
Sensitivity of *Aphanomyces cochlioides* from Sugar Beet to selected Fungicides

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ABSTRACT

Aphanomyces damping-off caused by *Aphanomyces cochlioides* Drechsler is one of the major diseases affecting sugar beet production worldwide. Growers rely mainly on hymexazol seed treatment to prevent infection by *A. cochlioides* at early stages of sugar beet growth. In vitro sensitivity of *A. cochlioides* isolates from Minnesota, North Dakota and Texas to pyraclostrobin, prothioconazole, tetraconazole, and hymexazol was determined using radial mycelial growth inhibition assay. The mean effective concentrations that reduced radial growth by 50% (EC₅₀) values were 0.52, 0.78, 2.39, and 3.48 µg ml⁻¹ for hymexazol, pyraclostrobin, prothioconazole, and tetraconazole, respectively. Hymexazol and pyraclostrobin had lower EC₅₀ values compared to prothioconazole and tetraconazole under in vitro conditions. In vivo fungicide efficacy was evaluated in the greenhouse using an in-furrow application method. *Aphanomyces cochlioides* zoospore suspension was used as inoculum on seed and 2-week-old plants to compare the relative effects of fungicide treatments on disease severity. Hymexazol was most effective at reducing *Aphanomyces damping-off* compared to the other fungicides at both stages under in vivo conditions. Pyraclostrobin, prothioconazole, and tetraconazole provided more effective disease reduction to the 2-week old inoculated plants compared to those inoculated at seed stage. Efforts should continue to find effective fungicide rotation partners for hymexazol that will effectively control *A. cochlioides*.

Additional Key Words: Damping-off, Oomycetes, hymexazol.

Aphanomyces cochlioides Drechsler is a fungus-like organism that belongs to the oomycetes. It is a soilborne pathogen, which causes damping-off and root rot in sugar beet and survives unfavorable conditions as dormant oospores in the soil and plant residues (Windels and Brantner, 2000; Windels and Nabben-Schindler, 1996). Warm temperatures (20 to 30°C for damping-off and 16 to 35°C for root rot) and wet soils are conducive for the development of *Aphanomyces* diseases (Windels and Engelkes, 1995; Windels and Nabben-Schindler, 1996; Harveson et al., 2009).

Aphanomyces root rot can be managed using Tachigaren® (hymexazol 70% active ingredient [a.i.], Mitsui Chemicals Agro, Japan) treated seeds (49g a.i. /100,000 seeds), use of tolerant varieties, early planting, improved field drainage, and elimination of alternative hosts (Windels and Brantner, 2000; Windels and Nabben-Schindler, 1996). Use of spent lime (precipitated calcium carbonate) added to the field soil at 5, 10, 15, and 20 ton A⁻¹ was found effective at reducing infection by *A. cochlioides* and increasing yield in sugar beet (Brantner and Chanda, 2017). Spent lime also affected zoospore and oospore production of *A. cochlioides* under in vitro conditions (Lien et al., 2015).

Few fungicides were found effective in vivo for controlling oomycete pathogens because the common fungicide targets are absent in this group of pathogens (Lee et al., 2008). The Fungicide Resistance Action Committee (FRAC) has classified fungicides into groups based on their mode of action (Muller et al., 2013). Currently the only fungicide used to effectively control *A. cochlioides* is Tachigaren (hymexazol, a hetero-aromatic compound that inhibits nuclear acid synthesis [FRAC group 32] which has been used as a sugar beet seed treatment since 1995 (Harveson et al., 2007). Tachigaren is also effective against *Pythium spp.* which is another oomycete pathogen. There are few studies conducted to evaluate the efficacy of other fungicides to manage *A. cochlioides*. Cyazofamid fungicide affects respiration by inhibiting quinone inside inhibitor (QiI) (FRAC 21). This fungicide was found effective against some oomycetes species, including *A. cochlioides*, and the EC₅₀ value of cyazofamid was 0.2 µg ml⁻¹ (Mitani et al., 2001).

Gaulin et al. (2010) found that *A. euteiches* has a cytochrome P450 sterol 14alpha-demethylase (*cyp51*) enzyme which is the target site for sterol demethylation inhibitor (DMI, FRAC 3) fungicides. Further, in vitro studies showed two DMI fungicides (tebuconazole and epoxiconazole) were effective at reducing mycelial growth of *A. euteiches* (Madoui et al., 2009). Pyraclostrobin (quinone outside inhibitor [QoI], FRAC 11) was found effective in vitro and in the field for members of oomycetes, including *Phytophthora spp.* and *Pythium spp.* (Kerns et al., 2009; Rebollar-Alviter et al., 2005; Rebollar-Alviter et al., 2007). These reports indicate the potential for DMIs and QoI fungicides for controlling *A. cochlioides*.

The objectives of this study were (i) to determine the EC₅₀ values of *A.*

cochlioides isolates to pyraclostrobin, prothioconazole, tetraconazole, and hymexazol using mycelial growth inhibition assay and (ii) to test the in vivo efficacy of these fungicides in the greenhouse.

MATERIALS AND METHODS

Aphanomyces cochlioides isolates

Fifty-six isolates of *Aphanomyces cochlioides* (Table 1) were obtained from Dr. Carol Windels at the Sugar beet pathology lab at the University of Minnesota Northwest Research and Outreach Center, Crookston, Minnesota, USA. These isolates were originally collected from Minnesota, North Dakota, and Texas. The isolates were kept as mycelium plugs in vials containing sterilized distilled water. The isolates were kept in dark at room temperature.

Table 1. Year of collection, state of origin, number of isolates, and isolates of *Aphanomyces cochlioides* used in the radial mycelial growth inhibition assay and in the greenhouse studies.

| Year | State | Number of isolates | Isolates |
|-------|--------------|--------------------|---|
| 1994 | Minnesota | 6 | 55-8-23, 105-5-5, 25-3-4, K4-4W, Soil 8R4#1, Soil 9R3#1 |
| 1997 | North Dakota | 14 | C10, C12,, C14, C16, C2, C32, C34, C54, C60, C64, C70, C84, C88, C5 |
| 1997 | Texas | 10 | 24sss, 24W, 31ss, 32ss, 35ss,3ss, 51ss, 56ss, 61ss, 64ss |
| 1997 | Minnesota | 12 | B18, B2, B22, B33, B35, B36, B39, B4, B44, B45, B48, B43 |
| 2010 | Minnesota | 3 | 10-15-2, 10-44-5, 10-54-7 |
| 2011 | Minnesota | 4 | 11-169-2, 11-169-4, 11-169-6, 11-1697 |
| 2011 | North Dakota | 3 | WL301, WL405, WL501 |
| 2012 | Minnesota | 4 | 12-26-3, 12-28-6, 12-28-7, 12-56-4 |
| Total | | 56 | |

In vitro sensitivity assays

Radial mycelial growth assay was done according to Mitani et al. (2001) with some modification. The isolates were grown on 10% potato dextrose agar (PDA, HIMEDIA, Kelton, PA, USA) and kept in dark at room temperature (20 ± 2 °C). Five-millimeter-diameter mycelial plugs were cut from the margins of 4-day old cultures of the *A. cochlioides* using a sterile cork borer. The plugs were placed inverted on fungicide amended

and non-amended 10% PDA media. Plates were kept at room temperature ($20 \pm 2^\circ\text{C}$) in the dark for 72 hours, two perpendicular diameters of the mycelial growth were measured and mean diameter was calculated for each plate. The concentration that caused 50% radial mycelial growth inhibition (EC_{50}) was determined by interpolation of the 50% intercept (Russell, 2004) using SAS (version 9.3, SAS Institute, Inc.; Cary, NC, USA).

Commercial formulation of hymexazol (70% a.i., Mitsui Chemicals Agro, China), and technical grades of pyraclostrobin (98% a.i., BASF, Research Triangle Park, NC, USA), prothioconazole (99.4% a.i., Bayer, Research Triangle Park, NC, USA), and tetraconazole (98% a.i., Sipcam Agro USA Inc., GA, USA) were used to prepare 100,000 $\mu\text{g ml}^{-1}$ stock solution in acetone (EM Science, NJ, USA). Ten-fold serial dilutions were prepared to have 0, 10, 100, 1000, and 10,000 $\mu\text{g ml}^{-1}$ fungicide concentrations. One liter of 10% PDA media was amended with 1 ml of one of the fungicide concentrations to get final concentrations of 0, 0.01, 0.1, 1, and 10 $\mu\text{g ml}^{-1}$. No salicylhydroxamic acid or glycerol were used since there is no report of alternative oxidase enzyme in *Aphanomyces cochlioides*. No special media was used for the QoI fungicide. Equivalent amount of acetone was added to non-amended media. The experimental design was a complete randomized design (CRD) with two replicates for each fungicide concentration and each isolate. The experiment was repeated once.

In vivo evaluation of efficacy of fungicides at controlling *A. cochlioides*

The experiment was conducted in the Agricultural Experiment Station greenhouse at North Dakota State University in Fargo, ND, USA. Fungicides evaluated, source, and concentration were: hymexazol (70% a.i. Tachigaren®, Mitsui Chemicals Agro, Japan), pyraclostrobin 672.3 ml ha⁻¹ (23.6 % a.i.; Headline®, BASF, Research Triangle Park, NC, USA), prothioconazole 416.5 ml ha⁻¹ (41% a.i.; Proline®, Bayer, Research Triangle Park, NC, USA), and tetraconazole 949.9 ml ha⁻¹ (11.6% a.i., Eminent®, SIPCAM Agro USA Inc., GA, USA). The application rates of pyraclostrobin, prothioconazole and tetraconazole were chosen based on their labeled rates for use in sugarbeet. (Friskop et al., 2021). Pyraclostrobin, prothioconazole, and tetraconazole were applied before inoculation as an in-furrow application (Noor and Khan, 2018) using a Generation III Research Sprayer (Devries Manufacturing Hollandale, MN) through a 4001E flat-fan nozzle calibrated to deliver the solutions at 138 kPal and 6.3 km hr⁻¹, and hymexazol was applied as a seed treatment at 45 g a.i./100,000 seeds.

Sugar beet plants were inoculated at the seed stage or two-weeks after planting using WL405 *A. cochlioides* isolate. Crystal 539RR (4.0 rating) a sugar beet cultivar susceptible to *A. cochlioides* was used (Niehaus, 2011). Sugar beet seeds were planted in 10 cm³ pots (T. O. Plastic Inc.; Clearwater, MN, USA) filled with Sunshine Mix LC1 (73 to 83%

Canadian sphagnum peat moss, perlite, and dolomite lime; Sun Gro Horticulture Distribution Inc.; Agawam, MA, USA). For the seed stage, 10 seeds were planted in each plastic pot, while for two-week-old plants, 15 seeds were planted and thinned (10 days after planting) to 10 plants per pot. Inoculation was done by pouring 50 ml of 2000 zoospores ml⁻¹ per pot (Schneider, 1954). Zoospores were prepared following the method published by Islam et al. (2007). The pots were placed in the greenhouse at 24±1°C (Argus Control Systems, Ltd.; British Columbia, Canada), and watered as needed. Three weeks after inoculation, disease severity was evaluated using a scale from 0 to 3 where 0 was healthy root and hypocotyl, 1 was light brown hypocotyl, 2 was moderate discoloration of hypocotyl, and 3 was where the hypocotyl rotted or the plant was dead (Windels and Nabben-Schindler, 1996). The experimental design was a completely randomized design (CRD) with fungicides as treatment; the experiment was repeated once and three replicates for each treatment were used. To confirm the causal agent of the symptoms on sugar beet plants, the pathogen was re-isolated from infected plants by plating small pieces (2 mm) of infected roots on water agar (WA) media after surface sterilization with sterilized water.

DATA ANALYSIS

For the *in vitro* study, the two runs were tested for homogeneity of variance using Levene's test for homogeneity of variance. For the *in vivo* study, the data were analyzed using non-parametric Kruskal-Wallis test (Shah and Madden, 2004). Median disease severity was calculated for each pot, and mean rank was calculated using Proc Rank with SAS. The ranked disease severities were used to estimate relative effects, standard errors, and the 95% confidence intervals using the SAS macro LD-CI (<http://www.ams.med.uni-goettingen.de/sasmakr-de.shtml>) to compare between different treatments (Shah and Madden, 2004).

RESULTS

For the *in vitro* sensitivity study of *A. cochlioides* to fungicides, the two experiments (four replicates) were combined based on lack of significance of Levene's test for homogeneity of variance (0.05). All tested fungicides inhibited radial mycelial growth *in vitro* although it varied depending on the fungicide. The mean EC₅₀ values were 0.52 ± 0.37, 0.78 ± 1.29, 2.39 ± 0.96 and 3.48 ± 1.79 µg ml⁻¹ for hymexazol, pyraclostrobin, prothioconazole, and tetraconazole, respectively. EC₅₀ range was 0.05-2 µg ml⁻¹ for hymexazol, 0.05-9.49 µg ml⁻¹ for pyraclostrobin, 0.24-5.75 µg ml⁻¹ for prothioconazole, and 0.44-8.11 µg ml⁻¹ for tetraconazole. Frequency of isolates with EC₅₀ values between 0.01 and 0.1 µg ml⁻¹ were 9.4, 9.4, 0, and 0 % for hymexazol, pyraclostrobin, prothioconazole, and tetraconazole, respectively (Table 2). Frequency of isolates with EC₅₀ values between 0.1 and 1 µg ml⁻¹ were 83.9, 71.9, 3.1, and 2.2 % for

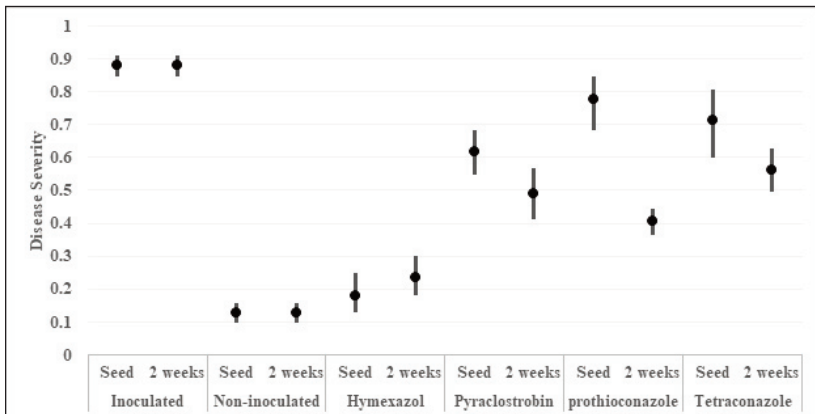
hymexazol, pyraclostrobin, prothioconazole, and tetraconazole, respectively. Frequency of isolates with EC_{50} values between 1 and 10 $\mu\text{g ml}^{-1}$ were 6.7 % for hymexazol, 18.8 % for pyraclostrobin, 96.9 % for prothioconazole, and 97.8% for tetraconazole (Table 2).

In greenhouse experiments, *A. cochlioides* was re-isolated from the infected plants and it was identified based on morphological characteristics. Hymexazol was found effective at reducing *Aphanomyces* damping-off and root rot when seeds or two-week-old plants were inoculated (Figure 1). Pyraclostrobin, prothioconazole, and tetraconazole were not as effective as hymexazol in reducing *Aphanomyces* damping-off when inoculation was done at seed stage or at the two-week-old stage, but these three fungicides were typically more effective in reducing *Aphanomyces* damping-off when inoculation was performed at the two-week-old stage compared to the seed stage (Figure 1).

Table 2. Frequency distribution of EC_{50} of *Aphanomyces cochlioides* isolates for hymexazol, pyraclostrobin, prothioconazole, and tetraconazole using mycelium radial growth assay.

| Fungicide | Isolate Frequency % | | |
|-----------------|---|---------|-------|
| | EC50 range (fungicide concentrations in $\mu\text{g ml}^{-1}$) | | |
| | 0.01-0.1 | >0.01-1 | >1-10 |
| Hymexazol | 9.4 | 83.9 | 6.7 |
| Pyraclostrobin | 9.4 | 71.9 | 18.8 |
| Prothioconazole | 0 | 3.1 | 96.9 |
| Tetraconazole | 0 | 2.2 | 97.8 |

Figure 1. Efficacy of pyraclostrobin (672.3 ml ha⁻¹), hymexazol, prothioconazole (416.5ml ha⁻¹), and tetraconazole (949.9 ml ha⁻¹) on damping off severity (*Aphanomyces cochlioides*) at three weeks after inoculation. Sugar beet plants (cultivar Crystal 539RR) were inoculated at seed stage and 2 weeks after planting. The plants were inoculated with 50 ml of 2000 zoospores ml⁻¹ concentration in water. The ranked disease severities were used to estimate relative effects and the 95% confidence intervals.



DISCUSSION

Aphanomyces cochlioides isolates obtained from ND, MN, and Texas were evaluated for fungicides sensitivity *in vitro*. Hymexazol, pyraclostrobin, prothioconazole, and tetraconazole fungicides were able to reduce radial mycelial growth *in vitro*, with hymexazol and pyraclostrobin giving lower mean EC₅₀ values. In the greenhouse, pyraclostrobin, prothioconazole, and tetraconazole were not effective as hymexazol at controlling *Aphanomyces* damping-off.

Pyraclostrobin was reported to reduce radial mycelial growth of other oomycetes, including *Phytophthora* and *Pythium* (Kerns et al., 2009; Rebollar-Alviter et al., 2005; Rebollar-Alviter et al., 2007), but no studies reported their effects on *Aphanomyces* spp. *Aphanomyces euteiches* was found to have the *cyp51* gene (Gaulin et al., 2010). The product of this gene is the target for triazole (DMI) fungicides. This may explain some reduction of radial mycelial growth of *Aphanomyces cochlioides* with the DMI fungicides reported in this study.

In the greenhouse study, hymexazol was the most effective at reducing *Aphanomyces* damping-off as previously reported (Payne and Williams, 1990). Pyraclostrobin was found ineffective at reducing *Aphanomyces* damping-off in sugar beet compared to hymexazol, which contrasts with

what was reported for *Phytophthora* and *Pythium* (Kerns et al., 2009; Rebollar-Alviter et al., 2005; Rebollar-Alviter et al., 2007).

Triazoles were effective in reducing radial mycelial growth of *A. eutichus* in vitro; there are no studies reporting in vivo testing (Madoui et al., 2009). In this study, triazoles were also found to be effective at reducing radial mycelial growth in vitro at 1 and 10 $\mu\text{g ml}^{-1}$. However, in the greenhouse study, the triazoles were found not as effective as hymexazol, probably because of binding of these fungicides to organic potting materials used in this study. It is known that the soil adsorption coefficient (Koc) for pyraclostrobin is 9,304 mL/g (US EPA, 2010), tetraconazole is 531 – 1922 mL/g, prothioconazole is 1765 mL/g and hymexazol is 12 -124 mL/g (European Food Safety Authority, 2007, 2008, 2010). Pyraclostrobin and the triazoles with higher Koc values will tend to be easily and highly adsorbed to the soil compared to hymexazol which has a lower adsorption and allows it to be more mobile. The DMI and QoI fungicides were more effective at reducing disease severity when inoculation occurred at the two leaf compared to the seed stage. This was probably a result of the seedlings being able to absorb the fungicides which may help to induce resistance in the seedlings, or the fungicides, with regular watering, being better distributed over time and providing better protection.

The results of this study indicate that hymexazol is one of the most effective fungicide that is currently labeled to prevent seedling damping-off and young plant infection from *A. cochlioides*. Although pyraclostroin, prothioconazole, and tetraconazole applied to soil reduced *Aphanomyces* damping-off disease severity, they were not as effective as the standard hymexazol seed treatment, and thus would not be considered as suitable alternative. However, efforts should continue to evaluate other products for controlling diseases caused by *A. cochlioides* so that they can be used in rotation with hymexazol to prevent development of possible fungicide resistance in the future.

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