

## Development of a Technique for the Recovery of Soilborne Sclerotia of *Botrytis cinerea*

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### ABSTRACT

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A differential flotation technique developed for the isolation of soilborne sclerotia of *Botrytis cinerea* is described. The average recovery of laboratory-reared sclerotia from five artificially infested soil samples was 96.7%. Laboratory-reared sclerotia had a higher specific gravity than naturally produced sclerotia. Recovery of sclerotia from vineyard soils

during two growing seasons revealed that the sclerotia can survive long enough to serve as a source of inoculum for the following season. Weed control practices (mechanical versus chemical) had no effect on the survival of sclerotia of *Botrytis cinerea*.

*Additional key words:* gray mold, *Vitis vinifera*.

Sclerotia of *Botrytis cinerea* Pers. ex Nocca & Balb. (5) were observed for the first time in South African vineyards during the winter of 1979 when they developed abundantly on infected vine leaves and pruned shoots on the soil surface (10). Sclerotia are considered to be the principal survival structures of *B. cinerea* (4,8). During cultivation, the sclerotia become detached from infected plant tissues and are dispersed through the soil. Although techniques are described for the recovery of the sclerotia of a number of soilborne fungi (1-3,7,9,11), a study of the literature revealed no simple technique specifically designed for the isolation of soilborne sclerotia of *B. cinerea*. Harrison (6) wet-sieved 7 kg soil and examined all material that passed a 2-mm, but not a 0.5-mm, sieve for the presence of sclerotia of *B. fabae*. Recovery of sclerotia by direct wet-sieving from muck soils was precluded by the presence of large amounts of debris in the same size range of sclerotia (11).

A reliable and simple method of assessing the number of soilborne sclerotia is required in studies on survival and epidemiology of *B. cinerea*. This report describes the development and testing of a simple and rapid differential flotation technique for the isolation of sclerotia of *B. cinerea* from vineyard soils.

### MATERIALS AND METHODS

**Sclerotia.** Naturally produced and laboratory-reared sclerotia were used for developing a technique to isolate sclerotia from soil. Naturally produced sclerotia were those that developed spontaneously on field-infected grapes during cold storage.

Laboratory-reared sclerotia were produced in 9-cm-diameter petri dishes containing 10 ml of Difco potato-dextrose agar (PDA) inoculated with mycelium. Cultures were incubated at 15 C under cool-white fluorescent lights (12 hr day light) for 28 days. At the end of the incubation period, sclerotia were removed from the plates and rubbed gently between the fingers to remove most of the adhering agar.

**Specific gravity and viability of sclerotia.** Sclerotia were soaked for 2 hr in water, blotted dry, and weighed. The volume of the weighed sclerotia was determined by measuring the volume of water displaced in a burette at 20 C. These data were used to determine the specific gravity (SG).

A saturated solution of technical grade  $\text{Ca}(\text{NO}_3)_2$  was filtered through Whatman No. 2 filter paper to obtain a clear, slightly yellow liquid with a SG of 1.54. This solution was diluted with tap water to obtain eight solutions with SG ranging from 1.18 to 1.54. The SG of each solution was determined with an SG flask at 20 C. Five replicates of 30 sclerotia each were placed in the  $\text{Ca}(\text{NO}_3)_2$  solutions and the number of sclerotia floating after 0.5, 1, 2, 3, 4, 5, and 6 hr were counted. The apparent specific gravity (ASG) was determined according to the method of Utkhede and Rahe (11).

The viability of the sclerotia after floating on a saturated  $\text{Ca}(\text{NO}_3)_2$  solution was determined. Five replicates of 15 sclerotia each were removed from  $\text{Ca}(\text{NO}_3)_2$  solutions after 1, 2, 3, 4, 5, and 6 hr, rinsed in tap water, surface sterilized with  $\text{Ca}(\text{ClO})_2$  (10), incubated on PDA at 15 C for 5 days, and examined for growth of *B. cinerea*.

**Separation of sclerotia from soil.** Air-dried soil samples (50 g) were screened through a 3-mm sieve to remove stones and plant debris. Water was added to the sample to form a slurry which was allowed to stand for at least 2 hr. A glass column (275 cm long  $\times$  3 cm ID) was filled to approximately 230 cm with water. A saturated aqueous solution of  $\text{Ca}(\text{NO}_3)_2$  was gravity-fed from below to form a

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layer about 25 cm deep at the bottom of the column. The soil sample was then transferred quantitatively to the top of the column. After standing for 5 min, the lower half of the  $\text{Ca}(\text{NO}_3)_2$  solution containing the sand was drained and discarded. The upper half of the  $\text{Ca}(\text{NO}_3)_2$  solution, together with about 30 cm (column length) of the water fraction, was passed through a 0.5-mm sieve. The residue in the sieve was rinsed with tap water and transferred quantitatively to a 9-cm-diameter petri dish. Sclerotia were removed from the petri dish using forceps and a dissecting microscope. Sclerotia were surface sterilized with  $\text{Ca}(\text{ClO})_2$ , transferred to PDA plates, and incubated for 5 days at 15 C for final identification under a dissecting microscope.

The effectiveness of the technique was tested by adding known numbers of laboratory-reared sclerotia, ranging from 6 to 18, to each of five 50-g soil samples. Analyses were carried out by a technician who had no knowledge of the number of sclerotia added.

**Field samples.** Soil samples were collected during the 1979–1980 and 1980–1981 seasons from vineyards in the De Doorns area where prunings are shredded with a chain mill and left as a mulch on the soil. The soil samples were analyzed for the presence of sclerotia. During the 1979–1980 season, composite soil samples of about 1 kg each were collected from each of four plots in a vineyard where mechanical weed control was practiced. Soil was collected at random from the top 10 cm of the soil profile and mixed thoroughly. Each plot was 50 × 10 m and each composite sample was analyzed in triplicate. Samples were collected from October 1979 to January 1980. During the 1980–1981 season, soil samples were collected from three adjacent vineyards where chemical weed control with paraquat, mechanical weed control, and no weed control were practiced, respectively. The soil in all three vineyards was a uniform dark sandy loam. Sampling techniques and plot sizes were the same as that of the previous season. Samples were collected from August 1980 to April 1981.

## RESULTS

**Specific gravity and viability of sclerotia.** The specific gravities of presoaked laboratory-reared and naturally produced sclerotia were  $1.18 \pm 0.006$  and  $1.17 \pm 0.01$ , respectively, as determined from its volume and mass.

The ASG of sclerotia of *B. cinerea* is presented in Fig. 1. Within 30 min some sclerotia sank in all the solutions with  $\text{SG} \leq 1.44$ . No sclerotia sank in the saturated  $\text{Ca}(\text{NO}_3)_2$  solution with  $\text{SG} 1.54$  for the duration of the experiment.

Flotation of sclerotia on a saturated  $\text{Ca}(\text{NO}_3)_2$  solution for 2 hr had no adverse effect on viability. After 3 hr 98.7% were viable and after 6 hr 93.3% were viable.

**Separation of sclerotia from soil.** Five soil samples (each 50 g) with sclerotia added were analyzed. The ratios of number of sclerotia recovered to the number added were: 18/18, 9/9, 11/11, 5/6, and 12/12. The mean percent recovery was  $96.7 \pm 3.4\%$ .

**Field samples.** Sclerotia were recovered from vineyard soils until January during the 1979–1980 season and until April during the 1980–1981 season (Table 1). Some of these sclerotia were attached

to small pieces of leaves. No sclerotia were found adhering to the debris floating on the water. Different weed control practices had no significant effect on the time and number of sclerotia isolated from the soil.

## DISCUSSION

A solution with  $\text{SG} = 1.54$  was required to float sclerotia of *B. cinerea* long enough for separation from soil. The wet-sieving flotation technique described by Utkhede and Rahe (10) for the isolation of soilborne sclerotia of *Sclerotium cepivorum* sclerotia from soil was tested for the recovery of soilborne sclerotia of *B. cinerea*. The high viscosity of the sucrose solution needed to float the sclerotia made this method impractical because the soil particles settled too slowly for effective separation from the sclerotia. This problem was solved by using a saturated  $\text{Ca}(\text{NO}_3)_2$  solution for the flotation of the sclerotia. The saturated  $\text{Ca}(\text{NO}_3)_2$  solution was mildly toxic to sclerotia of *B. cinerea* after contact for 3 hr or more. However, it is unlikely that it will be harmful to the sclerotia in the 5 min needed to separate them from the soil as exposure times of up to 2 hr did not affect sclerotium viability.

The separation of sclerotia from soil is based here on the difference in SG values between the sclerotia and the soil. The effectiveness of this technique may be adversely affected by grains of sand adhering to the sclerotia, thus altering the ASG values. This

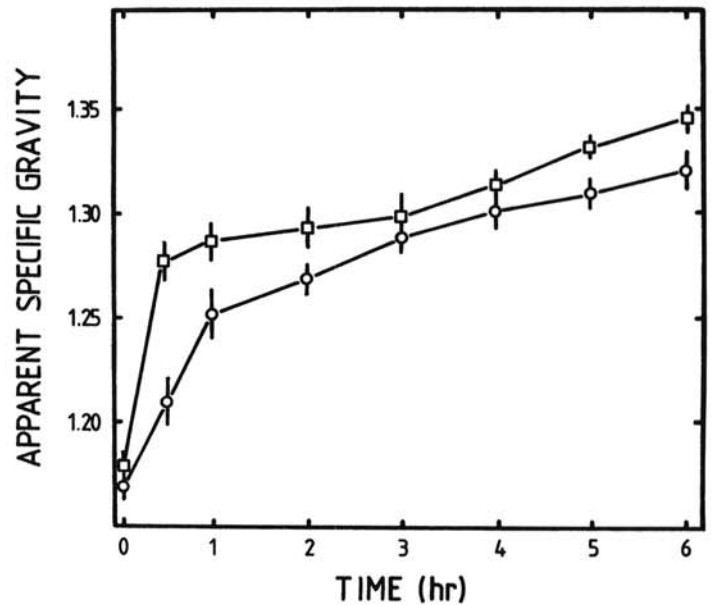


Fig. 1. Effect of length of exposure to saturated aqueous  $\text{Ca}(\text{NO}_3)_2$  solutions on the apparent specific gravity of hydrated field collected and laboratory-reared sclerotia of *Botrytis cinerea*.  $\square$  = laboratory-reared sclerotia.  $\circ$  = naturally produced sclerotia. Vertical lines represent the standard error of the mean of five replicates.

TABLE 1. Isolation of sclerotia of *Botrytis cinerea* from naturally infested vineyard soils during two seasons in which two types of weed control were employed

| Year      | Weed control treatment | Sclerotia per 50 g soil |                       |                                   |                       |                       |                       |                       |                       |                      |
|-----------|------------------------|-------------------------|-----------------------|-----------------------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|----------------------|
|           |                        | Aug                     | Sept                  | Oct                               | Nov                   | Dec                   | Jan                   | Feb                   | Mar                   | Apr                  |
| 1979–1980 | Mechanical             | NS                      | NS                    | 7.3<br>( $\pm 1.0$ ) <sup>a</sup> | 5.0<br>( $\pm 0.2$ )  | 5.3<br>( $\pm 0.9$ )  | 1.0<br>( $\pm 0.4$ )  | 0                     | NS <sup>b</sup>       | NS                   |
| 1980–1981 | Mechanical             | 30.0<br>( $\pm 2.2$ )   | 15.0<br>( $\pm 1.5$ ) | 9.3<br>( $\pm 2.1$ )              | 6.0<br>( $\pm 1.4$ )  | 14.0<br>( $\pm 3.9$ ) | 12.3<br>( $\pm 3.9$ ) | 11.5<br>( $\pm 4.2$ ) | 4.8<br>( $\pm 1.4$ )  | 4.8<br>( $\pm 1.1$ ) |
|           | Chemical               | 32.8<br>( $\pm 5.1$ )   | 25.5<br>( $\pm 3.8$ ) | 12.8<br>( $\pm 2.3$ )             | 11.5<br>( $\pm 1.3$ ) | 9.0<br>( $\pm 1.3$ )  | 17.8<br>( $\pm 4.5$ ) | 13.3<br>( $\pm 5.2$ ) | 6.3<br>( $\pm 1.3$ )  | 5.3<br>( $\pm 1.1$ ) |
|           | None                   | 34.8<br>( $\pm 5.0$ )   | 16.0<br>( $\pm 2.8$ ) | 10.3<br>( $\pm 1.4$ )             | 14.3<br>( $\pm 3.4$ ) | 10.5<br>( $\pm 1.7$ ) | 8.0<br>( $\pm 0.8$ )  | 17.3<br>( $\pm 5.6$ ) | 11.3<br>( $\pm 2.1$ ) | 4.0<br>( $\pm 0.9$ ) |

<sup>a</sup>Standard error of the means are shown in parentheses.

<sup>b</sup>NS = not sampled.

problem was minimized by washing the soil through a column of water above the  $\text{Ca}(\text{NO}_3)_2$  solution. A further advantage of the water column was that plant debris floated on the water. The high rates of recovery of sclerotia from artificially infested soil samples indicated that this method is effective and reliable for the isolation of soilborne sclerotia of *B. cinerea*.

Sclerotia of *B. cinerea* primarily are formed on infected vine leaves and shredded prunings that remain on the soil surface during the wet winter months (10). Results of this study indicated that sclerotia can survive in the soil for 5–9 mo. The numbers recovered decline appreciably from October onwards.

With this technique it was possible to show that viable sclerotia of *B. cinerea* were present in vineyard soils from winter until fall. Sclerotia must therefore be considered to be an important source of survival in South Africa. It was shown previously that *B. cinerea* can survive as mycelium in infected shoots (10). Sanitation measures, whereby prunings are removed from the vineyard, should reduce the inoculum level significantly as both survival sources can thus be minimized.

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