Factors Affecting Sclerotial Germination of *Sclerotium cepivorum*, Secondary Sclerotia Formation, and Germination Stimulants to Reduce Inoculum Density

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ABSTRACT

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Eruptive germination of sclerotia was stimulated by allyl sulfide (AS) and *Allium* extracts. Exposure to 2.5–5% AS (v/v) for 7 days was near optimal for stimulation of eruptive germination, whereas exposure to higher concentrations was somewhat inhibitory to total (hyphal + eruptive) germination. Incubation of sclerotia on substrates containing available nutrients also inhibited eruptive germination. Exposures to AS for 15 min were sufficient to stimulate eruptive germination, but the highest level of eruptive germination was attained after exposures of 24 hr or longer. A 6-hr exposure was required for subsequent secondary sclerotia formation. A maximum mean of three secondary sclerotia was produced per eruptively germinated primary sclerotium when sclerotia were incubated on purified agar and exposed to 2% AS for 1 wk. Applications of *Allium* extracts to fallow field soil induced germination of up to 60% of recovered sclerotia. Under conditions not conducive for secondary sclerotia formation and of low initial inoculum density, such reductions could reduce white rot incidence in the next crop.

Eruptive germination, as described by Punja and Grogan (13) for *Sclerotium rolfsii*, is characterized by the eruption of a compact, mycelial tuft(s) through the sclerotial rind. A hollow rind remains. Allyl cysteine sulfoxides present in *Allium* root exudates are precursors of the volatile allyl and propyl sulfides that stimulate eruptive germination of sclerotia of *S. cepivorum* (2–4,8,9,15). The controlled conditions under which this stimulative effect is maximal are defined in this study. Because eruptive germination is the potentially infective form of germination, extrapolation from the acquired data allows discussion of the feasibility of white rot control by preplant stimulation of sclerotial germination in nonsterile soil. Merriman et al (10) reported a reduction in onion white rot incidence when onion oil was applied to soil 1 mo before planting. We also describe the formation of secondary sclerotia of *S. cepivorum* and the conditions requisite for their formation under controlled conditions.

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MATERIALS AND METHODS

Sclerotia were recovered from naturally infested soil or diseased plants (natural sclerotia) collected in Salinas Valley (SV-3) or Tulelake (TLS-5), CA. They were recovered from infected bulbs by stripping the outer sclerotia-covered scales and blending them in water at low speed for 30 sec. Further separation was achieved by wet sieving as described by Crowe et al (6).

For mass production of SV-3 and TLS-5 sclerotia, the oat seed method was used. A mixture of oats, water, and nutrient broth was autoclaved and cooled before inoculation with mycelial disks (7 mm diameter) removed from the margin of an actively growing colony of *S. cepivorum* on potato-dextrose agar (PDA). Flasks were incubated at room temperature and gently shaken daily by hand to separate the oat seeds. Sclerotia were retrieved by wet sieving after 7–8 wk. Sclerotia, unless stated otherwise, from each source were dried on the laboratory bench and stored in open containers at room temperature (20–25 C). Sclerotia were used in experiments for a maximum of 12 mo only. Stock cultures were maintained on PDA slants and in dry soil.

To break constitutive dormancy before use in experiments, natural sclerotia and others produced in culture were buried in nonsterile field soil that was watered daily for 4–6 wk. Viability of recovered sclerotia was verified by the mycelial growth from cracked sclerotia and by the formation of spermatia on 1.5% Bacto water agar (WA), as described by Crowe and Hall (5). Sclerotia were always surface-sterilized for 3 min in 0.5% NaOCl immediately before use. In all experiments, both natural sclerotia and culture-produced sclerotia (of the same isolate) were used and compared. Incubation was in darkness.

Eruptive germination was considered to have occurred when mycelial tufts that emerged through the rind were macro-

scopically visible. Confirmation of eruptive germination was the remaining shell of rind cells, detected after light probing. Sclerotia that germinated hyphally maintained integrity and had mycelial strands that emerged individually from the rind.

Sclerotial treatment. The effect of washing and drying sclerotia on their germination response was examined when sclerotia not previously air-dried at room temperature were either dried for 24 hr over $CaCl_2$ in a desiccator at room temperature or washed for 5 hr under running water. All sclerotia were subsequently incubated at 15 C, partially submerged in WA. Sclerotia were incubated at 15 C in the presence or absence of 2% allyl sulfide (AS) contained in centrally placed microbeakers. Assessment of sclerotial germination was made 15 days later and weekly thereafter.

Incubation substrate. To determine the effect of incubation substrate (and nutrient status) on eruptive germination, sclerotia were partially submerged in 1% Difco Noble (NA), 1% Difco ion (IA), and 1% Difco purified agars (PA) (Difco Laboratories, Detroit, MI), 1.5% unpurified WA, and PDA. In addition, sclerotia were also placed on moistened, acid-washed (three washes in 0.1 N HCl followed by distilled water rinses; final pH 5.5) quartz sand (Ottawa Silica Co., Ottawa, IL) and sterilized and nonsterile silty loam field soil from King City, CA (20% moisture, v/v, pH 7.8, 1–2% organic content). Incubation and assessment of eruptive germination was as described.

AS exposure. An emulsion of AS and Tween 20 was diluted with distilled water to concentrations ranging from 2.5 to 20% AS. Sclerotia partially submerged in WA and positioned around a microbeaker containing AS were exposed to the AS concentrations for 7 days in sealed dishes (6 mm diameter) at 15 C. Controls were exposed to diluted Tween 20.

The duration of exposure to 2.5% AS necessary for elicitation of eruptive germination was determined for sclerotia partially submerged in WA. They were exposed at 18 C for time periods ranging from 15 min to 84 hr. After the varied exposure times, microbeakers that contained the AS were removed and sclerotia in the resealed dishes were kept at room temperature (20–22 C) for 5 days; thereafter, incubation resumed at 15 C.

For examination of the effect of incubation temperature, sclerotia exposed for 36 hr to 2.5% AS were incubated at 4 and 9–27 C (in three-degree increments from 9 C). Eruptive germination assessments were made twice a week for 70 days.

Effects of allyl extracts on soilborne sclerotia. Sclerotia were enclosed with 2 g of soil from Tulelake (unclassified, 14% organic content) in 3-cm-square nylon mesh bags. Bags were buried at Tulelake 10 or 20 cm below the soil surface and within 10 or 20 cm of sites at which garlic oil or garlic extract (50%, w/v) was injected at the 10-cm depth. Several sites received an additional injection of the extracts at the original application sites 15 days later. The effects on sclerotial germination of a single or double exposure to the stimulatory compounds were assessed 30, 45, and 60 days after the initial application of garlic oil or extract when bags were retrieved. Germination was determined after wet-sieving and microscopic examination of recovered sclerotia. Total germination counts included the sum total of those sclerotia with cracked rinds, evident only as empty shells, and those with extruded mycelial tufts or strands. Viability of apparently intact sclerotia was determined as described previously.

For examination of the effects of onion root exudates from actively growing or decomposing roots on germination of the sclerotia, bags were prepared and buried as before in an area naturally infested with *S. cepivorum*. Onions (cultivar Southport White Globe) were planted heavily (200 seeds per meter in four rows per bed) 1 day later in this area. Forty-three, 65, and 86 days after planting, the green onion tops were removed by cutting below the stem plate, thus leaving the root systems intact. At these times, and again 22 and 44 days after removal of the tops, bags were retrieved and seven soil samples (30-g) were taken from the top 20 cm of soil in each plot. Sclerotia were recovered directly from bags and from soil samples by wet sieving. Total germination of the introduced and natural sclerotia was then determined.

Secondary sclerotia. Secondary sclerotia are those derived from the internally stored contents of parent sclerotia after eruptive germination of parent sclerotia. The formation of secondary sclerotia was examined by placing sclerotia on glass coverslips thinly coated with the test substrates mentioned earlier. As a control, sclerotia were placed on plain glass coverslips but were moistened twice a week with a distilled water droplet (syringe size 25, Monoject 200). A microbeaker that contained 2% AS was placed centrally between the four coverslips enclosed in a glass petri dish (9 cm diameter). Sclerotia were also exposed to 2% AS en masse at 18 C and were removed after periods ranging from 15 min to 1 wk. After removal, they were placed on 1% NA and transferred to a different incubator set at 18 C.

Table 1. Effect of allyl sulfide concentration on total and eruptive germination of field-produced sclerotia of *Sclerotium cepivorum*[a]

Allyl sulfide concentration[b] (v/v)	Percent germination[c]	
	Total	Eruptive[d]
2.5	100	75
5.0	98	72
10.0	88	52
15.0	88	47
20.0	47	37
Control	85	5
LSD ($P = 0.05$)	5.03	10.9

[a] Sclerotia were recovered from infected *Allium* plants, conditioned in nonsterile soil, and stored at room temperature (20–25 C). Before use, sclerotia were moistened and surface-sterilized for 3 min in NaOCl.

[b] Dilutions were made in distilled water after preparation of an allyl sulfide (AS) emulsion with Tween 20. Exposure to AS was for 7 days.

[c] Germination on 1.5% Bacto water agar, assessed 20 days after removal of AS, was the average of three replicates of 20 sclerotia per replicate.

[d] Eruptive germination recorded as a percentage of total germination.

RESULTS

Eruptive germination of sclerotia. Individual sclerotia collected from field soil (or diseased tissue) or produced in culture on oat seeds could not be distinguished by their color, size, or shape. Clumped sclerotia produced on oats sometimes were misshapen. Sclerotia from either source responded similarly in all laboratory experiments. Washing or drying of sclerotia had no effect on the rate or percentage of eruptive germination achieved in the presence or absence of AS.

Eruptive germination was observed frequently on substrates devoid of available nutrients: NA, PA, and IA and acid-washed quartz sand. In contrast, few viable sclerotia germinated eruptively on PDA or unpurified WA. On WA in the presence of AS, however, more than 70% of sclerotia could be induced to germinate eruptively. Total germination of sclerotia on nonsterile soil increased from 4% in the absence of AS to 47% 20 days after exposure to AS. Sclerotia that did not germinate were visibly contaminated with bacteria, fungi, and/or actinomycetes, yet in some cases were viable because a 3-min surface sterilization in 0.5% NaOCl and incubation on NA resulted in a further 30% eruptive germination. On no medium did 100% of viable sclerotia germinate eruptively.

Exposure of sclerotia to AS concentrations of 2.5 and 5% stimulated eruptive and total germination and the rate of germination. Exposure to 20% AS, despite stimulating eruptive germination over that achieved in the absence of AS, inhibited total germination significantly ($P = 0.05$) (Table 1). Eruptive germination was stimulated after a short exposure to AS. Exposure of sclerotia to 2.5% AS for 15 min was sufficient to induce 32% eruptive germination (Fig. 1). Increasing lengths of exposure to AS from 1 hr up to 24 hr resulted in increased eruptive germination.

Eruptive germination of sclerotia was observed at all temperatures within the range of 4–24 C in the absence of AS and of 4–27 C when sclerotia had been

exposed to AS for 36 hr. AS was most effective in stimulating eruptive germination of sclerotia incubated at 15–18 C.

Germination of soilborne sclerotia. Applications of garlic oil and garlic extract to fallow field soil during April significantly ($P = 0.01$) increased total germination of buried sclerotia (Fig. 2). The number of recovered sclerotia that had germinated, or were in the process of germinating, increased over time after application of the stimulants but never exceeded 60%. The germination response of sclerotia buried 10 cm from the site of stimulant application(s) was not significantly ($P = 0.01$) different from that of sclerotia buried 20 cm from the source of the volatile. Total germination of sclerotia recovered from plots that received two applications of garlic oil or garlic extract (designated 10 + 10, 20 + 20, etc.) was not significantly enhanced over that of sclerotia exposed only to a single application. The viability of recovered, intact sclerotia ranged from 89 to 100%.

Actively growing onion roots stimulated germination of buried sclerotia produced in culture. This stimulation continued to increase as the plants aged, and presumably root mass and quantity of exudation increased. The germination of sclerotia exposed to decomposing onion roots did not differ significantly ($P = 0.01$) from that achieved by exposure to roots of intact plants. On average, germination of the soilborne sclerotia exposed to exudates from the onion roots was significantly ($P = 0.01$) lower than that observed in the buried, culture-produced sclerotia (14 and 45%, respectively). Differences in germination were not observed between the soilborne sclerotia exposed to root exudation and those in the control plots exposed only to water until after 86 days of exposure to onion roots. At completion of the trial, 130 days after planting, the average soilborne sclerotial populations in the areas that had remained fallow and in those with growing onions were 0.10 and 0.09 sclerotia per gram of soil, respectively, and were not significantly different ($P = 0.05$).

Secondary sclerotia formation. The secondary sclerotia of *S. cepivorum* are smaller (0.09–0.35 mm) but otherwise macroscopically similar in appearance to parent sclerotia (0.2–0.6 mm) (Fig. 3). The rinds of some, however, failed to melanize fully. The rind of secondary sclerotia was a one- or two-cell layer more pliable than that of parent sclerotia. Conditions conducive to eruptive germination of primary sclerotia were requisite for subsequent secondary sclerotia formation. Secondary sclerotia with melanized rinds developed within 30 days of a 6-hr exposure to 2% AS. After a 1-wk AS exposure, mycelial clumping and secondary sclerotia development continued for up to 70 days. An average of 2.8 secondary sclerotia per primary sclerotium was observed 68 days after a 1-wk exposure. Exposures of 1 hr or less, though sufficient to stimulate eruptive germination, resulted in subsequent formation of secondary sclerotia with partially melanized rinds. These secondary sclerotia failed to germinate. Overall, secondary sclerotia developed from an average 50% of eruptively germinated sclerotia. Optimal temperatures for their development were between 15 and 21 C. Secondary sclerotia were routinely produced on media devoid of nutrients and also on WA in the presence of AS. After a 1-wk exposure to AS, an average of 0.8, 1.2, 1.2, 3.0, and 0.7 secondary sclerotia per eruptively germinated sclerotium developed on the plain glass coverslips and on coverslips coated with 1% NA, IA, and PA and 1.5% WA, respectively. Delayed exposures of sclerotia to AS after placement on the incubation substrates and precracking of sclerotial rinds exposing medullary cells before AS exposures significantly ($P = 0.05$) reduced subsequent secondary sclerotia formation. Eight of 66 secondary sclerotia that germinated eruptively on NA after a 48-hr AS exposure produced tertiary sclerotia.

DISCUSSION

The duration of exposure to AS, the AS concentration, the incubation substrate's nutritional component, and incubation temperature influence the germination response, hyphal or eruptive, of exposed sclerotia. The results define parameters important for eruptive germination of sclerotia and formation of secondary sclerotia. They provide details necessary for evaluation of a white rot control strategy based on the application of germination stimulants to

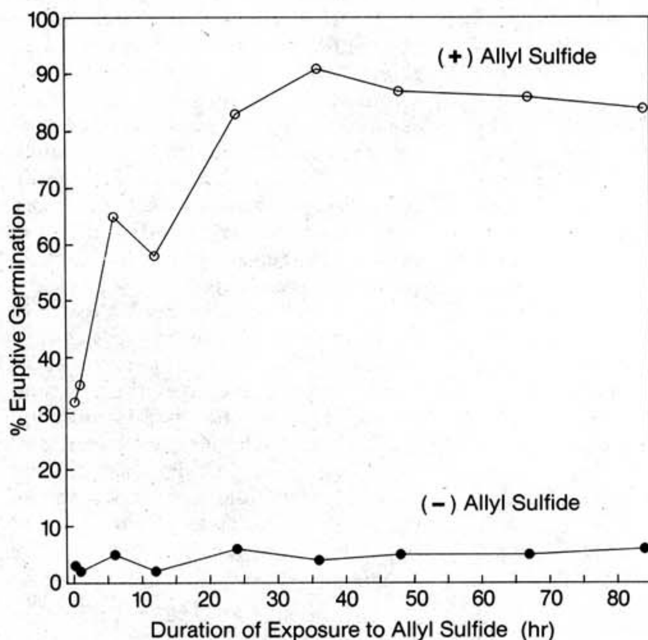

Fig. 1. Effect of duration of exposure to allyl sulfide (AS) on eruptive germination of sclerotia of *Sclerotium cepivorum*. Germination was assessed 30 days after removal of AS. LSD ($P = 0.01$) = 15.3. Sclerotia were incubated during exposure to 2.5% AS at 18 C. After removal of AS, they were placed at room temperature (25 C) for 5 days, then incubated at 15 C for the remainder of the observation period.

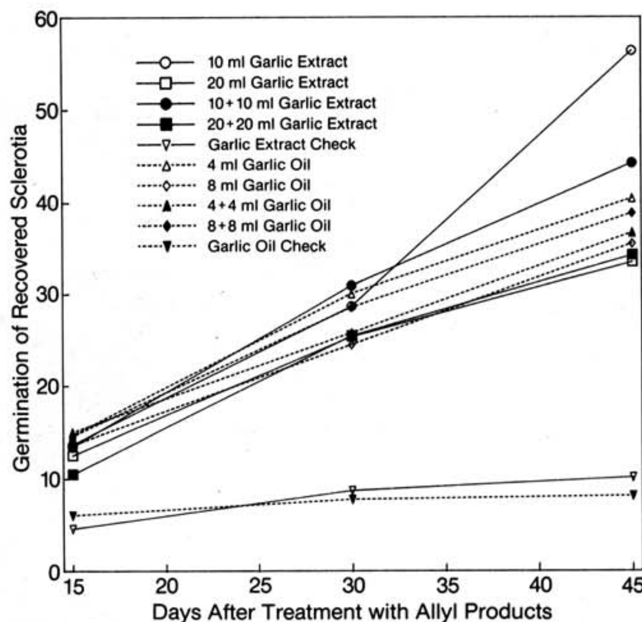

Fig. 2. Stimulation of germination of sclerotia of *Sclerotium cepivorum* with garlic oil and fresh garlic extract (GE) applied to nonsterile soil. Stimulants were injected at 10 cm deep in holes 16 cm apart and arranged linearly down the center of each bed. Additional injections were made 15 days after the initial application and at the same sites.

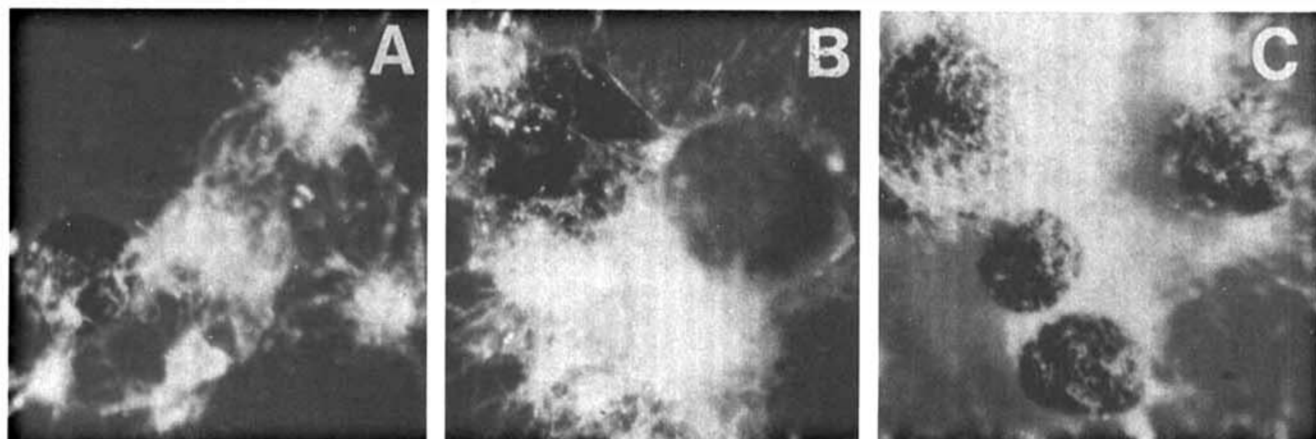

Fig. 3. Secondary sclerotia development on 1% Noble agar; isolate TLS-5 parent sclerotia produced on oats: **(A)** mycelial clumping (40×), **(B)** partially melanized rinds of secondary sclerotia 40 days after 1-min exposure to 2% allyl sulfide (AS) (70×), and **(C)** mature secondary sclerotia 40 days after 36-hr exposure to 2% AS (40×).

soil in the absence of a host crop.

The failure to induce a germination response by washing or drying sclerotia supports observations of other researchers (1,12). This phenomenon probably enhances the ability of such sclerotia to survive and remain potentially infective at depths of up to 30 cm in moist or dry soil (5). *S. rolfsii*, a fungus stimulated by drying to germinate eruptively, does not infect roots from depths greater than about 8 cm in soil, although other factors in addition to drying are involved (14,17). As observed with *S. cepivorum*, eruptive germination of the sclerotia of *S. rolfsii* is inhibited on media containing available nutrients (13).

Onions and garlic are susceptible to infection by *S. cepivorum* throughout their development. Soilborne sclerotia germinate, after overcoming their constitutive dormancy, at staggered intervals during a host's growing season, and a small percentage germinate in the absence of a susceptible host. The concentration of AS and duration of exposure to AS have a greater effect on sclerotial germination than the frequency of exposures, and this probably explains observed "staggered" germination response of sclerotia exposed to *Allium* root exudates or in repeatedly treated field soil. In addition, diffusible metabolites from microorganisms (bacterial and fungal) isolated from the rinds of sclerotia recovered from field soil inhibited the eruptive germination of sclerotia partially submerged in NA (18). This inhibition was overcome by the stimulatory effect of AS, except when metabolites of *Bacillus* strain (C-5) or *Trichoderma* isolate (TH-1) were present in the agar medium (18). Under field conditions, we have shown that onion root exudates and *Allium* extracts applied to the soil stimulated total germination of up to 60% of exposed culture-produced sclerotia. In fields infested with a sclerotial population of less than two per kilogram of soil, an inoculum density reduction of 50–60% would be sufficient to provide a significant reduction in white rot incidence in the next *Allium* crop (6). This inoculum density threshold would be reduced if secondary sclerotial formation and infectivity potential were shown to occur under field conditions. Sclerotial contaminants probably caused the reduced germination response of the natural soilborne population to onion root exudates. Bacteria isolated from sclerotial rinds were generally sensitive to 1% AS. The growth of two *Bacillus* strains, BC-1 and C-5, and a fluorescent pseudomonad, S-4, however, was not affected by exposures to 1 or 5% AS. The growth of *Trichoderma*, TH-1, appeared stimulated by 5% AS (18).

Although the potential for secondary sclerotia formation and disease production by them in nonsterile soil is unknown, it is conceivable that these sclerotia are important in the perpetuation of *S. cepivorum* in soil over long periods by allowing conservation of some of the energy lost when sclerotia germinate in the absence of a host. Secondary sclerotia are smaller than parent sclerotia. Sizing of sclerotia isolated from fallow soil over extended periods may provide further insight into the potential role of secondary sclerotia in *S. cepivorum* population maintenance and disease development. The formation and development of *S. cepivorum* secondary sclerotia, as observed under controlled conditions since 1982 (19), is comparable with that exhibited by *S. rolfsii*, yet unlike that described for *S. cepivorum* by Entwistle and Munasinghe (7). They observed secondary sclerotia fully developed within the parent rind.

For maximum benefit of preplant applications of stimulatory compounds, treatments should be made when soil temperatures favor germination of sclerotia (4–27 C, optimum 15–18 C) but are unfavorable for secondary sclerotia formation (<15 or >21 C). Our trials suggest applications should be made at 12–15 C, because the volatility of AS is reduced and the duration of exposure to AS extended under cool conditions. Field trials established for evaluation of such treatment, to be meaningful, should allow soil sampling and sclerotial sizing to be continued for at least 70 days after the last application. By this means, the germination response of sclerotia, which were observed to be staggered over a 70-day period, and possible secondary sclerotia formation could be determined.

Sclerotia can develop on crop residue in the presence of AS and absence of microbial competition (P. A. Somerville, *unpublished*), but under nonsterile conditions, *S. cepivorum* is not highly competitive and its saprophytic growth and colonization of organic matter is limited (5,16). Thus the search for an economically feasible compound to reduce initial inoculum density by stimulation of germination (10,11) remains a promising approach to white rot control in areas of low inoculum density. This study has shown, however, that despite exposure to stimulatory compounds under conditions near optimal for eruptive germination, only a portion of the sclerotia were stimulated to germinate and that some of these have the potential to produce secondary sclerotia. The residual population of viable sclerotia preserves the potential for white rot infection in a future crop. Dormancy of sclerotia, soil conditions, host genotype, and unidentified inherent sclerotial defense and survival mechanisms influence the survival of this residual population in the absence of susceptible *Allium* crops.

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