

Survival of Sclerotia and Conidia of *Botrytis squamosa*

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Portion of a thesis submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree, Cornell University.

This research was supported in part by the Orange County Vegetable Improvement Cooperative Association, Goshen, N. Y. 10924.

Accepted for publication 11 August 1976.

ABSTRACT

ELLERBROCK, L. A., and J. W. LORBEER. 1977. Survival of sclerotia and conidia of *Botrytis squamosa*. *Phytopathology* 67: 219-225.

Sclerotia of *Botrytis squamosa* survived for 21 months at rates of 7 and 66% when buried 3 and 15 cm, respectively, below the surface of an organic soil. Sclerotia placed on the soil surface in September 1971 were 96, 30, and 0% viable by 24 March, 21 May, and 25 July 1972, respectively. Myceliogenic germination rates for recovered sclerotia generally were higher than sporogenic rates. Large numbers of sclerotia of *B. squamosa* frequently were formed on blighted onion leaves before and after harvest. Formation was most prolific during wet periods in late August and September. Sclerotia also formed on onion bulb necks, seed

stalks, and the inner leaf sheaths in the neck region of bulbs. Conidia of *B. squamosa* survived for short periods (usually less than 2 months) in natural soil under both controlled and field conditions. Higher temperatures reduced conidial survival and populations of conidia declined rapidly in soil that was alternately dried and remoistened. Mycelium of *B. squamosa* failed to survive in leaf debris under field conditions. *Botrytis squamosa* survived for 17 months in association with onion seeds but could not be recovered after 25 months. Rates of infestation of seed by *B. squamosa* were low; 6% was the maximum rate detected.

Additional key words: *Allium cepa*, *Botryotinia squamosa*.

Botrytis squamosa Walker (*Botryotinia squamosa* Viennot-Bourgin) first was described as a neck rot pathogen of onion bulbs, primarily of white cultivars (20). In New York, *B. squamosa* is most important as the cause of Botrytis leaf blight of onion (8). During wet growing seasons, blighting of onions begins in early summer and continues until harvest in autumn. Information on survival of *B. squamosa* from one growing season to the next is lacking. Walker (20) stated that sclerotia and conidia survived in situ on onion bulbs on a window sill in Wisconsin from December to March, and others (12, 15) have suggested that sclerotia are important survival structures. There have been no detailed studies, however, of survival under field conditions.

Walker (20) observed many small sclerotia on the scales of bulbs infected by *B. squamosa*. Moreover, sclerotia form in abundance on artificial media in pure culture. There is much discrepancy, however, regarding formation of sclerotia in connection with Botrytis leaf blight of onion. Most investigators failed to observe sclerotia in the field (9, 17, 18) or they reported that sclerotia formed only on the neck region of bulbs in fields where Botrytis leaf blight occurred (8, 12). McLean (15) found that sclerotia formed on field-infected leaves when placed in a moist chamber. Whether or not sclerotia form on blighted onion foliage in the field previously has not been established.

This investigation was conducted to clarify questions concerning formation of sclerotia by *B. squamosa* and to determine whether or not sclerotia and/or conidia survive from season to season under field conditions. Other possible mechanisms of *B. squamosa* survival also were examined. A preliminary report of the selective medium that was developed during this investigation was published earlier (4).

MATERIALS AND METHODS

Production of conidia.—Large numbers of *B. squamosa* conidia were needed for the survival studies. Since *B. squamosa* sporulates very poorly on conventional artificial media, various media incorporating different types of onion material and soil extracts were tested for promoting formation of conidia by *B. squamosa*. An agar medium containing onion leaf straw gave satisfactory results. Bottoms of Pyrex glass petri plates (9-cm diameter) were covered with field-collected dried onion leaf straw (approximately 2 g), 20-25 ml of 2% Difco water agar was poured into each plate, and the plates were autoclaved for 20 minutes and allowed to cool. Plates then were seeded with mycelium from rapidly growing colonies of *B. squamosa* on potato-dextrose agar (PDA) and incubated under fluorescent light (Sylvania Cool-White F20T12-CW, 14-hour photoperiod) at 18 C. After 7-10 days the agar surface usually was covered with conidiophores and conidia.

Production of sclerotia.—Preliminary experiments

revealed that sclerotia produced on agar media survived for a shorter time than naturally formed sclerotia. Sclerotia typical of those formed naturally were produced by filling Pyrex petri plates (9-cm diameter) with 2 g of dried onion leaf straw, adding 20 ml of water, autoclaving the mixture for 30 minutes, adding 2 milliliters of a *B. squamosa* conidial suspension which then was spread over the straw in each plate, and incubating at 18 C in darkness for 3 weeks. To collect the sclerotia, leaf material from several plates was combined in a large beaker and rubbed together to separate sclerotia from the leaf tissue. Water then was added to the beaker and the mixture was stirred for several minutes. Sclerotia settled to the bottom, and water with floating leaf debris was decanted. After several washings, sclerotia free of leaf material remained. The sclerotia then were air-dried on paper towels and stored at 20-24 C until needed. Sclerotia

produced in this manner were undamaged and were similar in morphology and size to those formed naturally.

Development of a selective medium.—Soil assay by dilution-plating to determine the presence of *B. squamosa* required a medium at least partially selective for *B. squamosa*. Martin's rose bengal medium (14) was used as a basal medium for testing the effect of different commercial fungicides and antibiotics on conidial germination and mycelial growth of *B. squamosa* and for their ability to limit growth of background microorganisms present in organic soils. Conidial suspensions of *B. squamosa*, mycelial disks cut from rapidly growing *B. squamosa* colonies on PDA, and soil dilutions were plated on the basal medium amended with the different toxicants. In addition, the efficiency of recovery of *B. squamosa* from soils artificially infested with conidia was determined for some treatments. All

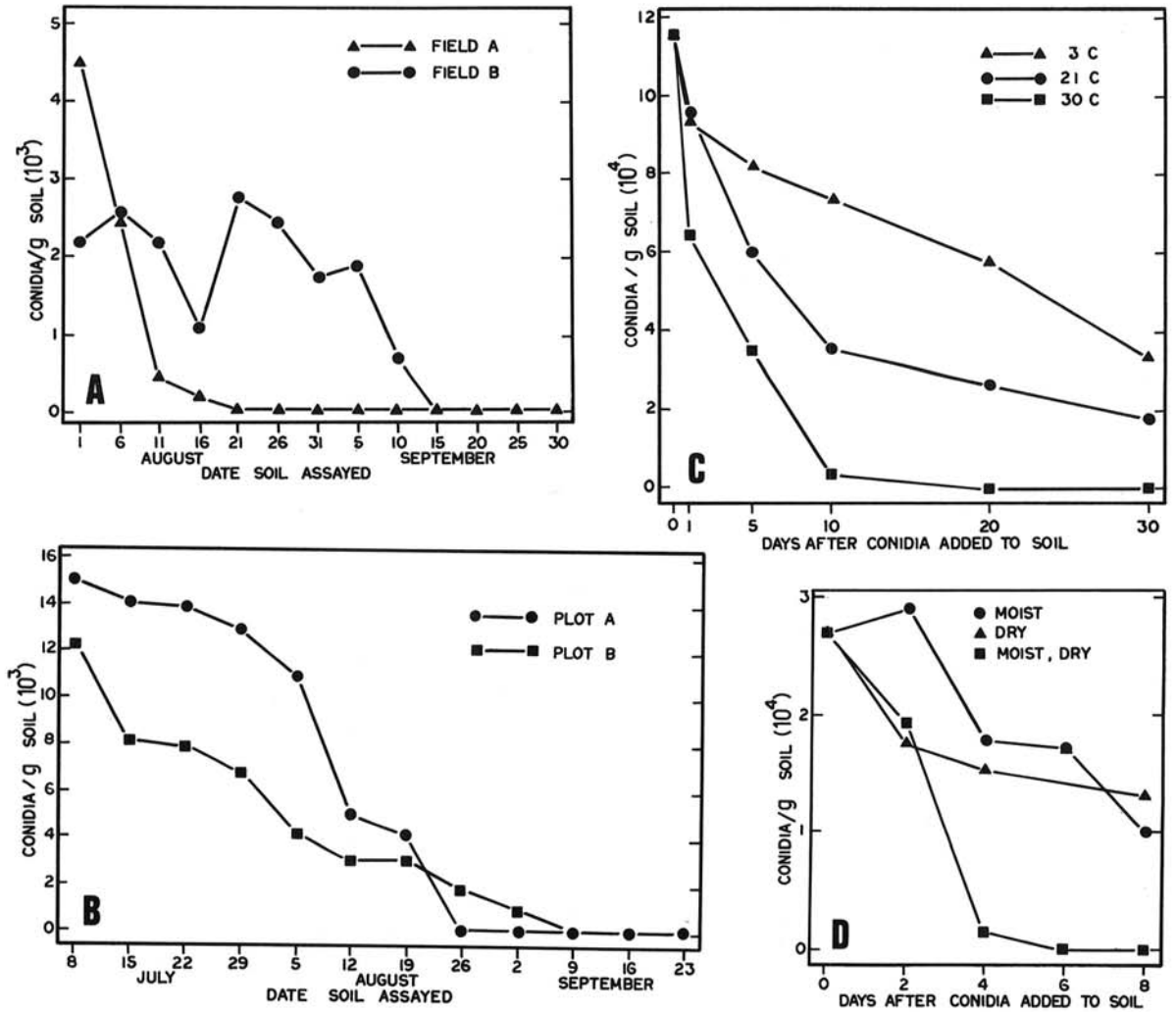


Fig. 1-(A to D). Survival of *Botrytis squamosa* conidia in natural organic soil. A) Survival of conidia in two fields cropped to onions in 1972. Onions were harvested on 2 August in field A and 6 September in field B. B) Survival of conidia in field plots artificially infested with conidia on 8 July 1971. C) Survival of conidia in artificially infested soil stored in plastic bags at 3, 21, and 30 C. D) Survival of conidia in soil maintained at different moisture levels.

plates were incubated in darkness at 16-18 C. Toxicants were added to the basal medium after it was autoclaved and cooled to 50-55 C.

Assay of populations of conidia in soil.—Populations of *B. squamosa* conidia in natural field soil were measured by dilution-plate assaying of soil samples on the selective medium (4). Unless stated otherwise, three composite samples, each consisting of 20 subsamples, were collected from the top 10 cm of soil in each field on the assay dates (Fig. 1-A, B). A suitable dilution (based on oven-dry weight) of each soil sample was prepared so that 1 ml of the dilution yielded 30-40 fungal colonies on a plate of the selective medium. Ten plates were prepared for each soil sample, giving 30 plates per field per assay. Colonies of *V. squamosa* were counted, and the number of conidia per gram of oven-dry soil was calculated.

In 1971, two plots (1 × 6 meters) were established in two fields of organic soil that had been cropped to lettuce earlier in the season (Fig. 1-B). Conidia were artificially incorporated into the top 15 cm of soil on 8 July by the following method. Conidia were blown from sporulating cultures of *B. squamosa* with a blast of air inside a large plastic bag containing 5 liters of soil. This soil then was thoroughly mixed into the soil in the plots, and assays were made at weekly intervals until 23 September. The soil in the plots was not disturbed during the investigation, except during sampling.

In 1972, soil was assayed in two fields (A and B) of

organic soil cropped to onions and in which many *B. squamosa* conidia were formed (Fig. 1-A). Soil was assayed from 1 August to 30 September. Fields A and B were harvested on 2 August and 6 September, respectively, but the soil was not disked until after the assays were discontinued.

To determine the effect of temperature on survival of conidia, 2 liters of natural organic soil (63% moisture based on oven-dry weight) was infested with conidia as described above. The soil then was placed in each of nine small plastic bags. Three bags each were stored in darkness at 3, 21, and 30 C, and soil from each bag was assayed at the indicated times (Fig. 1-C).

To determine the effect of fluctuating soil moisture on survival of conidia, natural organic soil (65% moisture based on oven-dry weight) was infested with conidia as described earlier. The soil then was placed in nine glass petri plates (20 g per plate) and maintained at 20-24 C. Three plates were enclosed in plastic bags to prevent evaporation. The lids were removed from six plates to allow the soil to dry. After 2 days, the soil in all plates was assayed for *B. squamosa* by preparing dilutions with 2 g of soil from each plate. Soil in three plates was restored to its original moisture level by adding distilled water, whereas soil in the three remaining plates was allowed to continue to dry. These procedures were repeated after 4, 6, and 8 days.

Determination of survival of sclerotia.—To determine

TABLE 1. Effect of depth and duration of burial in soil on the survival of sclerotia of *Botrytis squamosa* under natural conditions

Date sampled	Depth (cm)	Sclerotia recovered ^a (no.)	Germination (%)	
			Myceliogenic ^b	Sporogenic ^c
8 Nov 1971	0	297	97	93
	3	297	98	85
	15	296	98	88
24 March 1972	0	291	99	87
	3	298	100	92
	15	299	99	93
21 May 1972	0	114	80	81
	3	250	93	95
	15	296	96	83
25 July 1972	0	0
	3	213	85	92
	15	298	100	97
10 Sept 1972	0	0
	3	128	86	70
	15	283	98	91
18 March 1973	0	0
	3	117	81	53
	15	285	94	89
13 June 1973	0	0
	3	47	34	18
	15	237	83	51

^aValues indicate the total number of sclerotia recovered from the original 300 placed in the field (three bags, 100 sclerotia per bag.). Sclerotia were placed in the field in nylon mesh bags on 14 September 1971.

^bPercentage of the recovered sclerotia giving rise to *B. squamosa* colonies on acidified potato-dextrose agar (one-half of the recovered sclerotia were tested).

^cPercentage of recovered sclerotia becoming conidiated on moistened filter paper in petri plates (one-half of the recovered sclerotia were tested).

the duration of survival of sclerotia under natural conditions, sclerotia were placed in nylon mesh bags (100 sclerotia per bag) with 1 g of natural organic soil. The bags were placed in a small plot established along the edge of an onion field in Orange County on 14 September 1971. Bags were placed on the surface and at 3 and 15 cm

below the soil surface. Three bags were removed from each level on each sampling date (Table 1). Bags on the surface were anchored to small wood stakes. The soil surface in the plot was not disturbed except for periodic hand-weeding.

Upon sampling, the sclerotia present in each bag were

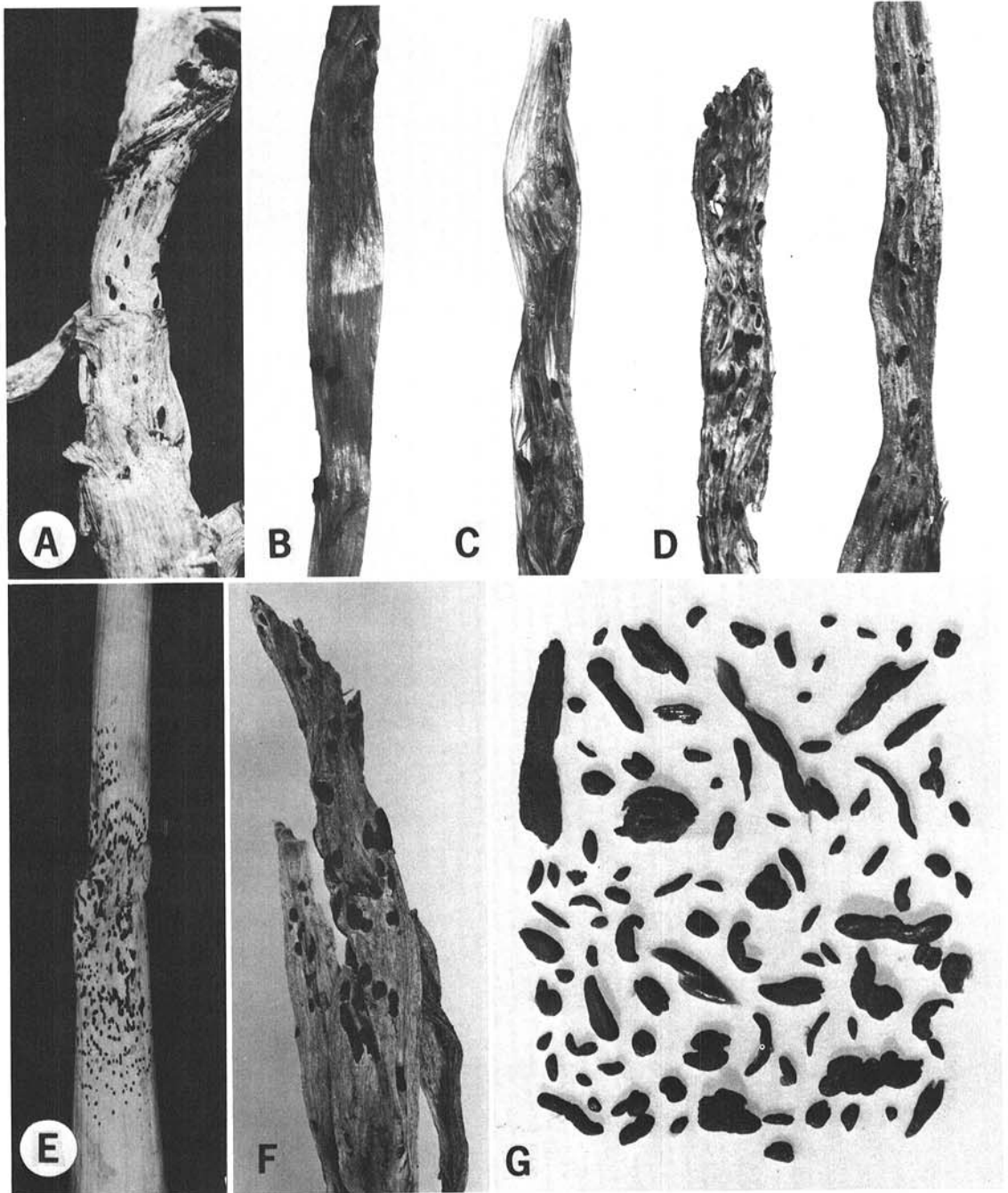


Fig. 2.-(A to G). Formation of *Botrytis squamosa* sclerotia under natural conditions. A) Sclerotia on outer leaf sheaths of an onion bulb neck. B-C) Sclerotia on inner leaf sheaths (several outer sheaths have been removed). D) Sclerotia formed on blighted onion leaves before harvest. E) Sclerotia on an onion seed stalk. F) Sclerotia formed after harvest on blighted leaves in contact with the soil. G) Variation in morphology of naturally formed sclerotia.

counted after the soil and other debris were removed by wet-sieving. Only intact sclerotia were counted. Sclerotia then were stirred in a 1% sodium hypochlorite solution for 3 minutes, washed in sterile distilled water, and air-dried on sterile paper towels. One-half of the sclerotia from each soil depth then were placed on acidified potato-dextrose agar (APDA) in petri plates (five sclerotia per plate). The plates were incubated at 16-18 C in darkness for 7 days and then examined for growth of *B. squamosa* mycelia. The remaining sclerotia were placed on filter paper in petri plates (approximately 20 sclerotia per plate) and incubated under continuous fluorescent lighting (Sylvania Cool-White F20T12-CW) at 15 C for 14 days. The filter paper then was moistened and the plates were placed at 9 C in darkness. Sclerotia were examined periodically for 4 weeks. Those that produced conidia were counted and removed.

RESULTS

Selective medium.—Recovery of *B. squamosa* conidia from artificially infested soil was most efficient when Martin's rose bengal medium was amended with a combination of the following materials at the rates (milligrams per liter) indicated: pentachloronitrobenzene (PCNB), 20; a mixture (5.2:1, w/w) of ammoniates of [ethylenebis (dithiocarbamate)]-zinc and ethylenebis [dithiocarbamic acid] bimolecular and trimolecular cyclic anhydrosulfides and disulfides (Polyram), 7; 2, 6-dichloro-4-nitroaniline (Botran), 2; streptomycin sulfate, 150; and chlortetracycline HCl (Aureomycin), 40. Germination of conidia of

representative isolates of *B. squamosa* on the medium was nearly 100%. The percent recovery of conidia from soil samples artificially infested with conidial suspensions ranged from 72-95%, with an average of 80%. Best results were obtained by drying plates (10-12 ml of medium per plate) 4-5 days at room temperature before placing soil dilutions over the agar surface. Colonies of *B. squamosa*, which were readily identifiable by their differential pigmentation, were counted by viewing the underside of the plates.

Survival of conidia.—Conidia of *B. squamosa* were recovered from soil in fields during Botrytis leaf blight epidemics, but conidia were no longer detectable 1-2 weeks after the onion crop was harvested (Fig. 1-A). Conidia survived longer in artificially infested field soil. By 2 months after infestation, however, conidia could not be recovered from soil in either plot (Fig. 1-B). Survival of conidia in soil was affected adversely by high temperature. Optimum survival occurred at 3 C, followed by decreasing survival at 21 and 30 C, respectively (Fig. 1-C). Alternate drying and remoistening of soil markedly reduced the survival of conidia; none was recovered after two drying and remoistening cycles (Fig. 1-D). Populations of conidia declined rapidly as the soil dried during the first 2 days after infestation, but survival in the continuously dry soil was similar to that in the moist soil during the remainder of the 8-day experiment.

The direct and cellophane-agar-diffusion methods (10) were used to determine if conidia of *B. squamosa* are sensitive to soil fungistasis. No germination occurred on natural soil, whereas almost 100% germination occurred after 24 hours on autoclaved soil.

Formation of sclerotia in the field.—Sclerotia of *B. squamosa* were found on the outer leaf sheaths of the necks of bulbs in fields where Botrytis leaf blight epidemics had occurred (Fig. 2-A). Although this was the most common location for formation of sclerotia, such sclerotia usually formed on a low percentage of bulbs within a field and constituted a small percentage of the total sclerotia observed in this study. Sclerotia occasionally were found on the inner leaf sheaths of the bulb neck (Fig. 2-B, C). Such sclerotia often formed after harvest, either on the neck portion left in the field or on the portion attached to the bulb in storage. By far the highest percentage of sclerotia observed were formed on blighted leaves in fields where severe epidemics had occurred (Fig. 2-D). Such sclerotia were most common on late crops during wet weather in late August and September. Sclerotia also formed after harvest on blighted leaves in contact with the moist soil surface (Fig. 2-F). During wet weather, sclerotia formed on onion flower stalks (Fig. 2-E). Sclerotia were observed occasionally on dry bulb scales of red and yellow cultivars and commonly on bulb scales of white cultivars. Naturally formed sclerotia varied greatly in size and morphology (Fig. 2-G). Those formed on leaf sheaths and bulb scales usually were larger and more laminar than the spindle-shaped sclerotia formed on blighted leaves.

Effect of temperature on formation of sclerotia.—The foliage of severely blighted plants was removed from bulbs in the field shortly before harvest. The foliage was placed in large paper bags (10 plants per bag), and three bags were placed in incubators at 3-degree intervals from 0-30 C. After 3 weeks of incubation, sclerotia that formed

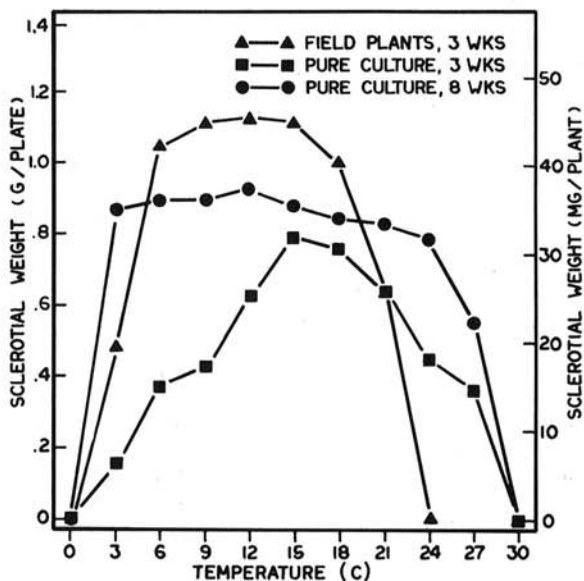


Fig. 3. Effect of temperature on formation of sclerotia by *Botrytis squamosa*. Sclerotial formation in pure culture was measured by growing *B. squamosa* on autoclaved onion leaf straw in 9-cm-diameter petri plates for 3 and 8 weeks. Sclerotial formation on naturally infected onion leaves was measured by placing leaves from naturally infected plants at the different temperatures for 3 weeks.

were separated from the leaves, and the average weight of sclerotia formed per plant was determined.

Sclerotia were produced on naturally infected foliage at 3-21 C, with maximum formation at 9-15 C (Fig. 3). Sclerotia formed on all parts of the onion foliage, but was most common on the inner leaf sheaths in the neck region. Sclerotia formed on the inner sheaths were much larger than those formed on more distal parts of the leaves.

The effect of temperature on sclerotial formation in vitro was tested by placing 1.5 g of a uniform mixture of onion straw in 9-cm diameter glass petri plates with 20 ml of water. The plates were autoclaved and cooled, 2 ml of a *B. squamosa* conidial suspension was spread over the surface of the straw, and the plates were sealed in small plastic bags and incubated at 18 C in darkness for 5 days. Ten plates then were transferred to incubators maintained at 3-degree intervals from 0-30 C. After 3 and 8 weeks, five plates were removed from each incubator. The sclerotia were collected as described earlier and allowed to air-dry for 8 hours, and the average weight of sclerotia formed per plate was determined.

In vitro formation of sclerotia by *B. squamosa* occurred from 3-27 C (Fig. 3). After 3 weeks, maximum formation had occurred at 15 C, and after 5 more weeks little additional sclerotial formation occurred at 15 C. In contrast, few sclerotia formed at 3 C by 3 weeks, but total formation at 3 and 15 C was about the same after 8 weeks. Total sclerotial formation after 8 weeks was similar from 3-24 C. Sclerotia failed to form only at 0 and 30 C.

Sclerotial survival.—Most of the sclerotia placed on the soil surface in September were recovered on 24 March, and 99% were viable (Table 1). By 21 May approximately one-third of the sclerotia were recovered, and 80% of those were viable. By 25 July, however, no sclerotia were recovered from the soil surface. Sclerotia survived longer in the soil; after 21 months, 237 sclerotia were recovered at the 15-cm depth and were 83% viable, in contrast to 47 sclerotia recovered at the 3-cm depth, of which 34% were viable (Table 1). Sclerotial numbers and viability declined most rapidly during the warmer months at all levels. Many sclerotia on the surface bore conidiophores and conidia when they were recovered in March and May. A few conidiophores were observed on sclerotia when they were recovered from the soil at 3 cm, but not on those from the 15-cm depth. In most cases, a higher percentage of recovered sclerotia germinated myceliogenically than sporogenically.

Survival of mycelia.—To ascertain if *B. squamosa* overwinters as mycelium in plant debris, onion plant residue including leaves, bulb necks, and outer bulb scales was collected during the winter and early spring from fields in which Botrytis leaf blight epidemics had occurred in the previous growing season. A portion of this residue was surface-disinfested for different time periods in 1% sodium hypochlorite, rinsed in sterile water, and then either incubated in moist chambers to promote *B. squamosa* sporulation or placed on plates of APDA to allow mycelial growth. A portion of the residue, not surface-disinfested, was treated similarly. In no case was growth of *B. squamosa* obtained, which indicated that *B. squamosa* mycelium probably does not survive very long in plant residue. This conclusion is supported by the fact that *B. squamosa* never has been observed to sporulate on plant residue in the field.

Seed-borne Botrytis squamosa.—To determine if *B. squamosa* survives on onion seeds, samples of seed grown in Orange County were placed on APDA in petri plates (five seeds per plate, 300 seeds per sample). Both untreated and surface-sterilized seeds (5 minutes in 0.5% sodium hypochlorite) were tested. The plates were incubated at 16 C in darkness for 7 days, and fungal colonies that grew from the seeds were counted. Seeds were tested 2-4 months after harvest and storage in plastic bags at 4-6 C.

Of 25 seed lots tested, *B. squamosa* was found in four; the highest rate of infestation was 6%. *Botrytis squamosa* was observed only when seeds were surface-sterilized, presumably because other microorganisms present on the untreated seeds obscured or prevented growth of *B. squamosa*. Seeds from the lot infested at the rate of 6% at the time of the initial test (2 months after harvest) were tested again at 6, 12, 17, 25, and 32 months after harvest. *Botrytis squamosa* was detected at rates of 4, 4.7, 1.7, 0, and 0% at these times, respectively. Thus, no growth of *B. squamosa* occurred from seeds after more than 17 months of storage.

DISCUSSION

Sclerotia are the important survival structures of *B. squamosa* in New York State. Sclerotia overwinter on the soil surface in association with plant residue, but survive for much longer periods when buried in the soil. Successful survival of sclerotia is important, since inocula produced on sclerotia can initiate Botrytis leaf blight epidemics (5).

The common occurrence of *B. squamosa* sclerotia in the field, especially on blighted leaves, has not been noted in other areas (9, 12, 15, 17, 18). This may be due to the different environmental conditions involved. Weather during much of the present investigation generally promoted severe epidemics of Botrytis leaf blight, which favored development of sclerotia. The wide temperature range at which sclerotia formed suggests that formation in the field seldom is limited by temperature.

Long-term survival of sclerotia in soil has been reported for other *Botrytis* spp. (3, 7, 13). Sclerotia of *B. squamosa* failed to survive through the summer on the soil surface because germination began in early spring, thus exhausting food reserves. Furthermore, once sclerotia germinated they probably were more rapidly degraded by other microorganisms. Willets (21) noted that sclerotia are more susceptible to mycoparasitism after they germinate. Mycoparasitism of *B. squamosa* sclerotia has been reported (19). Sclerotia buried in the soil apparently were sensitive to soil fungistasis which prevented or reduced germination and prolonged their survival. Sclerotia probably survived better at the 15-cm depth than at the 3-cm depth because they were subjected to fewer temperature and moisture fluctuations at the deeper level and/or to a higher level of fungistasis. These fluctuations may have enabled sclerotia at the 3-cm level to overcome fungistasis and to germinate sporogenically. Alternate wetting and drying causes leakage of nutrients from sclerotia (2, 11). Adams (1) reduced sclerotial populations of *Sclerotinia sclerotiorum* in soil by fluctuating the soil moisture level. Leakage of nutrients, in addition to exhausting food reserves, favors growth of antagonistic microorganisms. Under normal field

conditions, sclerotia would not be expected to survive as long as they did in the present study, because routine plowing and disking most likely would subject sclerotia to the fluctuating conditions that reduce survival.

Recovered sclerotia germinated both myceliogenically and sporogenically in vitro. Sporogenic and carpogenic germination both have been observed in nature (5). Myceliogenic germination, however, apparently does not occur under natural conditions. This agrees with Garrett's (6) observation that sclerotia of fungi that infect aerial plant parts usually are sporogenic and/or carpogenic, whereas sclerotia of root-infecting fungi are myceliogenic. The generally higher myceliogenic germination rates of recovered sclerotia in vitro were most likely due to the more specific conditions required for sporogenic germination.

The failure of conidia to survive for long periods in soil indicates that they probably are not significant in survival. Although the reasons for lack of long-term survival were not determined, it was demonstrated that alternate drying and remoistening of soil greatly reduced conidial populations. Fluctuation in soil moisture, especially near the surface where most conidia are concentrated, probably accounts for the rapid disappearance of conidia in the field. Although *B. squamosa* conidia are sensitive to soil fungistasis, it is possible that conidia germinate when dry soil is remoistened. Once germination takes place, lysis of the mycelium probably occurs.

Botrytis squamosa previously has not been reported as seed-borne (16). The nature of the association between *B. squamosa* and onion seeds was not determined, but it appears that *B. squamosa* is borne internally, at least to some extent, since surface-sterilization procedures did not eradicate the fungus. It is possible that small sclerotia may have been within or upon seeds that yielded *B. squamosa* colonies. Although seed-borne, *B. squamosa* on seeds probably does not function as primary inoculum (5). Furthermore, the low infestation rates observed make it doubtful that significant seed transmission occurs. If, on the other hand, seed transmission does occur, the fact that *B. squamosa* survived on seed for at least 17 months may be very significant.

Cultural practices that prevent formation and survival of sclerotia might disrupt the pathogen cycle in *Botrytis* leaf blight and afford some disease control. Long-term survival of sclerotia in the soil implies that inoculum of *B. squamosa* can build up from year to year and initiate epidemics when favorable weather occurs.

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