Resistance to fungicides in the plant pathogen *Microdochium nivale*

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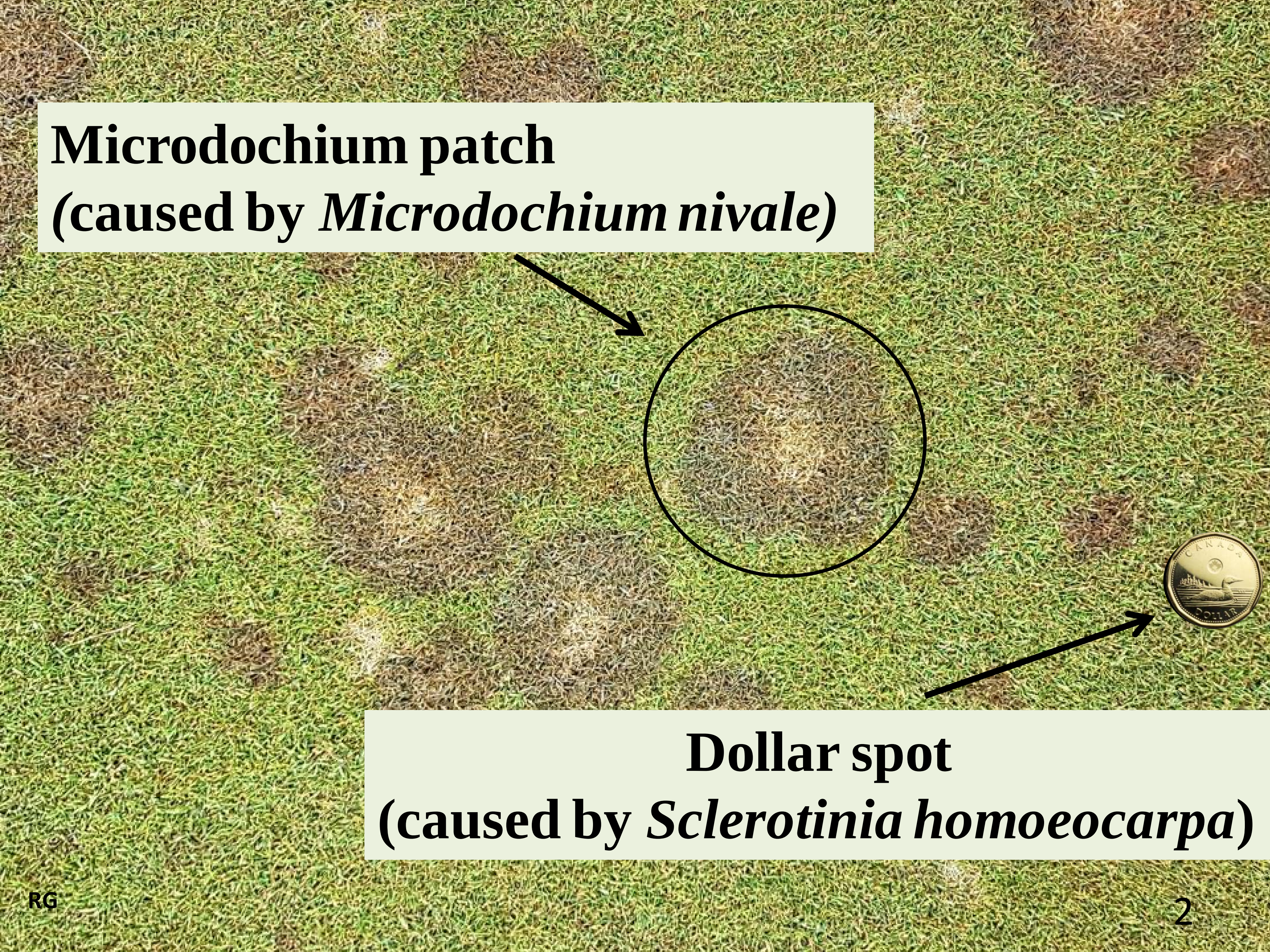

Microdochium patch
(caused by *Microdochium nivale*)
Dollar spot
(caused by *Sclerotinia homoeocarpa*)
RG

Pink snow mold on winter wheat

Murray, T. 2017. https://news.wsu.edu/2017/03/28/pink-snow-mold-wheat-fields/
Accessed: May 27, 2018

Table 1. Fungicides registered for *Microdochium nivale* in Canada; table adapted from Vincelli & Munshaw (2015) and PMRA website.

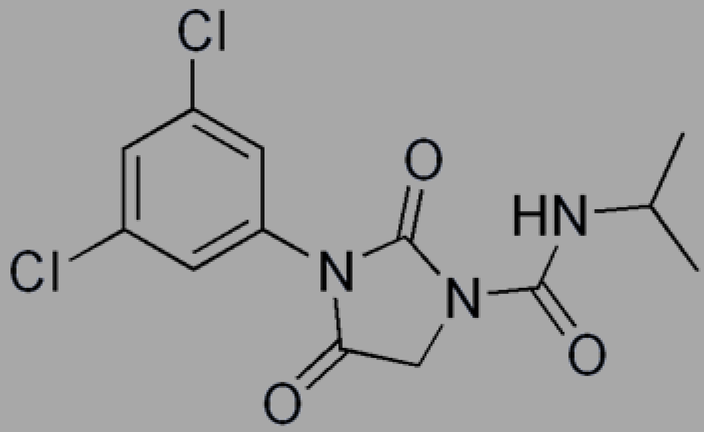
Fungicide	**Group**	**Resistance Risk**
Azoxystrobin	QoI	High
Benzovindiflupyr	SDHI	Medium to High
Chlorothalonil	CN	Low
Difenoconazole	DMI	Medium
Fludioxonil	PP	Low to Medium
Fluoxastrobin	QoI	High
Iprodione*	DC	Medium to High
Metconazole	DMI	Medium
Mineral oil	NC	Unrated
Penthiopyrad	SDHI	Medium to High
Propiconazole	DMI	Medium
Pyraclostrobin	QoI	High
Thiophanate-methyl	MBC	High
Trifloxystrobin	QoI	High
Triticonazole	DMI	Medium

* deregistered as of June, 2018 PMRA re-evaluation decision PRVD2016-09

Iprodione (dicarboximide)

(3-(3,5-dichlorophenyl)-*N*-isopropyl-2,4-dioxoimidazolidine-1-carboxamide)

- Disrupts osmotic signal transduction
- Target site thought to be an osmosensing histidine kinase encoded by *os-1* gene
- Two lab-verified cases of resistance in *M. nivale* (Washington & New Zealand)



Oshima, M., M. Fujimura, S. Banno, C. Hashimoto, T. Motoyama, A. Ichiishi, and I. Yamaguchi. 2002. A point mutation in the two-component histidine kinase BcOS-1 gene confers dicarboximide resistance in field isolates of *Botrytis cinerea*. *Phytopathology* 92(1): 75–80

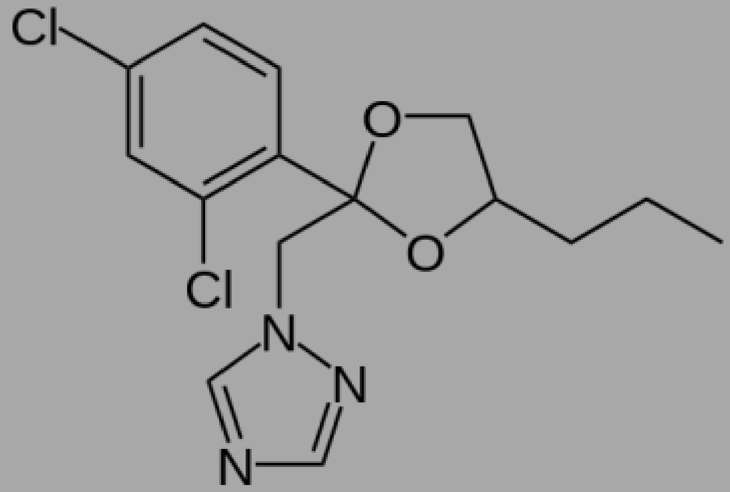
Chastagner, G. and Vassey, W. 1982. Occurrence of iprodione-tolerant *Fusarium nivale* under field conditions. *Plant Disease*, 66(2), pp.112–114

Pennucci, A., Beever, R. and Laracy, E. 1990. Dicarboximide-resistant strains of *Microdochium nivale* in New Zealand. *Australasian Plant Pathology*, 19(2), pp.38–41.

Propiconazole (DMI)

(1-[[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-yl]methyl]-1,2,4-triazole)

- Disrupts ergosterol synthesis
- Target site identified as C14-demethylase encoded by the *cyp51* gene
- No lab-verified cases of resistance in *Microdochium nivale* reported



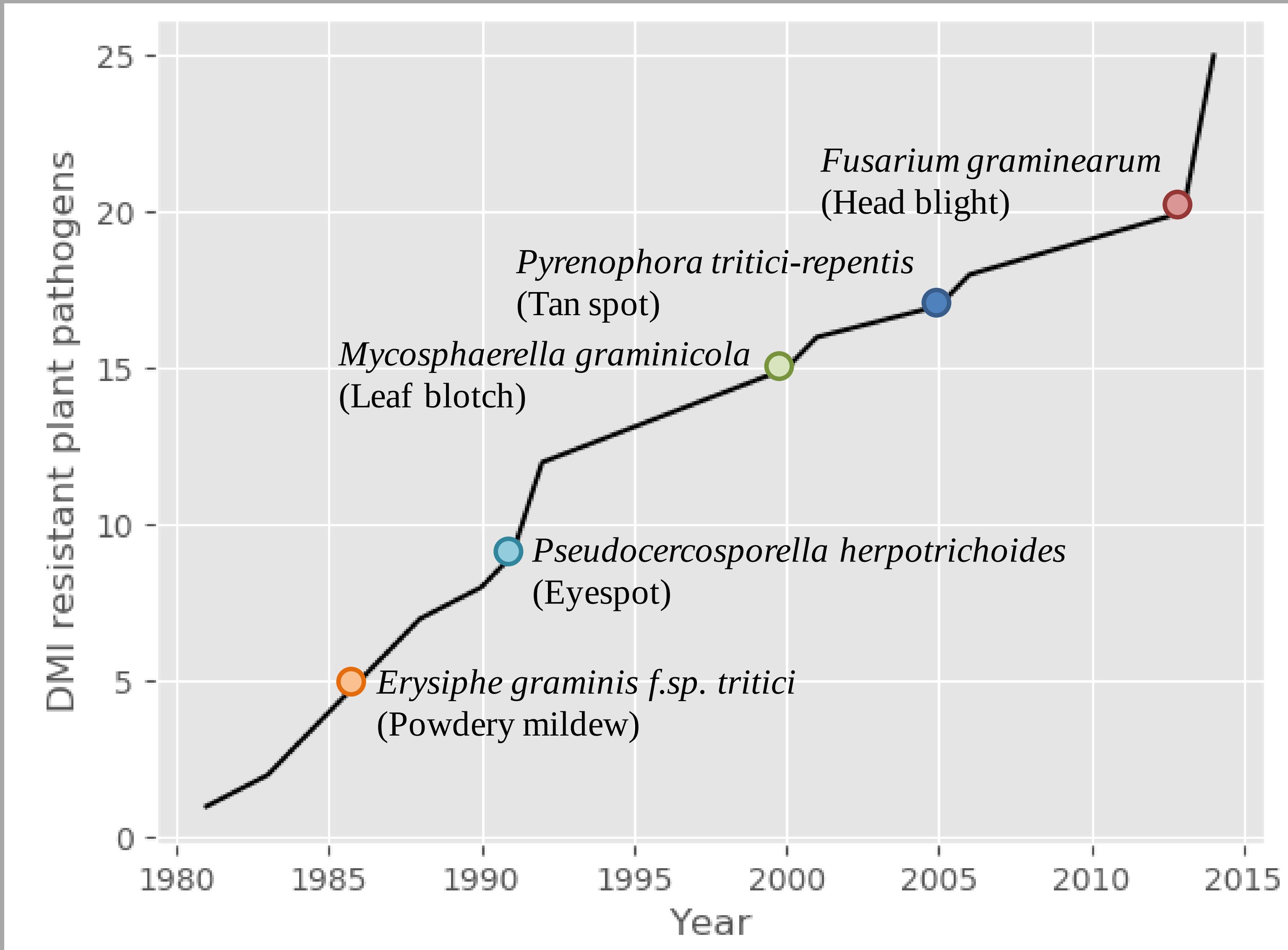
Siegel, M.R. 1981. Sterol-inhibiting fungicides: effect on sterol biosynthesis and sites of action. *Plant Disease*, 65, pp.986–989.

Dicarboximide resistance

Table 2. First instances of field resistance to dicarboximide fungicides, adapted from FRAC (2017).

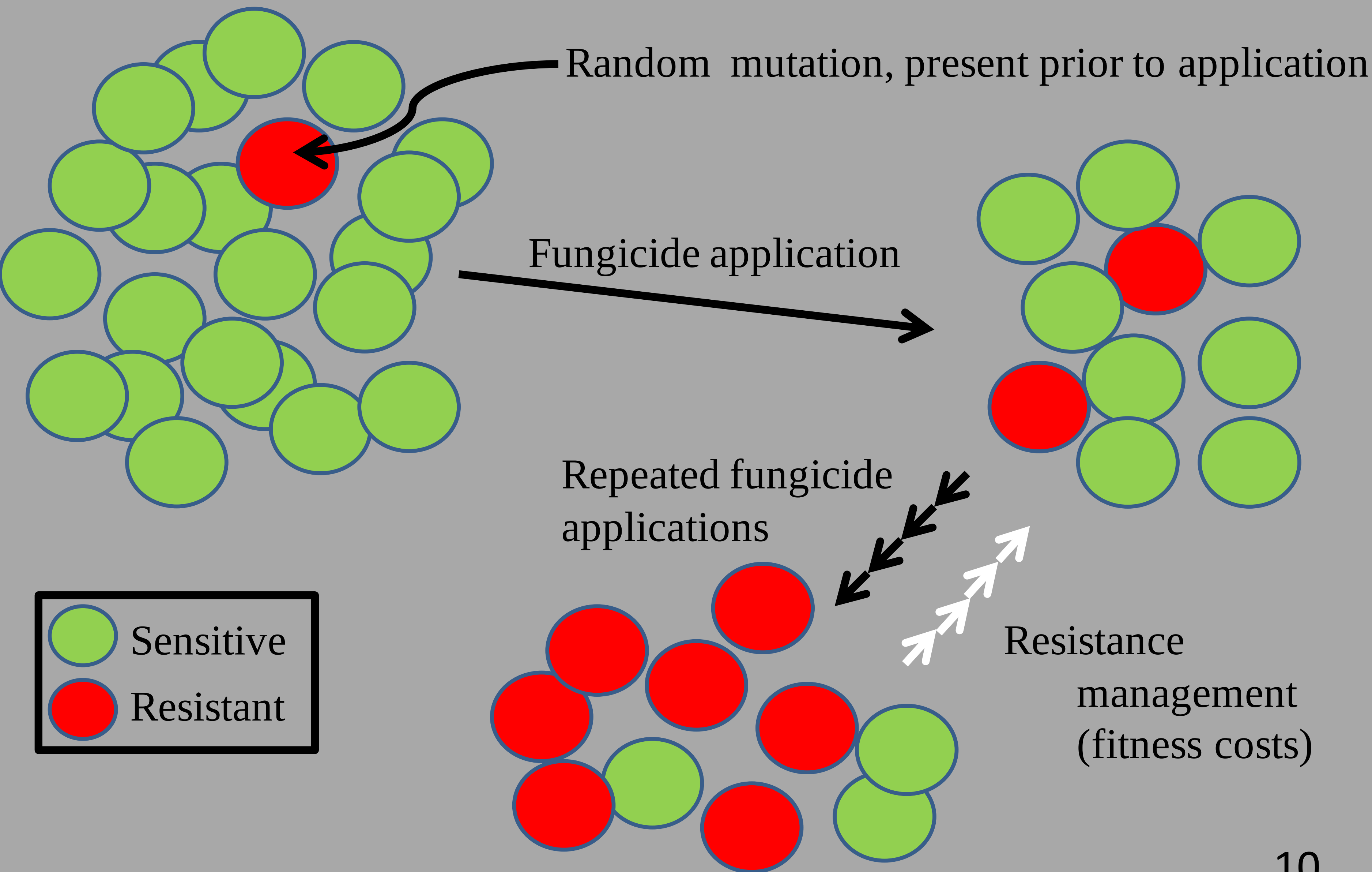
Pathogen	**Host**	**Reference**
Botrytis cinerea	Strawberry	(Dennis and Davis, 1979)
Sclerotinia homoeocarpa	Turfgrass	(Detweiler et al., 1983)
Monilinia fructicola	Stone fruit	(Penrose et al., 1985)
Botrytis tulipae	Tulip	(Chastagner and Riley, 1987)
Didymella bryoniae	Cucumber	(Van Steekelenburg, 1987)
Botrytis elliptica	Bulbs	(Hsiang and Chastagner, 1991)
Microdochium nivale	Turfgrass	(Pennucci et al., 1990)
Sclerotinia minor	Lettuce	(Hubbard et al., 1997)
Alternaria alternata	Pistachio	(Ma and Michailides, 2004)
Alternaria brassicicola	Brassicas	(Avenot et al., 2005)
Stemphylium vesicarium	Pear	(Alberoni et al., 2005)
Sclerotinia sclerotiorum	Soybeans	(Zhou et al., 2014)

Demethylation inhibitor resistance



*adapted from FRAC (2017)

Development of fungicide resistance



Fitness costs associated with insensitivity to fungicides

- Pleiotropic effects of mutations
- Fitness costs reported in 7 dicarboximide resistant species
- Fitness costs reported in 8 DMI resistant species
- Fitness metrics:
  - *in vitro* growth rate
  - Biomass (dry-weight)
  - Virulence
  - Spore survival and germ tube length
  - Competition in mixed inoculum experiments

Research objectives

1) Collect isolates of *M. nivale* from populations with different fungicide exposure histories.
2) Assess fungicide sensitivity using full concentration range tests and discriminatory concentration tests (larger sample set).
3) Assess relationship between fungicide sensitivity and virulence in field tests using representative *M. nivale* isolates (varying sensitivity profiles).
4) Sequence genomes of representative isolates and search for genetic differences which may underlie any observed insensitivities.

Pathogen isolation

- Symptomatic leaves surface sterilized with bleach
- Grown on PDA with streptomycin sulfate (0.2 μg/mL) and tetracycline hydrochloride (0.2 μg/mL)
- Compared to known *M. nivale* cultures visually (mycelia colour and presence of sporodochia) or sequencing of the ITS region

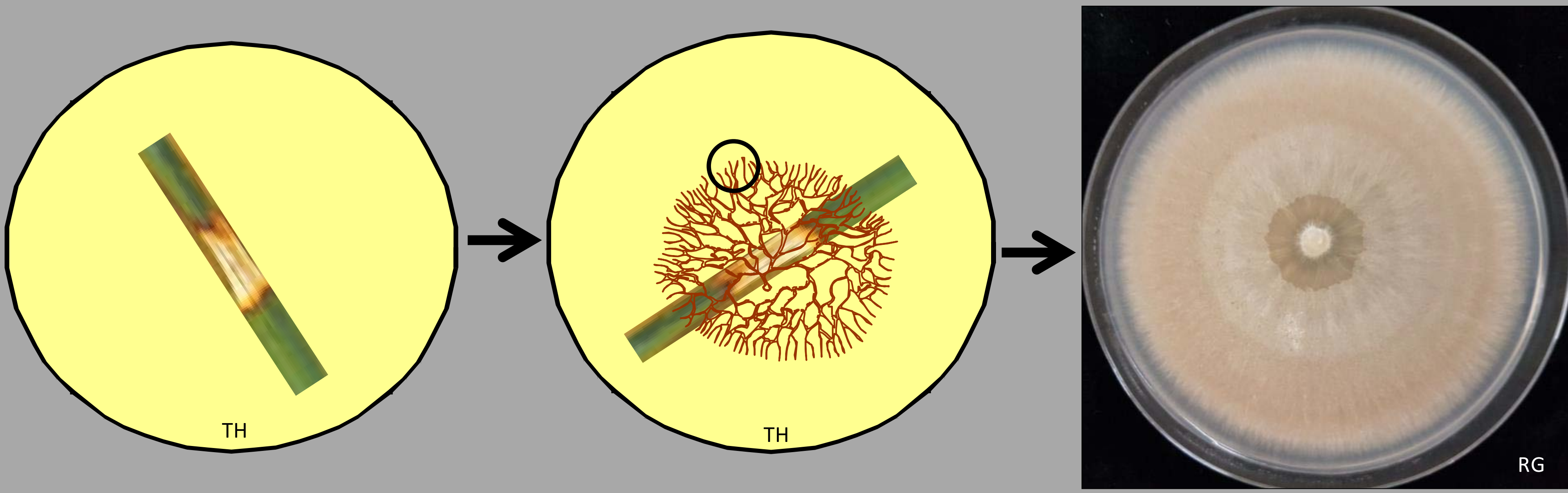
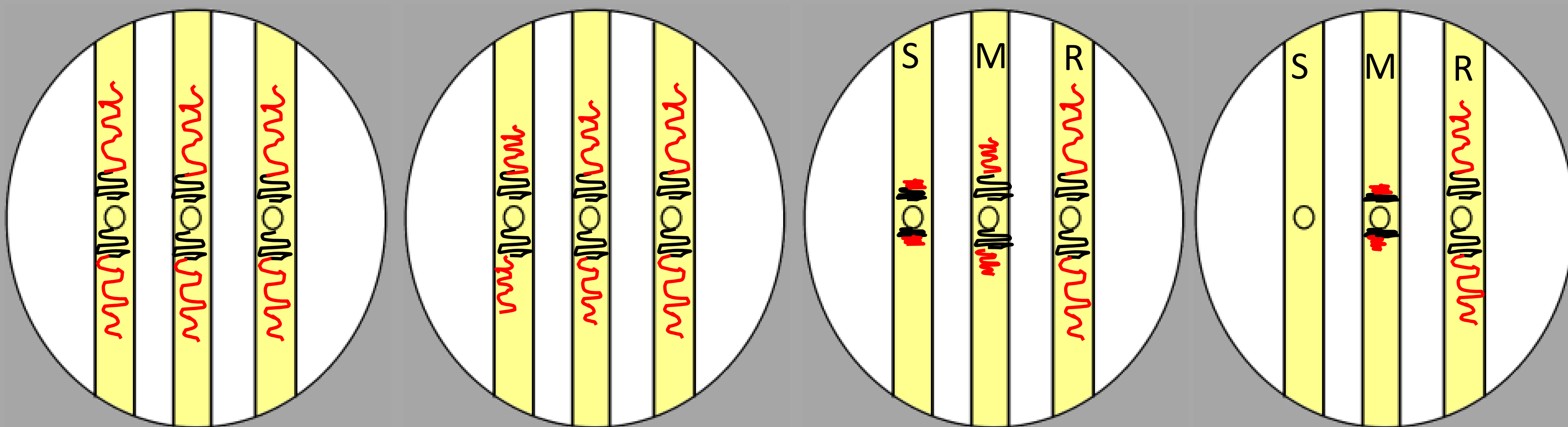


Table 3. Origin, host and number of *Microdochium nivale* isolates used in fungicide testing.

Location	Host	Number of isolates	
Along Highway 86 near Listowel, ON	*Triticum sp.*	6	
Graham Hall, Guelph, ON	*Poa pratensis*	4	
GTI, Guelph, ON	*P. pratensis*	7	
GTI, between green and road, Guelph, ON	*P. pratensis*	14	
GTI, hillside of upper green, Guelph, ON	*P. pratensis*	3	
GTI, native green, Guelph, ON	*Agrostis stolonifera / Poa annua*	15	
GTI, pathology green fringe, Guelph, ON	*P. pratensis*	10	Various exposures
GTI, pathology green, Guelph, ON	*A. stolonifera*	7	
GTI, roadside of upper green, Guelph, ON	*P. pratensis*	7	
GTI, roadside, Guelph, ON	*P. pratensis*	9	
GTI, roadway, Guelph, ON	*Lolium perenne*	26	
Highway 131, near Atwood, ON	*Triticum sp.*	4	
Ottawa Experimental Farm, Ottawa, ON	*Triticum sp.*	11	
ON-1, Guelph, ON	*A. stolonifera / P. annua*	17	
ON-2, Guelph, ON	*A. stolonifera / P. annua*	15	
ON-3, Guelph, ON	*A. stolonifera / P. annua*	14	
ON-4, Guelph, ON	*A. stolonifera / P. annua*	22	
BC-1, Victoria, BC	*P. annua*	9	Collected for this study
BC-2, North Vancouver, BC	*P. annua*	40	
BC-3, Victoria, BC	*A. stolonifera / P. annua*	37	
BC-C, Victoria, BC	*A. stolonifera / P. annua*	7	
Various	*Various*	6	

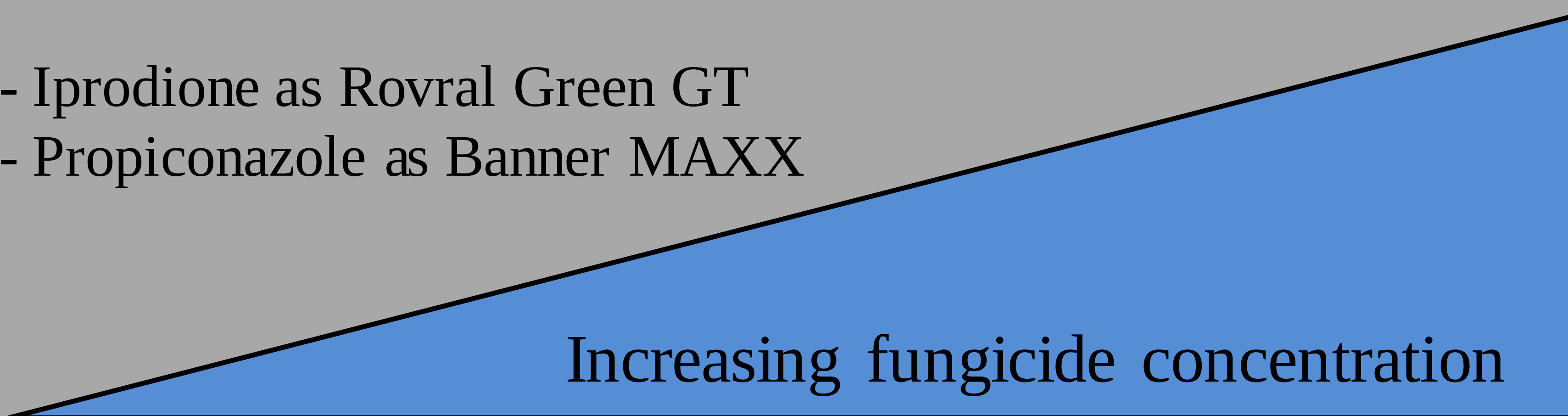
GTI – Guelph Turfgass Institute

Strip agar assay sensitivity testing



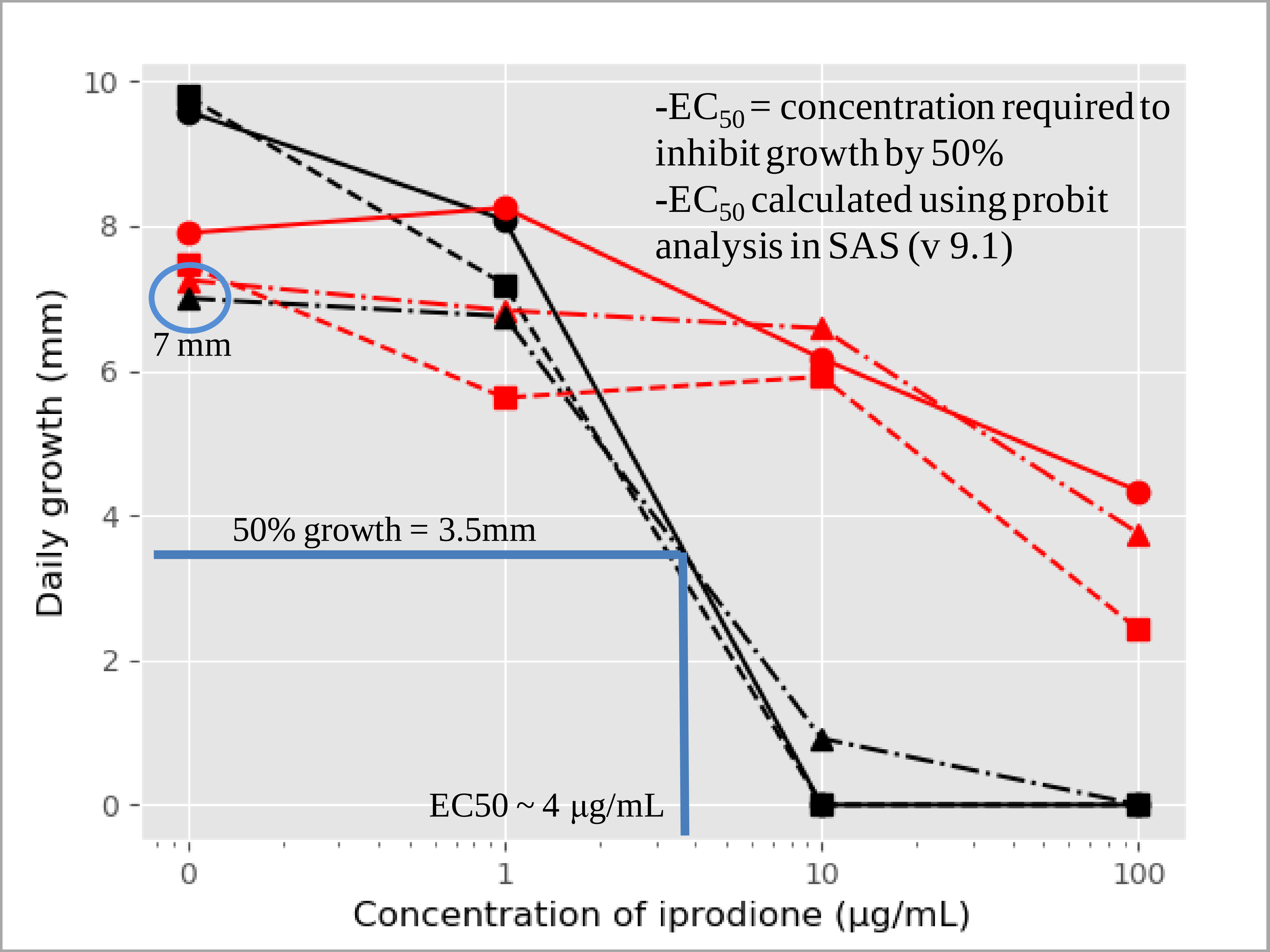
0 µg/mL | 1 µg/mL | 10 µg/mL | 100 µg/mL

- Iprodione as Rovral Green GT
- Propiconazole as Banner MAXX



Hsiang, T., Yang, L. and Barton, W. 1997. Baseline sensitivity and cross-resistance to demethylation-inhibiting fungicides in Ontario isolates of *Sclerotinia homoeocarpa*. *European Journal of Plant Pathology*, 103(5), pp.409–416.

Example of full concentration range data



Full concentration range results

Table 4. Iprodione EC_{50} ranges and resistance factors (RF) for Ontario and B.C. isolates

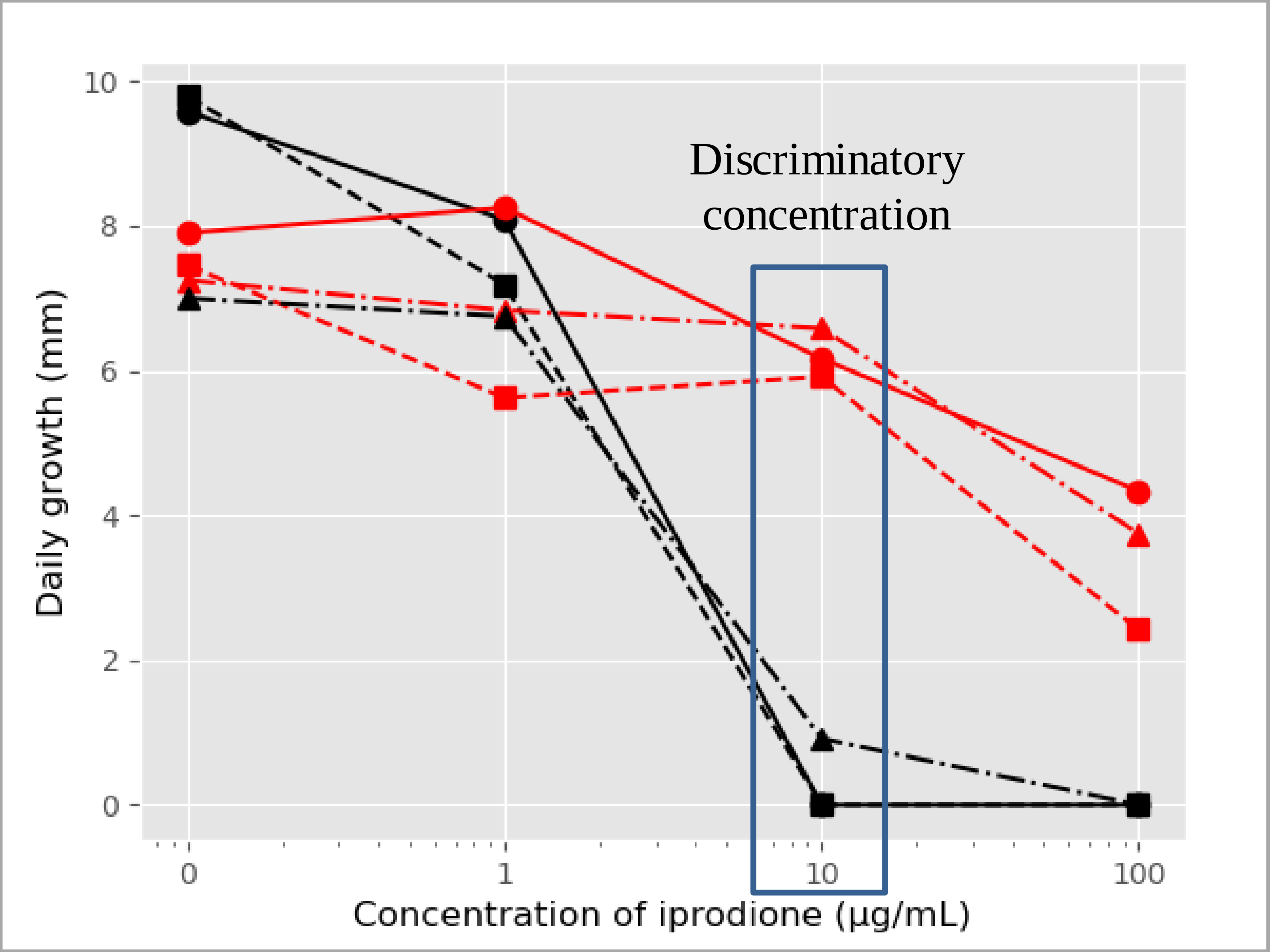
Population	EC_{50} (µg/mL)	RF
Ontario	1.2 to 32	2.6
British Columbia	1.5 and 542.6	38.2

Table 5. Propiconazole EC_{50} ranges and resistance factors for Ontario and B.C. isolates

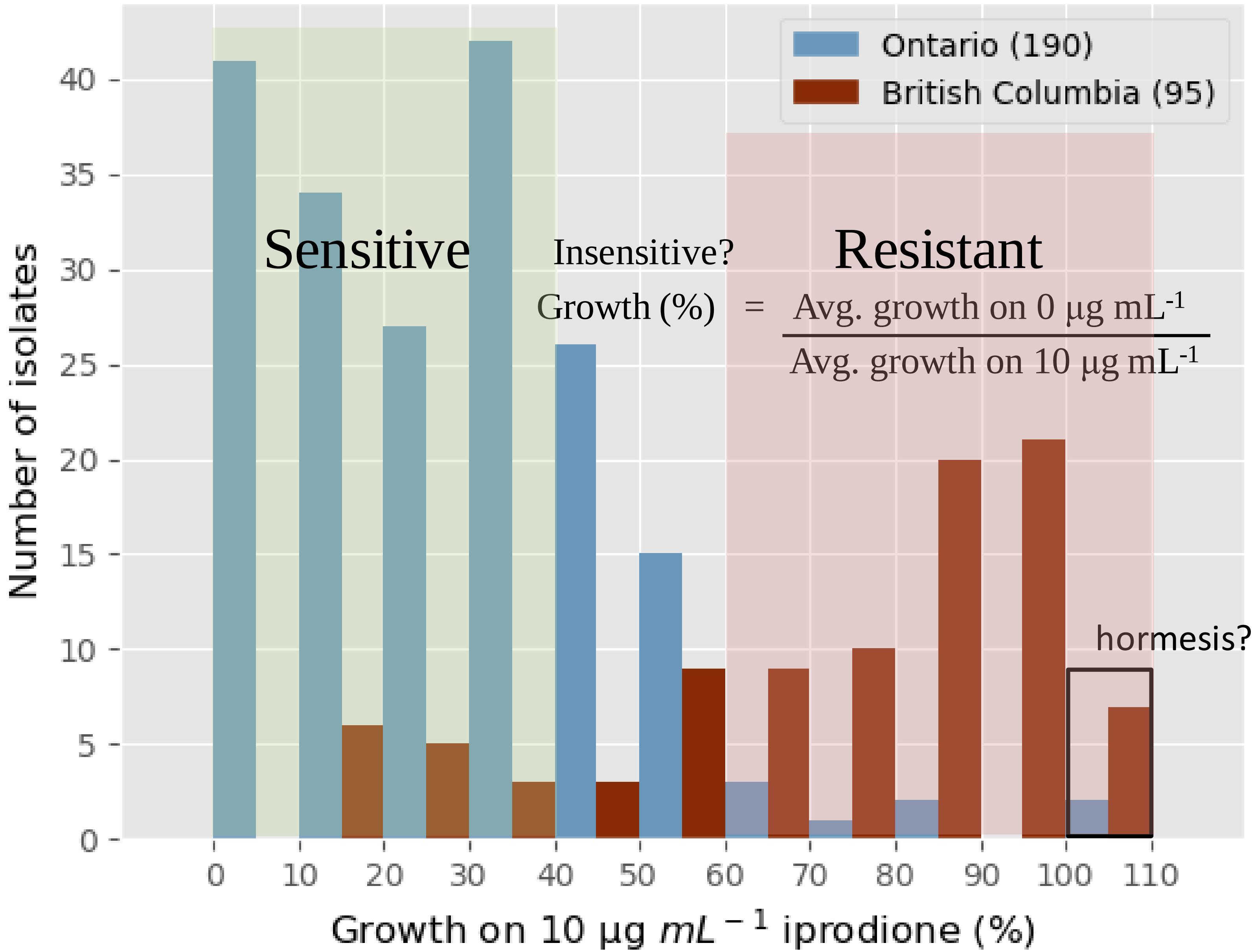
Population	EC_{50} (µg/mL)	RF
Ontario	<0.001 to 0.89	7.9
British Columbia	0.02 to 8.7	20.4

Resistance factor = (Avg. EC_{50} resistant isolates / Avg. EC_{50} sensitive isolates)

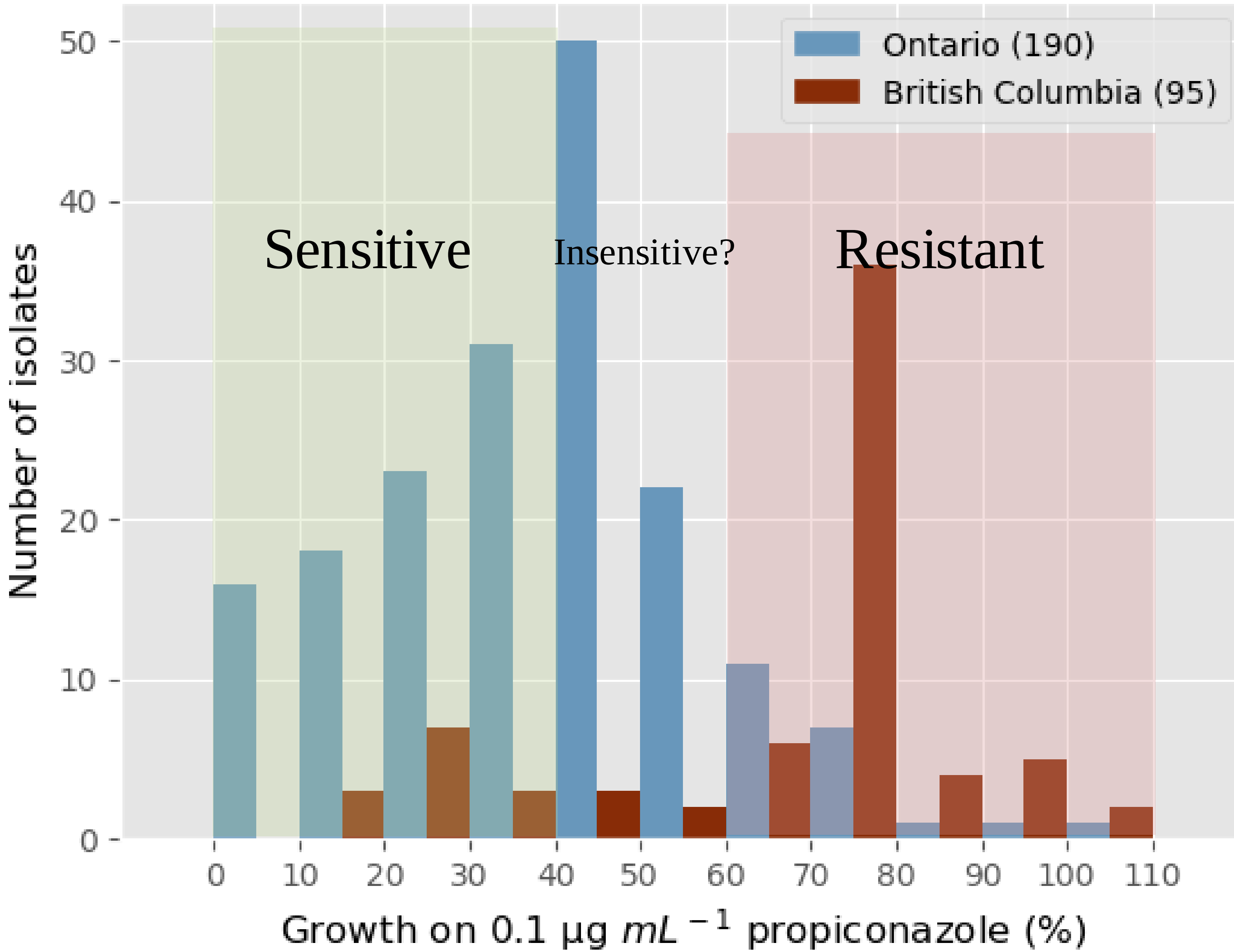
Example of full concentration range data



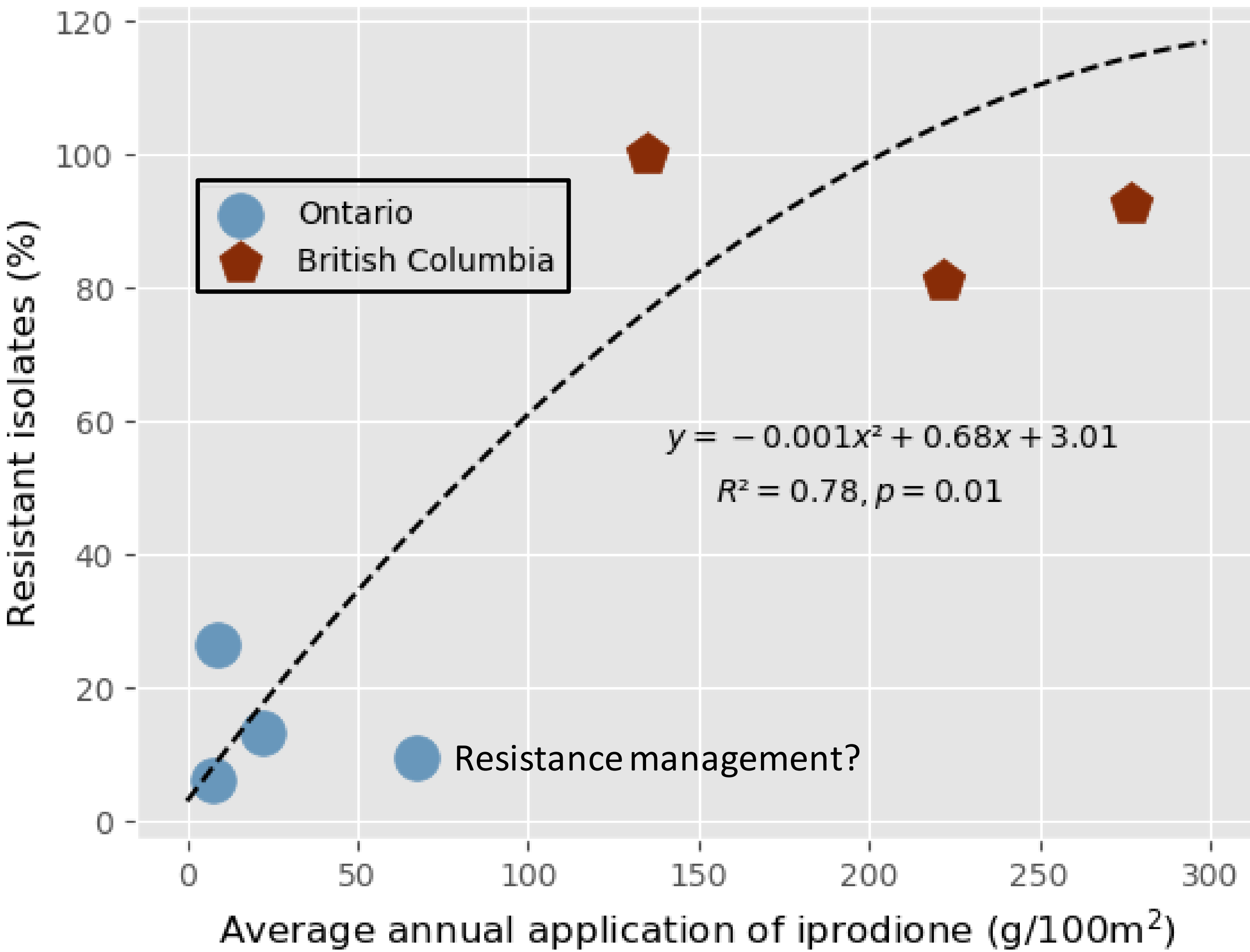
Iprodione discriminatory concentration results



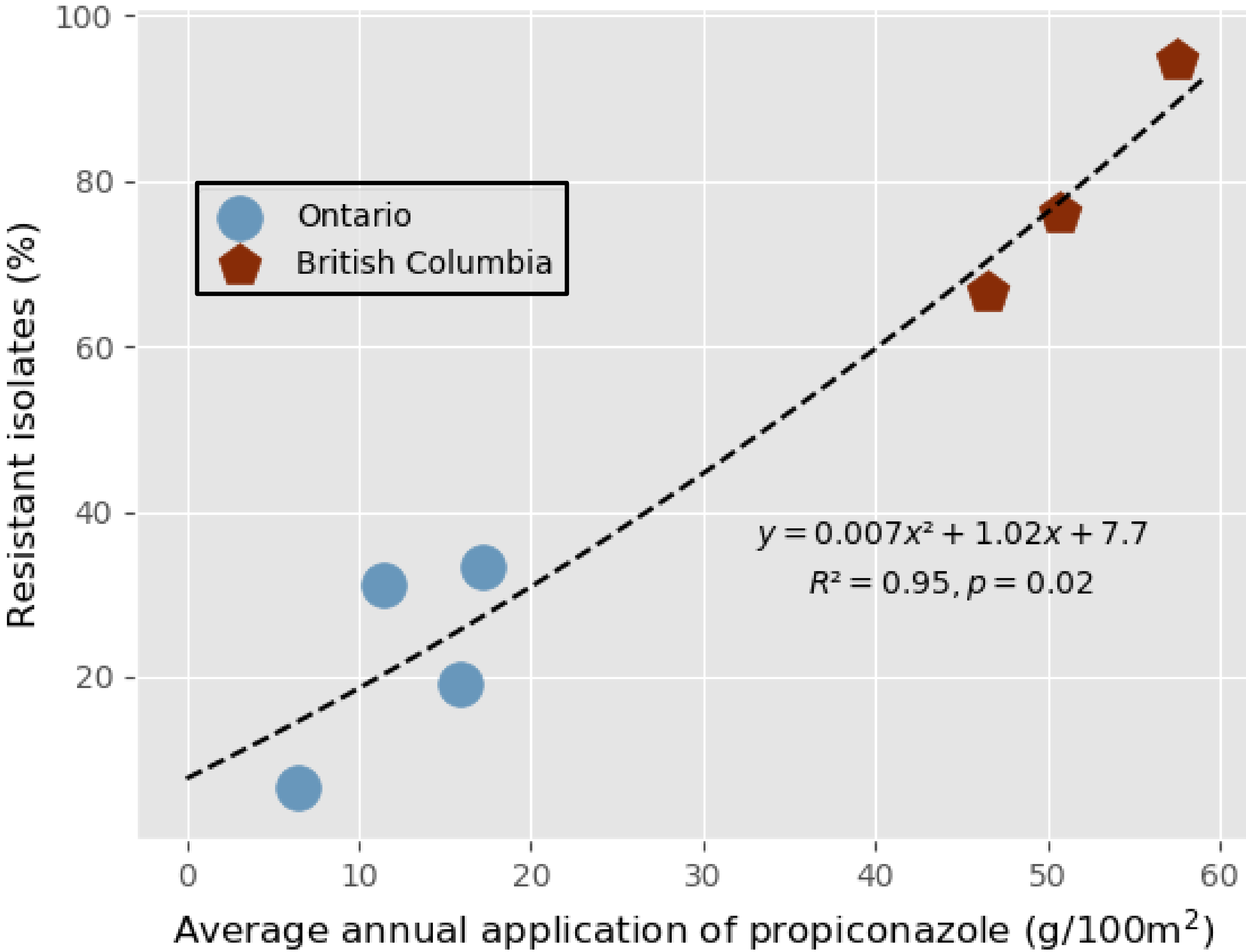
Propiconazole discriminatory concentration results



Iprodione application rate vs. sensitivity



Propiconazole application rate vs. sensitivity



Growth rate and biomass associated with sensitivity results

Table 6 Daily growth rate and biomass production associated with different sensitivity to the fungicides iprodione and propiconazole in *Microdochium nivale*. Sensitivity groups based on discriminatory concentration testing, where isolates with growth >50% on the discriminatory concentration were deemed resistant. An asterisk (*) indicates significant difference from the fully sensitive group based on ANOVA tests (p = 0.05).

Sensitivity	Growth (mm/day)	Dry weight (mg/day)
Fully sensitive	6.9	2.9
Iprodione-resistant	5.7*	3.0
Propiconazole-resistant	5.7*	2.5
Resistant to iprodione and propiconazole	7.0	3.5

Field trials at Guelph Turfgrass Institute

'California Green' at GTI

-complete randomized block design

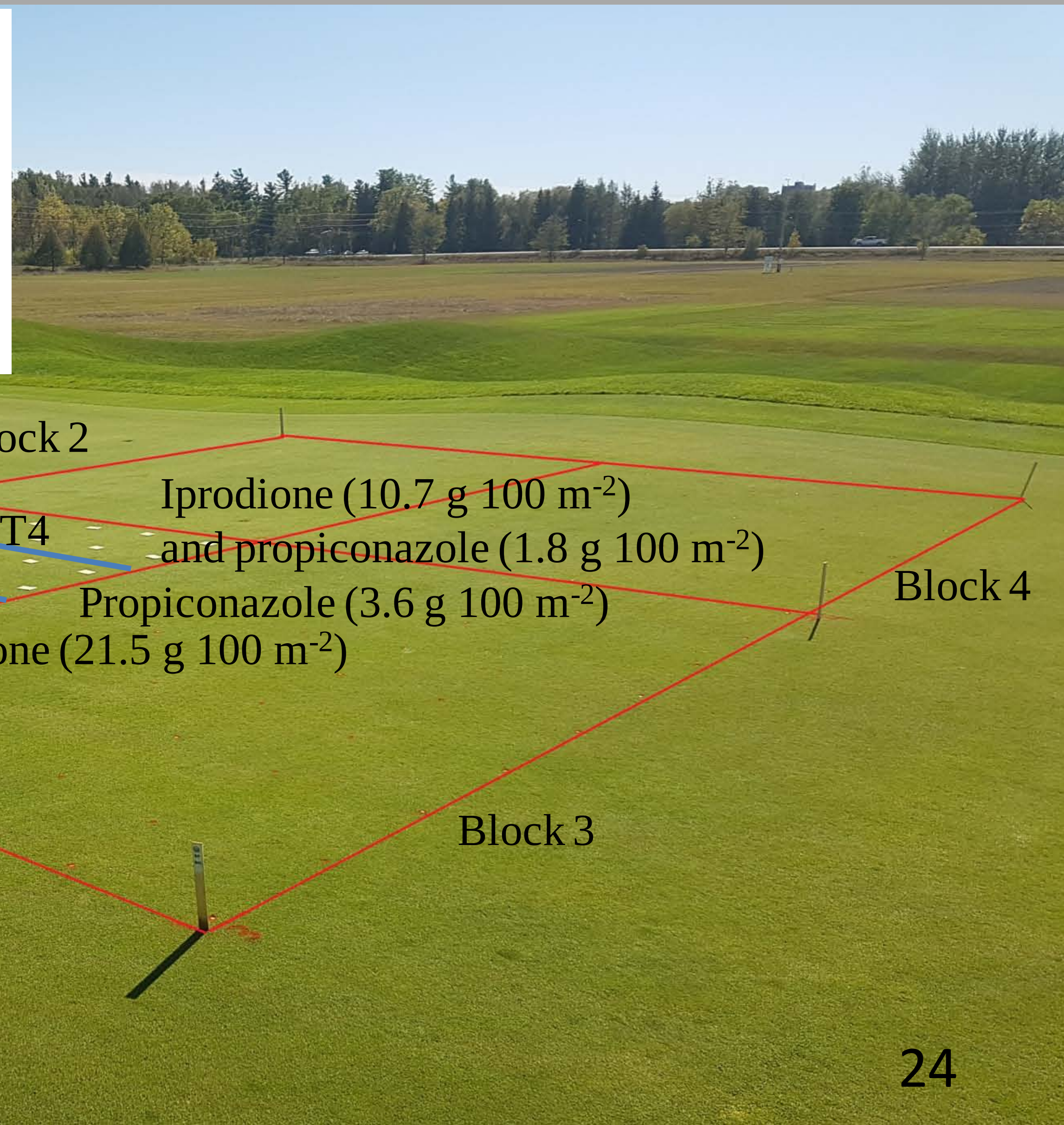
-0.5 m by 0.5 m plots

-5 g inoculum per plot

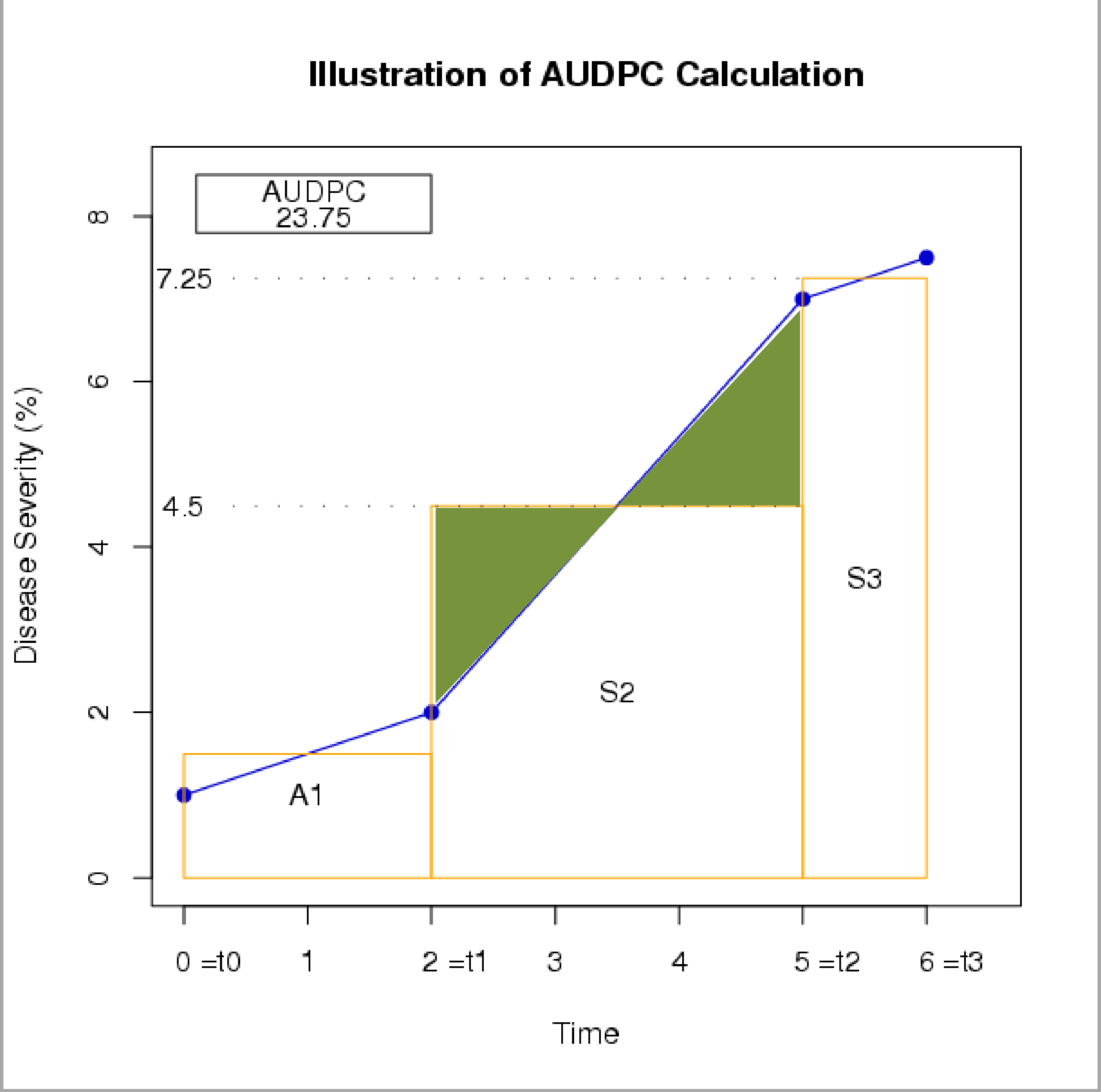
-treatments applied with CO_2 sprayer

-disease rating (% yellowing) weekly

-area under disease progress curves

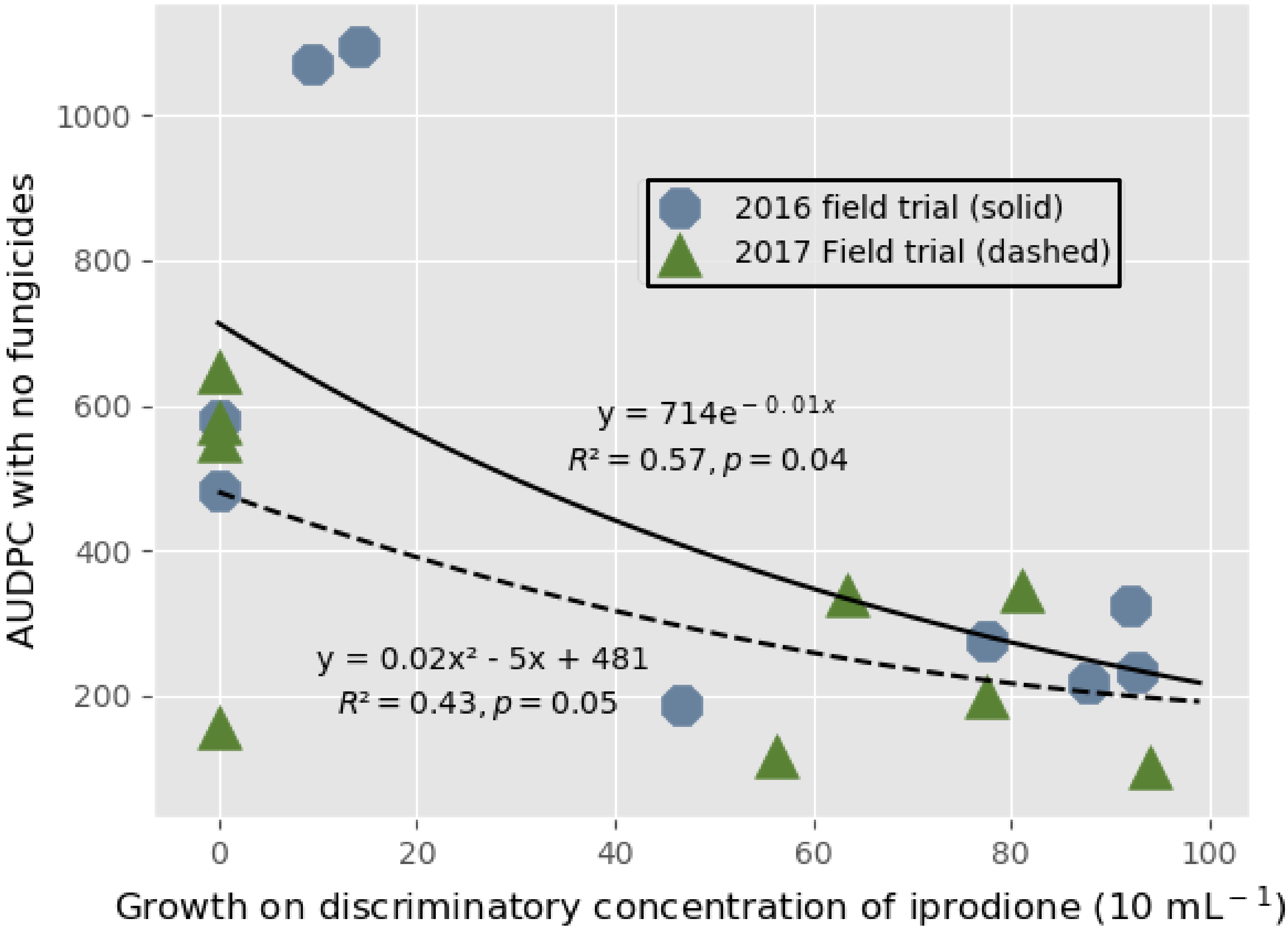


Area under disease progress curves

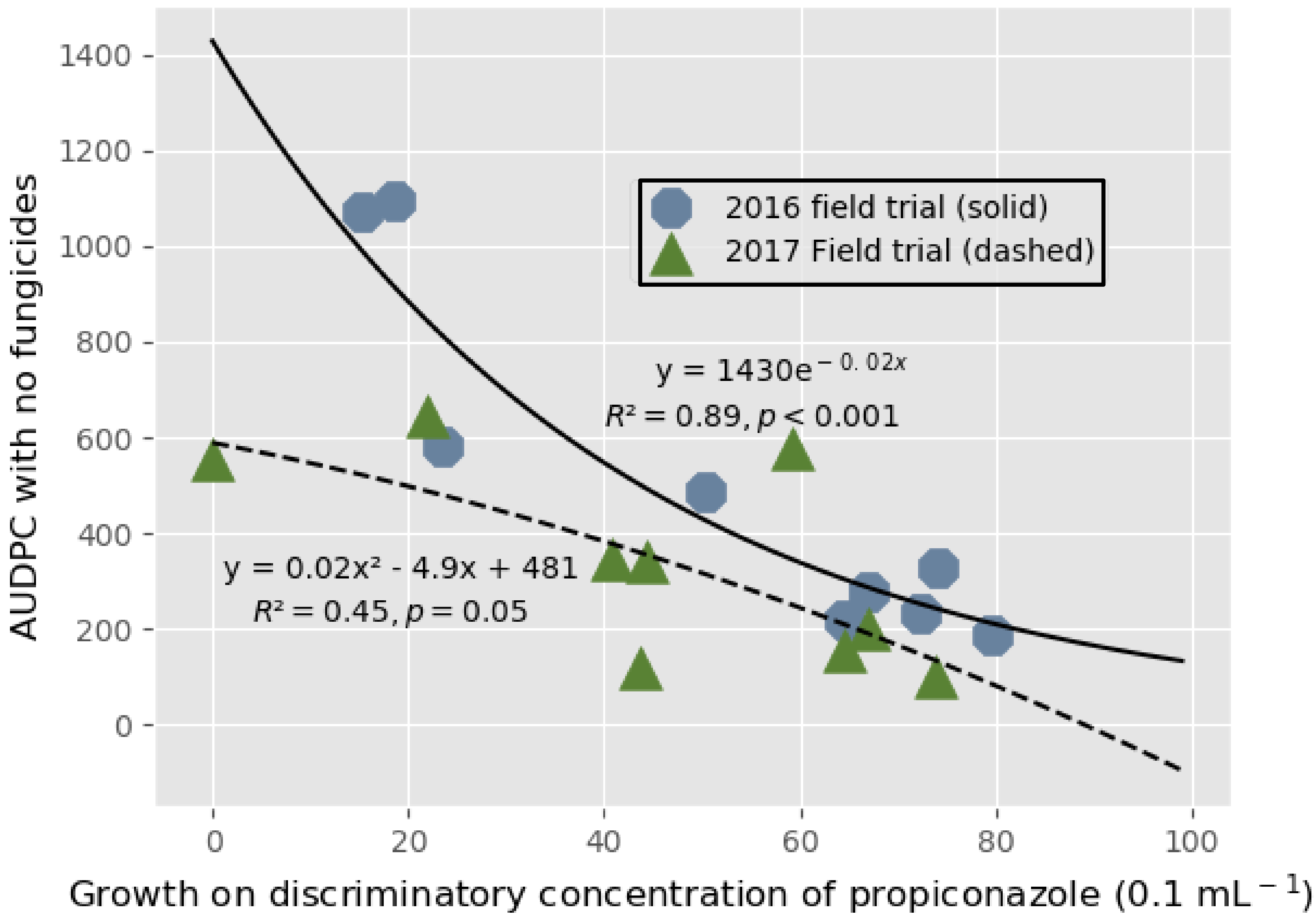


http://www.apsnet.org/edcenter/advanced/topics/EcologyAndEpidemiologyInR/DiseaseProgress/Pages/AUDPC.aspx

Virulence associated with iprodione sensitivity



Virulence associated with propiconazole sensitivity



Identification of mutations associated with insensitivity

DNA extraction →

Sequencing →

-Isopropanol precipitation (modified Edwards et al. (1990))

-Genomic DNA sent to Genome Quebec

-Illumina HiSeq 2000

-Library inserts approximately 400 bp

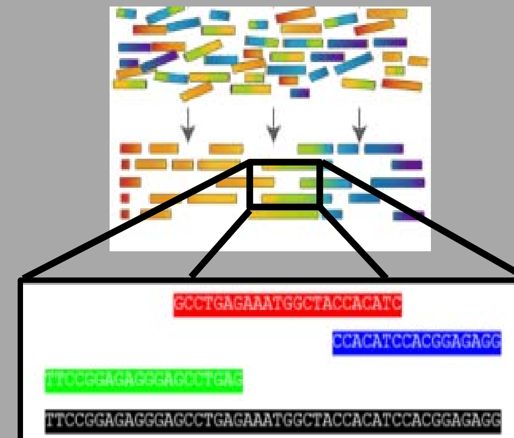
-Sequencing was 100 bp paired-end

Edwards, K., C. Johnstone, and C. Thompson. 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. NucAcidRes. 19: 1349

Haridas, S., C. Breuill, J. Bohlmann, and T. Hsiang. 2011. A biologist's guide to de novo genome assembly using next-generation sequence data: A test with fungal genomes. J. Microbiol. Methods 86: 368–375

Identification of mutations associated with insensitivity

de novo genome assembly



-Setup network server (Ubuntu server, 32 gb RAM)
-Reads assembled with three programs, each using a range of kmer values (SOAPdenovo, ABySS, Velvet)
-Assembly with highest N50 value selected
-Gene prediction using AUGUSTUS based on *F.graminearum*, *M. grisea*, and *N. crassa* gene models
-Model producing highest number of genes selected
-Genome completeness assessed with BUSCO

Identification of mutations associated with insensitivity

-Genes associated with resistance to dicarboximide and DMI fungicides retrieved from GenBank

-Standalone BLAST databases setup for sequenced *M. nivale* isolates (raw assembly and predicted gene sets)

-Genomes queried for select genes using BLAST (e-50)

-Top hits extracted from databases and aligned using Muscle(v3.8) or MEGA7 (v7.0.26)

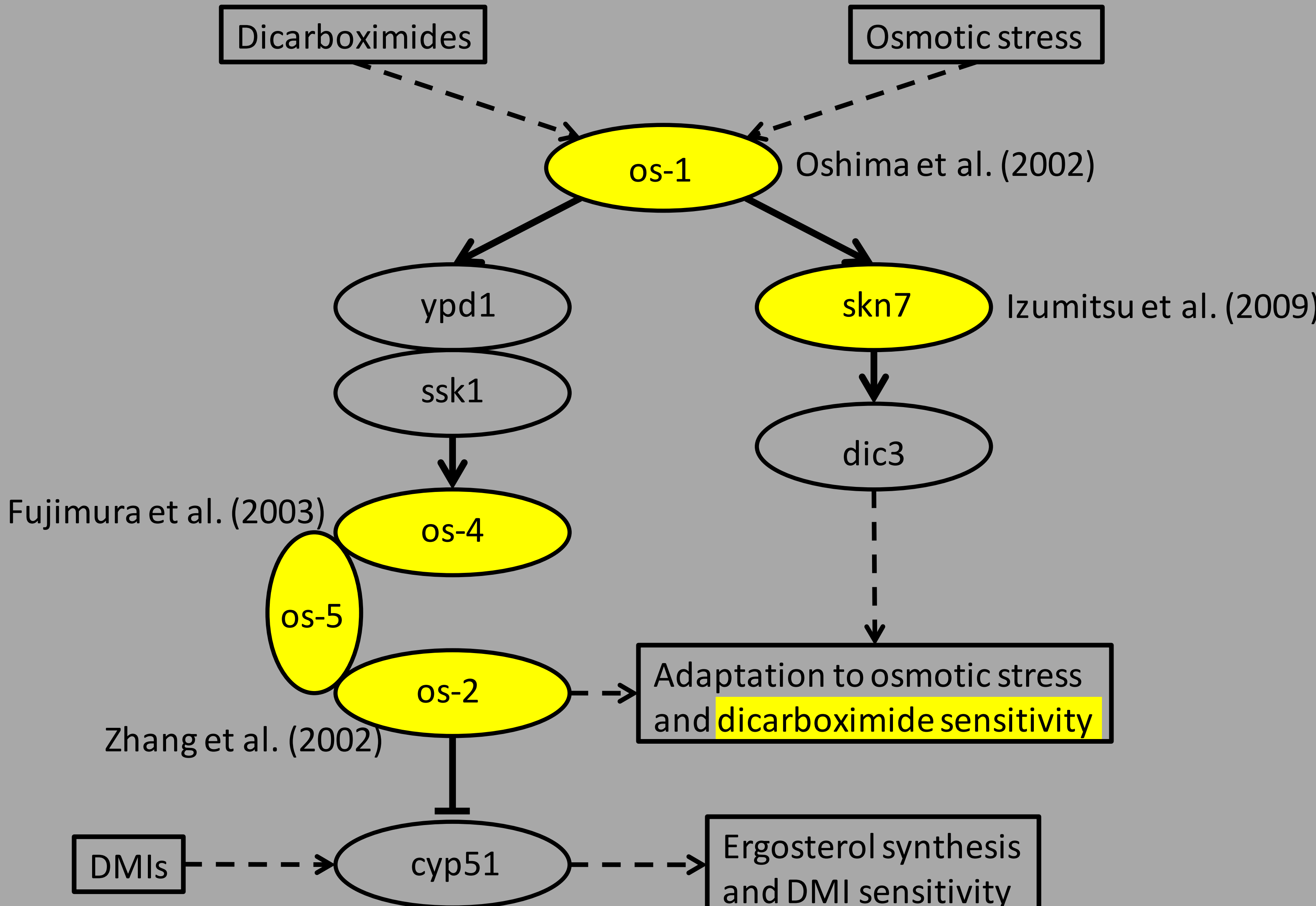
Sequencing and assembly results

Table 7. Sequencing and assembly details of *Microdochium nivale* isolates.

Isolate	Location	EC_{50} (µg mL^{-1})		Reads (millions)	Program	N50 (kb)	Contigs
		Iprodione	Propiconazole				
10082*	United Kingdom	1.4	0.31	22.7	ABySS	356	8732
10106*	Medicina, ITL	1.3	0.56	22.2	Velvet	477	3741
11037*	GTI, Guelph, ON	1.6	0.28	70.0	SOAP	155	2642
12099*	GTI, Guelph, ON	2.3	0.03	19.1	SOAP	467	3943
12262*	Ottawa, ON	1.9	0.16	28.1	ABySS	383	7527
13172	GTI, Guelph, ON	10.4	0.14	9.6	SOAP	241	2035
13407*	Upland Res. Center, JPN	4.3	0.06	20.2	SOAP	526	4482
13408*	Kitasato Univ. Farm, JPN	2.6	0.06	20.6	SOAP	381	10170
15109	BC-1, BC	26.7	0.11	16.7	SOAP	235	806
15110	BC-1, BC	40.8	0.81	15.7	ABySS	281	791
15141	BC-3, BC	5.6	1.83	9.1	SOAP	108	23726
15165	BC-3, BC	115	0.36	8.2	SOAP	121	3669
15170	BC-3, BC	191	0.44	11.5	SOAP	207	3974

*assembled previously by Hsiang Lab, other downstream analysis by Gourile

Genes previously associated with resistance



Kim, S.Y. et al., 2011. Hrk1 plays both Hog1-dependent and -independent roles in controlling stress response and antifungal drug resistance in *Cryptococcus neoformans*. *PloS one*, 6(4), p.e18769.
Izumitsu et al., 2009. Dic2 and Dic3 loci confer osmotic adaptation and fungicidal sensitivity independent of the HOG pathway in *Cochliobolus heterostrophus*. Mycological Research, 113, 1208-1215

Mutations associated with iprodione insensitivity

Isolate	Location	EC_{50} ($\mu g\ mL^{-1}$)	*os-1*	*os-2*	*os-4*	*os-5*	*skn7*	efflux transporters *mfsM2*	*mrr1*
10106	Medicina, ITL	1.3	-	-	-	-	-	-	-
10082	United Kingdom	1.4	-	-	-	-	-	-	-
11037	GTI, Guelph, ON	1.6	-	-	-	-	-	-	-
12262	Ottawa, ON	1.9	-	-	-	-	-	-	-
12099	GTI, Guelph, ON	2.3	-	-	-	-	-	-	-
13408	Kitasato Univ. Farm, JPN	2.6	-	-	-	-	-	-	-
13407	Upland Ag Res. Center, JPN	4.3	-	-	-	-	-	-	-
15141	BCGC-3, BC	5.6	-	-	-	-	-	-	-
13172	GTI, Guelph, ON	10.4	-	-	-	-	+[4]	-	-
15109	BCGC-1, BC	26.7	+[1]	-	-	-	-	-	+[5]
15110	BCGC-1, BC	40.8	+[1]	-	-	-	-	-	+[5]
15170	BCGC-3, BC	115	+[2]	-	+[3]	-	-	-	+[5]
15165	BCGC-3, BC	191	+[2]	-	+[3]	-	-	-	+[5]

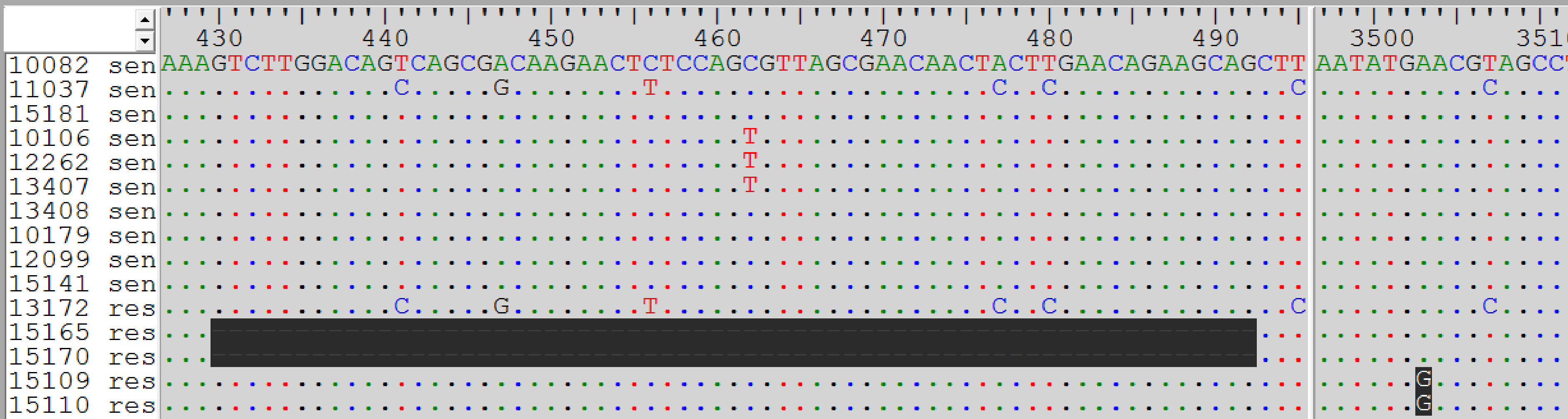
[1] point mutation A3503G; [2] deletion Δ430-462; [3] point mutations T182C, T395C;

[4] several deletion, insertions, and many point mutations;[5] point mutations G2237A, G2392A

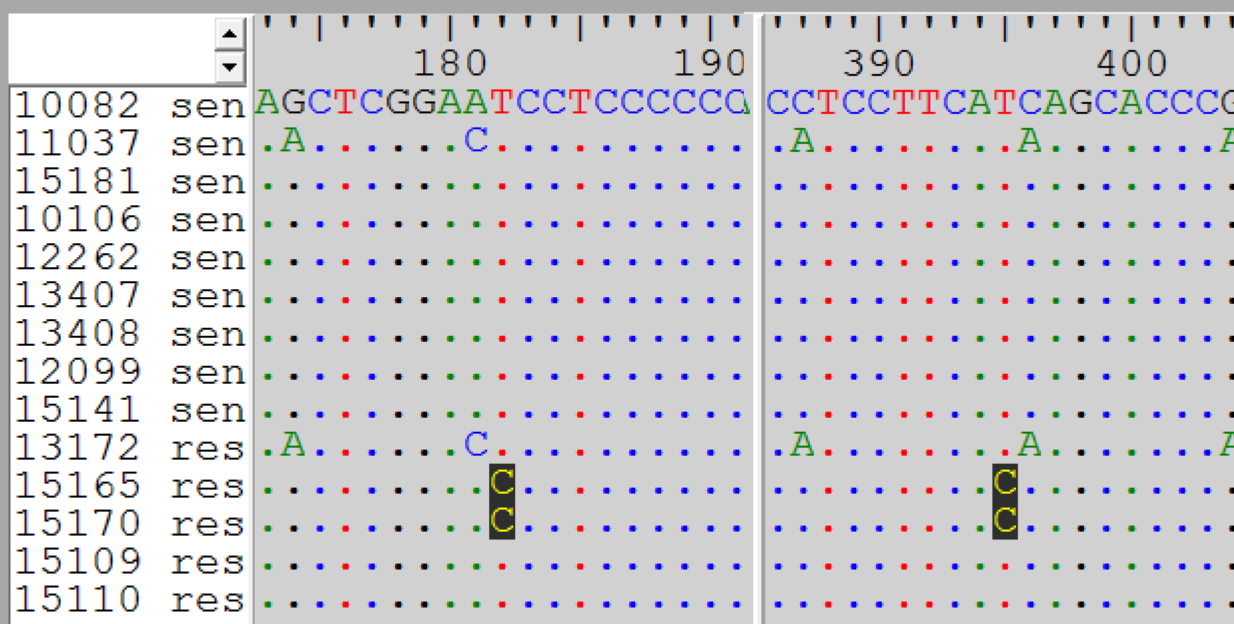
*None of the 54 point mutations previously associated with dicarboximide resistance were correlated with insensitivity in *M. nivale*

Mutations in the genes *mnos-1* and *mnos-4*

mnos-1



mnos-4



<u>Mutations in the coding region of *skn7* gene of resistant isolate 13172</u>

- 214 point mutations, 6 deletions, and 2 insertions

Δ8-130	T930C	A1134G	T1266C	A1455G	T1536G	C1677A	A1833C	A1944G	C2188A	G2367A	A2523T	G2625A
G34T	T936A	A1143T	C1269T	T1464C	C1545T	T1686C	T1842C	C1968T	T2199C	T2371G	C2526T	A2636C
Δ141-286	A948G	C1167T	A1296G	A1482G	T1563C	T1692A	C1845G	T1974C	A2229C	G2373A	C2532T	A2637G
												G2638C
C288T	C591G	A1176G	G1311A	C1490G	A1569T	C1698A	G1848A	T1980C	G2232A	2388_*ins*CATGGC	T2538C	
Δ292-294	C993G	C1179T	T1315C	C1491A/T	C1572T	C1716T	A1858G	A1989G	G2235T	T2392A	G2553A	A2640G
							1863_*ins*					
A296G	C999T	G1191C	C1353T	T1503A	C1578T	C1725T	GAACAA	C2013T	C2247T	A2403G	G2568A	C2641A
C302A	G1002C	T1194A	G1359A	C1506T	G1596A	C1746T	A1867T	C2019T	G2250T	G2404C	C2571A	G2648C
C303G	C1017T	C1197T	T1365G	A1508T	T1614C	T1749C	A1879T	T2049C	T2277C	C2406T	G2574C	A2655G
C305G	C1132T	A1203G	T1389C	T1509C	T1617C	G1758A	A1881G	A2070G	T2280C	C2409T	T2589G	G2659A
C309A	T1045C	T1212C	G1395A	T1510C	T1623C	G1761A	T1885C	C2085T	T2283C	T2415C	G2592A	A2673G
C310A	C1059T	A1221G	T1404C	T1515C	G1626A	T1773C	G1891A	A2109G	C2286T	G2419A	T2601C	T2676C
G312A	T1062C	G1226C	C1410G	G1519A	A1633G	A1776G	C1893T	G2115T	A2293G	A2424G	A2607C	G2688A
T314C	A1074G	G1230C	T1419G	A1521T	T1641G	C1782G	T1896C	T2118A	A2294T	C2427T	G2608A	C2706G
Δ316-339	T1080C	T1242C	G1423A	T1522G	A1644G	A1794C	G1908A	T2154C	A2311C	A2436G	C2609T	G2708A
Δ351-655	A1095T	T1257C	C1438A	C1527T	G1647A	T1812C	T1915C	T2157C	C2316A	G2502A	T2616A	C2709T
Δ662-804	A1110C	C1260T	A1439T	G1530T	A1656G	G1821C	G1917T	C2178T	G2331C	T2508C	T2619G	T2717A
T894C	C1116T	G1263A	C1451A	A1534G	A1657G	G1831A	A1929C	G2181A	A2346G	A2519C	C2624G	

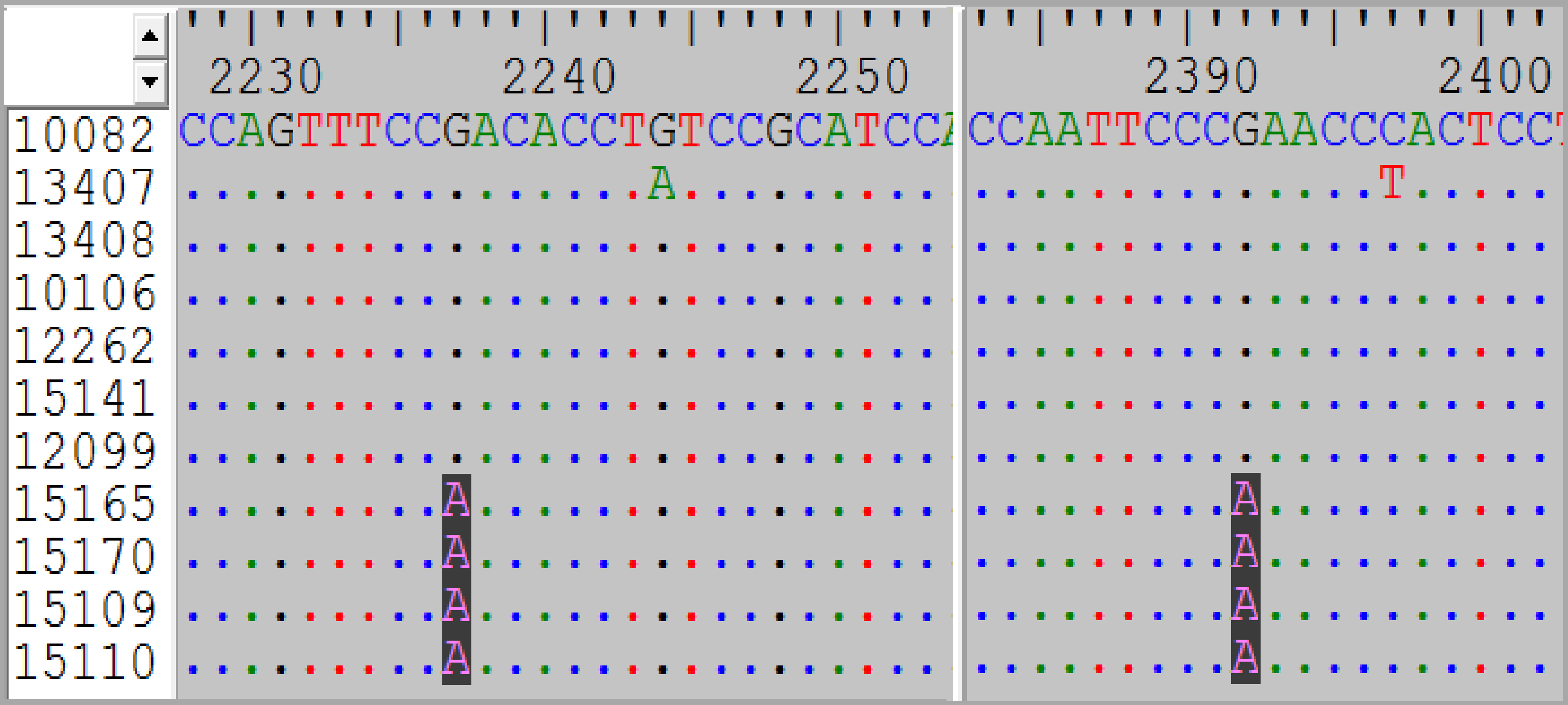
Mutations associated with propiconazole insensitivity

Isolate	Location	EC$_{50}$ ($\mu g\ mL^{-1}$)	*cyp51A*	*cyp51B*	efflux transporters *atrD*	*mfsM2*	*mrr1*	*mfs1*
12099	GTI, Guelph, ON	0.03	-	-	-	-	-	-
13407	Upland Ag Res. Center, JPN	0.06	-	-	-	-	-	-
13408	Kitasato Univ. Farm, JPN	0.06	-	-	-	-	-	-
15109	Victoria, BC	0.11	-	-	-	-	+[1]	-
13172	GTI, Guelph, ON	0.14	-	-	-	-	-	-
12262	Ottawa, ON	0.16	-	-	-	-	-	-
11037	GTI, Guelph, ON	0.28	-	-	-	-	-	-
10082	United Kingdom	0.31	-	-	-	-	-	-
15165	Cordova Bay, BC	0.36	-	-	-	-	+[1]	-
15170	Cordova Bay, BC	0.44	-	-	-	-	+[1]	-
10106	Medicina, ITL	0.56	-	-	-	-	-	-
15110	Victoria, BC	0.81	-	-	-	-	+[1]	-
15141	Cordova Bay, BC	1.83	-	-	-	-	-	-

[1] point mutations G2237A, G2392A

*None of the 25 mutations (point mutations, insertions, and promoter mutations) previously associated with DMI resistance were correlated with insensitivity.

Mutations in the genes *mrr-1*



Major Conclusions

1. Annual use of iprodione (>75 g / 100 m^2 / year) and propiconazole (>35 g / 100 m^2 / year) increases the proportion of *Microdochium nivale* isolates with decreased sensitivity.
2. First report of DMI resistance in this economically important plant pathogen.
3. Resistance to iprodione and propiconazole is associated with decreased *in vitro* growth rate and decreased virulence in the field.
4. Recommended field application rates may still be effective for the control of isolates exhibiting *in vitro* insensitivity.
5. Novel mutations in the *mnos-1* and *mnos-4* coding regions may be associated with iprodione resistance.
6. Novel mutations in the transcription factor *mrr1* may be associated with resistance to both iprodione and propiconazole.

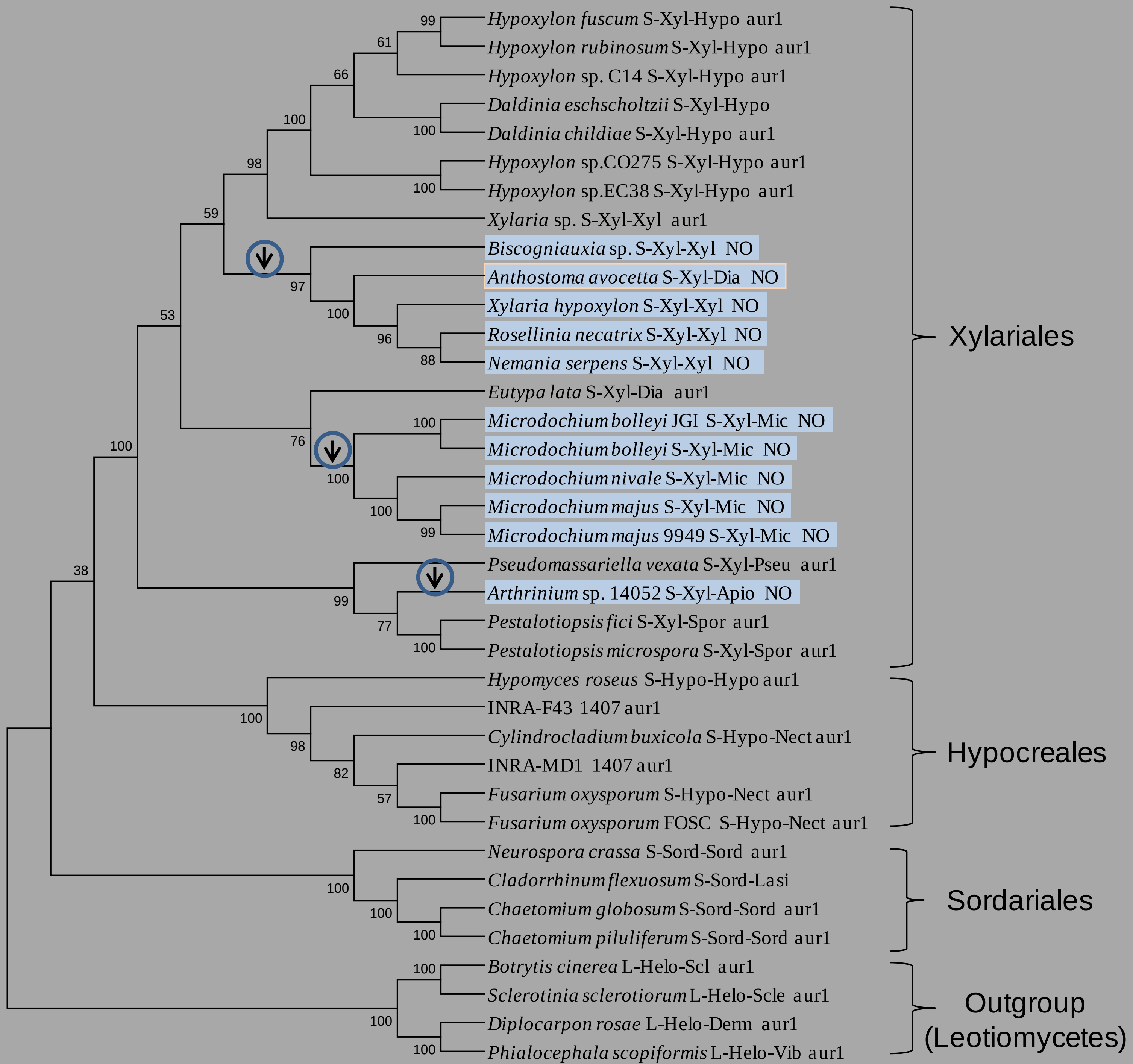
Future Work

- Cross-resistance to other DMI fungicides
- Resistance to other modes of action (e.g SDHI)
- More extensive field trials
- Gene replacement for *mnos-1*, *mnos-4*, and *skn7*
  - Confer resistance to sensitive isolates
  - Restore sensitivity in resistant isolates
- Gene expression analysis (RT-PCR or RNAseq)
  - *cyp51A* or *cyp51B* over-expression?
  - over-expression of efflux transporters?
  - confirm *mrr1* is up-regulating efflux transporter *atrB*

Gene loss in the order Xylariales

(side project)

- BUSCO assessment of a *M. nivale* genome appeared to lack a conserved gene
- Confirmed by checking 12 other *M. nivale* genomes
- Gene identified as *aur1*, involved in sphingolipid production and associated with aureobasidin resistance
- Expanded to genus, family, and order (total of 44 genomes)


Hypoxylon fuscum S-Xyl-Hypo aur1
Hypoxylon rubinosum S-Xyl-Hypo aur1
Hypoxylon sp. C14 S-Xyl-Hypo aur1
Daldinia eschscholtzii S-Xyl-Hypo
Daldinia childiae S-Xyl-Hypo aur1
Hypoxylon sp.CO275 S-Xyl-Hypo aur1
Hypoxylon sp.EC38 S-Xyl-Hypo aur1
Xylaria sp. S-Xyl-Xyl aur1
Biscogniauxia sp. S-Xyl-Xyl NO
Anthostoma avocetta S-Xyl-Dia NO
Xylaria hypoxylon S-Xyl-Xyl NO
Rosellinia necatrix S-Xyl-Xyl NO
Nemania serpens S-Xyl-Xyl NO
Eutypa lata S-Xyl-Dia aur1
Microdochium bolleyi JGI S-Xyl-Mic NO
Microdochium bolleyi S-Xyl-Mic NO
Microdochium nivale S-Xyl-Mic NO
Microdochium majus S-Xyl-Mic NO
Microdochium majus 9949 S-Xyl-Mic NO
Pseudomassariella vexata S-Xyl-Pseu aur1
Arthrinium sp. 14052 S-Xyl-Apio NO
Pestalotiopsis fici S-Xyl-Spor aur1
Pestalotiopsis microspora S-Xyl-Spor aur1
Hypomyces roseus S-Hypo-Hypo aur1
INRA-F43 1407 aur1
Cylindrocladium buxicola S-Hypo-Nect aur1
INRA-MD1 1407 aur1
Fusarium oxysporum S-Hypo-Nect aur1
Fusarium oxysporum FOSC S-Hypo-Nect aur1
Neurospora crassa S-Sord-Sord aur1
Cladorrhinum flexuosum S-Sord-Lasi
Chaetomium globosum S-Sord-Sord aur1
Chaetomium piluliferum S-Sord-Sord aur1
Botrytis cinerea L-Helo-Scl aur1
Sclerotinia sclerotiorum L-Helo-Scle aur1
Diplocarpon rosae L-Helo-Derm aur1
Phialocephala scopiformis L-Helo-Vib aur1
Xylariales
Hypocreales
Sordariales
Outgroup
(Leotiomycetes)

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