2×10^6 b	43.9±3.8 d
RB10S	RB 10 ppm + SS 1 g l^{-1}	0.5±0.1 f	6.3×10^6 d	16.1±1.3 i
RB50S	RB 50 ppm + SS 1 g l^{-1}	0.4±0.1 g	ND	ND
RB150S	RB 150 ppm + SS 1 g l^{-1}	0.4±0.1 g	1.3×10^7 a	41.3±1.6 d
FRBS	Folpet 1 ppm + RB 60 ppm + SS 0.2 g l^{-1}	0.7±0.1 e	8.9×10^6 a	18.2 ±3.21 hi
Chipco	Iprodione 1 ppm	0.3±0.1 g	none	9.1±3.4 j
Switch	CIP 1 ppm + FLU	0.1±0.1 h	none	23.4±3.3 gh

[a] SS, streptomycin sulphate; RB, rose bengal; CIP, ciprodinil; FLU, fludioxonil. Basal medium for all formulations: potato-dextrose agar (PDA) for the mycelial growth and sporulation tests, and water agar (WA) for the conidial germination tests.
[b] Data given are the means of five replicates ± standard error.
[c] Data given are the means of three replicates ± standard error.
ND, not determined.

elial growth rates were obtained on folpet at 10 ppm without streptomycin sulphate, followed by those on PDA and folpet at 0.5 and 1 ppm (Table 1), but growth rates greater than 65% of the control were also obtained on RB10, F1S0.5, F10S and F0.5S and on PDA amended with streptomycin sulphate. Mycelial growth rates on RB50, RB150 and RB10S were not significantly different from each other, nor were those on F0.5 and F1, or on RB50S and RB150S. The benomyl and captan formulations completely inhibited growth of *Pa. chlamydospora*, and Switch did so almost completely (Table 1).

Sporulation of *Pa. chlamydospora*

Colonies obtained from the mycelial growth experiments were used to test for sporulation. ANOVA revealed significant effects (F=298.88, $P<0.001$) for the variable formulation. The best sporulation occurred on media supplemented with high concentrations of rose bengal and folpet, and on these media amended with streptomycin sulphate (RB150S, FRBS, RB150, F1S0.5, F10S and F10) (Table 1).

Spore germination of *Pa. chlamydospora*

All amended media significantly decreased (F=2051.32, $P<0.001$) spore germination of *Pa. chlamydospora*, although germination on F10 and F10S, and on RB150 and RB150S, was still more than 50% and 40–45% of conidia respectively, compared to the control (Table 1).

The formulations F10S and RB150S were selected for further testing with the accompanying microflora because of their low inhibitory effects on *Pa. chlamydospora*, combined with their broad spectrum antibacterial activity.

Mycelial growth of accompanying microflora

Mycelial growth of the accompanying fungi studied was tested on PDA, F10S and RB150S for 20 days. Only *Rhizopus, Trichoderma* and *Alternaria* reached the edge of the control dishes, after 2, 5 and 14 days respectively (Table 2). ANOVA detected a significant effect for the factors formulation (F=1157.17, $P<0.001$) and fungal genera (F=4616.01, $P<0.001$). *Rhizopus* had a significantly faster growth rate on all the media tested, being followed by *Trichoderma* and *Alternaria* (Table 2). Analysis of the formulation effect revealed that all fungal genera had a significantly slower growth rate in the amended media than in PDA, except for *Rhizopus, Trichoderma and Penicillium* on F10S, whilst with RB150S the reductions of these fungi were 36, 38 and 73% respectively. There were also differences between the two semi-selective media, except with *Epicoccum, Coniothyrium* and *Botryosphaeria*. F10S particularly decreased the growth rate of *Botryosphaeria* (by 77%), *Epicoccum* (62%) and *Fusarium* (60%). RB150S caused the greatest mycelial growth reduction on *Botryosphaeria* (80%), *Penicillium* (73%), *Epicoccum* (63%), *Phaeoacremonium* (56%) and *Fomitiporia* (55%) (Table 2).

Spore germination of accompanying microflora

Spore germination of the accompanying microflora was examined on F10S and RB150S (Table 2). Spore germination strongly responded to formulation (F=12387.74, $P<0.001$). The two media did not differ significantly in their effect on *Phaeoacremonium, Rhizopus* and *Epicoccum*, decreasing spore germination by close to 60% for *Phaeoacremonium*, 66% for *Rhizopus* and 94% for *Epicoccum*. F10S inhibited sporulation more strongly than RB150S in *Cylindrocarpon*, *Fusarium* and *Penicillium* (86, 94 and 94% vs. 54, 61 and 60% respectively). RB150S was more effective against *Alternaria* and *Trichoderma* than F10S (96 and 20% vs. 86 and 0%). Neither medium enabled spore germination of *Botryosphaeria* or *Coniothyrium*.

Isolation from vine wood and soil samples

F10S and RB150S increased the isolation of *Pa. chlamydospora* from wood samples of naturally infected grapevines by 50 and 40% respectively, compared with the control on PDA. On F10S the contamination rate decreased by 48%, and on RB150S by 42%.

These two media were also tested for *Pa. chlamydospora* reisolation from artificially-inoculated vineyard soils. In the two assays, re-isolation of *Pa. chlamydospora* was 64 and 59% higher on F10S, when compared with PDA; on RB150S the first assay recovered *Pa. chlamydospora* from 70% of the dishes, 57% more than on PDA. Both these media dramatically reduced growth of bacteria (F10S by 96% and RB150S by 100%) and of saprobic fungi (49% on F10S, and 39% on RB150S) as compared with the control (Fig. 1). The fungi isolated consisted predominantly of *Fusarium* and *Penicillium* species.

Table 2. Daily mycelial growth rate (mm diameter) of accompanying fungi on three culture media (PDA, F10S and RB150S) and spore germination on FS10 and RB150S media.

Genus	Mycelial growth rate (mm day^{-1})[a]						Germination (%)[b]	
	PDA		F10S		RB150S		F10S	RB150S
Rhizopus	40.0±15.3	aA	40.0±15.7	aA	25.5±9.6	aB	32.9	35.6
Epicoccum	4.4±2.3	dA	1.7±0.9	eB	1.6±1.2	dB	5.4	7.8
Fusarium	3.2±1.6	dA	1.3±0.7	fC	1.8±0.6	cB	6.4	39.4
Coniothyrium	3.0±1.1	eA	2.0±0.9	dB	2.0±1.1	cB	0.0	0.0
Botryosphaeria	2.5±1.0	eA	0.6±0.5	gB	0.5±0.5	fB	0.0	0.0
Cylindrocarpon	2.6±1.1	eA	2.1±0.9	dB	1.8±0.9	dC	14.0	46.4
Alternaria	6.5±3.8	cA	4.9±2.3	cB	4.0±1.6	bC	13.7	3.7
Trichoderma	16.9±0.0	bA	16.9±0.0	bA	10.5±0.3	bB	100.0	80.0
Penicillium	3.8±1.2	dA	3.2±1.5	cdA	1.0±0.1	eB	6.0	39.6
Fomitiporia	3.5±0.1	dA	2.9±0.2	dB	1.6±0.2	dC	N.A	N.A
Phaeoacremonium	2.6±0.3	dA	1.8±0.1	eB	1.2±0.4	eC	43.3	39.0

[a] Means followed by different letters differ statistically (Tukey's test, $P<0.05$). Small letters compare mycelial growth rates of the different fungi on the same medium (columns) and capital letters compare the three media for the same fungal genus (rows). Data given are the means of five replicates ± standard error.
[b] Data given are means of three replicates.

N.A., not applicable.

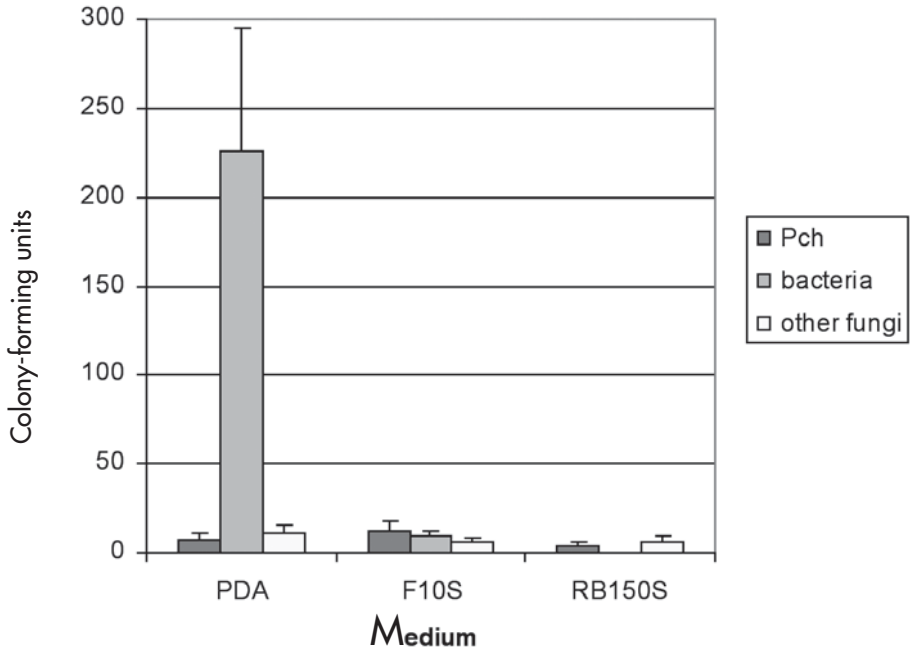

Fig. 1. Colony-forming units (CFUs) of *Phaeomoniella chlamydospora*, bacteria and saprobic fungi per Petri dish, grown on three culture media (PDA, F10S and RB150S) after inoculation with soil suspensions and incubation at 25°C in darkness for 20 days. Data given are the means of 54 replicates. Bars represent standard errors.

Discussion

The chemicals tested in this study were chosen to formulate a semi-selective medium for the isolation of *Pa. chlamydospora*, which would partially control contaminant microflora. Mycelial growth, sporulation and conidial germination of *Pa. chlamydospora* were assessed on PDA amended with fungicides having the following active ingredients: folpet (phthalimmide), captan (dicarboximide), benomyl (benzimidazol), iprodione (dicarboximide), cyprodinil/fludioxonil (anilopyrimidines), rose bengal (dye) and streptomycin sulphate (aminoglycoside), with different modes of action. Jaspers (2001) tested the efficacy of twenty-two fungicides against *Pa. chlamydospora*. Of the fungicides we selected folpet and iprodione on account of their low control of the pathogen. Captan and benomyl were chosen because of their broad spectrum action. Captan in particular controls *Alternaria*, which is frequently isolated as a contaminant, especially from soil samples. Rose bengal is generally added to inhibit bacteria and to restrict fungal growth (Tsao, 1970). Streptomycin sulphate is a broad-spectrum antibiotic commonly used in culture media with also some inhibitory effect on fungal growth.

The effect of penicillin G and streptomycin sulphate at 1, 2, 3 and 4 g l^{-1} against *Pa. chlamydospora*, *Pm. aleophilum*, *Pm. inflatipes*, *Fomitiporia punctata, Cylindrocarpon destructans, Stereum hirsutum, Fusarium equiseti, Clonostachys rosea*, *Aspergillus, Penicillium, Trichoderma, Cladosporium,* and *Rhizoctonia* species was compared by Redondo (2003), who found that streptomycin alone is a suitable component of a semi-selective medium for *Pa. chlamydospora* isolation. Redondo *et al.* (2004) also tested *in vitro* the effects of triadimephon (triazol) and hymexazol (dicarboximide) on the mycelial growth and conidial germination of *Pa. chlamydospora, Phaeoacremonium* spp. and *F. punctata*; these fungi were markedly inhibited by the two fungicides.

The medium amended with a high concentration of folpet (F10) stimulated *Pa. chlamydospora* growth. Other formulations which increased *Pa. chlamydospora* radial growth by more than 75% were F0.5, F1, RB10, F1S0.5 and F10S. These media were considered to be the most appropriate for mycelial growth. The formulations with rose bengal moderately inhibited *Pa. chlamydospora*, to at least 33% of the controls. This property, which rose bengal also exhibits against other fungi (Edwards and Seddon, 2001), could facilitate the counting of colony-forming units when necessary.

A chemical antagonism seems to exist between rose bengal and streptomycin sulphate since when they were applied mixed they were not twice as effective in reducing mycelial growth as when each was applied singly. None of the benomyl or the captan formulations supported fungal growth, the results obtained with benomyl confirming Groenewald *et al.* (2000). Cyprodinil almost completely inhibited mycelial growth.

Sporulation tests were conducted to determine whether these chemicals interfered with the conidiogenesis of *Pa. chlamydospora*. Several formulations actually stimulated sporulation. Toubia-Rahme *et al.* (1995) found a stimulatory effect with sulphur and carbendazim. In our work, rose bengal in combination with streptomycin (RB150S) and folpet (FRBS) were the most effective, nearly doubling the number of conidia obtained on PDA. Sporulation on F1S0.5, RB150, F10S and F10 was also more abundant than on PDA.

A semi-selective medium for *Pa. chamydospora* should support not only the mycelial growth of this fungus but also its spore germination, because spores also initiate colonies on the isolation medium, especially in the case of soil samples. Although the conidial germination of *Pa. chlamydospora* decreased in all the tested media compared with the control, an acceptable response was achieved with the highest concentrations of both folpet and rose bengal, and with these agents when combined with streptomycin.

To select the best culture media in terms of all three parameters of fungal development (mycelial growth, sporulation and spore germination), these parameters were examined together. Although streptomycin sulphate moderately reduced mycelial growth of *Pa. chlamydospora*, it was retained in the formulation because it effectively controlled the numerous bacteria frequently occurring together with *Pa. chamydospora*. Folpet and rose bengal were most effective at the highest concentrations. The inhibitory action of rose bengal at 150 ppm has been demonstrated against a range of fungi (Baggerman, 1981). For these reasons, F10S and RB150S were tested as potential inhibitors of the interfering microflora.

The eleven fungal genera included in our assays (*Alternaria*, *Fusarium*, *Epicoccum*, *Rhizopus*,

Coniothyrium, *Botryosphaeria, Cylindrocarpon, Trichoderma, Penicillium, Fomitiporia* and *Phaeoacremonium*) were selected because, in our experience and that of several authors (Serra *et al*., 2000; Fourie *et al*., 2001; Larignon *et al*., 2001; Hallen *et al*., 2003) these are some of the most frequent natural contaminants or pathogens involved in esca, Petri disease and other grapevine declines (Phillips, 2002; Hallen *et al*., 2004). The two media selected in the study inhibited mycelial growth of these genera except for *Rhizopus, Trichoderma* and *Penicillium*, whose growth rate was significantly reduced only by RB150S.

When spore germination on F10S and RB150S was analyzed, both media strongly inhibited *Epicoccum* and totally inhibited *Botryosphaeria* and *Coniothyrium*, while they achieved a lower but still significant level of control of *Phaeoacremonium* and *Rhizopus*. F10S inhibited germination of *Cylindrocarpon*, *Fusarium* and *Penicillium* better than RB150S. In contrast, RB150S was more effective against *Alternaria* and *Trichoderma*.

To test the effectiveness of these media with natural samples, two isolation assays were performed: direct isolation of *Pa. chlamydospora* from wood samples coming from naturally infected grapevines, and re-isolation from artificially inoculated vineyard soils. The amount of *Pa. chlamydospora* isolated from surface-sterilized wood samples incubated on RB150S and F10S was 40 and 50% greater respectively than that on PDA, while the contaminants were reduced by 42 and 48% respectively. Fungi that grew in the semi-selective media did so only to acceptable levels.

Soils from different vineyards were collected to provide a more diverse soil composition and microflora for testing. These soil samples were artificially inoculated with *Pa. chlamydospora* at two inoculum concentrations, and the pathogen was successfully reisolated on both F10S and RB150S. Rose bengal-amended agar has been used before to analyse soil fungi (numerous papers after Martin, 1950). For *Pa. chlamydospora* and *Phaeoacremonium inflatipes* rose bengal-amended agar was used with good results by Rooney *et al*. (2001). In our work, the *Pa. chlamydospora* count increased by 64 and 57% in F10S and RB150S respectively when the initial inoculum concentration was high (10^4 conidia per ml). At the lowest inoculum concentration (10^2 conidia per ml), the pathogen count in F10S increased by 59%, but the number of colonies in RB150S decreased by 50%. This shows the importance of inoculum density. Both media effectively limited bacterial growth and fungal contamination.

It is concluded that the semi-selective medium F10S (PDA + folpet 10 ppm + streptomycin sulphate 1 g l^{-1}) allowed satisfactory growth of *Pa. chlamydospora* and gave acceptable control of most of the representative interfering microflora, thus improving *Pa. chlamydospora* isolation from vine wood and soil samples. *Pa. chlamydospora* has still not been isolated from a naturally contaminated soil, even though Damn and Fourie (2005) demonstrated its presence there with a PCR-based molecular method. RB150S (PDA + RB 150 ppm + streptomycin sulphate 1 g l^{-1}) was also suitable, especially when *Pa. chlamydospora* had to be isolated from samples containing *Rhizopus, Penicillium, Alternaria, Trichoderma* or samples with a heavy bacterial contamination.

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