

Selective media for the specific isolation and enumeration of *Botrytis cinerea* conidia

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Aims: To develop selective media for the enumeration of *Botrytis cinerea*.

Methods and Results: Two new media, *Botrytis* Selective Medium (BSM) and *Botrytis* Spore Trap Medium (BSTM), were developed and compared with currently available media for the enumeration of *B. cinerea* conidia from the environment. The new *Botrytis* media proved advantageous over previous media because they were easier to prepare, had greater selectivity and allowed enumeration when a greater number of colony-forming units were present on individual plates.

Conclusions: BSM and BSTM were shown to be suitable selective media for the enumeration of *B. cinerea* conidia from the environment.

Significance and Impact of the Study: The media developed can be used to monitor the population of the pathogen *B. cinerea* and will allow detailed studies within the crop environments.

INTRODUCTION

Botrytis cinerea is a polyphagous fungal plant pathogen that causes extensive damage to a wide range of economically important crops world-wide (Jarvis 1977; Coley-Smith *et al.* 1980) and is the focus of many epidemiological and biocontrol studies (Elad *et al.* 1996; Seddon *et al.* 1997; Walker *et al.* 1998). The identification, isolation and enumeration of a plant pathogen in the environment are of great importance in these studies. The easiest method available is the use of a selective medium. Several selective media for the isolation of *Botrytis* spp. have been developed and the most commonly used is that developed by Kritzman and Netzer (1978). This medium contains fungicides that are non-toxic towards *Botrytis* spp. and tannic acid, which is oxidized to produce a brown pigment (Ribéreau-Gayon *et al.* 1980). Kerssies (1990) modified this medium for use in spore traps within greenhouses. The medium developed was highly selective towards conidia of *Botrytis* spp. within the airborne microflora. Both of these media were tested and found to

be inadequate when isolating *Botrytis cinerea* from soil and plant material. Firstly, the media allowed the growth of several other fungi, other than *Botrytis* spp., including some which could oxidize tannic acid. Secondly, the radial growth of *B. cinerea* colonies was diffuse and therefore enumeration was difficult when a large number of colonies were present.

To check that *Botrytis* Selective Medium (BSM) allowed the growth of all *B. cinerea* conidia present it was also compared with a general mycological medium, Malt Extract Agar.

MATERIALS AND METHODS

Botrytis selective medium contained (g l⁻¹): glucose, 2; NaNO₃, 0.1; K₂HPO₄, 0.1; MgSO₄·7H₂O, 0.2; KCl, 0.1; chloramphenicol (Sigma, Poole, UK), 0.2; pentachloronitrobenzene (Sigma), 0.02; Maneb 80 (80% manganese ethylene bisdithiocarbamate; Agrichem, Whittlesey, UK), 0.02; rose bengal (Sigma), 0.05; tannic acid (BDH, Poole, UK), 5; agar no. 3 (Oxoid, Basingstoke, UK), 20. The pH was adjusted to 4.5 with 1 mol l⁻¹ NaOH prior to addition of the agar. The medium was brought to the boil with constant stirring and poured whilst still hot. For practical purposes this medium was found not to require autoclaving due to its high selectivity. The colour of BSM varies with pH and should be opaque pink when poured. *Botrytis* spore trap

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medium (BSTM) differed from BSM in that Rubigan (12% fenarimol; Dow Elanco, Hitchin, UK), 0.1 ml l⁻¹, replaced rose bengal. Diluted, supplemented Malt Extract Agar (dsMEA) contained (g l⁻¹): Malt Extract Broth (Oxoid), 4; chloramphenicol, 0.2; rose bengal, 0.05; agar no. 3, 15. This medium, with an unadjusted pH of approx. 5.4, was autoclaved at 121 °C for 15 min. Kritzman and Netzer (K & N) and Keressies (KERS) media were prepared as detailed in previous publications (Kritzman and Netzer 1978; Keressies 1990).

Botrytis cinerea conidia were produced on X + 10% Sucrose medium (Harrison 1978). Mycelial plug-inoculated plates were incubated at 25 °C until mycelia had reached the edge of the plates (4–5 d). Plates were then transferred to 25 °C under near-u.v. light for 2 d after which they were returned to the laboratory and left for 14 d at room temperature before spores were harvested. Conidia were harvested by the addition of 10 ml sterile distilled water (SDW) to each plate and conidia dislodged using a sterile blunt-ended spatula. The conidial suspension was poured through two layers of sterile lens tissue (105; Whatman, Maidstone, UK) and centrifuged at 3000 g for 10 min, resuspended in 15 ml SDW and centrifuged at 3000 g for 10 min. This washing procedure was repeated twice. The conidial suspension was stored at –20 °C until required. On thawing the concentration of conidia was determined using an Improved Neubauer counting chamber (Weber, Scientific International, Teddington, UK) and diluted with Ringer's solution (Lab M, Manchester, UK) to give a stock suspension of approximately 10⁴ conidia ml⁻¹. Further dilutions were made using two soil suspensions. The first was prepared by adding 5 g fresh soil to Ringer's solution to give a final volume of 50 ml. This was mixed and sterilized at 121 °C for 15 min and allowed to cool. A second soil suspension was prepared in the same manner but not sterilized. Aliquots of these suspensions were used to make three decimal dilutions of the *B. cinerea* conidial suspension. Controls were prepared which contained the soil suspensions but no *B. cinerea*. Aliquots (0.1 ml) of these dilutions were spread on triplicate plates of dsMEA, BSM, K & N and KERS. The numbers of *B. cinerea* colony-forming units (cfu) were counted on the plates after 5–7 d incubation at 20 °C in the dark. This method was repeated as above with a sample of Chinese cabbage (*Brassica campestris* subsp. *pekinensis* var. Granaat) leaves grown in a glasshouse. In this method, 5 g leaf samples were homogenized (Lab-Blender 400; Seward Medical, London, UK) for 2 min with 50 ml Ringer's solution to form a leaf suspension and BSM was compared with dsMEA only.

The selectivity of BSTM was tested against BSM, KERS and dsMEA. A 10⁴ spore ml⁻¹ stock of *B. cinerea* was prepared as described above and diluted in three

decimal dilutions in Ringer's solution. Triplicate plates of BSTM, BSM, KERS and dsMEA were spread with 0.1-ml aliquots of the three dilutions and a control set of plates was spread with SDW. The plates were left uncovered below an open window for 24 h, except the dsMEA plates that remained covered and incubated at 25 °C in the dark. Plates were covered and incubated at 25 °C in the dark for a further 4 d before the number of *B. cinerea* cfu were enumerated. The number of other fungal species present was also noted.

RESULTS

Botrytis cinerea colonies on BSM were surrounded by a dark brown halo clearly visible against the pink colour of the medium (Fig. 1). K & N and KERS media are both brown in colour and consequently the dark brown haloes surrounding *B. cinerea* colonies are not as distinct. All media gave similar counts from the sterile suspensions, showing that there was no reduction in *B. cinerea* viability on any of them (Table 1). However, at the 10⁻¹ dilution the cfu could not be counted on K & N or KERS due to the diffuse growth of *B. cinerea*. K & N was the least selective of the three *Botrytis* media with more than 10 other fungal species present on non-sterile soil plates resulting in an inability to count *B. cinerea* cfu on these plates. KERS had greater selectivity than K & N with only three other fungal species present and this selectivity was

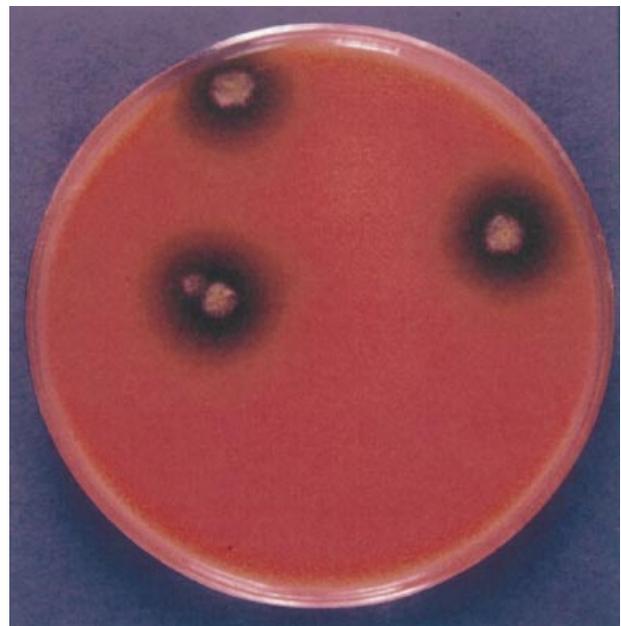


Fig. 1 Appearance of *Botrytis cinerea* colonies with a dark brown halo on *Botrytis* selective medium when plated out from a soil suspension seeded with *B. cinerea* conidia

enough to allow the enumeration of *B. cinerea*. Enumeration was easier on BSM, which had only one other fungal species present, whose growth was restricted on this medium. No *B. cinerea* colonies developed on the control plates, indicating that the soil sample was free of *B. cinerea* conidia. Results from the leaf suspension experiment indicated that BSM was also suitable for sampling plant material as well as soil (Table 1). KERS and BSTM gave a similar cfu count, when exposed to daylight for 24 h, as the unexposed dsMEA plates, indicating that neither medium affected the viability of *B. cinerea* conidia (Table 2). However, BSM became inhibitory to *B. cinerea* when exposed to daylight for 24 h, hence the need to modify the medium. Both KERS and BSTM had two other morphologically distinct fungal species

Table 1 Colony-forming units of *Botrytis cinerea* from three decimal dilutions of *B. cinerea* conidia in sterile and non-sterile soil and leaf suspensions incubated on various media

Media	Suspension	Decimal dilution		
		1	2	3
dsMEA	Sterile soil	93 (12)	12 (3.6)	2.7 (2.9)
	Non-sterile soil	PS	PS	PS
BSM	Sterile soil	79 (14)	13 (5)	1.7 (1.5)
	Non-sterile soil	94 (17)	16 (2.1)	2.0 (1.7)
KERS	Sterile soil	RRG	11 (3.1)	1.7 (0.6)
	Non-sterile soil	RRG	11 (4.2)	0.3 (0.6)
K & N	Sterile soil	RRG	10 (2.6)	1.3 (0.3)
	Non-sterile soil	PS	PS	PS
dsMEA	Sterile leaf	102 (23)	9.7 (3.5)	0
	Non-sterile leaf	PS	PS	PS
BSM	Sterile leaf	86 (15)	13 (5.3)	1.3 (2.3)
	Non-sterile leaf	83 (10)	10 (2.3)	1.3 (1.2)

RRG, No count possible due to rapid radial growth of *B. cinerea*; PS, no count possible due to poor selectivity; BSM, *Botrytis* selective medium; dsMEA, diluted, supplemented malt extract agar; KERS; K & N.

Data given are the mean (S.D.) from triplicate plate counts.

Table 2 Colony-forming units of *Botrytis cinerea* on various media from three decimal dilutions of *B. cinerea* conidia exposed to the air for 24 h

Media	Decimal dilution		
	1	2	3
BSM	0	0	0
KERS	84 (14)	13 (4.2)	1.7 (1.5)
BSTM	72 (17)	15 (5.3)	1.0 (1.0)
dsMEA (not exposed)	79 (17)	14 (3.1)	1.7 (1.5)

BSM, *Botrytis* selective medium; KERS; BSTM, *Botrytis* spore trap medium; dsMEA, diluted, supplemented malt extract agar.

Data given are the mean (S.D.) from triplicate plate counts.

present on exposed plates. These species had restricted growth (more so on BSTM) and did not oxidize tannic acid. Enumeration was easier on BSTM where restricted fungal growth allowed counts to be made with larger numbers of cfu and tannic acid oxidation was easier to distinguish.

DISCUSSION

The results show that BSM is an appropriate medium for the enumeration of *B. cinerea* from soil and plant material. There were fewer species of fungi able to grow on BSM plates from both soil and leaf suspensions compared with K & N and KERS media. *Botrytis* selective medium also enabled higher numbers of *B. cinerea* cfu to be enumerated on individual plates due to the restricted radial growth of colonies. This was not possible with the other two media and was a proven asset to easier and more accurate enumeration of this important plant pathogen.

Essentially the modifications made to the K & N medium were as follows. Rose bengal was added to inhibit bacteria and to restrict fungal growth (Tsao 1970). This resulted in small, isolated fungal colonies, which allowed a greater number of *B. cinerea* cfu to be enumerated over a longer period of time. The concentration of chloramphenicol, pentachloronitrobenzene and Maneb were increased to improve the selectivity of the medium. Copper sulphate was removed from the medium as it was no longer necessary as a bactericidal agent and it had a detrimental effect on the solubility of tannic acid. All the selective agents used are heat-stable and as such they could all be mixed together prior to boiling. As BSM did not require autoclaving, the tannic acid was added to the medium and the pH adjusted prior to heating without significant acid hydrolysis of the agar. This made BSM much easier to prepare than K & N and KERS that required the addition of tannic acid and pH adjustment after autoclaving. Precautions are necessary when rose bengal is used within media as photo-oxidation of this compound increases its fungal toxicity (Pady *et al.* 1960) and therefore plates must be stored and incubated in the dark.

Botrytis selective medium was tested as a spore trap medium but the increased toxicity due to the photo-oxidation of rose bengal could not be overcome by reducing its concentration. Consequently, BSTM was developed by the replacement of rose bengal with fenarimol for the enumeration of airborne conidia. *Botrytis* spore trap medium is similar in selectivity to KERS but has the advantages that it is lighter in colour which makes tannic acid oxidation easier to distinguish, restricts fungal growth allowing more accurate enumeration when a large number of cfu are present and is easier to prepare. *Botrytis* selective medium has been successfully used in our laboratory over the past few years when monitoring *B. cinerea* from soils and plant

material and BSTM has been used when monitoring airborne conidia of *B. cinerea* (Edwards 1993; Seddon *et al.* 1997). Their use has provided easier and more accurate monitoring than either K & N or KERS media and therefore their adoption for studies on the enumeration and population dynamics of *B. cinerea* is recommended.

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