DEVELOPING TESTS TO DETERMINE VIABILITY OF *CIBORINIA CAMELLIAE* (KOHN) SCLEROTIA

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ABSTRACT

Two methods were developed to assess the viability of *Ciborinia camelliae* (Kohn) sclerotia for subsequent use in assays of sclerotial parasitisation. In the first method, external contaminants were removed by washing the sclerotia twice in 13.5% NaOCl and soaking them in antibiotics. Bisected sclerotia grown on potato dextrose agar for 9 days at 20°C produced identifiable colonies of *C. camelliae*. In the second method, sclerotial softness, which is proposed as an indicator of decay, was measured. The compression energy required to push a 2.76 mm diameter penetrometer with a force of 40 N into a healthy sclerotium gave an indication of sclerotial softness.

Keywords: *Ciborinia camelliae*, camellia blight, sclerotial viability, disinfestation.

INTRODUCTION

Camellia flower blight, caused by the sclerotial-forming fungus *Ciborinia camelliae* (Kohn), is widespread in North America, Japan, Europe and most regions within New Zealand (Taylor *et al.* 1999). The disease survives between seasons as sclerotia, which are the only source of inoculum for infection of camellia blooms in spring. Disease management by eradication of sclerotia is currently being investigated, using a range of sclerotial mycoparasites reported to be effective against *Sclerotinia sclerotiorum* (Rabeendran *et al.* 1998), a pathogen closely related to *C. camelliae* (Kohn and Nagasawa 1984). Before the efficacy of these biocontrol agents in killing sclerotia could be evaluated, a method was needed to reliably assess sclerotial viability. Mycelial production from sclerotia was selected as an indicator of viability in preference to stipe production because it was considered to be more reliable under laboratory conditions. However, many contaminants were found within the rinds of sclerotia of *C. camelliae* which confounded results. Because the contaminants originated from the decaying flower petals which had been trapped within the outer 1-2 mm of each sclerotium, they could not be eliminated by commonly used surface sterilisation techniques and, therefore, more stringent methods were needed. This paper reports on the development of a method for surface-cleansing sclerotia and stimulating mycelium production. A method for assessing sclerotial viability by measuring their firmness using a penetrometer is also described.

METHODS

Sclerotia were collected from the Wellington Botanical Gardens on 14 August 1999, washed over a 4 mm sieve to remove soil and loose sclerotial fragments, air-dried and sorted into size classes by mean weight, 0.16 g (0.005), 0.49 g (0.008) and 1.10 g (0.020) for small, medium and large sizes, respectively (standard error of the means shown in parentheses).

Surface-cleansing of sclerotia

In all washing and rinsing operations, sclerotia were vigorously agitated using a KS250 IKA Labortechnik table shaker at 300 rpm at room temperature. Sclerotia were washed in 2 ml of the disinfectant solutions and rinsed three times in total; once in

15 ml of sterile distilled water (SDW) containing 0.04% Tween 80 and twice in 15 ml of SDW. Sclerotia were then air-dried at room temperature within a laminar flow cabinet before determining viability.

Preliminary tests evaluated washes of 4.8% NaOCl with and without ethanol, for a single 30 min wash and two 10 min washes. Because these washes did not prevent contamination, a series of wash treatments with higher concentrations of available chlorine were evaluated. These comprised 30% CaOCl (Scientific Supplies, Auckland) or 13.5% NaOCl (Hypostat 135, Wilson Chemicals), alone or combined with absolute ethanol (50/50 v/v) in single washes for the two time periods, 5 or 20 min. In addition, 3 and 30 min double washes, using 13.5% NaOCl with and without ethanol (50/50 and 70/30 v/v) in a number of permutations (Table 1) were also tested.

Dissection and incubation of sclerotia

After disinfection treatments, sclerotia were stimulated to produce mycelium by two aseptic dissection techniques: by bisecting sclerotia or by totally removing their rinds. The sclerotia whose rinds were removed had their last cut faces placed onto growth medium. For the bisected sclerotia, one half of each was soaked for 5 min in a solution of 20 ppm streptomycin sulphate (SIGMA Chemical Co., St. Louis, USA), 40 ppm aureomycin (5.5% chlortetracycline, Cyanamid Australia PTY Ltd, West Victoria) and 250 ppm ampicillin (Ampesco, Solon, Ohio, US). The antibiotic-treated and untreated halves of each sclerotium were placed 40 mm apart, medulla side down onto growth medium.

In an attempt to limit microbial contamination, four growth media were tested: (1) potato dextrose agar (PDA, DIFCO), (2) PDA containing 20 ppm streptomycin, 20 ppm aureomycin and 250 ppm ampicillin, (3) number 3 Whatman filter paper moistened with 2 ml 0.1% Murashige and Skoog (MS) solution and (4) *S. sclerotiorum*-selective medium (Bourdôt *et al.* 2000). Plates containing the treated sclerotia were arranged in a randomised block design of 20 replicates, and incubated at 15 or 20ºC and 12 h diurnal light. After 4-13 days incubation, plates were assessed, with the aid of a Zeiss binocular microscope at 15-40 x magnification, for contaminants and for *C. camelliae* mycelium growing out from the sclerotia. Mycelium of *C. camelliae* was recognised by its densely-compacted colony morphology. The method which gave greatest recovery of *C. camelliae* mycelium was selected for a final test.

Sclerotium firmness

Because parasitised and decaying sclerotia had regularly been observed to be very soft (A. Stewart, pers. comm.), it was postulated that sclerotial firmness could be used as an indicator of parasitism. Medulla segments, 6 mm thick, were cut from 300 medium and 300 large healthy sclerotia and their moisture content standardised by storing at 85% relative humidity, in a desiccator bowl containing saturated sodium chloride, at room temperature for 8 days. Sclerotial firmness was then determined using an Instron 4444 penetrometer (Instron USA). This measured the force on a 2.76 mm diameter probe travelling at 50 mm/min, from initial contact with the face of the sclerotium up to a compression force of 40 N. The compression energy (Nmm) was calculated from the area under standard force-deformation curves.

Statistical analyses

Sclerotial viability data was analysed by generalised linear model with a logit link function and penetrometer data by analysis of variance.

RESULTS AND DISCUSSION

Surface-cleansing of sclerotia

Surface cleansing tests aimed to eliminate microbial contamination so that viable *C. camelliae* mycelium could be identified growing from healthy sclerotia. Preliminary experiments followed the method described by Hoes and Huang (1975), where sclerotia of *S. sclerotiorum* were considered viable when mycelia grew from sclerotia which had been surface-cleansed in 4.8% NaOCl for 3 min and bisected onto PDA. However, this wash treatment resulted in a high level of fungal contamination which created confusion with identification of *C. camelliae* mycelium.

Results from the range of surface-disinfection treatments (Table 1) showed that including ethanol in the wash reduced development of mycelia from the treated sclerotia. The method providing the highest incidence of *C. camelliae* mycelium (80%) and lowest incidence of microbial contamination (20%) was the 3 min double wash in 13.5% NaOCl.

TABLE 1: Viability of Ciborinia camelliae sclerotia and occurrence of bacterial and fungal contaminants for sclerotia treated with different surface cleansing treatments.

Wash and duration[1]	*C. camelliae* mycelium (%)	Contaminants Bacteria (%)	Fungi (%)
Single – 5 min			
NaOCl	95 (78-99)[2]	75	70
NaOCl/ethanol (50/50)	70 (51-84)	100	10
CaOCl	85 (67-94)	40	75
Single – 20 min			
NaOCl	60 (41-76)	60	20
NaOCl/ethanol (50/50)	50 (32-68)	80	20
CaOCl	70 (51-84)	60	80
Double – 3 min			
NaOCl & NaOCl	80 (61-91)	20	20
NaOCl/ethanol (50/50) & NaOCl	80 (60-89)	25	30
NaOCl/ethanol (70/30) & NaOCl	10 (3-29)	15	15
NaOCl/ethanol (70/30) & NaOCl/ethanol (70/30)	5 (1-23)	20	10
Double - 30 min			
NaOCl/ethanol (50/50) & NaOCl/ethanol (50/50)	0	13	3

[1]Concentrations used throughout were 13.5% NaOCl and 30% CaOCl.
[2]Lower and upper 95% confidence limits in parentheses.

Dissection and incubation of sclerotia

Sclerotia with rinds removed gave slightly higher incidence ($P>0.05$) of *C. camelliae* mycelium in surviving segments than bisected sclerotia, and there was a lower incidence of bacterial contamination ($P<0.05$). However, because the method was extremely time-consuming and carried a high risk of extracting dead portions of sclerotia, it was not considered a feasible option for assessment of sclerotial viability. Bisection of sclerotia was used in subsequent tests. There were no differences ($P>0.05$) in incidence of mycelia from *C. camelliae* sclerotia incubated at 15 and 20°C and so the higher incubation temperature was used in further tests.

Antibiotic dips were found to have no effect ($P>0.05$) on the incidence of *C. camelliae* mycelia. However, because the mean incidence of bacterial contamination was reduced from 96 to 89% by this treatment, the use of antibiotic dips was continued. A similar effect was observed on PDA ammended with antibiotics, where incidence of *C. camelliae* mycelia remained the same and incidence of bacterial contamination was reduced slightly. Recovery of *C. camelliae* mycelium on *S. sclerotiorum*-selective medium and on sterile MS filter paper, was reduced ($P<0.05$) and their use was discontinued.

Testing the method to determine viability of sclerotia

One thousand apparently healthy sclerotia, equally represented in the small, medium and large size classes, were washed in 13.5% NaOCl at room temperature for 3 min followed by one rinse of SDW containing 0.4 ml/litre Tween 80 and two rinses

in SDW, and the cycle repeated. The sclerotia were then immersed in 20 ppm streptomycin, 40 ppm aureomycin and 250 ppm ampicillin for 5 min, aseptically bisected wet and each was placed medulla face down onto PDA. After incubation at 20°C in 12 h diurnal light for 9 days, 79% of sclerotia had produced *C. camelliae* mycelium from at least one segment, 3% had produced no growth and 18% were contaminated mainly by fungi which resembled *Trichoderma* spp. and *Clonostachys rosea* (Schroers). These contaminated sclerotia were re-washed in 15 ml 13.5% NaOCl followed by two rinses in SDW, and after incubation for 8 days an additional 2.5% of sclerotia were found to have produced *C. camelliae* mycelium. Because the increase in incidence of *C. camelliae* mycelium was small, re-washing of sclerotia was not considered to be warranted.

Sclerotium firmness

Measurements of sclerotial firmness showed that segments of medium-size sclerotia were harder to penetrate ($P<0.05$) than segments of large-size sclerotia. Resistance was measured in the centre of the medulla and since the rind of a medium-size sclerotium was closer to that area than that of a large-size sclerotium, it therefore seemed likely that the rinds had greater influence on resisting penetration in smaller sclerotia than in larger ones. The energy expended when the probe exerted a 40 N force was 17.3 Nmm (15.9-18.6) for medium sclerotia and 14.7 Nmm (13.3-16.0) for large sclerotia (95% confidence limits in parentheses). These narrow confidence limits confirm that measurements of firmness were uniform. If energy measurements from a candidate population of prepared sclerotia sections are below the 95% confidence limits for the 40 N force, for medium or large sclerotia, then the sclerotia are softer than normal. Therefore, the technique can be used to detect differences in firmness in sclerotia treated with biocontrol agents to measure effects of their parasitic activity.

CONCLUSIONS

The double concentrated chlorine wash and antibiotic dip should provide a reliable method for determining the viability of sclerotia of *C. camelliae* after treatment with biocontrol agents. Those sclerotia producing *C. camelliae* mycelium may be designated as viable and those producing no growth can be considered dead. In situations where sclerotia are badly contaminated with mycelium from other fungi, measurements of tissue firmness can be taken and compared to those of healthy sclerotia to ascertain parasitism.

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