

Survival of *Didymella rabiei* in chickpea straw debris in Spain

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Didymella rabiei grew saprophytically on pieces of infested chickpea stems and pods, and formed pycnidia and pseudothecia. The extent of saprophytic growth and production of viable spores were determined by the incubation conditions. On debris left on the soil surface under natural conditions, the fungus rapidly colonized the tissues, formed abundant pseudothecia and pycnidia, and remained viable throughout the 2 years of the study. When plant debris was buried, *D. rabiei* was restricted to the original lesions, in which it formed new pycnidia and was viable for 2 to 5 months. Under controlled conditions in the laboratory, *D. rabiei* extensively colonized plant debris spread over the soil surface. On the other hand, the fungus did not grow on buried debris, or showed only very limited development when the artificially infested debris was buried between two layers of sterilized soil. Incubation temperature was the principal factor associated with the production of conidia and especially ascospores.

INTRODUCTION

Ascochyta blight of chickpeas (*Cicer arietinum*), caused by *Ascochyta rabiei*, causes severe crop losses in those areas of the world where chickpeas are cultivated (Nene & Reddy, 1987). The characteristic symptoms of the disease are necrotic lesions, which are circular on the leaflets and pods and elongated and irregular on the stems and petioles. Necrotic lesions weaken the stems and petioles, which then break easily. Inspection of the lesions often reveals concentric arrangements of *A. rabiei* pycnidia (Nene & Reddy, 1987).

A. rabiei survives in chickpea seed and in infested crop residues (Nene & Reddy, 1987), and the seed provides an efficient means of survival and dissemination of the fungus (Kaiser, 1984). However, there is less published data on the ability of *A. rabiei* to survive and remain active in the debris of infested plants. Authors from India (Luthra *et al.*, 1935), Iran (Kaiser, 1973) and Greece (Zachos *et al.*, 1963) have reported that the fungus can survive for at least 2 years in debris of infested chickpeas lying on the soil surface. However, studies conducted in Syria reported a maximum survival period of 8 months (ICARDA, 1982, 1983). Nevertheless, when debris of plants infected by this pathogen was

buried at depths that ranged from 5 to 40 cm, the fungus quickly lost viability, and maximum survival periods were from 2 to 4 months (Luthra *et al.*, 1935; Kaiser, 1973; ICARDA, 1982, 1983). Under controlled conditions, *A. rabiei* remained viable for more than 2.5 years in debris of infested chickpea plants kept at temperatures in the range 4–35 °C with relative humidities (RH) in the range 30–40% (Kaiser, 1973; Kaiser *et al.*, 1987). However, fungus survival was drastically reduced to 2–18 weeks when RH was in the range 65–100% (Kaiser, 1973).

In the debris of infected plants that lies on the soil surface in winter, *A. rabiei* forms the sexual stage, or teleomorph, *Didymella rabiei* [= *Mycosphaerella rabiei*]. The teleomorph was first described in Bulgaria in 1936 (Kovachevski, 1936). Subsequently, its occurrence was confirmed in Russia (Gorlenko & Bushkova, 1958), Greece (Zachos *et al.*, 1963), Hungary (Köveci *et al.*, 1986), the United States (Kaiser & Hannan, 1987), Spain (Jiménez-Díaz *et al.*, 1987) and Syria (Haware, 1987).

Knowledge of how the pathogen survives and develops in infested plant debris would be of importance in the design of effective control strategies for ascochyta blight. The objective of this work was to identify and determine, both in

the laboratory and under the field conditions of Andalusia (S. Spain), the environmental factors that affect survival and growth of *D. rabiei* on chickpea debris.

MATERIALS AND METHODS

Field experiments

In July 1986, infested debris of chickpea plants cv. Blanco Lechoso was gathered from a crop in Córdoba province that had been subject to a severe attack by ascochyta blight in the spring of that year. Pieces of stems and pods that showed the characteristic lesions of blight were placed in nylon-net bags (14 × 20 cm) with a 1.6-mm mesh permeable to air. In September 1986, bags were secured on the soil surface or were buried at a depth of 10 or 25 cm in two plots, one at the 'Alameda del Obispo' research farm near Córdoba, and the other at a farm in Montilla (Córdoba province). The same procedure was repeated at the 'Alameda del Obispo' farm in October in each of the four years 1987 to 1990, where bags of infested stem debris were secured on the soil surface or buried at a depth of 10 cm. The soils of the farms at Córdoba and Montilla are, respectively, alfisol (sandy loam soil, pH 8.5) and vertisol (clay soil, pH 8.3), with 1.4 and 2.1% organic matter, respectively, which are representative of the soils of the two principal chickpea cultivation areas of Andalusia. For each experimental plot and period, the content of one bag for each burial depth was sampled at monthly or 15-day intervals for 2 years, except for those in the Córdoba experiment started in September 1986, which were sampled for 1 year only.

In order to assess the extent of colonization and to determine the fungal structures produced and their viability, 12 pieces of stem were taken from each bag and split into 1.5-cm fragments. The samples were washed with tap water, surface-sterilized in 0.25% NaOCl for 5 min, and incubated in a moist chamber at $20 \pm 1^\circ\text{C}$ in darkness for 48–96 h.

The extent of saprophytic colonization of debris by *D. rabiei* was assessed on a scale of 0–5 according to the percentage of surface tissue covered by pycnidia and pseudothecia of the fungus (0, 0%; 1, 1–25%; 2, 26–50%; 3, 51–75%; 4, 76–99%; 5, 100%). Eight 1.5-cm stem fragments randomly selected from each sample were used to determine *D. rabiei* viability. The fungal structures on the surface of the fragments were placed in a drop of sterile water on a sterile

microscope slide. The material was macerated with a sterile needle in order to give a homogeneous suspension, and this was spread evenly on the surface of acidified water agar (AWA; 20 g agar, 1 l deionized water, 0.25 ml 85% lactic acid) in Petri dishes. In addition, selected stem fragments were wiped on the AWA surface of other dishes. The two sets of dishes were incubated in darkness at $20 \pm 1^\circ\text{C}$ for 7–14 days before inspecting them to determine the occurrence of *D. rabiei* colonies.

Laboratory experiments

Two types of stem debris of chickpea plants cv. Blanco Lechoso were used in all the experiments: (a) naturally infested debris; and (b) sterilized debris of healthy plants. The dried infested stems were cut into 6-cm-long pieces which had at least two *D. rabiei* lesions. The stem pieces were washed in running tap water for 60 min, surface-sterilized in 1% NaOCl for 5 min, and then dried with sterile filter paper. The stems from healthy plants were also cut into 6-cm-long fragments, sterilized with propylene oxide, and then inoculated with a conidial suspension (10^5 conidia/ml) from two sexually compatible monoascosporic isolates of *D. rabiei* (ATCC-76501 and ATCC-76502) (Trapero-Casas & Kaiser, 1992). The soil used in these experiments was from the 'Alameda del Obispo' research farm at Córdoba. The matric potential/gravimetric humidity relationship was determined in the Regional Agricultural Laboratory at Córdoba. The soil was sieved to remove the coarser elements and homogenized with a wooden roller. The water content of an aliquot of this homogenized soil was determined on the basis of percentage weight lost after drying in a desiccating oven at 110°C for 24 h. Water was added to the homogenized soil to reach 24.6% water content by weight, the equivalent of a matric potential of 0.03 MPa (water retention capacity). For the experiments with sterilized soil, the soil was autoclaved at 121°C for 2.5 h before moistening.

The moistened soil was placed in 9-cm Petri dishes (60 g per dish), and six 6-cm-long stem fragments were placed on the soil surface of each dish and then covered evenly with an additional 30 g of soil. A further six stem fragments were placed on the surface of this layer and left uncovered. The dishes were placed on a grill over a film of distilled water inside hermetically sealed plastic boxes and incubated in the dark at 5, 10, 15, 20 or 25°C for a period of 12 weeks. Samples

of three stem fragments were collected from each experimental combination at 1-week intervals. The artificially inoculated stem fragments were incubated in either sterilized or unsterilized soil, while the naturally infested stem fragments were incubated only in unsterilized soil. The extent of colonization of the fragments was established using the 0–5 scale described previously. In addition, the production of viable conidia in the pycnidia formed on the fragments collected at 4 and 8 weeks was quantified. A total of 60 pycnidia were taken at random from each fragment, macerated as described previously, and a serial dilution of a homogeneous suspension of the macerate was spread evenly on the surface of AWA in Petri dishes. The dishes were incubated in darkness at 20°C for 8–12 days, and the number of *D. rabiei* colonies growing on the agar plates was then counted. The production of asci was determined for 60 selected pseudothecia in which the asci appeared to be mature, with the ascospores well differentiated (Trapero-Casas & Kaiser, 1992). Pseudothecia were squashed on microscope slides, stained with lactophenol-acid fuchsin, and examined under the microscope.

Data analysis

The data were analysed by standard analysis of variance (ANOVA). For the field experiments, the extent of colonization was analysed separately for each field plot and yearly period. The experiments consisted of a completely randomized design with three burial depths in soil, 24 or 48 sampling units (months or 15-day intervals), and 12 replications (chickpea stem pieces). In the laboratory experiments, temperature was the main factor, and subordinate factors were the type of debris (naturally infested or artificially inoculated), the location of the debris in the plates (buried or placed on the surface) and the soil treatment (natural or sterilized). Because of the high number of factors involved and of significant interactions between them, and because there were treatments in which *D. rabiei* did not grow, separate analyses were carried out for each type and position of debris, and for each soil treatment. In each analysis, temperature was the only factor considered. In the analysis of the extent of saprophytic colonization, the weekly observations were the sample units. For the numbers of conidia per pycnidium, the observations made at 4 and 8 weeks were analysed as blocks and there were three replicates, each with 60 pycnidia. For

numbers of asci per pseudothecium, the 60 pseudothecia sampled at maturation constituted the replicates. Means were compared using the Fisher protected least significant difference test at the 5% probability level (Steel & Torrie, 1985).

RESULTS

Survival of *Didymella rabiei* in the field

At the time when the debris of the chickpea stems and pods was placed on the soil surface or buried, *D. rabiei* was restricted to the lesions developed during the growing period of the crop; some pycnidia had already formed. The subsequent development of the fungus on the surface debris differed markedly from that of the fungus on the buried debris (Table 1). In all the experimental plots, the stem debris on the surface showed a significantly higher ($P < 0.05$) level of colonization than that of the debris buried at either 10 cm or 25 cm. There was no significant difference ($P = 0.05$) between the two depths of burial, and the viability of the fungus was lower in the buried debris.

The fungus colonized the tissues most extensively on plant debris secured on the soil surface. After October, many fruiting bodies covered the entire debris surface. The extent of saprophytic colonization of debris ranged from 50 to 80% of the tissue surface, and was found to depend on both experimental plots and periods. Stereomicroscopic examination of surface debris, both untreated and incubated in a moist chamber, revealed that it was extensively covered with numerous pycnidia and pseudothecia of *D. rabiei*. From November to January, pseudothecia were immature and their contents were completely undifferentiated, or the lumen of pseudothecia developed thin, septate and hyaline hyphae arranged in compact packets. These pseudothecia matured at the end of February or the beginning of March, and produced many asci and ascospores. In the viability tests, all the fungal structures formed on surface debris gave rise to abundant colonies of *D. rabiei* on AWA (Table 1).

In the debris buried at 10 and 25 cm, *D. rabiei* was confined to the original lesions, and new pycnidia formed in these during the first 2 months of the study. In the subsequent samples, the outermost tissues of the buried debris were progressively disintegrating and the debris was profusely colonized by saprophytic fungi. These fungi occupied the entire tissue in the later samples. In general, only pycnidia and not

Table 1. Survival of and colonization by *Didymella rabiei* in stem debris of naturally infected chickpeas on the soil surface, and buried at depths of 10 cm and 25 cm in two experimental plots

| Place | Experimental period | Depth (cm) | | | | | |
|----------|---------------------|---------------------------|--------------------|---------------------------|--------------------|---------------------------|--------------------|
| | | 0 | | 10 | | 25 | |
| | | colonization ^a | viability (months) | colonization ^a | viability (months) | colonization ^a | viability (months) |
| Córdoba | 1986/87 | 4.3 ^b a | >12 | 1.0 b | 2 | 1.0 b | 2 |
| | 1987/88 | 3.0 a | >24 | 1.7 b | 3 | — | — |
| | 1988/89 | 3.4 a | >24 | 1.0 b | 2 | — | — |
| | 1989/90 | 2.8 a | >24 | 1.2 b | 2 | — | — |
| Montilla | 1986/87 | 3.5 a | >24 | 1.7 b | 5 ^c | 1.5 b | 5 |

^a The extent of colonization was assessed on a scale of 0–5 according to the percentage of tissue covered by pycnidia and pseudothecia.

^b For each place and experimental period, values followed by the same letter do not differ significantly ($P = 0.05$).

^c The fungus was only reliably recovered during the first 5 months of the study; however, one sample was recovered after 8 months.

pseudothecia of the fungus developed in the buried debris. However, in the debris buried at 10 cm on the Córdoba research farm in 1987, 15–20% of the plant tissue surface was covered with pseudothecia and, to a lesser extent, with pycnidia. Even here the growth of the fungus was uneven, and pseudothecia had degenerated, ascospore production being extremely scarce.

D. rabiei pycnidia in buried debris rapidly lost their viability. The fungus could not be isolated from viability-test samples from the Córdoba research farm 2 or 3 months after burial (Table 1). When debris buried at 10 cm and 25 cm at the Montilla farm was sampled, colonies of *D. rabiei* grew from macerated fungal structures or stem wipings up to 5 months after burial, but the number of colonies produced by the last sample was considerably lower than that produced by earlier samples. Almost all viability tests of subsequent samples were negative, but one sample of debris buried at 10 cm for 8 months generated a very small number of colonies.

Colonization of chickpea stems under controlled conditions

Both pycnidia and pseudothecia developed in most of the experimental treatments in which chickpea debris was colonized by *D. rabiei*. The exception was debris artificially inoculated and incubated at 5°C, which produced only pycnidia. The extent of colonization of chickpea debris by *D. rabiei* varied with incubation temperature,

and was found to depend primarily on soil treatment and whether the debris was on the soil surface or buried (Fig. 1a).

In unsterilized soil, debris incubated at either 20°C or 25°C was rapidly colonized by saprophytic fungi, which effectively limited the growth and spread of *D. rabiei* on the debris. These fungi included species of *Pleospora*, *Stachybotrys* and *Stemphylium*, but *Chaetomium* was the most common genus. On debris incubated at 10°C or 15°C, this saprotrophic colonization was less marked and *D. rabiei* developed. Although the extent of *D. rabiei* colonization of the debris incubated on the surface of unsterilized soil was found to depend on the type (naturally infested or artificially inoculated) of chickpea stems used in the experiment, it was significantly ($P < 0.05$) influenced by incubation temperature.

On the plant debris placed between two soil layers, the development and spread of *D. rabiei* was observed only on those pieces of artificially inoculated stem that were incubated in sterile soil. The extent of colonization of these pieces depended significantly ($P < 0.05$) on incubation temperature. No fungal growth or development occurred, either on artificially inoculated or on naturally infested stem pieces incubated in natural soil, at any of the five temperatures studied.

Spore production under controlled conditions

In all cases in which *D. rabiei* colonized chickpea debris, the fungus remained viable for the entire

3-month period of the experiments. The number of viable conidia in the pycnidia on stem debris varied significantly ($P < 0.05$) with the following four factors: temperature, time, position of debris and soil treatment (Fig. 1b). On the surface debris, conidial production decreased significantly as temperature increased from 5°C to 25°C. Overall, the highest numbers of conidia were observed on the artificially inoculated debris incubated on the surface of sterilized soil, and in debris incubated on the surface of natural soil at 5°C. Soil treatment and the type of debris influenced conidial production differently. However, the type of debris on the surface of natural soil did not significantly affect the number of conidia that developed in the pycnidia.

In buried debris, the number of conidia per pycnidium showed no significant variation with incubation time, but it was significantly influenced by incubation temperature. The number of conidia produced was highest with incubation at 10°C, and decreased with incubation at 5, 15, 20 and 25°C. Except on artificially inoculated debris incubated at 5°C, pseudothecia of *D. rabiei* developed in all the experimental combinations that gave rise to fungal growth on debris (Fig. 1c). Nevertheless, only those pseudothecia formed at 10°C in any incubation conditions, and those developed on artificially inoculated debris incubated on sterilized soil at 15°C, matured and produced asci and ascospores. Pseudothecial development was abnormal on debris placed between two soil layers; many pseudothecia degenerated and the number of asci per pseudothecium was significantly ($P < 0.05$) lower than the value for pseudothecia formed on surface debris.

DISCUSSION

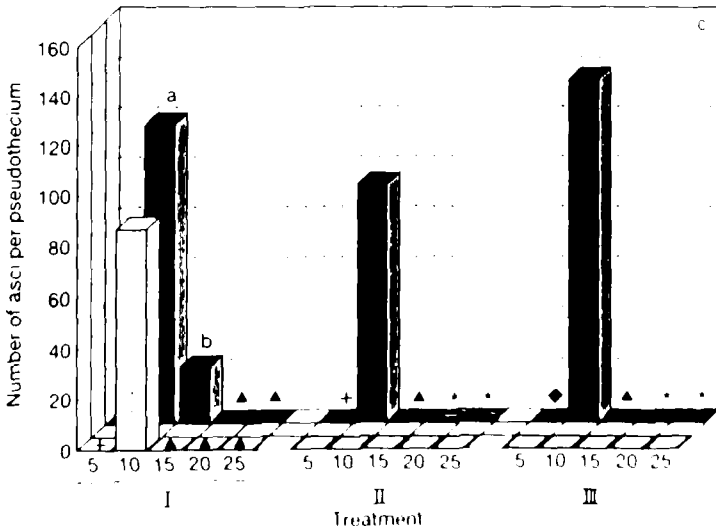
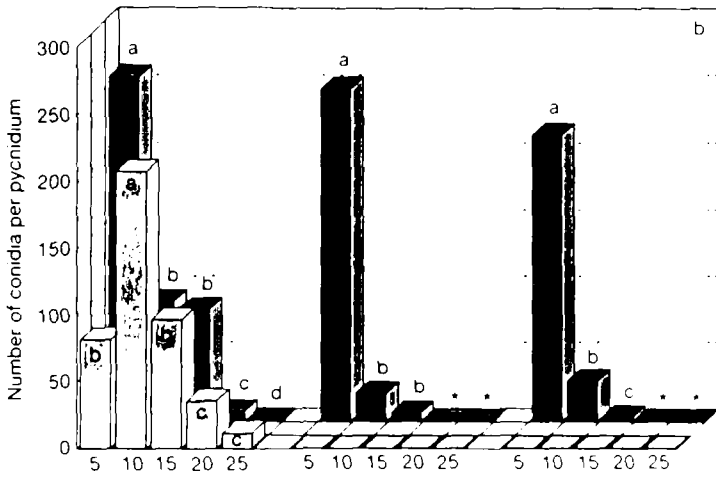
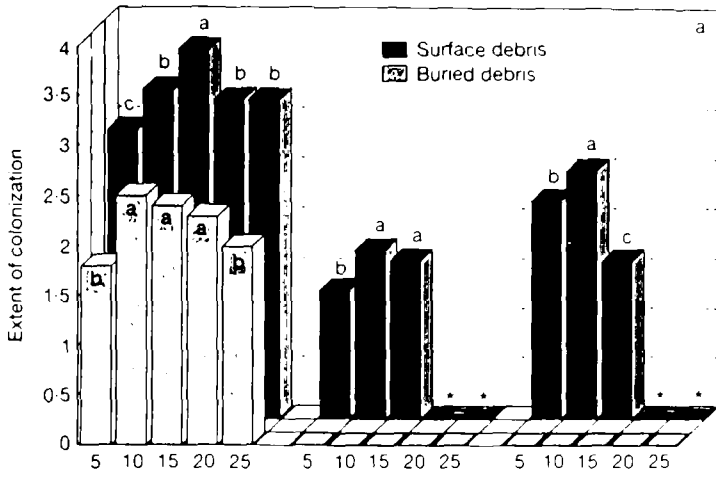
During the 2-year period of each experiment, *D. rabiei* survived and extensively colonized chickpea debris left on the soil surface, with the formation of both pycnidia and pseudothecia that produced viable spores. However, this capacity to produce inoculum decreased sharply after the first year on the soil surface. Furthermore, the viability of the fungus was much reduced and more erratic in debris buried at 10 cm or 25 cm. Other investigators have reported that *D. rabiei* survives on chickpea debris on the soil surface in other parts of the world where the crop is grown (Luthra *et al.*, 1935; Zachos *et al.*, 1963; Kaiser, 1973; Kaiser *et al.*, 1987). In the present study, the saprophytic

colonization of buried debris by *D. rabiei* was slight. The fungus was viable for only 2–5 months, and its viability depended on both the locality and the year. In buried debris the fungus usually produced pycnidia, except in the 1987/88 period, when it formed some pseudothecia. However, these had degenerated, extending the survival period only slightly, and were probably of little epidemiological significance. In this context, our results are very similar to those of previously published investigations on ascochyta blight of chickpeas in other geographical areas (Kaiser, 1973; ICARDA, 1982, 1983), although none of these investigators reported the formation of pseudothecia. Kaiser *et al.* (1987), working in Washington State, USA, reported that ascospores and conidia lost viability after 2 and 3.5 months, respectively, when the debris containing both pycnidia and pseudothecia was buried at a depth of 16 cm.

In our study, no specialized fungal survival structures such as chlamydozoospores of sclerotia, as described for *Ascochyta pinodes* or *Ascochyta pisi*, were found on either surface or buried debris. Such structures confer on these species the ability to survive in soil for more than 1 year after the complete destruction of the host tissues (Wallen *et al.*, 1967; Dickinson & Sheridan, 1968). Although, in the course of this work, we often observed brown, thick-walled, swollen hyphae in the infested tissues, the fact that these hyphae were always associated with fruiting bodies suggests that their survival role is limited, and that it is linked in some way with the development of the sexual stage of the fungus.

The more rapid growth rates and more extensive spread on surface debris on sterilized compared to non-sterilized soil suggests that the growth and spread of *D. rabiei* in chickpea debris is influenced by the presence of other soil micro-organisms. On debris lying on sterilized soil, the production of pycnidia and pseudothecia was uniform and extensive, and very similar to that observed in experiments under controlled conditions without soil (Navas-Cortés, 1992). On natural soil, however, fungal growth and spread were very uneven, and the numbers of pycnidia and pseudothecia were very much smaller than those on sterile soil. This supports field and laboratory observations which suggest that the presence of other saprophytic micro-organisms, particularly fungi, limits *D. rabiei* growth and spread.

When stem debris was buried, growth of *D.*



rabiei was completely prevented unsterilized soil and considerably reduced in sterilized soil. The numbers of pycnidia and pseudothecia produced on debris in sterilized soil were much lower than on the surface of the same soil. The development of pseudothecia was abnormal, and those that predominated either had degenerated contents or very few asci and ascospores. This development pattern is very similar to that observed in buried debris under field conditions, especially during the 1987/88 period, when the colonization of debris gave rise to both pycnidia and pseudothecia.

The results of our experiments on the survival of *D. rabiei* under natural conditions support some of those measures recommended for control of the disease which aim to reduce the initial inoculum (Luthra *et al.*, 1935; Zachos *et al.*, 1963; Nene & Reddy, 1987). Specifically, these measures involve either removal or deep burial of the debris from infected chickpeas, combined with crop rotation, so that there is an interval of at least 2 years between chickpea crops. However, the efficacy of disease control measures may be curtailed by the formation of the teleomorph stage of *D. rabiei*. This sexual reproductive stage was first reported in Spain in 1987 (Jiménez-Díaz *et al.*, 1987). The discovery greatly modified our previous picture of the epidemiology of ascochyta blight of chickpea (Navas-Cortés, 1992) because, until that time, all the available data suggested that, in Spain, only the imperfect stage of *D. rabiei* was involved in the disease cycle (Trapero-Casas, 1986; Nene & Reddy, 1987).

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Fig. 1. Extent of (a) colonization, (b) number of viable conidia produced per pycnidium and (c) number of ascospores produced per pseudothecium by *Didymella rabiei* in surface and buried chickpea stem debris. Treatments: I, artificially inoculated debris incubated in sterilized soil; II, artificially inoculated debris incubated in natural soil; III, naturally infested debris incubated in natural soil. Treatments were incubated in the dark at 5, 10, 15, 20 or 25 °C. The extent of colonization was assessed on a scale of 0-5. Conidia were counted after 4 and 8 weeks of incubation with three replicates at each count date. (*) Treatment combinations in which the debris was colonized rapidly by saprophytic fungi that impeded growth of *D. rabiei*; (+), treatment combinations in which pseudothecia did not form; (◆), treatment combinations in which pseudothecia did not develop mature asci; (▲), treatment combinations in which pseudothecia degenerated without fully developing. For each treatment and debris position, bars with the same letter did not differ significantly ($P = 0.05$)

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