UNDERSTANDING THE EFFECTS OF SPENT LIME ON *APHANOMYCES COCHLIOIDES*

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Throughout Minnesota and North Dakota, major loss of sugarbeet yield occurs due to seedling damping-off and root rot caused by *Aphanomyces cochlioides* (= *A. cochlioides)*. This is an oomycete soilborne pathogen that favors warm and wet conditions and active over a wide range of soil pH. Spent lime is a by-product of the sugar purification process from the sugar factory and is available to growers at very little cost. Soil amended with spent lime has been shown to reduce stand loss and root rot caused by *A. cochlioides* while increasing yields, sugar percent, recoverable sucrose per acre (RSA), recoverable sucrose per ton (RST), and revenue per acre (1). In addition, an application rate of 10 tons per acre or more has been shown to last for 10 years (1). However, the effects of spent lime on *A. cochlioides* have not been understood.

OBJECTIVES

The objectives of this study were 1) to determine the effects of spent lime on the formation of sporangia, asexual zoospores and overwintering oospores and 2) to determine the effects of spent lime on infection of seedlings inoculated with zoospores or oospores.

MATERIALS AND METHODS

Extracts were made from non-limed and limed field soils near Breckenridge, MN and spent lime. One hundred mL of deionized water was added to a 100 g sample of each non-limed soil (NLS), soil limed at 10 tons per acre (LS10) and soil limed at 20 tons per acre (LS20). Assuming that lime is incorporated into the top four inches of soil, 100 mL of deionized water was added to each of the four lime samples weighed to 3 g and 6 g, representing a rate of 10 (BL10) and 20 (L20) tons per acre, respectively. All extracts were autoclaved, allowed to cool down, and filtered using a vacuum pump. All extracts were made in 4 replications. The pH of each extract was measured (Table 1).

Water controls were also made to have an equal pH to each of the extract treatments. A solution of 10 g sodium bicarbonate in 250 mL deionized water was further adjusted by 1N NaOH until the pH of the solution was 8.79. Fifty milliliter aliquot of the solution was then transferred into a separate flasks and pH was adjusted using lactic acid to match each of the extract treatments. All water and pH adjusted controls were autoclaved. All extracts and controls were autoclaved and stored at 38 ˚F until further use.

Zoospore production. A 0.4 cm (#2) cork borer was used to cut plugs from the margin of an actively growing *A. cochlioides* culture on PDWA-*rp* media (1/10 potato dextrose water agar amended with rifampicin and penicillin G). One plug was placed into 35 mm x 10 mm plates filled with 5 mL of each extract and water controls. Growth and production of sporangia was evaluated 24 hours after the plug had been submerged into the extract. 48 hours after, the production of zoospores was quantified by counting several samples using a Speirs-Levy Eosinophil counting slide. The specialized hemocytometer contains four separate 2 mm^3 chambers, each contain ten (1 mm^2 x 0.2 mm) squares that are subdivided into 16 smaller squares.

Oospore production. The protocol used was a modified hypocotyl inoculation procedure for *A. cochlioides* oospore production (2). Two week old sugarbeet hypocotyls were excised at 2 cm and surfaced treated with 0.5% sodium hypochlorite followed by a double rinse with deionized water. Two hypocotyls were placed in a 35 mm x 10 mm plates with 3 mL of each extract and water controls. A 0.4cm (#2) cork borer was used to cut plugs from the margin of an actively growing culture of *A. cochlioides* culture PDWA-*rp* media. One plug was placed into each plate containing the two hypocotyls and extract or water controls and incubated in the dark at 62-73 ˚F. Growth and production of oospores were evaluated 24 hours, 48 hours, 72 hours, 1 week, and 2 weeks after incubation. After two weeks, oospores were harvested using a tissue macerator and quantified by counting several samples using a Speirs-Levy Eosinophil counting slide.

Soil assay. Two soil samples were used in this study; one from Breckenridge lime plots and other from NWROC East Range. Breckenridge soil was autoclaved before planting.Soil from the NWROC East Range is known to be *Aphanomyces*-free, therefore, this soil was not autoclaved. For each soil, treatments included non-limed soil and limed soil at 10 tons per acre, inoculated by either zoospores or oospores. Non-inoculated controls were also included. Soil from the Breckenridge lime plot was amended with lime in 2004. The East Range soil had been limed with 13 g of lime per pot and incubated one month prior to inoculation; non-limed soil was treated similarly regarding incubation. Twenty four sugarbeet seed were planted per pot and were grown for 2 weeks. Sugarbeet seedling are most susceptible to *A. cochlioides* when they are 2 weeks old.

Zoospore infection. A procedure from Mitchell and Yang (3) modified by Malvick (4) was used to produce zoospores of *A. cochlioides*. Several days before inoculation, *A. cochlioides* cultures were transferred to PDWA-*rp*. Several flasks with 30 mL of sterile Peptone Glucose broth were inoculated with three 6 mm^2 plugs from the margin of an actively growing *A. cochlioides* culture. A mineral salt solution (MSS) containing calcium chloride, magnesium sulfate, and potassium chloride was used. The mycelial mats were then rinsed with a 50 % MSS and left in full strength MSS for two days, allowing production of zoospores. Zoospores were confirmed to be motile, quantified, and added into a suspension of sterile deionized water, and adjusted to a concentration of 2.8 x 10^6 zoospores in 1,400 mL. Using a graduated cylinder, 50 mL of the zoospore suspension was evenly applied to the top of the soil, distributing 10^5 zoospores into each pot. Non-inoculated pots were treated in a similar way using 50 mL of sterile deionized water. The initial baseline stand count had been done prior to inoculation. Stand counts were taken regularly, dying seedlings were plated in deionized ultra-filtered water to observe and validate infection by *A. cochlioides*.

Oospore infection. Oospores were produced using the modified hypocotyl inoculation procedure for *A. cochlioides* oospore production (2). Two week old sugarbeet hypocotyls were excised at 2 cm and surfaced treated with 0.5% sodium hypochlorite followed by a double rinse with deionized water. A 0.4 cm (#2) cork borer was used to cut plugs from the margin of advancing *A. cochlioides* cultures that had been growing on PDWA-*rp*, and placed into deionized water with several sugarbeet hypocotyls. Oospores were harvested with a tissue macerator, quantified, and added into a suspension of sterile deionized water and adjusted to a concentration of 1.2 x 10^5 oospores in 6 ml. Oospores that were counted appeared to be viable. Finally, a pipette tip was used to create a small hole in the center of each pot approximately ¼ inch deep in which 250 ul of the oospore suspension was placed. The initial baseline stand count had been done prior to inoculation. Stand counts were taken regularly, dying seedlings were plated in deionized ultra-filtered water to observe and validate infection by *A. cochlioides*.

RESULTS AND DISCUSSION

Table 1. pH of each of various extract treatments. Each of the water control treatments were made to have an equal pH..

Treatment	pH[z]
Lime @ 20 tons/A	8.79
Lime @ 10 tons/A	8.75
Limed soil @ 20 tons/A	6.97
Limed soil @ 10 tons/A	6.16
Non-limed soil	5.57

[z] Data are mean of four replications

Zoospore production. Observations were taken within the first 24 hours after the plugs of *A. cochlioides* had been submerged in each extract. The deionized water control without pH adjustment produced many sporangia around the perimeter of the plug, while the L10 extract produced a few sporangia around the perimeter and on top of the plug. Extract treatments produced mycelium, varying in size while all of the pH adjusted water control treatments did not have any growth from the plug.

After 48 hours, only the deionized water control and the L10 extract had produced sporangia and zoospores. There was high variability in number of zoospores produced in the L10 extract; replicates that had a low number of zoospores typically had zoospores that germinated while still in their clusters. When zoospores were motile, away from the sporangia, many large clumps of zoospores were seen.

Table 2. Number of zoospores and oospores produced by *A. cochlioides* in various extract treatments.

Treatment	No. of zoospores/mL[Z]	No. of oospores/mL[Z]
DI Water	22,688 ± 6,673	25,625 ± 4,923
Lime @ 20 tons/A	None	5,125 ± 1,750
Lime @ 10 tons/A	12,328 ± 7,288	7,750 ± 2,328
Limed soil @ 20 tons/A	None	None
Limed soil @ 10 tons/A	None	None
Non-limed soil	None	None
DI water pH equivalent to Lime @ 20 tons/A	None	None
DI water pH equivalent to Lime @ 10 tons/A	None	None
DI water pH equivalent to Limed soil @ 20 tons/A	None	None
DI water pH equivalent to Limed soil @ 10 tons/A	None	None
DI water pH equivalent to Non-limed soil	None	None

[Z] Data are mean of 8 replicates ± standard deviation

The Speirs-Levy Eosinophil counting slide was used to count multiple samples. The DI water control without pH adjustment had an average of 22,688 zoospores/mL, while the L10 extract had an average of 12,328 zoospores/mL, and no zoospores were observed in L20 extract (Table 2). There was no significant difference between these two treatments because of the lack of zoospores in L20 extract. However, a linear regression analysis showed a strong negative relationship between lime rate and production of zoospores.

Oospore production. Observations of oospores 24 hours after submergence into the treatments were similar to those during the observations of the zoospores regarding mycelial growth and sporangia formation. After 48 hours, the L20 extract began production of sporangia and zoospores. In addition, sugarbeet hypocotyls in contact with the mycelial growth started to become infected.

72 hours after, oogonia and antheridia had been observed on one of the hypocotyls in the DI water control without pH adjustment. Other hypocotyls were exhibiting the production of oospores within the plant tissue. After one week, sugarbeet hypocotyls in both of the lime extract treatments were producing sporangia in addition to oospores within the tissue. The pH adjusted DI water controls did not produce any growth from the plug while the limed soil extracts (LS10 and LS20) only produced mycelial growth.

After two weeks, no significant changes occurred in any of the treatments. The DI water control without pH adjustment and the lime extracts were evaluated for the number of oospores. A tissue macerator was used to break apart the sugarbeet hypocotyls and harvest the oospores. The Speirs-Levy Eosinophil counting slide was used to count multiple samples. The DI water without pH adjustment yielded an average of 25,625 oospores/mL (Table 2). The L10 and L20 extract yielded an average of 7,750 and 5,125 oospores/mL, respectively. There was a significant difference between the DI water control without pH adjustment and the lime extracts L10 and L20. In addition, a linear regression analysis showed a strong negative relationship between lime rate and production of oospores.

Breckenridge Soil assay. As early as 3 days after infestation with zoospores, seedlings began showing symptoms of damping-off in the non-limed soil, and stands were significantly different between limed and non-limed soils (Fig. 1). By the end of the first week, all seedlings in both limed and non-limed soils had been infected by the zoospores (Fig. 1). Soils infested with oospores began showing symptoms 10 days after inoculation (Fig. 1). Overall, there was no significant difference in stands between lime-amended soils and non-limed soils.

NWROC East range Soil assay. Seedlings began to show damping-off symptoms in the non-limed and limed zoospore infested soil as early as 5 days after inoculation (Fig. 2). Seedlings in the soil infested with oospores did not exhibit any symptoms of infection. This could be due to a variety of possibilities, one being the soil was not autoclaved and microbes within the soil interfered with the ability of oospore to locate or infect sugarbeet seedlings. There was no significant difference in stands between lime-amended soils and non-limed soils.

Although the growth chamber soil assay did not show any significant difference in stands between lime-amended soils and non-limed soils, field studies have shown excellent benefit from spent lime (1). Future studies will be needed to further understand this phenomenon and to look further into different methods of oospore inoculation and understand the mechanism of infection.

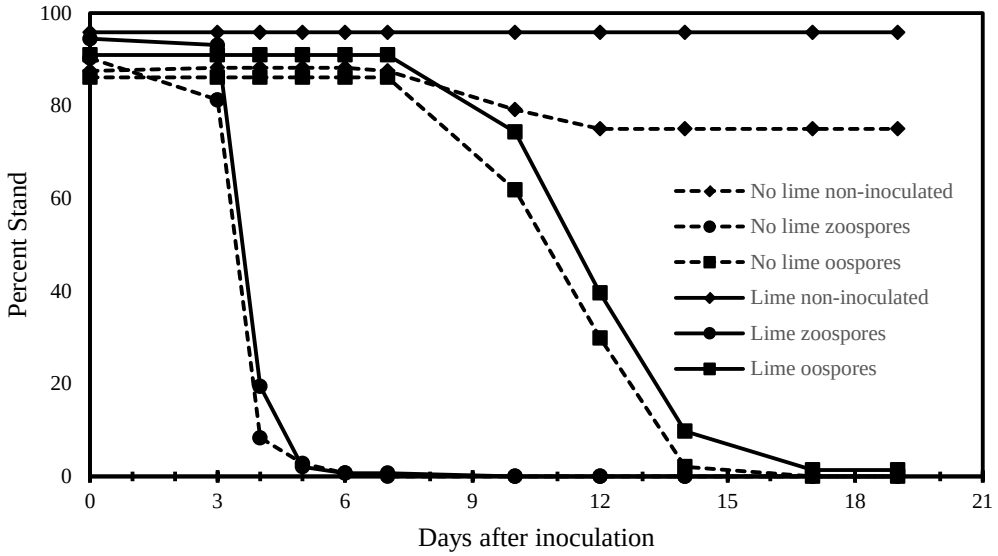

Fig. 1. Rate of *A. cochlioides* infection in the limed and non-limed Breckenridge soils by percent stand over days after inoculation. Data are mean of 4 replications. Non-limed non-inoculated soil had a small amount of soil in which the sugarbeet seedlings were infected by *Rhizoctonia solani*.

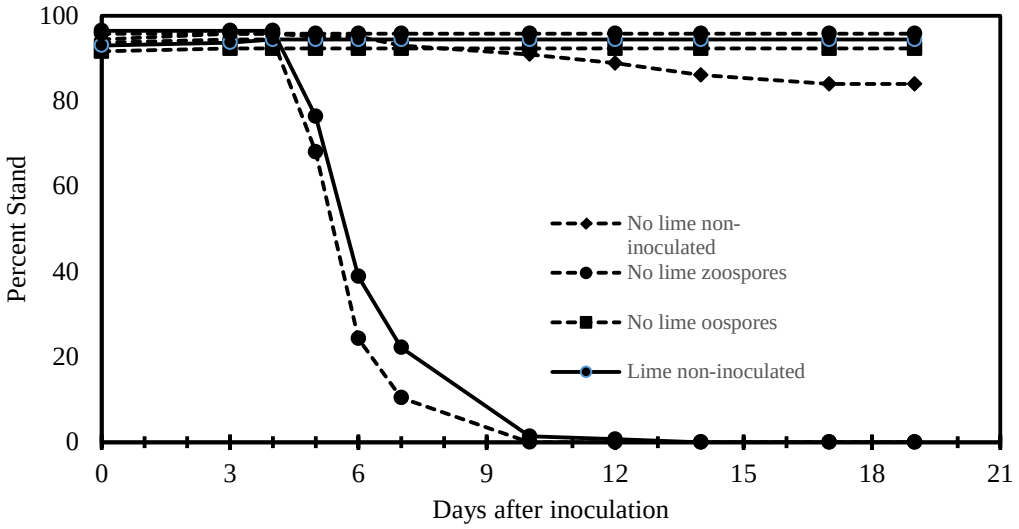

Fig. 2. Rate of *A. cochlioides* infection in the limed and non-limed East Range soils by percent stand over days after inoculation. Data are mean of 4 replications. One replicate pot in non-limed non-inoculated soil had been possibly contaminated by *A.cochlioides* inoculum from adjacent inoculated pots.

CONCLUSIONS

1. Production of zoospores and oospores was reduced by lime extracts compared to the deionized water control without pH adjustment.
2. Amending soil with lime did not significantly reduce stand loss in the growth chamber soil assay when inoculated by either zoospores or oospores.
3. Sugarbeet seedlings inoculated with zoospores began showing symptoms as early as 3 days after inoculation while infection from oospores was observed 10 days after inoculation.

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