Use of Oospore Inoculum of *Aphanomyces cochlioides* to Initiate Blackroot Disease in Sugarbeet Seedlings[1]

C. L. SCHNEIDER[2]

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Introduction

The beet water mold *Aphanomyces cochlioides* Drechs., is one of the most important pathogens associated with the black root disease complex of sugarbeet (*Beta vulgaris* L.) seedlings. Since 1955, sugarbeet cultivars with resistance to *A. cochlioides* have been grown in the Great Lakes area of the USA, where the disease limits sugarbeet production.

Increasing resistance to black root disease is a major objective in breeding programs for development of improved sugarbeet cultivars for the Great Lakes area. Since 1957, breeding lines have been screened for resistance to *A. cochlioides* in greenhouse tests with zoospore inoculum (4)[3]. The development of techniques for *in vitro* production of *A. cochlioides* oospores and the demonstration that oospores are infective (5) prompted me to investigate the possibility of using oospore inoculum in screening tests. Methods of producing inoculum in bulk were developed and I studied the effects of inoculum storage time, oospore density, inoculum placement, and type of potting mix on the development of seedling infection from oospore inoculum. The results of the study, reported herein, provide the basis of a methodology for use of oospore inoculum of *A. cochlioides* to initiate blackroot disease.

Materials and Methods

Inoculum production

The *A. cochlioides* culture had been isolated from a sugarbeet seedling and used for production of zoospore in inoculum in greenhouse screening tests. For production of oospores, the fungus was

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[2]Plant Pathologist, Agricultural Research Service, U.S. Department of Agriculture, Box 1633, East Lansing, Michigan 48823.

[3]Numbers in parentheses refer to literature cited.

cultivated in 0.5% homogenized oatmeal broth. Preparation of the broth, based on the method of Gooding and Lucas (1), consisted of comminuting rolled oats in 300 ml of distilled water in a blender for 5 minutes, adding the appropriate amount of water, and heating to 50°C. Before autoclaving, the broth was adjusted to pH 6.6 with N/20 HCl.

I developed and tested two kinds of oospore inocula, designated nutrient vermiculite (N-V) and spore suspension-vermiculite (SS-V). The N-V inoculum was prepared from vermiculite carrier saturated with oatmeal broth in which the fungus had been cultivated. the SS-V inoculum was prepared from vermiculite carrier mixed with an aqueous suspension of oospores.

Medium grade vermiculite carrier with particles of about 2-5 mm in diameter was used in most cases. Fine grade (with particles about 0.5-2 mm in diameter) was used in the latter part of the investigation when the medium grade became unavailable from our usual suppliers. The fine grade proved as satisfactory as the medium grade, and had the advantage of being less bulky. Preliminary tests had indicated that seedlings grown in soil with sterile vermiculite carrier emerge and develop normally.

The N-V inoculum was prepared according to the method of Varney (6). The fungus was incubated for about 30 days in wide-mouthed Erlenmeyer flasks containing oatmeal broth:medium grade vermiculite mixture (1:2.5, v:v) at 25°C. Upon removal from the flasks, the vermiculite cultures were air dried on paper-covered screens for two weeks, then stored in cans with plastic covers at 4°C for periods from 30 days to 52 months. Microscopic examination revealed oospores and mycelial fragments associated with the vermiculite particles. Previous studies (5) indicated that mycelial fragments are unable to survive desiccation. The oospores could not be readily separated from the opaque vermiculite particles; so determinations of oospore concentration in the inoculum were not attempted.

For preparation of SS-V inoculum, mycelial mats, previously incubated in flasks of oatmeal broth, were comminuted in water in a blender for 5 minutes. The concentration of oospores in the resulting suspension was determined with a Howard spore counting chamber. Usually 10-low-power microscope fields were counted in each of three to six samples. The 95% confidence limits for the mean number of spores/ml were subsequently computed. The suspension was then diluted with water to the desired spore concentration and vermiculite was added at the rate of 2.5 parts (medium grade) or 2 parts (fine grade):1 part of suspension (v:v). To reduce the likelihood of spores settling excessively, spore suspension and

vermiculite were mixed in wide, shallow vessels such as photographic developing trays, and the depth of the suspension was limited to about 5 mm. After mixing, the inoculum was air dried for 14 days, then stored in cans at 4°C.

Inoculation tests

Greenhouse temperature was 25°C and ranged from approximately 18-43°C. In most experiments seedlings were grown in plastic pots (10.2 cm upper diameter, 7.1 cm lower diameter, and 9.5 cm depth) containing a steam-sterilized mixture of medium grade vermiculite:peat:arcillite (a granular calcined montmorillinite clay) 1:2:1 (v:v:v). Later, when medium grade vermiculite was no longer available, I used fine grade vermiculite in a 2:1:1 mixture. At planting, 500 cc of growth mixture were added per pot, the seed balls were placed on the soil surface, and a measured volume of inoculum was added; the seed was then covered with an additional 100 cc of growth mixture.

Emerged seedlings were counted 10-15 days after planting. In some experiments, I determined the number of days from inoculation until the first symptom of blackroot disease appeared in each pot (incubation period). The incidence and severity of seedling infection were determined 4 to 6 weeks after planting. Each plant was rated according to a disease-severity index (DSI) ranging from 0 (no symptoms) to 5 (dead). A mean DSI for each pot of seedlings was then computed.

In each experiment the significance of differences among treatments was tested by means of the t-test or by the analysis of variance. The least significant difference (LSD) among treatments and the coefficient of variation (the standard deviation in percent of the general mean) were also computed.

Most of the inoculation tests included cultivars US 401 or US H20. Both cultivars are moderately resistant to black root disease under field conditions and have been used as standards for comparison in greenhouse screening tests (4). Since 1968, US H20 has been grown as a commercial variety in the Great Lakes area.

Results

Effect of inoculum density

Disease severity increases with increasing density of *A. cochlioides* zoospore inoculum (3); I investigated the possibility of a similar relationship with oospore inoculum. Earlier, MacWithey (2) showed that blackroot disease severity varies with numbers of *A. cochlioides* oospores/mg of barley residue in soil.

With N-V inoculum, for which oospore concentration was unknown, inoculum density was measured as volume added per pot. Preliminary tests with NV inoculum showed 4 cc/pot to be the minimum density that consistently produced 100% incidence of disease. In a subsequent series of 10 tests with cultivar US H20, each test comprising two pots of each of two inoculum treatments, inoculum densities of 5 and 10 cc/pot produced D.S.I.'s of 2.8 (2.1-3.8) and 3.2 (2.3-4.4) respectively. This difference is significant at the 5% level according to a t-test of paired variates. Seedling emergence in the inoculated pots did not differ significantly from that of the uninoculated control. At inoculum density levels of 5 and 10 cc/pot, disease incidence was 100%.

In tests with SS-V inoculum, inoculum density was measured by the concentration of oospores in the suspension mixed with vermiculite and the volume of vermiculite carrier/pot. A series of six experiments was conducted to compare disease development at densities (mean $\pm$ 95% confidence limits, x 10^3) of 5 $\pm$ 3, 11 $\pm$ 5, 23 $\pm$ 11, and 53 $\pm$ 15 oospores/pot. A total of 13 batches of inoculum with oospore concentration ranging from (0.4 $\pm$ 0.3) to (10.0 $\pm$ 3.0) x 10^3 oospores/cc of vermiculite carrier was employed. The volume of vermiculite carrier ranged from 1 to 10 cc/pot.

In each experiment, seedling emergence at the various inoculum densities did not differ significantly from that of the uninoculated control and at each inoculum density, disease incidence was consistently 100%. In each experiment, the DSI increased with increasing inoculum density (Table 1). In five of the experiments, differences in DSI's at different inoculum densities were statistically significant.

Effect of depth and time of inoculum placement

Disease development was compared among pots in which inoculum had been applied at different levels in the growth mixture. Preliminary tests showed no significant differences among the DSI's resulting from application of N-V inoculum at seed ball level (12 mm below the surface), application 6 mm above, and application 6 mm below the seed ball level.

In a subsequent experiment, I compared the effects of applying SS-V inoculum at three depths: on the surface of the growth mixture and 12 and 36 mm below the surface; I also tested surface applications made 6 days after planting. The inoculum, containing (2.9 $\pm$ 0.8) x 10^3 oospores/cc of vermiculite, was applied at the rate of 7 cc/pot.

Table 1.—Effect of density of *Aphanomyces cochlioides* SS-V* oospore inoculum on severity of blackroot disease in seedlings of sugarbeet cultivar US H20 in six experiments.

Mean no. of† oospores/pot x 10^3	Disease severity index‡ in experiment no. 1	2	3	4	5	6	Mean
O (Control)	0	0	0	0	0	0	0
5 (±3)	1.9 a	2.1 a	3.2 a	—	—	—	2.4
11 (±5)	2.8 b	2.5 ab	—	2.4 a	3.2 a	3.6 a	2.9
23 (±11)	—	3.1 b	3.5 a	2.9 b	3.5 b	3.6 a	3.3
53 (±15)	—	—	4.1 b	—	—	4.1 a	4.1
No. of replicated pots/treatment	8	4	6	8	21	3	
Coefficient of variation, %	23.9	16.8	10.2	21.0	14.9	16.3	

*Spore suspension — vermiculite.
†Confidence limits (95%) are indicated in parentheses.
‡At about 30 days after inoculation; 0 (no symptoms) to 5 (dead); within an experiment values followed by a letter in common do not differ significantly at the 5% level according to the LSD test.

None of the inoculation treatments significantly reduced seedling emergence, and each inoculum treatment produced 100% incidence of disease. The two sub-surface applications produced significantly higher DSI's than the surface applications (Table 2). The incubation period was significantly longer and the DSI's lower for the surface application 6 days after planting than for the surface application at planting.

Effect of potting mix composition

I studied the effects on disease development of varying the proportions of several potting mix components in general use including

Table 2.—Effect of depth and time of placement of *Aphanomyces cochlioides* SS-V* oospore inoculum on development of blackroot disease in seedlings of sugarbeet cultivar US H20.

Placement of inoculum: Depth below surface (mm)	Time (days after planting)	Incubation† period	DSI‡
0	0	14.5 b	3.0 ab
0	6	21.3 a	2.3 a
12 (seed level)	0	14.5 b	4.2 c
36	0	15.0 b	3.7 bc
Uninoculated control	—	—	0
Coefficient of variation, %		15.4	17.2

*Spore suspension — vermiculite, applied at the rate of 20 ± 6 x 10^3 spores/pot.
†Number of days from placement of inoculum until first appearance of blackroot disease symptoms.
‡Data are expressed as means of four pots. In each column, means followed by a letter in common do not differ significantly at the 5% level according to LSD test.

vermiculite (medium and fine grades), arcillite, peat, and siliceous volcanic rock. I also tested two commercially prepared mixes containing tree bark compost in addition to some of the previously listed components.

In tests with SS-V inoculum at (21 $\pm$ 12) x 10^3 oospores/pot there were no significant differences in seedling emergence among mixes, but there were significant differences in DSI's. Disease severity increased when the proportions of mineral conponents:peat were increased from 1:1 to 2:1 or more. Disease severity with the two commercial mixes was significantly lower than that with the standard mix used in these studies.

Effect of storage of inoculum

Most batches of oospore inoculum were used within a few months after preparation. My studies on the effect of age of inoculum on infectivity and of disease severity was restricted to the relatively few batches that were kept for a year or more.

Preliminary tests showed that storage temperature affects the ability of oospore inoculum to retain infectivity for prolonged periods. Three lots of N-V inoculum from the same batch were stored at 23, 4, and -9°C and infectivity was periodically tested in greenhouse inoculations. After 6, 12, and 18 month's storage, each lot was equally and highly infective to US H20 sugarbeet seedlings. After 24 and 30 month's storage, however, the mean DSI of seedlings exposed to inoculum stored at 23°C was significantly lower than that of inoculum stored at 4 and -9°C.

Batches of SS-V inovulum were infective after storage periods exceeding one year. Among six batches tested in December, 1974, after 13-16 months in storage, the DSI of US H20 seedlings exposed to 20-30 x 10^3 oospores/pot ranged from 2.4 to 3.7 (mean. = 3.3). Among five of the batches, the DSI's did not differ significantly from DSI's produced 12 months previously. One batch produced a significantly lower DSI (2.4) in 1974 than in 1973 (3.5). A batch tested in June, 1975, after the longest storage period (22 months) of SS-V inoculum involved in this experiment, proved highly infective, producing a DSI of 3.9 on seedlings of US H20.

Oospore and zoospore inocula compared

The results of screening tests of breeding lines for resistance to black root disease indicate little difference in variability between tests with zoospore inoculum and with oospore inoculum. In a series of 14 tests with zoospore inoculum, each test comprising 12 entries replicated 5 times, the mean and the range of the coefficient of

variation was 14.0% (9.3-20.2%). In a subsequent series of 15 similar tests with N-V oospore inoculum and 18 tests with SS-V oospore inoculum, the mean and the range of the coefficient of variation was 13.9% (8.0-20.9%) and 13.6% (5.7-29.4%) respectively.

Discussion

Aphanomyces cochlioides oospore inoculum can be used to initiate experimental infection of sugarbeet seedlings, producing incidence and severity of disease that permit evaluation of black root resistance. Some desirable features of oospore inoculum are simplicity of preparation, ease in application, and relatively long storage life.

In greenhouse testing of sugarbeet breeding lines for resistance to blackroot disease, I have used SS-V oospore inoculum because it is easier to prepare than N-V inoculum and the number of propagules (oospores) per unit volume can be readily determined. The space, equipment, and labor requirements for producing SS-V inoculum are moderate — even for tests involving several hundred entries. My records indicate that one mycelial mat, produced in a 500 ml flask containing 100 ml of nutrient broth, can be expected to yield 2 to 5 x 10^6 oospores — a quantity sufficient for inoculating 66-165 pots at a density of 30 x 10^3 spores/pot.

In the greenhouse, density of oospore inoculum influences the subsequent severity of disease. However, the optimum density of oospore inoculum may vary from one location to another because of differences in prevailing environmental conditions, differences in susceptibility of material to be inoculated, and differences in virulence of fungus isolates.

Normally, oospore inoculum is placed below the soil surface at seed ball level because such placement is most conducive to disease. When a moderating of disease development is desired, applications of inoculum on the soil surface at planting or several days later may be preferable.

The researcher using oospore inoculum should be aware of the effect of some potting mix components on development of black root disease. Choosing a potting mix to be used with oospore inoculum might well include comparing its composition with those of mixes known not to inhibit disease development, such as the mixes used in this investigation.

Although oospore inoculum remains infective for periods exceeding one year, the severity of the disease produced may progressively decline with storage. After storage for several months, therefore, preliminary testing of inoculum before use in critical experiments is advisable.

Summary

Methods for preparation and use of oospore inoculum to initiate experimental infection of sugarbeet seedlings with the beet water mold, *Aphanomyces cochlioides,* are described. Increased density of oospore inoculum produced increased severity of blackroot disease in seedlings. Oospore inoculum applied below the soil surface near seed level produced a more severe disease than did inoculum applied on the soil surface, and inoculum applied at planting produced a more severe disease than did inoculum applied 6 days later. Disease severity increased when the proportion of mineral components:peat in potting mixes was increased from 1:1 to 2:1 or more. Oospore inoculum usually remained infective after storage for more than 1 year at 4 or -9°C.

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