FUSARIUM GRAMINEARUM AT THE INTERSECTION OF WHEAT AND WILD GRASS COMMUNITIES

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Fusarium graminearum poses a significant challenge to the production of staple cereal crops around the world and causes animal and human health risks by contaminating grain with toxin. Primarily understood as an agricultural pathogen, *F. graminearum* is also associated with dozens of non-cultivated or wild hosts. The pathogen's relationship with wild hosts is poorly understood and may have an underestimated influence on epidemiology and evolution. This dissertation broadly examined the population biology and ecology of *F. graminearum* at the interface of wheat and grass communities. A field survey associated pathogen incidence in grasses with cumulative host density and rainfall. Two distinct *Fusarium* communities differing in diversity and structure were connected to variation in annual and regional environmental conditions. *Fusarium graminearum* population structure was characterized using microsatellite markers and loci associated with the production of toxin variants. Three sympatric North American populations were present in New York, but in remote, fragmented host communities, extensive admixture was detected between populations with different toxin production profiles. Wild grasses in non-agricultural settings supported a different balance of populations than was found in wheat fields, and even

small pockets of wild hosts provided overwintering sites for a large number of diverse isolates. The competency of six common grasses and wheat were contrasted in controlled experiments, and though several grasses were less suitable than wheat during some stages in the pathogen life cycle, the majority were comparable as hosts. Isolates derived from wheat and grasses were phenotyped for fitness related traits and displayed a difference in mean asexual reproductive traits. Conidium length appeared to be under selection, and evidence of a trade-off with mycelial growth was observed. Cumulatively, these studies implicated regional environmental factors as the driving forces behind the epidemiology, community ecology and population structure of *F. graminearum*, highlighted the potential for inoculum produced on grasses to contribute to crop infections, and suggested wild grasses play a greater role in promoting pathogen diversity than previously recognized.

BIOGRAPHICAL SKETCH

The author was born January 2, 1992 to Robert and Diane Fulcher. He lived in Annandale, Virginia with his siblings Samantha and Paul until matriculating at Virginia Polytechnic Institute and State University in 2010. There he earned a Bachelor of Science in Environmental Horticulture and developed interests in fungi and crop diseases. During that time, he also worked as a gardener and on an organic vegetable farm. Before beginning doctoral studies at Cornell University in June 2015, the author conducted research in the Virginia Tech Plant Disease Clinic under the supervision of Mary Ann Hansen, at the Agricultural Experiment Station in Geneva, NY with Chris Smart, and at the USDA Fungal Biology and Systematics Lab in Beltsville, MD headed by Lisa Castlebury. At Cornell, he majored in plant pathology with minors in ecology and international agriculture.

Beth

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PREFACE

If in agricultural science "true Theory is the surest guide to successful Practice," then designing the best approach to plant disease management demands a complete understanding of pathogen biology[1]. No plant pathogen being confined strictly to agricultural production, an essential part of the Theory is recognition of relationships between naturally occurring hosts, cultivated hosts, and pathogens. In some cases, the significance of wild hosts to disease in crops is easily seen, for example, in the heteroecious rusts, whose life cycles often require non-cultivated plants. Impacts on the wellbeing of natural hosts by pathogens spreading from cultivated plants are likewise easy to observe in certain situations, for instance forest trees visibly cankered by the oomycete *Phytophthora cinnamomi*. On occasion, the impact of agriculturally significant pathogens on wild hosts is less clear, obscured by partial tolerance or the ambiguity of symptoms, see barley yellow dwarf virus in prairie grasses. Or from the opposite perspective, the cryptic infection of natural hosts can contribute to the spread of crop disease with devastating effects, illustrated well by the bacterium *Xylella fastidiosa*. The system considered in this dissertation involves an impactful agricultural pathogen with a cryptic and ambiguous relationship to wild plants, consisting of subtle, poorly understood interactions that are perhaps consequential in both agricultural and natural environments.

Each chapter in this thesis examines a different facet of the ecology and population biology of the plant pathogenic fungus *Fusarium graminearum* as it exists at the intersection of wheat fields and natural grass communities. The infection of wheat, and other staple grain crops, causes a sizeable economic impact and, due to the

pathogen's toxigenic abilities, also brings animal and human health risks. Capable of infecting dozens, if not hundreds, of plant species from over 20 taxonomic families, the pathogen's geographic distribution is equally wide ranging, extending across six continents. Unsurprisingly, this is accompanied by significant genetic and phenotypic diversity. The following chapters contain further background information as needed, and the rest of this introduction serves only to outline a brief history of *F. graminearum* research involving non-cereal hosts.

The earliest formal description of this pathogen dates to 1822, when von Schweinitz defined the species *Sphaeria zeae* from a sample of corn stubble collected in North Carolina[2]. The preferred name of *F. graminearum* was provided in 1839 by Schwabe, who described a specimen from European beach grass (*Ammophila arenaria*). His choice of specific epithet, literally meaning "of the grasses," reflected a habitat he described simply as *in floribus graminum*, "in the flowers of grasses," which suggests the range of hosts was already clear to him[3]. Head blight (FHB), or scab, on grain crops did not feature prominently in the literature until 1880-1890[4,5], and the lynchpin of the agricultural disease cycle, the connection between inoculum from corn stalks and scab on grain spikes, was not made clear for some time[6]. Even then, the frequent colonization of wild grasses was proposed as a challenge for effective disease management in wheat[7].

The bulk of mid-century research was related to livestock health, in particular the estrogenic compound zearalenone found in infected corn and the emetic effects of molded grain[8]. However, one instance of pathogen biology being advanced through research on a non-cereal host is worth noting. A floricultural disease led to the

development of carnation leaf agar as a standard medium for the morphological identification of *Fusarium* spp. and the study of perithecia production by *F. graminearum*[9]. During this time, mycologists also continued updating the catalogue of wild hosts[10], though no further work on their relationships is apparent.

Research output in North America dwindled until Fusarium head blight emerged as a major concern in the United States during the 1990s. Jenkinson and Parry conducted the first modern survey of wild hosts for *Fusarium* pathogens[11] but did not ascribe much importance to wild hosts in their review of "Fusarium ear blight" of cereals the following year[12]. Since then, a number of studies have recorded the incidence of FHB pathogens in wild hosts. Only recently have more incisive questions been asked about the interactions between *F. graminearum* and wild hosts[13,14]. Possibly this has been motivated by the splitting of *F. graminearum* into many sub-species, with some found on hosts very distantly related to cereal crops (e.g. acacia trees), and recognition that non-cereals may have more interesting connections to the pathogen than previously acknowledged.

Despite the time elapsed since wild grasses were identified as common hosts of *F. graminearum*, many questions remain. Among these are: (1) what determines pathogen distribution in wild grasses and do these hosts have epidemiological relevance to crop epidemics, (2) do different grass species influence the formation of *Fusarium* communities and species diversity, (3) are pathogen populations inhabiting grasses distinct from those infecting crops, (4) how similar are host-fungus interactions in wheat and grasses, and (5) has pathogen evolution been significantly influenced by wild grasses or do these hosts continue to influence pathogen evolution?

By starting to answer these questions, this dissertation attempts to make the Theory surrounding *F. graminearum* a bit sounder so that future Practice will be better informed.

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CHAPTER 1

THE INCIDENCE OF A BROAD HOST RANGE, AGRICULTURAL PATHOGEN IN GRASSES IS ASSOCIATED WITH RAINFALL AND CUMULATIVE HOST DENSITY

ABSTRACT

The movement of plant pathogens between cultivated and natural host communities can result in lost agricultural production and altered biodiversity. These interactions are shaped in part by host distribution and diversity but are largely dependent on pathogen life-history. The ecology of the fungus *Fusarium graminearum* was examined using host community and environmental data to better understand these interactions in a broad host range, widely dispersed pathogen. Pathogen incidence was recorded in a three-year survey of wild grasses in New York. In order to assess the likelihood of pathogen spillover between environments, research sites were spread between regions of high and low agricultural production and included the margins of agricultural fields, remote grass communities and a 10,000-acre wildlife preserve bordered by intensive agriculture. Pathogen incidence in living grass spikes and senesced, overwintered stems was lowest in a region of low agricultural production ($P = 0.001$), but equal across agricultural and non-agricultural sites. The groundcover of agricultural and non-agricultural hosts within 1 km of sample sites were similarly effective predictors of pathogen incidence, indicating both classes of host may drive pathogen spread and unidirectional spillover is unlikely. Rainfall in the weeks preceding sample collection was strongly correlated with *F. graminearum* incidence in

grasses, as well as an increased prevalence of *F. graminearum* in *Fusarium* spp. communities ($P = 0.001$). Host species diversity was not associated with a reduction in pathogen incidence, and *F. graminearum* incidence did not vary between the most well-sampled grasses, providing no support for a dilution effect or the underlying conditions required for a dilution effect. These results indicate the pathogen spreads in non-cultivated grasses in a manner consistent with the existing understanding of crop disease epidemiology. Increasing host acreage, whether cultivated or not, could drive the infection of grasses in remote or protected environments, altering their microbial communities.

INTRODUCTION

The movement of plant pathogens across the interface of crop land and natural spaces has consequences for agricultural productivity and the maintenance of biodiversity. Pathogens can move from cultivated plants to unmanaged wild hosts[1], uncultivated hosts can act as pathogen reservoirs that contribute to disease in managed host communities[2], and in some instances, pathogen movement occurs in both directions[3].

The 'spillover' of pathogens from one host to another can be an important determinant of pathogen prevalence[4,5] and have significant impacts on host communities[6–10]. Understanding cross-environmental interactions may inform disease management, environmental conservation efforts, and land use decisions. While these interactions have been observed in some plant-pathogen systems, several referenced above, phytopathogen lifestyles are diverse and remain underrepresented in this field

of study. Accounting for the breadth of their life histories is important to our understanding of specific cases as well as our general understanding of disease ecology[11].

Dilution and amplification effects stemming from varying levels of host biodiversity are also known to impact disease incidence[12,13]. A dilution effect is predicated on variation in the susceptibility of different host species, but generalist pathogens well adapted to diverse hosts may not be subject to this effect. The interaction of biodiversity and disease risk should continue to be evaluated in different pathosystems to determine when generalized rules may apply[14,15], and the interface of cropland and natural plant communities is a particularly relevant setting to explore how altered biodiversity impacts the spread of disease in both managed and unmanaged environments.

This project examined pathogen-host community interactions, including potential spillover and dilution effects, in the broad host range fungal pathogen *Fusarium graminearum* Schwabe. A cosmopolitan species primarily understood as a pathogen of small grains and maize[16], *F. graminearum* is capable of infecting dozens of plant species, the majority of which are true grasses in the family Poaceae[17]. Many of these are non-cultivated species, ubiquitous across landscapes and often found in close proximity to susceptible crops.

In addition to directly damaging crops, the pathogen contaminates grain and stover with toxins that can render it unfit for human and animal consumption[18]. Annual economic losses are estimated at one billion dollars in the United States[19]. Current disease management practices rely heavily on fungicides[20] and are largely based on

our understanding of pathogen biology and ecology in an exclusively agricultural context. Airborne spores capable of kilometer scale dispersal[21,22] incite disease on flowering cereal spikes annually, and the pathogen persists from year-to-year on the dead tissue of its hosts, most importantly corn stalks, until environmental conditions are appropriate for the discharge of propagules.

While the occurrence and pathogenicity of *F. graminearum* on many grasses is well documented, and much is understood about the pathogen's behavior in agricultural systems, population level interactions with wild hosts and the connection between non-cultivated and cultivated hosts are not thoroughly studied. The impact on agriculture and the life-history of this microbe increase its value for ecological study.

Through a survey of naturally occurring grasses, this study attempted to identify host community characteristics and environmental conditions that influence *F. graminearum* incidence in non-cultivated hosts. Host density data and land use categories were used to look for evidence of pathogen spillover between environments, and grass host diversity was used to assess the potential for a dilution effect in this system. The ratio of *F. graminearum* to other *Fusarium* spp. inhabiting grass spikes was also recorded to detect any impacts the pathogen might have on other microbes inhabiting the same ecological niche.

METHODS

Research sites

Wild grass debris and inflorescences were collected from the borders of small grains fields and natural preserves in two regions of New York during the 2015-2017

growing seasons. Twenty unique locations were sampled, and one wildlife refuge was revisited in all three years (Figure 1.1). In 2015, three agricultural fields and a wildlife refuge in central NY were sampled. In 2016, the same refuge was sampled along with five different agricultural fields. In 2017, research locations were divided between central and northeastern NY to include environments from regions with different levels of agricultural production. The agricultural landscape in central NY is dominated by corn and soybean, while northeastern NY has a lower field crop acreage and is characterized by the wilderness of the Adirondack Mountains. Three agricultural fields and four natural spaces were sampled in the northeast region, and three agricultural fields and two natural spaces were sampled in the central region. These regions also differed in altitude and temperature. Northeastern NY has lower mean temperatures and several sites were situated at a higher altitude than those found in central NY.

Winter wheat was grown at the majority of agricultural sites. One location contained winter barley, and two others were Cornell University research farms growing a variety of small grains. Fields ranged in size from 8 – 154 acres ($\overline{x}$ = 32), and all but two had a history of wheat-corn-soybean rotations. Crop management practices varied, but fungicide applications were rare. Site borders were separated by a minimum distance of 1 km and the furthest distance between sites was 346 km. The natural preserves sampled were primarily public wildlife management areas selected for the presence of grass hosts and their proximity to agricultural sampling sites. For the duration of this study, these areas were subject to minimal management practices aimed at protecting wildlife habitat.

Sample collection

Inflorescence sampling focused on grass species that flower simultaneously with winter wheat (Table 1.1) and was timed to coincide with the 'early grain filling' growth stage in nearby wheat crops[23], by which time the majority of crop infections are thought to have occurred. Grass spikes were collected at a single time point from each site, and all sites within a region were sampled in a single week. In 2015, wild grass spikes were collected haphazardly from each site. In 2016 and 2017, 1 m^2 quadrats were laid at regular intervals on transects following the grassy margins of agricultural fields or accessible portions of natural grasslands. Quadrats were separated by 10 m, so the number of quadrats per site increased with the boundary length of agricultural fields or the size of natural preserves (n = 8-50). Spikes of each grass species present in a quadrat were collected (n = 5-20). Only spikes between flowering and maturity were taken to ensure infection could have occurred but the fungus was not present as a saprophyte on senesced tissue. Senesced, overwintered grass stems were collected in early May before disease epidemics began in field crops. Sampling was conducted in quadrats laid as described above but independent of spike samples. All senesced stems within a quadrat and containing at least one node or joint were collected. No stem samples were collected in 2015, and one site in 2017 did not contain enough stem material for adequate sampling.

Samples were stored at -20°C for at least one week to remove arthropods and inhibit tissue degradation, then kept at 4°C during processing. Ten to twenty pieces of stem tissue from each quadrat and five to ten grass spikes per species from each quadrat

were chosen at random and tested for *F. graminearum* incidence. Plant tissues were rinsed in sterile water to remove coarse debris, surface sterilized in 1.65 % NaOCl for two minutes, rinsed again in sterile water, and placed on artificial growth media amended with streptomycin (5 g/L) and neomycin (5 g/L) to inhibit bacterial growth. *Fusarium* spp. colonies growing from plant tissue were subcultured, *Fusarium graminearum* was identified morphologically[24], and pathogen incidence was recorded.

Host Community and Environmental Data

The host species richness of each site was determined by recording species presence in quadrats laid during spike sampling. Grass species identification was performed in the field based on floral morphology and growth habit. Grasses that could not be identified in the field were compared to preserved specimens at the Liberty Hyde Bailey Conservatory (Ithaca, NY).

Figure 1.1. Wild grass spikes were collected from 23 field sites over three years. During 2016-17, wild grass stems that had overwintered naturally were collected from 18 of these sites. Sampling locations were divided between two regions of New York and two land uses, agricultural and natural.

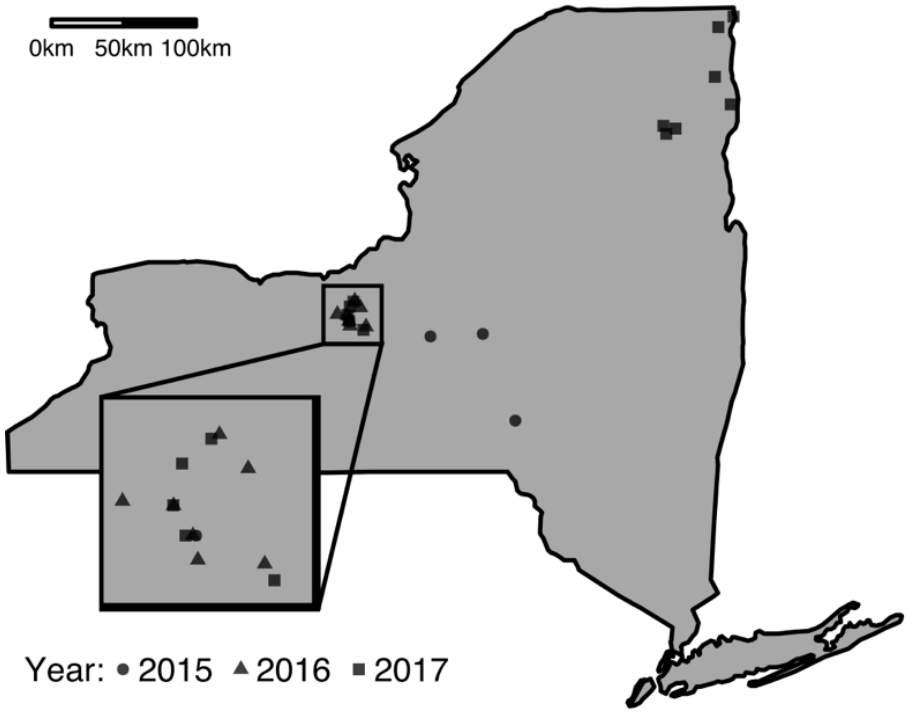


Table 1.1. Host species sampled with percent incidence of *Fusarium graminearum*.

Year	Grass Species	Sites†	Spikes	Incidence (%)
2015	*Bromus inermis* Leyss.	3	64	76.60
	Elymus canadensis L.	1	5	40.00
	Hordeum jubatum L.	1	20	15.00
	Phalaris arundinacea L.	1	7	28.60
	Phleum pretense L.	2	45	35.60
2016	*B. commutatus* L.	2	36	0.00
	B. inermis	4	247	2.02
	Dactylis glomerata L.	6	288	3.47
	E. repens	5	133	0.00
	Festuca spp.	4	98	1.02
	H. jubatum	1	29	0.00
	P. arundinacea	5	188	1.06
	P. pratense	4	101	0.00
	Poa annua L.	2	70	1.43
2017	*Alopecurus arundinaceus* Poir.	1	14	0.00
	Brachypodium sylvaticum (Huds.) P. Beauv.	2	21	0.00
	B. commutatus	2	46	30.43
	B. inermis	7	201	17.91
	B. secalinus L.	1	5	20.00
	D. glomerata	9	287	52.26
	E. repens	10	392	15.56
	Festuca spp.	11	259	16.22
	H. jubatum	1	10	10.00
	Lolium perenne L.	2	15	0.00
	Panicum spp.	5	55	5.45
	P. arundinacea	11	332	23.80
	P. pratense	10	332	11.14
	P. annua	8	130	10.77
	Typha latifolia L.	1	5	0.00

† Number of field sites where each species was found

Local host density was measured as the percent ground cover of corn, aggregated small grains, grasses or all three hosts within a 1 km radius of each field site during the year preceding sample collection. This host density was assumed to correlate with the amount of pathogen inoculum being produced on debris during the year spike samples were collected. The acreage estimates were taken from publicly available ground cover data downloaded in a georeferenced TIFF format[25]. Pixels were counted and converted to acres in QGIS version 3.4.7[26]. Cumulative rainfall estimates for the two months prior to spike sampling were calculated from the interpolated climate data available from the PRISM climate group at Oregon State[27].

Statistical analysis

All analyses were performed in RStudio version 1.1.453[28]. Pathogen incidence in stems and spikes was analyzed with a series of generalized linear models. First, pathogen presence or absence in individual pieces of host tissue was analyzed in response to fixed effects for year, land use, and region plus a random effect for sampling sites. Wald's X^2 statistic was used to determine predictor significance. Following this analysis, spike incidence was modelled again, using continuous predictors. Rainfall, host density, an interaction between the two, and rarefied species richness were included as fixed effects. The random effect of sample site was retained. This model was run four times, once for each class of host acreage (corn, small grains, grass, and total host acreage). The model with the lowest AIC value was used to generate confidence intervals and estimated probabilities for pathogen presence across different host density and rainfall values. A model containing the same predictors was

then used to analyze the ratio of *F. graminearum* to other *Fusarium* species at each site. The response variable was coded as a binomial with success being a *Fusarium* isolate identified as *F. graminearum* and a failure being the identification of any other *Fusarium* sp. The R^2 of all models was calculated with the 'piecewiseSEM' package[29]. Effective species numbers, or Hill numbers (qD), were calculated from observed species richness using the 'iNEXT' package at orders from 0-2[30]. Values for each order were correlated with *F. graminearum* incidence to assess whether disease dilution occurred with increases in host diversity.

RESULTS

Wild grass stems collected from 19 sites over two years were infested with *F. graminearum* at a rate of 13.4 % (n = 3671). Year and region were significant predictors of infestation ($P \leq 0.02$), but no difference was detected between land uses (Table 1.2, Figure 1.2a-b). Pathogen incidence in spikes collected from 23 sites over three years was 15.4% (n = 3435). Year and region were again significant predictors of pathogen incidence ($P \leq 0.001$), while land use had no effect (Table 1.3, Figure 1.2c-d).

Table 1.2. *Fusarium graminearum* incidence in grass stems, analysis of variance output.

Predictor	X^2	Degrees of Freedom	*P*-value
Year	5.155	1	**0.023**
Land use	0.050	1	0.822
Region	11.918	1	**0.001**

Table 1.3. *Fusarium graminearum* incidence in grass spikes, analysis of variance output.

Predictor	X^2	Degrees of Freedom	*P*-value
Year	55.987	2	**<0.001**
Land use	0.746	1	0.388
Region	11.089	1	**0.001**

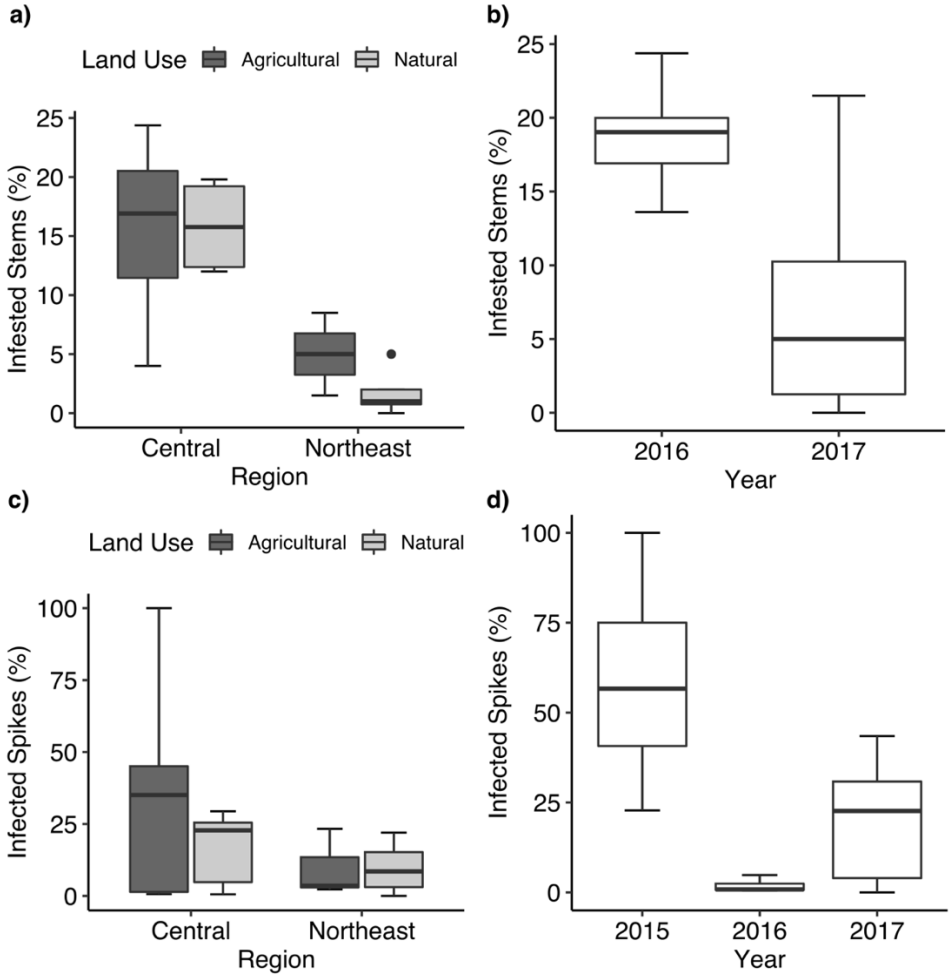


Figure 1.2. *Fusarium graminearum* recovery rates from a-b) infested grass stems and c-d) infected grass spikes. Lower pathogen incidence was recorded in a region with little agricultural production as compared to a region with intensive crop production. Differences between land uses were not detected, and year-to-year variation was high.

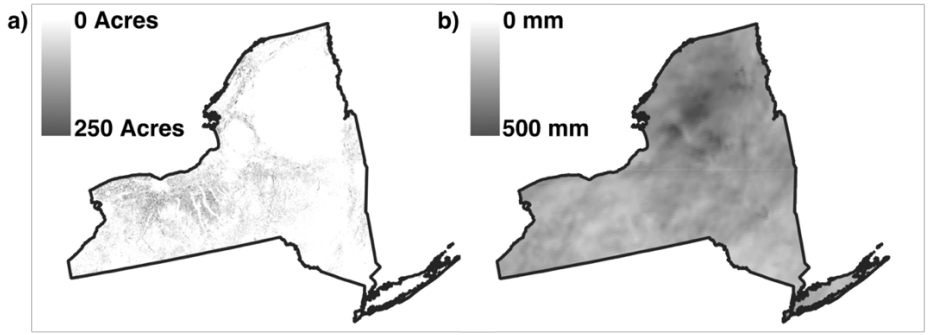


Figure 1.3. Maps of the 2017 environmental data used to model pathogen incidence in grass spikes. a) Crop density within 1km of sample sites was lower in northeastern New York. b) Cumulative rainfall in the weeks prior to sample collection varied between sites. Interpolated rainfall data was taken from the PRISM Climate Group at Oregon State.

Table 1.4. Model comparison using continuous predictors of *F. graminearum* spike incidence in non-cultivated grass.

Host Class	Model AIC	R^2 (fixed)	R^2 (fixed and random)
Corn	141.865	0.34	0.47
Small Grains	144.008	0.34	0.48
Grasses	140.331	0.37	0.48
All Hosts	139.296	0.36	0.47

Table 1.5. *Fusarium graminearum* incidence in grass spikes modeled with continuous predictors, analysis of variance output.

Predictor	X^2	Degrees of Freedom	*P*-value
Effective Species	2.285	1	0.131
Host Density	1.052	1	0.305
Rainfall	35.244	1	**<0.001**
Density:Rainfall	5.98	1	**0.014**

Table 1.6. *Fusarium graminearum* incidence in grasses with n > 100 and found at 10 or more field sites.

Host Species	Sites	Spikes	Incidence (%)
Bromus inermis	14	512	17.58
Dactylis glomerata	15	575	27.82
Elymus repens	15	525	11.62
Festuca spp.	15	357	12.05
Phalaris arundinacea	17	527	15.56
Phleum pratense	16	478	11.30
Poa annua	10	200	7.50

Host density and rainfall varied between locations and years (Figure 1.3). Models of spike incidence using corn, small grains, grass, and total host acreage within 1 km of sampling sites performed similarly (Table 1.4). The model using total host acreage had the lowest AIC value so was chosen for use in further analyses. A significant interaction was detected between rainfall and host density ($P = 0.014$), and a high probability of spike infection was expected when both rainfall and host density were greatest (Figure 1.4). The same model was applied to the probability of a recovered *Fusarium* species being identified as *F. graminearum*. The estimated probabilities are displayed in Figure 1.5 and were significantly impacted by the interaction of rainfall and host ($F_{23,1} = 7.172$; $P < 0.001$). Host richness did not have a significant effect on pathogen incidence and showed a slight positive correlation with *F. graminearum* incidence in spikes (Figure 1.6). The incidence of *F. graminearum* in grasses sampled at 10 or more sites with a sample size greater than 100 spikes ranged from 7.50 – 27.82 % (Table 1.6).

DISCUSSION

This study characterized the incidence of a widely dispersed, broad-host range pathogen in non-cultivated hosts across different environments. Our survey found that *Fusarium graminearum* is present in common, non-cultivated grasses throughout New York. Pathogen incidence was lower in a region with low agricultural production than in a region with high agricultural production but did not vary between agricultural and non-agricultural sites.

Figure 1.4. The probability of spike infection with *F. graminearum* was predicted by the interaction of host density and rainfall in the two months prior to sampling. Confidence intervals (95%) around least-squares means are displayed for increasing rainfall and host density. The range of host density and rainfall values displayed represents field conditions observed during the course of this study.

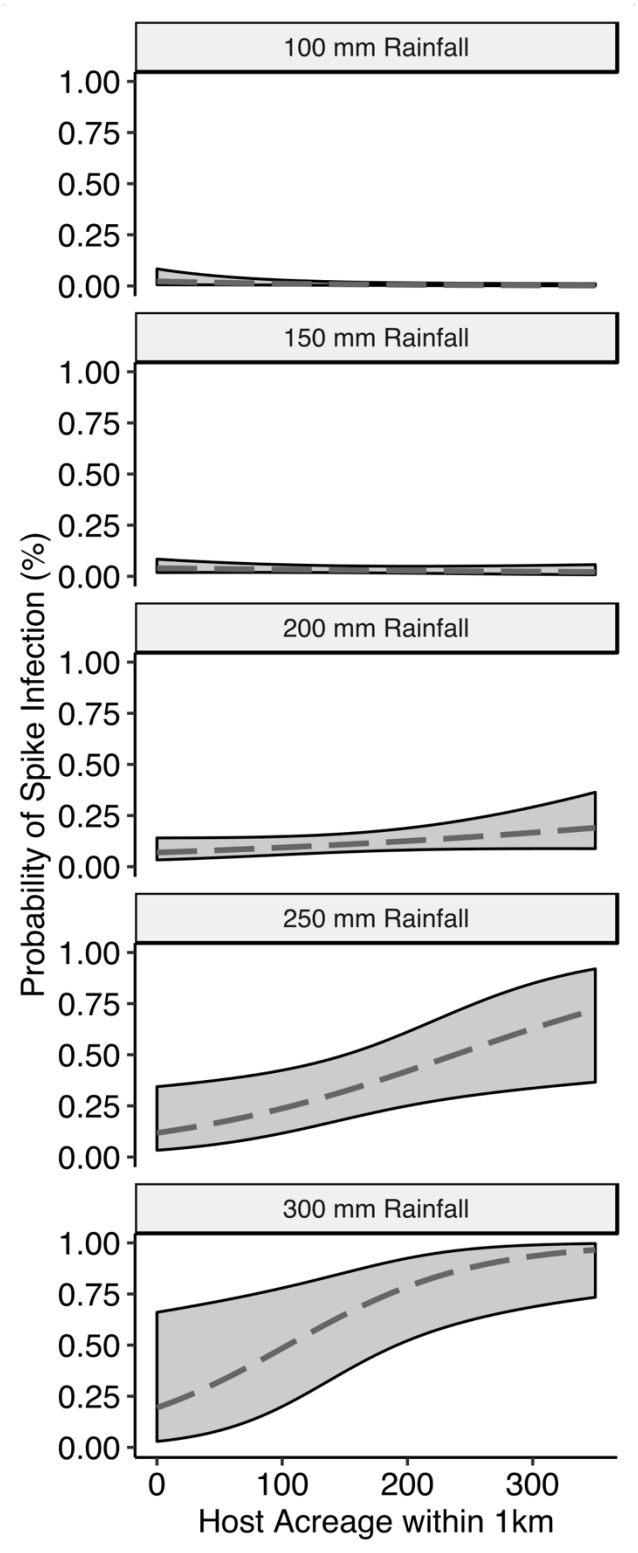

100 mm Rainfall
150 mm Rainfall
200 mm Rainfall
250 mm Rainfall
300 mm Rainfall
Probability of Spike Infection (%)
1.00
0.75
0.50
0.25
0.00
0
100
200
300
Host Acreage within 1km

Figure 1.5. The probability of a *Fusarium* sp. isolated from wild grass being *F. graminearum* increased as host density and rainfall increased. The incidence of non-graminearum *Fusaria* did not increase alongside the incidence of *F. graminearum*. Confidence intervals (95%) around least-squares means are displayed for increasing rainfall and host density.

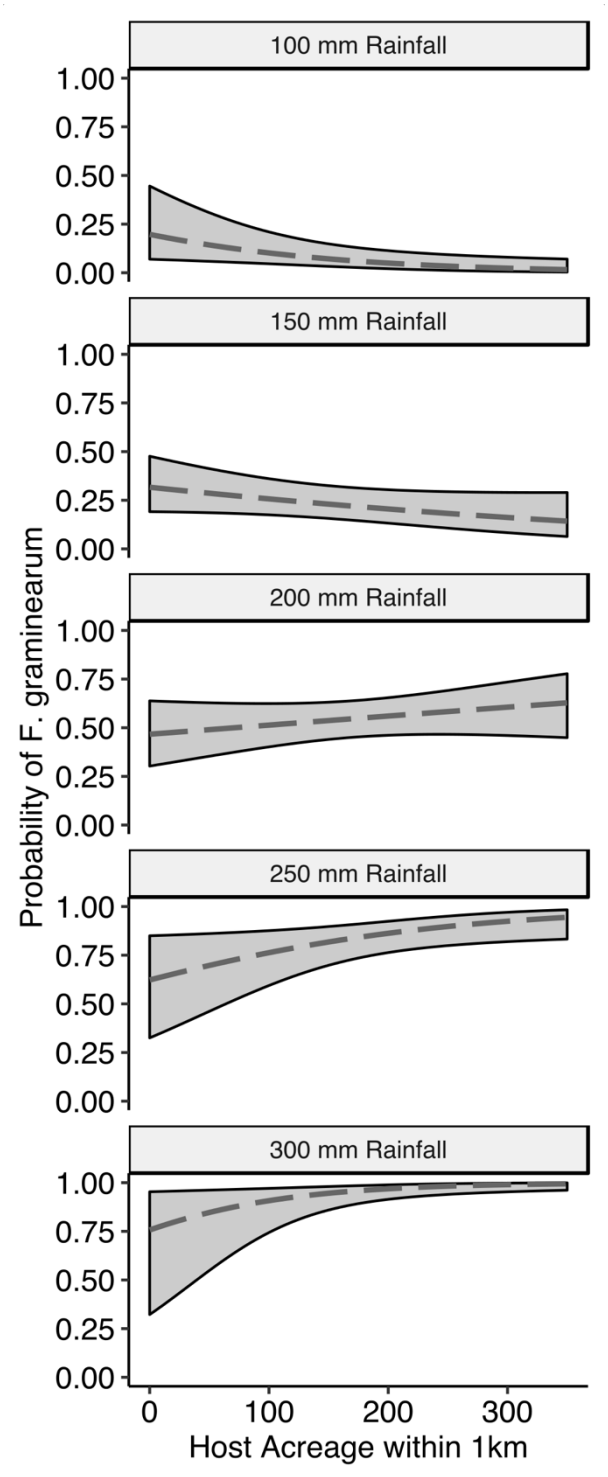
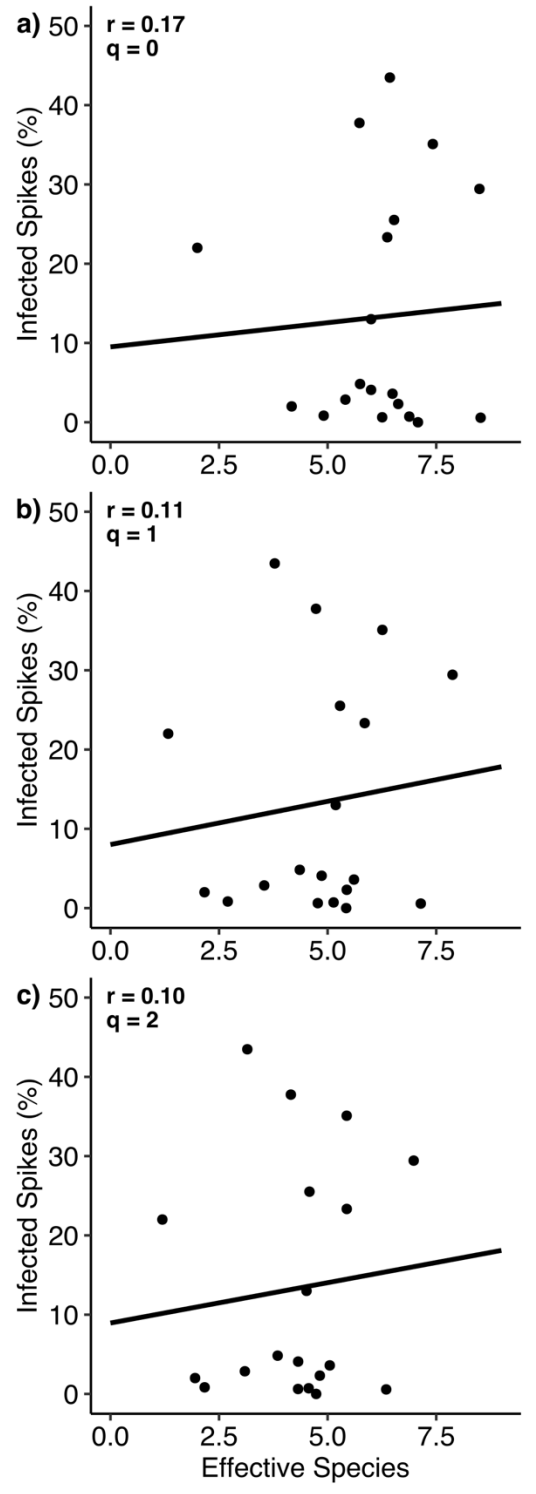

100 mm Rainfall
150 mm Rainfall
200 mm Rainfall
250 mm Rainfall
300 mm Rainfall
Probability of F. graminearum
1.00
0.75
0.50
0.25
0.00
0
100
200
300
Host Acreage within 1km

Figure 1.6. Host species diversity was not associated with a decline in pathogen incidence as predicted by a dilution hypothesis. The correlation of *F. graminearum* incidence and effective species number was low and insignificant. Effective species numbers, or Hill numbers (qD), were calculated on orders a) q=0, b) q=1, and c) q=2, which vary in the weight given to rare species.


a)
r = 0.17
q = 0
b)
r = 0.11
q = 1
c)
r = 0.10
q = 2
Infected Spikes (%)
0
10
20
30
40
50
0.0
2.5
5.0
7.5
Effective Species

The incidence of this pathogen in grass spikes was positively correlated with cumulative host density and rainfall. Agricultural and non-agricultural host density were both implicated as supporting *F. graminearum* presence, which does not suggest one-sided spillover events drive infection in either group of hosts. The dispersal of *F. graminearum* is favored by high rainfall and may lead to shifts in *Fusarium* spp. communities inhabiting non-cultivated grasses. Grass host diversity did not influence the incidence of the pathogen, providing no evidence of a host diversity driven dilution effect.

On average, the rate of pathogen incidence recorded here agrees with past observations about *F. graminearum* colonization of grasses, with estimates ranging from 15-30%[31–34]. We observed high incidence in wild grass inflorescences only under conditions similar to those resulting in disease epidemics in crops. The weather in 2015 and 2017 was conducive to infection, while a severe drought in 2016 likely limited disease pressure because both propagule dispersal and the infection process require moisture[35,36]. The low rates of disease in 2016 also may have reduced pathogen colonization of plant stem tissue, resulting in the low recovery rates from overwintered stems in 2017.

An increase in corn residues left in agricultural fields and the adoption of reduced tillage agriculture has been connected to the re-emergence of Fusarium head blight as a disease of concern in crop plants[37]. Previous observational evidence about pathogen incidence in grasses generated our hypothesis that spillover occurs from crops to non-cultivated grasses. Inch and Gilbert (2003) found no *F. graminearum* when sampling wild grasses from a single urban environment and suggested this could be a result of

distance from agricultural production. Turkington (2011), sampling senesced grass stems, found only those in a corn producing region contained *F. graminearum*. Crop to grass spillover would have been supported by low pathogen incidence in a region with less agricultural production, which we observed, and lower incidence in non-agricultural sites compared to agricultural sites, which was not observed. Spillover occurrence would also have been supported by a strong correlation between disease incidence and agricultural host acreage. Our measures of host density, limited to within 1 km of sample sites, did not support that conclusion. Pathogen incidence appeared driven by the presence of both classes of host, whose combined acreage produced a marginally better explanatory model of incidence than either group alone. While pathogen movement is most frequently examined in the context of non-cultivated hosts contributing to disease in cultivated communities[38,39], we found the density of both crops and non-cultivated hosts drive disease in grasses. Determining when local conditions sufficiently predict disease risk is important[40], and while local context can be a stronger driver of disease than regional context in some cases[10], this study found local land use less relevant than regional host density when predicting pathogen incidence.

No evidence of a dilution or amplification effect was seen in our study. The dilution of disease by increasing host diversity is predicated on having sufficient variation in host competence and the abundance of susceptible hosts decreasing with increased host diversity[13]. Estimated species numbers varied across sites, but increasing diversity was not associated with a change in pathogen incidence. While we observed a wide range of pathogen recovery rates from different grass species, those most well-

represented in our study showed similar levels of disease incidence, indicating little variation in host competency among these species. When sampled from the margins of agricultural fields not treated with fungicide, grasses had a similar rate of infection to cereal crops found at the same site. For example, cereal rye at one location had less than 1 % incidence of *F. graminearum* and grasses from this location had an incidence of 3.6 %. Spring malting barley at another site had no detectable head blight caused by *F. graminearum* while the incidence in grass was 1.9 %. Winter wheat planted in small variety trial plots, including susceptible and moderately resistant varieties, had 5-(12)-23 % incidence, while the average for all grasses collected there was 12.4 %. Experimental comparisons of host competency are lacking, though our observations suggest many hosts are equally competent. The recovery of *F. graminearum* from overwintered grass stem debris revealed levels of pathogen survival that indicate persistence in non-cultivated host communities is possible, further suggesting these grasses are capable of driving infection cycles. Because of this, pathogen movement between grasses and crops may readily occur in both directions.

This study also presents evidence that regional host density and rainfall have an impact on grass dwelling microbial communities. The prevalence of *F. graminearum* in *Fusarium* spp. communities increased along with host density and rainfall. This was the case in both agricultural environments as well as in conserved, natural environments. *Fusarium graminearum* is unique among its congeners for its ability to disperse long distances via airborne spores. All *Fusarium* spp. are able to generate asexual propagules that are wind or splash-dispersed over short distances[41], but *F. graminearum* sexual spores are dispersed on a kilometer scale[22,42]. Rainfall is crucial

to the production and dispersal of these spores as well as to eventual host infection. The benefit *F. graminearum* receives in high rainfall environments is unlikely to extend to other *Fusarium* spp., explaining the corresponding change in community composition. While the incidence of non-*graminearum* species did not decline with increased *F. graminearum* incidence, there could be impacts on species diversity or community structure over time. A more detailed study of changes in these communities is warranted because *Fusarium* spp. are responsible for many crop diseases and the production of toxins in agricultural commodities. They may also have variable interactions with wild hosts that lead to changes in natural plant communities.

The combined findings of this study have implications for the preservation of microbial biodiversity and the management of natural grasslands as well as crop fields. Situated in a region of moderate agricultural production, the 10,000-acre wildlife preserve sampled in all three years had *F. graminearum* incidence in grasses and prevalence in *Fusarium* communities comparable to that of grasses found in nearby agricultural fields. *Fusarium graminearum* can infect the roots and seedlings of some hosts, and another fungal seed pathogen, *Pyrenophora semeniperda*, has been shown to alter host community composition in grasses[7]. A related species, *Fusarium palustre*, is implicated in the dieback of native phragmites and shapes community interactions that include an organism from a higher trophic level[43]. Changes occurring in microbial communities due to increased host density, such as that resulting from agricultural expansion, deserve further study, particularly when any crop pathogens involved have broad host ranges and can disperse over medium to long distances.

We recommend further use of *Fusarium graminearum* as a model for research on

the ecology of plant pathogens. The pathogen's global distribution provides a number of geographically distinct regions with complex host environments that could be studied in parallel, and the pathogen life cycle, including pathogenic and saprophytic phases, is representative of many other fungal species. In addition to being ecologically attractive, the system is tractable. *Fusarium graminearum* is readily cultured in a laboratory setting and there are considerable genetic resources available.

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CHAPTER 2

STRUCTURE AND DIVERSITY OF FUSARIUM COMMUNITIES INHABITING NON-CULTIVATED GRASS INFLORESCENCES IN NEW YORK

ABSTRACT

The structure and diversity of grass spike-inhabiting *Fusarium* communities are not well understood. Fifteen common, non-cultivated grasses were surveyed across two years, regions, and land uses for spike-dwelling *Fusaria*. Eleven fungal species were identified from 857 isolates, including two not recorded previously in New York state or in grass hosts. Species diversity and community structure varied by year and region. Land use and host community did not influence *Fusarium* communities, and no species-specific grass-*Fusarium* associations were detected. *Fusarium* communities were divided into two categories, those dominated by *F. graminearum* and those dominated by *F. sporotrichioides*, that differed in species composition. The community formation process is relevant to disease prediction and toxin monitoring in agricultural systems as well as land management practices at the intersection of agricultural and natural spaces.

INTRODUCTION

Fusarium is a cosmopolitan genus containing many plant pathogen species with significant economic impacts[1]. A number of *Fusarium* spp. have wide host ranges that include non-cultivated grasses[2]. While previous surveys have recorded the incidence of various *Fusarium* spp. in wild grasses[3–6] none have explicitly considered the

influence of different environments or host plants on community composition. Because these fungal pathogens often afflict staple crops, like wheat and maize, and contaminate grain with diverse mycotoxins[7], the factors driving species diversity and structuring communities have relevance to disease prediction and monitoring in agricultural systems. Grasses growing in close proximity to crops may serve as pathogen reservoirs, contributing disease-inciting propagules and providing opportunities for survival between cropping cycles. Understanding the ecology of multi-host pathogens may also lead to land management practices that benefit natural host plants where agricultural and non-agricultural environments meet. The spillover of plant pathogens from one host to another can result in changes in host species abundance[8]. Multiple grass inhabiting fusaria are capable of causing seedling blights and root rots in crops, and if *Fusarium* communities change as a result of proximity to agricultural production, there may be an impact on natural host communities. Understanding grass-*Fusarium* community dynamics requires understanding drivers of species diversity and community structure. In particular, the role of environment and hosts in shaping wild grass spike-inhabiting *Fusarium* communities are of interest because both are known to be important in agricultural contexts[9–11].

In this study, we leveraged the presence of diverse host communities found across New York (i) to compare *Fusarium* species diversity across regional and local environments, (ii) to identify factors structuring these communities, (iii) and to relate host communities to pathogen communities. Species diversity was expected to be highest in diverse plant communities remote from agricultural production, whereas proximity to agricultural production was expected to cause a decrease in species

diversity and changes to community structure.

MATERIALS AND METHODS

Fungal cultures and species identification

The isolates used in this study were recovered in the course of a field survey recording *Fusarium graminearum* incidence in wild grasses (Fulcher, in review). Briefly, non-cultivated grass spikes (Table 2.1) were collected in June 2016 and 2017 from 19 field sites in New York (Figure 1.1). Sampling was performed in two regions, Central and Northeastern New York, that differed in host density and level of agricultural production. Within each region, both agricultural fields and unmanaged, natural spaces were sampled. Only the Central region was included in 2016, while both regions were included in 2017. Plant tissue was taken from 1 m^2 quadrats laid on transects following the margins of crop fields or placed at random in natural grass communities. Grass species richness was also recorded in quadrats at the time of sampling. Quadrats were separated by 10 m, and sites were no closer than 1 km from one another.

Fusarium spp. colonies were recovered from surface sterilized host tissue, and only a single culture was saved from each grass spike. Single-spore derived isolates were stored as conidial suspensions at -80°C until further use. *Fusarium graminearum*, the only homothallic member of the genus, was identified morphologically based on the production of perithecia in culture[12]. Taxonomic placement was confirmed for a subsample of these isolates using the molecular methods detailed below.

All non-*graminearum* isolates were grown on potato dextrose agar for one week under 12 hr fluorescent light cycles at room temperature. Mycelia were scraped from the agar's surface and placed into 2 ml microcentrifuge tubes with 1 g of garnet beads. Tubes were frozen at -20°C and tissue was ground using a VortexGenie2 (Scientific Industries, Bohemia NY). DNA extraction proceeded using a commercial kit and manufacturer instructions (DNeasy Plant Mini Kit, QIAGEN, Hilden, Germany). Molecular species identification was based on partial sequences of either the translation elongation factor 1-alpha (*TEF-1α*) or RNA polymerase II subunit (*RPB2*) genes[4,13]. While a fragment of either gene is able to resolve *Fusarium* to the species level, the *RPB2* locus was used for the majority of samples because PCR amplification was more consistent. Amplified DNA was visualized with gel electrophoresis, cleaned with a commercial silica spin column kit (QIAquick PCR Purification Kit, QIAGEN, Hilden, Germany; Monarch PCR & DNA Cleanup Kit, New England Biolabs, Ipswich, MA), and submitted to the Cornell Biotechnology Resource Center for sanger sequencing (ABI 3730xl, Applied Biosystems, Foster City, CA). Sequences were managed in Geneious Prime version 2019.0.4 (Biomatters, Auckland, New Zealand), trimmed for quality, and compared to existing NCBI accessions using BLAST[14]. Species identification was considered positive when sequence homology was ≥ 98 % to a single species.

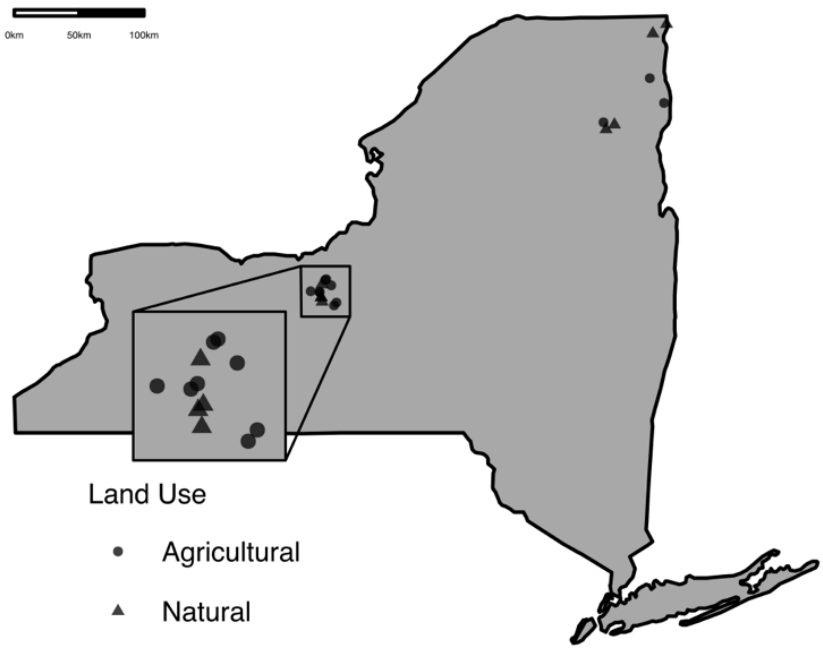

Figure 2.1. Grass inflorescences were sampled from 19 locations over two years. Field sites were divided between two regions of New York and two land-use categories: agricultural and natural.

Statistical analyses

All statistical analyses were performed in RStudio version 1.1.453[15]. Individual *Fusarium* species incidence was analyzed with a multinomial regression model using the 'nnet' package[16]. A full model was built using year, region, land use, site, and grass host as predictors. Automated, stepwise model selection was performed to minimize Akaike information criterion (AIC)[16,17]. The final model, with the lowest AIC, was subjected to analysis of variance to identify the variables that most effectively explained the data, and these were included in further analyses. The probability of each species occurring given region and land use was estimated with 95% confidence intervals using the 'emmeans' package[18].

Alpha diversity of *Fusarium* spp. was measured using Hill numbers, or effective species numbers[19]. Rarefaction and estimation of effective species numbers at orders 0, 1, and 2 was performed using the 'iNEXT' package[20]. Following Shapiro-Wilk tests for normal distributions[21], mean *Fusarium* species numbers were contrasted between years, regions, and land uses with linear models and analysis of variance. Beta diversity was measured and analyzed using the 'vegan' package[22]. Community dissimilarity, using Bray-Curtis distances, was contrasted by year, region and land use with a permutation analysis of variance. Dispersion within each group was assessed with analysis of variance to ensure any PERMANOVA significance was the result of differences in mean not dispersion[23]. Community dissimilarity was then visualized in nonmetric multi-dimensional scaling plots using k=2 dimensions.

Fusarium community structure was described with a principle components analysis of species abundance data, a heatmap, and a Mantel test (10,000 random

permutations) comparing community dissimilarity and physical distance matrices. The diversity and structure of grass communities were also examined using the above methods. The relationship between grass and *Fusarium* species was assessed by correlating their effective species numbers with a linear model and their community dissimilarities with a partial-Mantel test accounting for spatial autocorrelation. Preferential associations between grasses and *Fusarium* species were assessed with a permutation test using 10,000 simulations to generate a X^2 null distribution.

RESULTS

Species identification

There were 51 isolates discarded after DNA extraction, PCR amplification or sequencing failed. In total, 857 *Fusarium* isolates were identified as belonging to 11 species (Table 2.2). Partial gene sequences were deposited to NCBI GenBank. Year, region, and land use were retained in a multinomial model of species incidence (Table 2.3). Sample site and grass host identity were discarded during the model selection step because they lacked power to explain *Fusarium* species occurrence. The differences in the probability of each species given region and land use is shown in Figure 2.2.

Alpha and beta diversity of Fusarium *communities*

Effective species number (qD) for order 0 ranged from 1.34 to 3.00, with a mean of 2.12. Increasing to orders 1 and 2 resulted in slightly lower estimates of qD (Figure 2.3). Year and region had significant effects on effective species number at all three

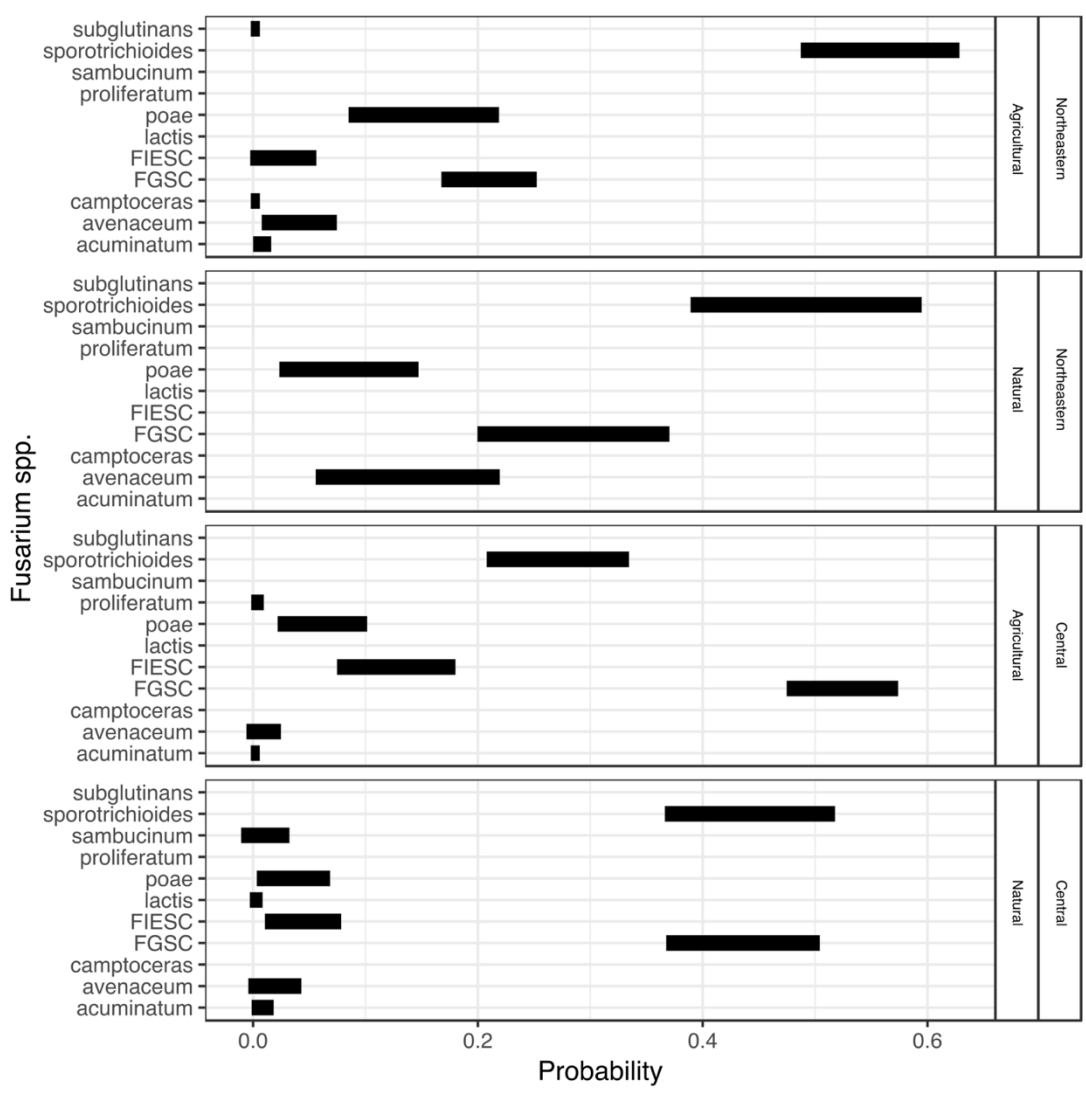

Figure 2.2. The probabiltiy of a given *Fusarium* species occuring in a region and land use. Hortizontal bars represent 95% confidence intervals from multinomial logistic regression estimates.

Table 2.1. Grass hosts examined, and number of isolates collected

Grass species	Samples (n)
Alopecurus arundinaceus	1
Brachypodium sylvaticum	1
Bromus commutatus	19
Bromus inermis	118
Bromus secalinus	2
Dactylis glomerata	232
Elymus repens	114
Festuca spp.	88
Hordeum jubatum	3
Lolium perenne	3
Panicum spp.	5
Phalaris arundinacea	134
Phleum pratense	101
Poa annua	35
Typha latifolia	1

Table 2.2. *Fusarium* species identified

Fusarium species	Isolates (n)
acuminatum	8
avenaceum	25
camptoceras	1
FGSC [a]	458
FIESC [b]	31
lactis	1
poae	41
proliferatum	2
sambucinum	1
sporotrichioides	288
subglutinans	1
Total	857

a. *Fusarium graminearum* species complex (FGSC) or *Fusarium graminearum sensu lato*
b. *Fusarium incarnatum-equiseti* species complex (FIESC)

Table 2.3. Multinomial regression output for *Fusarium* species occurrence.

Predictor	X^2	Degrees of freedom	*P*-value
Year	167.059	10	**<0.001**
Region	240.270	10	**<0.001**
Land use	29.205	10	**0.001**

orders (Table 2.4). Natural sites in Northeastern New York had the highest species diversity, while agricultural sites in Central New York had the lowest. Community dissimilarity was associated with differences between year and region, but not land use (Table 2.5). The nMDS plots showed moderate differentiation based on these categories (Figure 2.4).

Fusarium *Community structure*

The principle components analysis showed nearly all variation in *Fusarium* populations was attributable to the relative abundance of *F. graminearum* and *sporotrichioides*, the two most frequently recovered species (Figure 2.5). The communities found in Central New York during 2016 grouped together with the communities found in Northeastern New York during 2017, and these two groups were associated with the two most prevalent species (Figure 2.6). No spatial correlation in community dissimilarity was detected with a Mantel test (r = 0.106, P = 0.117).

Relating Fusarium *and grass communities*

The effective species estimates for grass and *Fusarium* communities were not correlated (r = -0.13, P = 0.599 for qD of order 0). Community dissimilarity was not correlated according to a partial Mantel test (r = 0.56, P = 0.281). A permutation X^2 test failed to find differential association between *Fusarium* species and grass species (P = 0.061).

Table 2.4. Analysis of variance output for models of effective species numbers.

Order of Hill Number / Predictor	Sum of squares	Degrees of freedom	*F* statistic	*P*-value	Adjusted R^2
0					0.441
Year	0.950	1	8.286	**0.011**	
Land Use	0.275	1	2.397	0.142	
Region	1.439	1	12.554	**0.003**	
Residuals	1.719	15			
1					0.421
Year	0.804	1	7.129	**0.017**	
Land Use	0.229	1	2.032	0.174	
Region	1.369	1	12.133	**0.003**	
Residuals	1.692	15			
2					0.400
Year	0.626	1	6.092	**0.026**	
Land Use	0.189	1	1.839	0.195	
Region	1.181	1	11.499	**0.004**	
Residuals	1.541	15			

Table 2.5. Permutation analysis of variance output for community dissimilarity.

Predictor	Sum of squares	Degrees of freedom	R^2	F statistic	*P*-value
Year	0.757	1	0.167	4.200	**0.004**
Land Use	0.247	1	0.054	1.368	0.236
Region	0.824	1	0.182	4.575	**0.003**
Residuals	2.703	15	0.597		
Total	4.531	18	1		

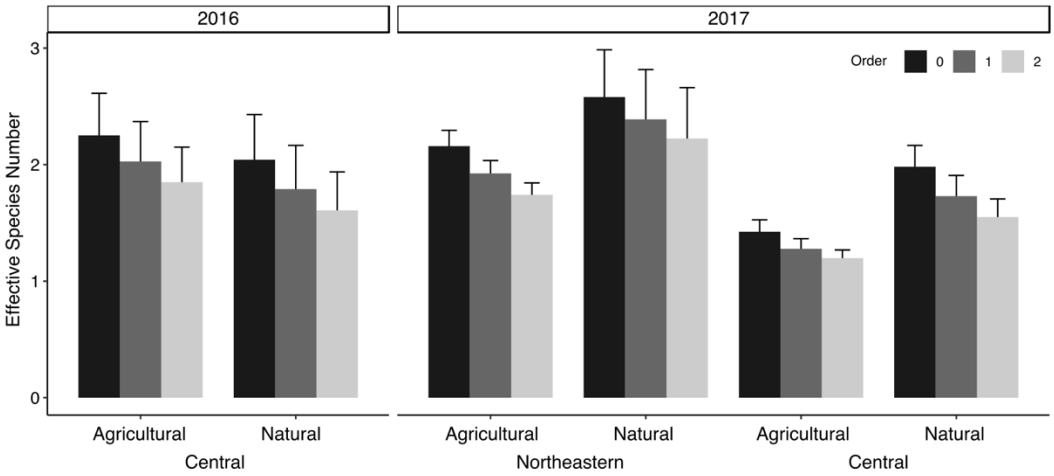

Figure 2.3. Effective species, or Hill numbers (qD), were obtained by rarifying species abundance and compared across year, region, and land use. *Fusarium* species diversity differed significantly between years and regions but not between land uses.

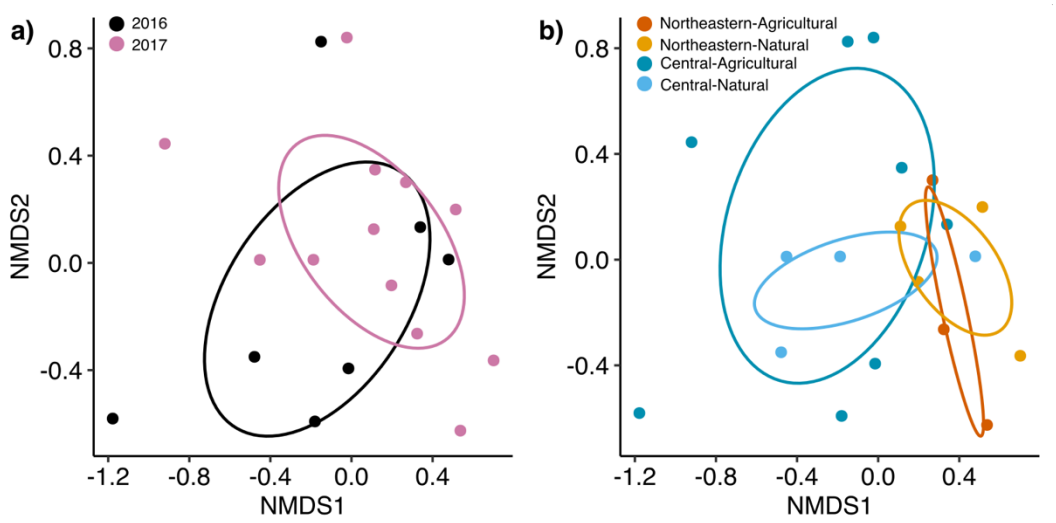

Figure 2.4. Community dissimilarity visualized with a non-metric multidimensional scaling plot. Ellipses mark one standard deviation around group means. According to a PERMANOVA of Bray-Curtis dissimilarities, the effect of year and region were significant. a. Sites color coded by year. b. Sites color coded by region and land use (Stress = 0.14).

DISCUSSION

This is the first study to catalogue *Fusarium* species diversity in non-cultivated grasses in New York, including those found in the Adirondack Mountain wilderness and a 10,000-acre national wildlife refuge. The geographic or host ranges of several uncommon *Fusarium* spp. were expanded by this study. Our findings also show communities are likely structured by annual and regional factors, rather than local environment or host community. This contrasts with findings for soil-borne *Fusarium* communities, which vary significantly between hosts[24], and does not support our hypothesis of positive correlation between fungal pathogen diversity and grass diversity, which has been shown for foliar fungal pathogens[25]. Our results may help predict pathogen population diversity and by extension toxin potential in different environments, specifically those with varying levels of rainfall and host density, which is potentially useful for monitoring disease and toxin content in crops. The findings also suggest altered land management practices at the intersection of crop and natural host communities, like adding buffer strips or restricting crop production, would have little influence on pathogen populations in nearby grasses.

The most common *Fusarium* species, such as *F. graminearum* and *sporotrichioides*, were well distributed across hosts, evidenced by a lack of any particular host-*Fusarium* associations. Most of the *Fusarium* species recorded here have previously been reported from numerous hosts, particularly plants in the true grass family, Poaceae[2]. However, several singleton isolates were recovered that

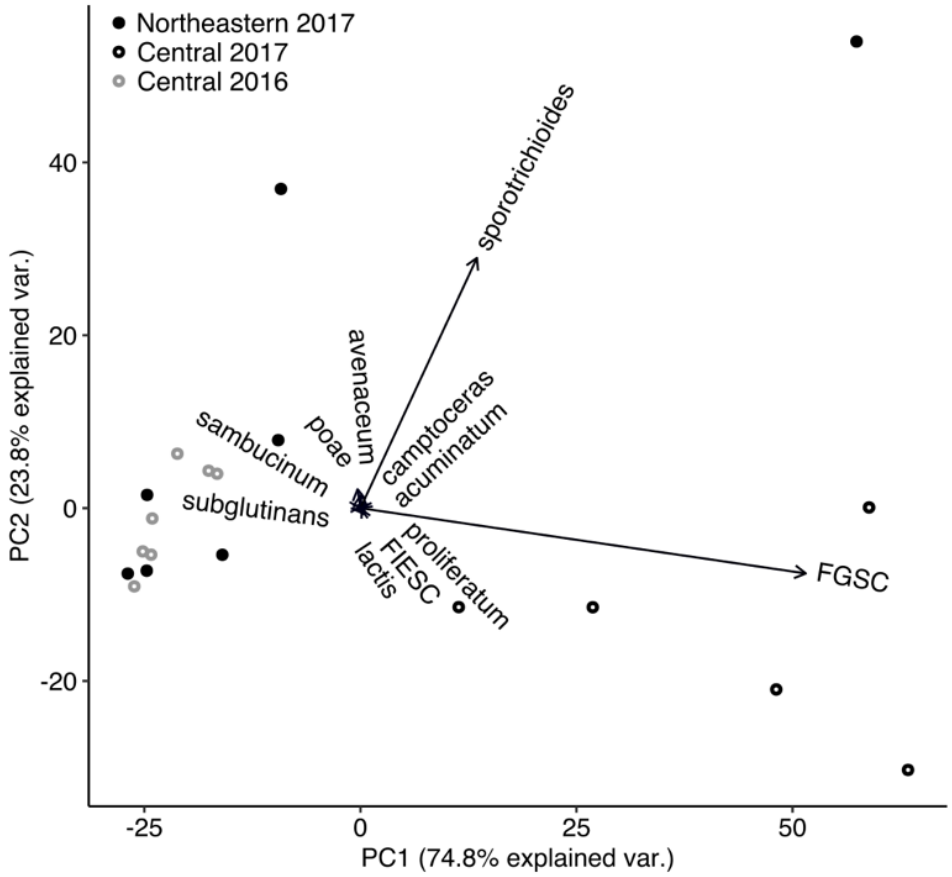

Figure 2.5. Principle components analysis of *Fusarium* species abundance at 19 sites. Community differences were attributable to large variation in the occurrence of two *Fusarium* species, *sporotrichioides* and *graminearum*. Central New York sites in 2016 experienced a drought, which led to communities more similar to those in low host density Northeastern New York during 2017 than to nearby communities in 2017 during a year of average rainfall.

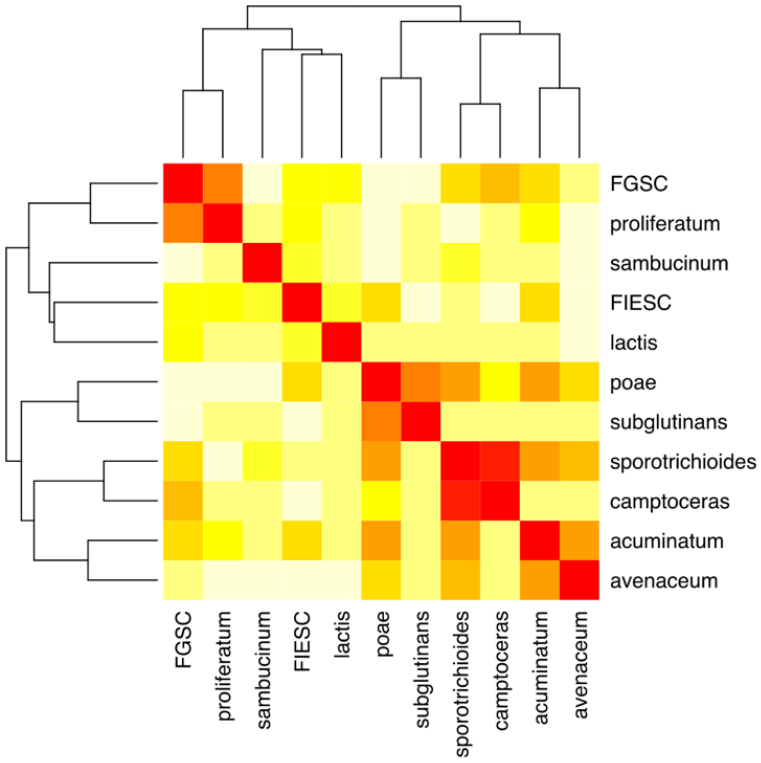

Figure 2.6. A heatmap shows loose co-occurrence of species and defines two communities, corresponding to those dominated by either *F. graminearum* (FGSC) or *F. sporotrichioides*.

represent the first instances of certain species occurring in New York or in association with a grass host. *Fusarium camptoceras* is most often found in association with rotting tropical fruits[26,27] but was once recorded from shattered wild rice (*Zizania palustris*) in Minnesota[28]. This is the first recorded occurrence in New York and from smooth brome (*Bromus inermis*). *Fusarium lactis* causes internal fruit rots in sweet pepper and fig[29,30]. This isolate is the first from any grass host (*Phalaris arundinacea*) and the first found in New York.

Annual and regional environments were more important to *Fusarium* species diversity than local environments or grass communities. It is likely that differences in rainfall and host density are important factors. Work focusing on *F. graminearum* shows these are strong predictors of pathogen incidence in grasses (Fulcher, in-review), and these factors were already recognized as driving crop disease epidemics [31,32]. The occurrence of various *Fusarium* species in crop hosts has also been related to regional differences in host community composition[33], and the regions sampled in this study did vary in both host density and the ratio of agricultural to non-agricultural hosts.

Within *Fusarium* communities, two species were dominant, and different species co-occurred with them. Alongside *F. graminearum*, a number of less common or saprophytes were found. Environments more favorable to *F. sporotrichioides* also had a greater incidence of other crop pathogens, like *F. avenaceum* and *poae*. These differences in composition could be linked to individual life-histories. Because *F. graminearum* is the only homothallic member of the genus, and the only species observed to reproduce sexually under natural conditions, it is more readily spread via

airborne ascospores than the other species only capable of producing asexual conidia. Conidia are splash or wind dispersed short distances, while ascospores are capable of kilometer scale movement[34,35].

It was somewhat surprising to find no spatial correlation in community dissimilarity matrices after seeing strong differentiation of communities based on regional location. The Mantel test used to assess spatial structure has been the subject of criticism because of its low power, among other things[36]. The Mantel test is unable to account for certain co-factors, in this case annual variation, which might mask the spatial variation in a rough analysis but are easily incorporated into linear models and related analyses.

To summarize, this study recorded significant regional and annual variation in *Fusarium* communities and species diversity that was not influenced by local land use or individual host species. Two distinct communities, corresponding to a high and low incidence of the major agricultural pathogen *F. graminearum*, were detected and contained different species capable of producing distinct mycotoxin cocktails. Understanding when these different communities form will aid crop disease management and toxin monitoring efforts. The potential impact of these different communities on grasses in natural spaces should be further investigated, especially at the level of individual plant-fungus interactions.

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CHAPTER 3

POPULATION GENETICS OF FUSARIUM GRAMINEARUM AT THE INTERFACE OF WHEAT AND NON-CULTIVATED GRASS COMMUNITIES IN NEW YORK

ABSTRACT

Fusarium graminearum is primarily understood as an agricultural pathogen affecting staple crops like wheat and maize, but the pathogen's host range includes diverse, non-cultivated grasses ubiquitous across agricultural and natural environments. These hosts may serve as reservoirs of genetic diversity and sources of disease inciting inoculum. The diversity and structure of *F. graminearum* populations found at the intersection of wheat and grass communities was described in New York using isolates collected from three sources (wheat spikes, grass spikes, and overwintered grass stems), two regions (high and low agricultural production), and two land uses (agricultural and natural). The production of toxin variants, or chemotypes, was predicted from two loci (n = 909), and multilocus genotypes (MLGs) were determined using eight microsatellite loci (n = 734). Chemotype frequencies were similar in wheat and grass derived isolates, genotypic and allelic diversity were comparably high ($\lambda = 0.99$; $\lambda \geq 0.78$ respectively), and differentiation between hosts was low ($G'_{st} = 0.029$). Duplicate MLGs were rare (54/734), even in samples collected from a single square meter ($\overline{x}$ = 4% chance of duplicates), though could be found in multiple hosts (MLGs = 19, n = 42), environments (MLGs = 18, n = 38), regions (n = 2) and years (n = 3). Chemotype frequencies differed between region and land use ($P < 0.001$), and removal from agricultural production was associated with higher

proportions of 3-ADON and NX-2 genotypes. Admixture between chemotype defined populations, which likely correspond to three previously described sympatric North American populations, was detected, and the proportion of admixed individuals was highest in a region with remote host communities and little agricultural production. Non-agricultural environments may favor toxin production profiles different from those found in wheat fields and provide an opportunity for recombination between *F. graminearum* populations. A lack of structural barriers suggests gene flow is uninhibited between wheat and grass communities, and the recovery of putative clones from multiple hosts and environments provides limited evidence that non-cultivated grasses are a source of local and regional inoculum causing disease in crops.

INTRODUCTION

Fusarium graminearum Schwabe is best known for causing Fusarium head blight of small grains and maize seedling blight but has also been recovered from dozens of non-cultivated plants. Able to inhabit plants from 26 families, *F. graminearum* is most often associated with species in the true grass family, Poaceae[1]. Non-cultivated grasses are a ubiquitous feature of both agricultural and unmanaged landscapes, often found in the margins of crop fields, occupying fallow land, or dominating natural spaces in close proximity to agricultural production[2]. Despite their widespread distribution and the high frequency at which *F. graminearum* colonization has been reported[3–5], the role these hosts play in pathogen evolution or crop disease epidemics is unknown. Understanding pathogen populations at the intersection of crop and non-crop host communities may help determine the importance of these hosts.

Fusarium graminearum sensu lato is a globally distributed species complex[6], and *F. gramimearum* sensu stricto is the dominant species in North America[7]. Three sympatric North American populations exist with overlapping distributions and can be differentiated largely by their production of toxin variants, which are commonly referred to as chemotypes[8]. The chemotypes corresponding to North American populations are the 15- and 3-acetylated forms of deoxynivalenol (15ADON, 3ADON) and the more recently described toxin NX-2[9]. The NX-2 chemotype, linked to the NA-3 population of *F. graminearum*, has been found with unusually high frequency in Northeastern New York[10]. The distribution of *Fusarium graminearum* chemotypes and populations has been the subject of many studies[6,11–18], though the role of diverse, non-cultivated hosts in the maintenance of chemotype diversity and structuring of populations has not been investigated.

Pathogen overwintering in crop debris, primarily corn stubble, is implicated as the most significant source of disease-causing inoculum[19]. Pathogen survival on infested crop residues results in both local and regional disease pressure[20], but the importance of local inoculum production on the tissue of segetal grasses and of regional inoculum resulting from long-term pathogen build-up in unmanaged grasslands where host tissue remains in situ has not been explored. Direct tracking of propagule movement is possible though challenging[21,22], but drawing inferences about pathogen movement from population genetics is another approach to studying the connection between grass debris and disease incidence in crops.

This study was conducted to determine (i) whether grasses serve as reservoirs of *F. graminearum* genetic diversity and (ii) how New York populations are structured at

the intersection of host communities. If grasses are reservoirs of *F. graminearum* diversity, we would expect to find higher genotypic or allelic diversity in grass spikes than in wheat spikes. By extension, we might find higher genetic diversity in non-agricultural field sites or environments than in a wheat field or a region containing intensive agricultural production. While many factors can influence population structure, this study was primarily concerned with the influence of host, physical distance, and membership in previously defined North American populations. Because *F. graminearum* is capable of kilometer scale dispersal, it was hypothesized that physical distance would only be associated with differentiation between the most separated isolate sources. This dispersal coupled with the well-mixed nature of aerial populations[23], led to the expectation that grass and crop derived isolates would show signs of gene flow, but that slight population sub-division could occur due to the persistence of local populations surviving between years in accumulated grass debris or crop residues. While sympatric populations can be clearly distinguished from one another, admixture has been recorded[24,25]. The frequency of 3-ADON and NX-2 producing isolates is greater in northern United States and eastern Canada, implicating these regions as the points of radiation for these populations, whether endemic or introduced[26,27]. For this reason, we hypothesized that while population structure would be evident based on chemotypes, this structure would be weaker in a region of New York where all three populations may have coexisted for a longer period of time.

MATERIALS AND METHODS

Cultures

The isolates used in this study were collected during a three-year survey of wild grass spikes, naturally senesced and overwintered wild grass stems, and winter wheat spikes (Fulcher, in-review). During the summers of 2015-17, asymptomatic grasses and symptomatic wheat were collected from 23 field sites in two regions of New York that differ in host density[2] and *F. graminearum* chemotype frequencies[28] (Figure 3.1). Two land uses, agricultural and non-agricultural or natural, were included in the survey. Host tissue was collected from within 1 m^2 quadrats spaced 10 m apart along the grassy margins of wheat fields or along transects randomly placed in fields of wheat or grass. Grass stem debris was collected early in the spring to capture only individuals overwintering in place. The collection of spikes from wheat and 12 common grass species that have flowering phenology similar to winter wheat was timed to capture the pathogen population after the primary infection cycle occurred and before secondary infection was likely to take place (Supplemental Table 3.S1). The isolates chosen for this study (n = 909) were selected to represent these hosts, regions, land uses and sampling sites as equally as possible (Table 3.1). Of these isolates, 150 were included specifically because they were collected within single 1 m^2 quadrats and could be used to observe fine scale population structure.

Genotyping

Cultures were grown for two weeks on potato dextrose agar (PDA) under 12 hr fluorescent light, mycelium was scraped from the surface of PDA, and samples were

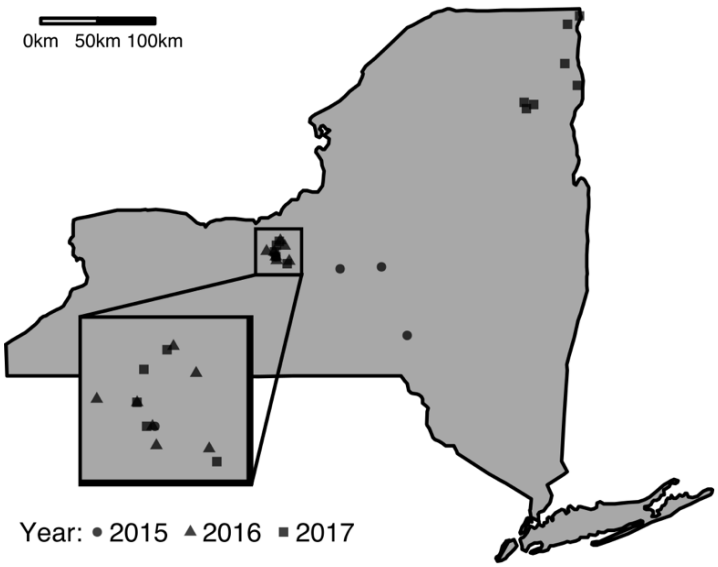

Figure 3.1. Isolates used in this study were collected over three years from 23 field sites situated in two regions of New York.

Table 3.1. Isolate collection by region, land use and host source.

Region	Land Use	Host	Isolates
Northeastern	Agricultural	Grass Debris	18
		Grass Spikes	12
		Wheat	29
	Natural	Grass Debris	4
		Grass Spikes	38
Central	Agricultural	Grass Debris	289
		Grass Spikes	115
		Wheat	134
	Natural	Grass Debris	138
		Grass Spikes	132
Total			909

frozen at -20°C before tissue disruption. DNA extraction was performed using the commercial QIAGEN DNeasy Plant Mini Kit according to the manufacturer's instructions (Holden, Germany). The chemotype of all 909 isolates was predicted using two loci. First, a portion of the *TRI12* gene was amplified and fragment size was visualized with gel electrophoresis to determine whether isolates had a NIV, 15-ADON, or 3-ADON genotype[29]. Following this, isolates with a 3-ADON genotype were used in a *TRI1* PCR-digestion assay to differentiate 'true' 3-ADON chemotypes and NX-2 chemotypes[30].

A subset of 800 isolates was genotyped at eight previously described microsatellite loci (Supplemental Table 3.S2)[31–33]. Fluorescently labelled microsatellite primers (Applied Biosystems G5 dye set) were split evenly between two multiplex reactions. The QIAGEN Multiplex PCR Plus Kit (Holden, Germany) was used, and reaction mixtures were 25 µl with 0.2 µM primer concentrations. The same cycling conditions were used for both reactions: 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 58°C for 1 min 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 10 min. Amplified products were diluted 1:10 in water, and 1 µl of diluted product was mixed with 10 µl of HiDi Formamide and 0.2 µl of GeneScan 500 LIZ size standard (Life Technologies, Woolston, United Kingdom). Fragments were separated on an ABI 3730xl DNA Analyzer at the Cornell Biotechnology Resource Center, and alleles were sized in Geneious Prime version 2019.0.4 (Biomatters, Auckland New Zealand) using the Microsatellite Analysis plugin version 1.4.6.

Data analysis

All analyses were performed in RStudio version 1.1.453[34]. Chemotype frequency was analyzed with a multinomial logistic regression implemented in the 'nnet' package[35] and analysis of variance, using chemotypes as the response variables and a three-way interaction between region, land use, and host source as the predictor. Chemotype probabilities were contrasted with 95% confidence intervals around least-squares means using the 'emmeans' package[36].

Microsatellite data was formatted in GenAlex version 6.503[37,38], and individuals with missing allele data were removed prior to analysis in R with the 'poppr', 'adegenet', and 'mmod' packages[39–41]. A genotype accumulation curve was generated to check the completeness of genotype discovery. Populations were defined based on host source: wheat spikes, grass spikes, or grass debris. Genotype diversity was measured using Shannon's H[42], Stoddart and Taylor's G[43], Simpson's λ[44], and evenness[45]. Allele diversity was measured in mean alleles per locus, Simpson's λ, Nei's gene diversity H_{exp}[46], evenness, and unique alleles. Population structure was first analyzed with fixation or differentiation indices similar to F_{st}[47], including Hedrick's $G`_{st}$, Jost's D, and Meirmans φ_{st}[48–50]. Bruvo's genetic distance[51] was calculated between all pairs of isolates and used to build a minimum spanning network[52]. The genetic distance matrix was also compared to a physical distance matrix to check for spatial correlation using a Mantel test and a null distribution generated with 10,000 random permutations[53]. In order to measure population structure on a finer scale, a subset of the data was used to determine the proportion of duplicate multi-locus genotypes collected from within 1 m^2 sampling quadrats. The probabilities were

determined based on 11 grass debris quadrats from which five or six isolates were recovered and 12 grass spike quadrats from which 5-10 isolates were recovered.

To further examine structure, populations were redefined using predicted chemotypes. Five isolates with a NIV genotype were removed before further analysis because of the small sample size. Pairwise differentiation statistics were calculated. A discriminant analysis of principle components was used to visualize the genetic similarity of isolates and to determine the posterior probability of isolate membership to predefined chemotype populations[54]. An arbitrary threshold of 80% assignment probability was used to define isolates showing admixture.

RESULTS

Chemotype frequencies

The production of 15-ADON was predicted for 679 isolates, 3-ADON for 201 isolates, NX-2 for 24 isolates, and NIV for five isolates. Predicted chemotype frequencies were contrasted between region, land use, and host source. No variation in chemotype frequency was detected between wheat spikes, grass spikes, and grass debris (Table 3.2). The probability of a given chemotype occurring was significantly affected by both region and land use ($P \leq 0.001$). The occurrence of 3-ADON and NX-2 genotypes was greatest in Northeastern New York, where agricultural and natural sites contained similar chemotype frequencies (Figure 3.2). In Central New York, the 15-ADON genotype was most common, but a significant increase in 3-ADON genotypes was detected at natural sites compared to agricultural sites (Supplemental Figure 3.S1).

Table 3.2. Analysis of variance output from a multinomial logistic regression of chemotype frequencies.

Predictor	X^2 Statistic	Degrees of Freedom	*P*-value
Region	62.345	3	**0.001**
Land use	45.416	3	**0.001**
Host	7.514	6	0.276
Region : Land use	6.987	3	0.072
Region : Source	2.49	6	0.870
Land use : Source	5.689	6	0.459
Region : Land use : Source	4.567	6	0.600

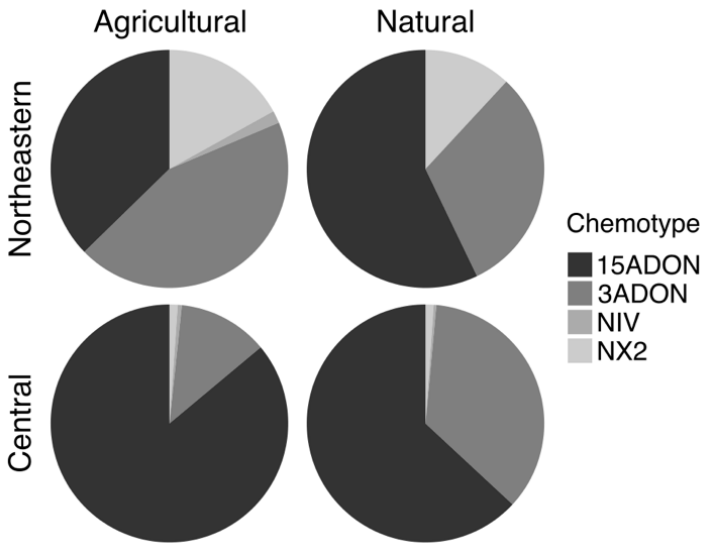

Figure 3.2. Predicted chemotype frequencies varied by land use and region.

Genetic diversity

Microsatellite genotypes were successfully determined for 734 isolates. A genotype accumulation curve indicated almost all genotypes could be detected with seven of the loci chosen (Supplemental Figure 3.S2). Genotypic diversity was high across all isolate sources, and 680 multi-locus genotypes (MLGs) were recorded (Table 3.3). Duplicate MLGs were found in isolates collected from different host sources (n=42, MLGs=19), land uses (n=38, MLGs=18), regions (n=2, MLG=1), and years (n=3, MLG=1). Allele diversity was even across host sources (Table 3.4).

Population structure

Little genetic differentiation was seen between isolates collected from grass spikes, grass debris, and wheat spikes ($G`_{st}$ = 0.029, D=0.017, φ_{st} = 0.015). Plotting the genetic distance between isolates as a minimum spanning network showed no clustering based on host source (Figure 3.3). A slight, significant positive correlation was found between genetic and physical distance (r = 0.03, $P < 0.038$). The chance of recovering duplicate MLGs from within 1 m^2 quadrats ranged between 0-10% for grass debris and 0-20% for grass spikes, with each averaging 4%.

Differentiation between chemotype defined populations was greater than for host sources (Table 3.5), and chemotypes were clearly separated by a DAPC (Figure 3.4). The NX-2 population was less differentiated than the 3- and 15-ADON populations. The posterior probability of isolate assignment to predicted chemotype population is displayed in Figure 3.5. The NX-2 genotype was highly admixed, and for all chemotypes admixture was greater in Northeastern NY than Central NY (Table 3.6).

Table 3.3. Genotypic diversity.

Host	N	MLG	Shannon's H	Stoddart and Taylor's G	Simpson's λ	Evenness
Wheat Spikes	150	148	4.99	146.10	0.99	0.99
Grass Spikes	285	274	5.59	264.57	0.99	0.97
Grass Debris	299	277	5.59	259.13	0.99	0.96
Total	734	680	6.49	632.34	0.99	0.95

Table 3.4. Allelic diversity.

Host	Alleles per locus	λ	H_{exp}	Evenness	Unique alleles
Wheat Spikes	14	0.80	0.80	0.74	13
Grass Spikes	16	0.78	0.78	0.71	13
Grass Debris	17	0.79	0.79	0.72	20
Total	16	0.78	0.78	0.72	--

Table 3.5. Differentiation between predicted chemotypes, Hedrick's G'_{st} (Jost's D).

	15-ADON	3-ADON
3-ADON	0.31(0.26)	
NX-2	0.17(0.14)	0.19(0.15)
Universal Meirmans φst	0.28	

Table 3.6. Admixture rates of chemotype defined populations.

Predicted Chemotype	Region	Admixed proportion†
3-ADON	Central	0.35
	Northeastern	0.53
15-ADON	Central	0.12
	Northeastern	0.19
NX-2	Central	0.67
	Northeastern	0.72

† Having ≤ 80 % assignment probability to the predicted chemotype group.

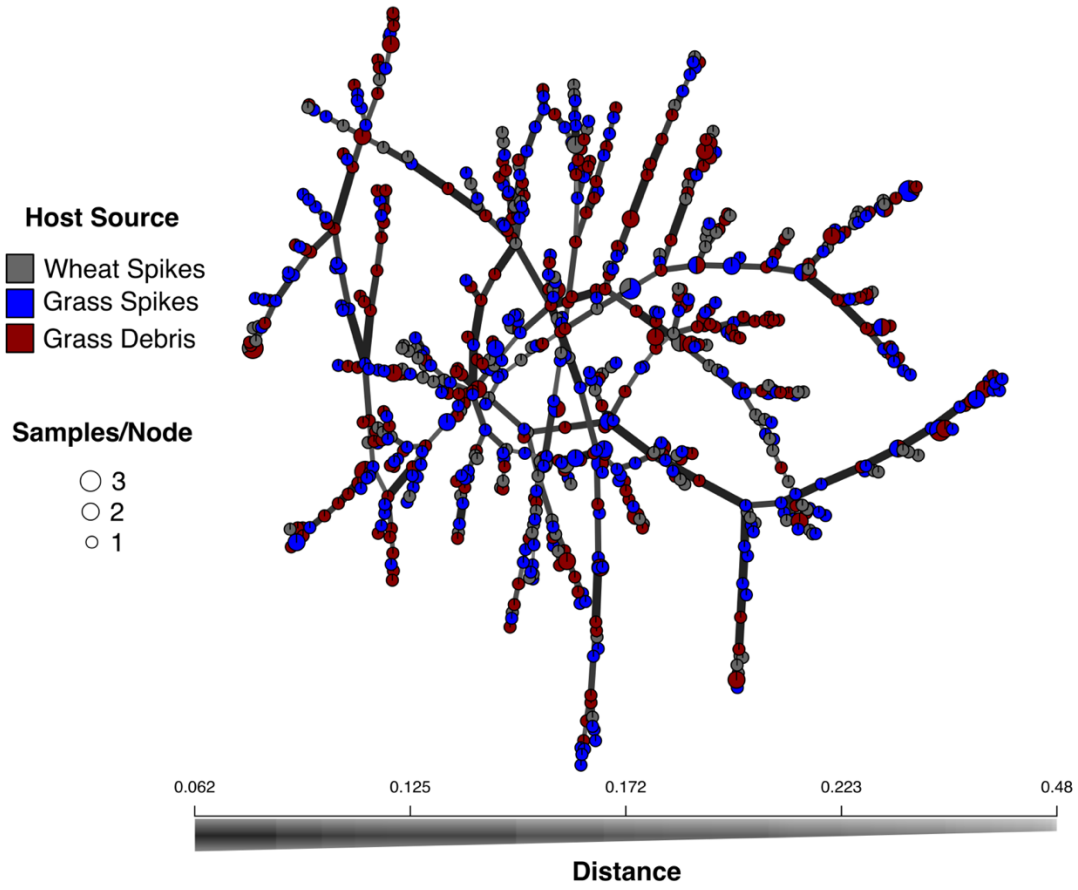

Figure 3.3. A minimum spanning tree using Bruvo's distance between isolates showed no clustering based on host of origin.

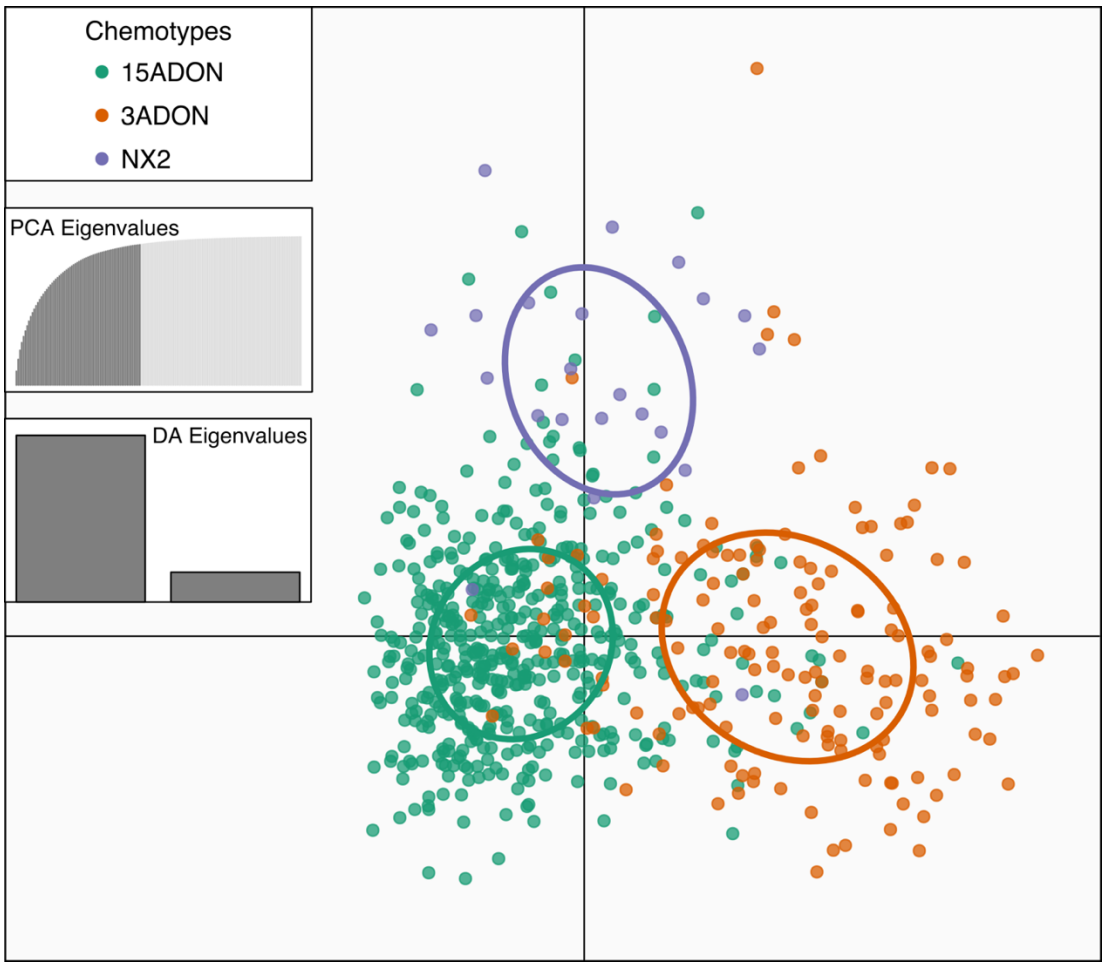

Figure 3.4. A scatterplot from a discriminant analysis of principle components separated isolates into three overlapping groups defined by predicted chemotype.

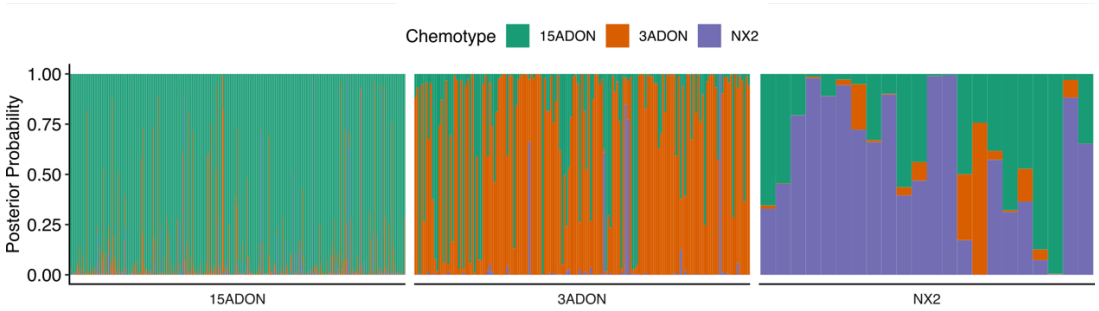

Figure 3.5. The posterior probabilities of isolate assignment to chemotype groups by DAPC indicated significant admixture.

DISCUSSION

This is the first study to consider in-depth the relationship between grass and wheat derived *F. graminearum* isolates. The primary goal of this work was to determine whether grasses serve as reservoirs of *Fusarium graminearum* diversity. Because pathogen diversity was high, with most individuals containing a unique MLG, wheat and grasses contained similar levels of genotypic and allelic diversity. However, the grasses found in non-agricultural sites did harbor a different ratio of chemotypes from both the grasses and wheat found at agricultural sites in Central New York. The grasses found in remote, isolated sites in Northeastern New York also harbored a different mixture of chemotypes, and these isolates showed signs of significant admixture. This is evidence grasses found in non-agricultural sites may support different pathogen populations than are found at agricultural sites, and that hosts found in remote areas could harbor recombined genotypes and serve as a source of novel genotypic diversity.

The genetic diversity recorded in this study was high and comparable to that found in previous studies focused on crop infecting *F. graminearum* populations[55] and in one study containing isolates collected from various non-cultivated hosts[56]. This high genotypic diversity is a result of outcrossing by *F. graminearum*, and the mutation rate associated with some markers, like the microsatellites used at present. Duplicate MLGs were rare even within 1 m^2 patches of grass debris and grass spikes. On this fine scale, little spatial structure was found and multiple MLGs could be recovered from the spikes or stems of individual host plants. Under such a scenario, even small patches of non-cultivated grasses may harbor multiple pathogen genotypes from year

to year or allow unique isolates to recombine.

The balance of *F. graminearum* chemotype frequencies has been the subject of many investigations[6,11–18]. It has been suggested that hosts, environmental factors such as temperature, or the virulence of isolates from different populations are determinants of chemotype distribution. In this study, it was clear that pathogen populations collected from winter wheat, grass spikes, and grass stems occurring in the same field did not differ in chemotype composition. This mirrors the results of an earlier study in New York that compared chemotype frequencies in isolates collected from wheat, corn and aerial populations[28]. The reason for the difference in chemotype frequency between grasses in non-agricultural environments and grasses in agricultural fields separated by only 3-5 km is not clear. The increased 3-ADON frequency in these non-agricultural sites in central New York reflects a similar increase in 3-ADON frequency in Northeastern New York, observed at both agricultural and non-agricultural sites and in all host sources. The similar chemotype distributions in these two areas implicates certain commonalities between their environments, such as lower host density or greater host diversity, as potentially important factors in shaping chemotype distribution. This interpretation is bolstered by previous work associating the relative abundance of different hosts with *F. graminearum* sensu lato species and chemotype distributions[57–59]. A shortcoming of this study was an explicit focus on predominantly cool season grasses. Another explanation for differences in chemotype frequency in natural spaces could be that warm season grasses flowering later in summer are infected with higher levels of 3-ADON producing isolates and contribute these to local cool weather grasses in subsequent years.

The second goal of this study was to assess pathogen population structure. *Fusarium graminearum* populations in New York were not structured by host but, on the basis of predicted chemotype, corresponded to three previously defined North American populations. A weak spatial correlation was found that may relate to the regional difference in population distributions, with higher 15-ADON (NA1) occurrence in Central New York and higher 3-ADON (NA2) and NX-2 (NA3) occurrence in Northeastern New York. The higher levels of admixture observed in this region could be a result of more even chemotype frequencies and prolonged co-occurrence of these populations allowing for more recombination events. Recent work has identified genomic regions under selection or prone to recombination events[60]. Individuals from non-crop hosts may be of particular interest in future genome analysis projects attempting to identify genes with adaptive function. *Fusarium graminearum* infected wild grasses are typically asymptomatic under natural conditions and do not accumulate mycotoxins to the extent observed in crop infection[4], indicating successful colonization is facilitated by yet to be discovered traits. For this reason, the host-fungus relationship between *F. graminearum* and grasses should be further characterized, particularly with respect to the selective pressures that exist in non-agricultural environments and how they contribute to the maintenance of agriculturally relevant pathogen phenotypes, for instance fungicide resistance or toxin production.

Grasses may also be important for their contribution of inoculum to crop disease epidemics. The movement of pathogen propagules between hosts and land uses does not appear inhibited by population structure, and several putative clones were found

across multiple hosts and land uses. Because the number of 'putative clones' identified based on isolates containing identical MLGs can be inflated due to size homoplasy of microsatellite alleles, this finding should be considered with caution[61]. Despite this, the combination of direct and indirect evidence suggests non-cultivated grass hosts are able to contribute inoculum at local and regional scales. The relative importance of this process compared to inoculum arising from agricultural crop residues, like corn stubble, remains to be determined.

Table 3.S1. Grass species sampled and number of isolates used in this study.

Grass species	Number of isolates
Bromus commutatus	5
Bromus inermis	60
Bromus secalinus	1
Dactylis glomerata	66
Elymus canadensis	1
Elymus repens	21
Festuca spp.	17
Hordeum jubatum	4
Panicum spp.	2
Phalaris arundinacea	56
Phleum pratense	31
Poa annua	13

Table 3.S2. Microsatellite markers used in this study.

Microsatellite	Primers	Reference
Multiplex 1		
HK1073	TATGATGCAGCGAATGCAAC	Suga 2004
	TAGAGACCTGGCCCATACCA	
HK913	GCAGGACCTGGATGATGAA	Suga
	ATGTGTGCAGCCATGAGATT	
HK1043	ACAGGCATCCAAGGACATTT	Suga
	GTTTGATGGCGCATTCAAAG	
msFG60	GAGCCATTCACATGTACCCC	Naef 2006
	TCCTCTCGCAAGTGTTGTTG	
Multiplex 2		
HK977	AAACGTAAACGGATCAACGG	Suga
	AGATTCGCAACTTTGTGCTG	
HK917	ATCTCCCAAGCTGGCTAATT	Suga
	AGAACCGGCAAAGTTCGATT	
HK957	TCCGAAGGTAGAAGCGTTGT	Suga
	TCAAGCCCATCTATGCTGTT	
FusSSR19	AGCCGGACATGAGACAAAGTAG	Vogelgsang 2009
	TGTTGTTCCCTCCAGTACTCG	

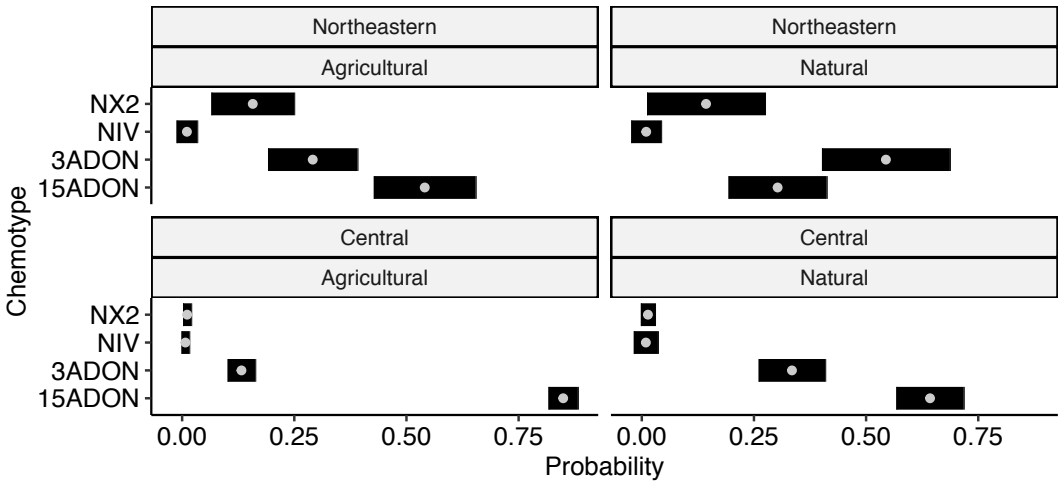

Figure 3.S1. Probability of chemotype occurrence given region and land use. Bars represent 95% confidence intervals around least-squares means estimates from a multinomial regression of chemotype frequencies.

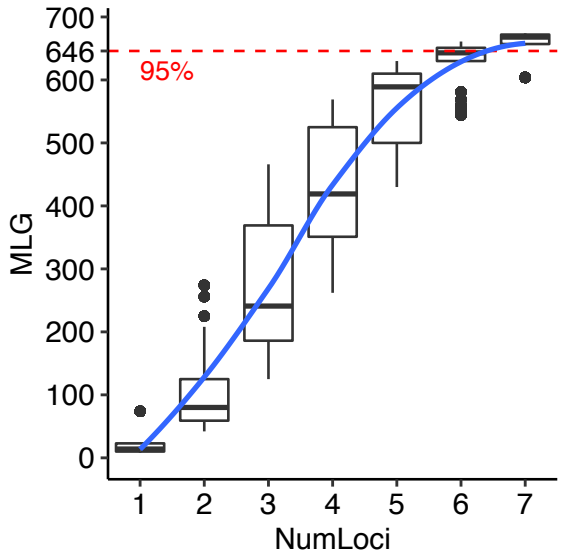

Figure 3.S2. Genotype accumulation curve for microsatellite loci shows near saturation of multilocus genotypes after seven markers are included.

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CHAPTER 4

VARIABLE INTERACTIONS BETWEEN NON-CEREAL GRASSES AND FUSARIUM GRAMINEARUM

ABSTRACT

In addition to causing disease in wheat and other grain crops, *Fusarium graminearum* is associated with a wide range of forage, weedy, and non-cultivated gramineous species. The nature of these associations is often unclear, and interactions between *F. graminearum* and these hosts are not well characterized, despite their prevalence in and around cereal fields. We observed differences in the host suitability (competency) of spring wheat and six common grasses during three stages of the pathogen life cycle. Pathogen survival in artificially infested stem tissue was comparable between grasses and wheat over two years with the exception of one host. Sexual and asexual spore production on host stems under laboratory conditions varied significantly but exceeded 1 x 10^5 spores per dry gram of tissue for all grass species. A seedling root infection assay revealed the apparent resistance of two grasses to root rot and indicated that host preference could vary between pathogen isolates. These findings are relevant for how the role of wild grass hosts in disease epidemics of proximate crops and in pathogen evolution is viewed. Managing weedy grasses in field margins and avoiding gramineous pasture – wheat rotations may reduce local sources of *F. graminearum* inoculum. Changes in the relative pathogenicity of isolates depending on host species suggest that wild grasses could exert selective pressure on *F. graminearum* and requires further investigation.

INTRODUCTION

Fusarium graminearum is a globally distributed pathogen of cereal crops responsible for lost revenue estimated at one billion dollars annually in the United States[1]. Known for causing head blight and crown rot of wheat and ear rot of corn, *F. graminearum* has been isolated from dozens of other gramineous hosts[2]. Many of these are wild or weedy species covering large areas of land and can be found readily in pastures, grasslands, and the margins of agricultural fields. Even in regions with intensive cereal production, the acreage of wild grasses and grassy pasture can be greater than that of susceptible field crops. For example, in 2017, the state of Kansas contained approximately 2.7 million ha of corn and 2.2 million ha of winter wheat but also 8.9 million ha of grass and mixed pasture[3]. Wild grass spikes and residues containing *F. graminearum* have been consistently reported from diverse locations[4–10]. Despite the distribution, extensive interface with crops and near universal ability of wild grass species to harbor *F. graminearum*, their interaction with the pathogen remains poorly understood. The host competency of these grasses, or how well they support the life cycle and successful spread of *F. graminearum*, has implications for disease epidemics and the evolution of the pathogen.

The role these wild grass hosts play in crop disease cycles and whether they are capable of contributing substantial amounts of inoculum to disease epidemics is unknown. Perithecia and sporodochia can be observed on senesced grass stems in the spring[11], indicating that the pathogen may overwinter in wild grass residue. How much inoculum is produced on these grasses and their importance in the context of multiple

inoculum sources originating from field crop residues and airborne propagules is not clear[12]. The relative suitability of these grasses for pathogen overwintering and spore production also is unknown.

In addition to epidemiological considerations, wild grass hosts could be influencing pathogen evolution. *Fusarium graminearum* sensu lato is a phylogenetic species complex whose members produce an array of toxic secondary metabolites[13,14], and passage through different agricultural hosts is reported to alter the pathogenic fitness of *F. graminearum*[15]. The presence of diverse gramineous hosts could be important to the maintenance of broad range pathogenicity or the emergence of new pathogen variants.

Previously observed interactions of *F. graminearum* with wild grass spikes indicate host-variable lifestyles and adaptations. Grass spikes are colonized asymptomatically under natural conditions[4,9,10,16], in contrast to the typical scab symptoms and 'tombstone' kernels that develop during the infection of small grains[17]. However, exceptions are known[18], and artificial inoculation of some grasses results in recognizable symptoms[10,19]. Further, little mycotoxin contamination is detected in grass hosts colonized by toxigenic *Fusarium* spp., indicating no clear role for these crop virulence factors and providing evidence of variable host interactions[10,20–22].

In order to better understand this variability and the potential role of wild grasses in crop disease epidemics, we compared the host competency of wheat and several grass species common to cereal production sites in New York state, USA. Host-pathogen interactions were characterized at three points in the pathogen life cycle: winter survival, reproduction and seedling infection.

MATERIALS AND METHODS

Grass and inoculum preparation

Senesced stems used in the winter survival and spore production assays were taken from plants grown in a greenhouse for two months. The spring wheat variety 'Norm' and five non-cereal grasses were included in these experiments. Four were grown with commercially available seed (Great Basin Seed, Ephraim, UT) (*Bromus inermis* Leyss. [smooth brome], *Phalaris arundinacea* L. [reed canarygrass], *Dactylis glomerata* L. [orchardgrass], *Lolium perenne* L. [perennial ryegrass]), and the stems of one grass were collected from mature, natural stands because seed was unavailable (*Elymus repens* (L.) Gould [quackgrass]). Stems were stripped of leaves and air dried for one week, trimmed into 5 cm long pieces containing one internode each, and autoclaved at 121°C for 20 min on two consecutive days before use. The seedling root rot assay included the above grasses with the exception of *E. repens* and the addition of *Phleum pratense* L. (timothy). The inocula for all three experiments were obtained by washing conidia from two-week-old pathogen colonies grown on potato dextrose agar (PDA) under 12 hr blacklight (F40/350BL, Sylvania, Wilmington, MA). All spore suspensions were adjusted to 1 x 10^4 conidia/mL. Ten isolates of the pathogen previously collected from several hosts, locations and years were used in the winter survival and spore production assays to incorporate between isolate variability in estimates of host competency. A different set of isolates, including four of *F. graminearum* and one of *F. cerealis*, were used in the seedling root rot assay so multiple pathogen chemotypes would be represented and the reaction of grasses to a

different *Fusarium* species would be included (Table 1).

Overwintering

The ability of *F. graminearum* to overwinter on the senesced stems of wheat and five grass species was examined by artificially infesting sterile plant tissue and observing year-to-year survival rates. A random complete block trial with six replicates per grass was conducted under field conditions in Tompkins County, New York from September 2016 to March 2018. Stems were dip inoculated in an equal-parts mixture of all 10 isolates to simulate multiple infections that may occur in nature, wrapped in cheesecloth and incubated for two weeks in darkness at room temperature. Each replicate packet contained 10 tissue segments. Packets were staked to the soil surface and after overwintering under natural conditions, three bundles of each grass were collected in March 2017 and the remainder in March 2018. Recovered stems were surface sterilized in 1.65% NaOCl for 60 s, rinsed in sterile distilled water for 30 s, and left on PDA amended with streptomycin (5 g/L) and neomycin (5 g/L) for four days under 12 hr white light. The resulting fungal colonies were identified morphologically, and the number of stems infested with *F. graminearum* was recorded[23].

Table 4.1. Fusarium isolates used in this study.

Isolate	Host	Chemotype[b]	Overwintering	Spore Production	Root Rot
Fusarium graminearum					
Gz5668NY15[a]	*Bromus inermis*	15-ADON	X	X	
Gz5673NY15	*Hordeum jubatum*	15-ADON	X	X	
Gz5676NY15	*Elymus canadensis*	15-ADON	X	X	
Gz6033NY16	*Triticum aestivum*	15-ADON	X	X	
Gz6039NY16	*T. aestivum*	3-ADON	X	X	
Gz6046NY16	*T. aestivum*	15-ADON	X	X	
Gz5953NY16	Grass residue	15-ADON	X	X	
Gz5911NY16	Grass residue	15-ADON	X	X	
Gz5933NY16	Grass residue	3-ADON	X	X	
Gz448NY13	*Zea mays*	15-ADON	X	X	X
Gz830NY11	*Z. mays*	NX-2			X
Gz014NY98	*T. aestivum*	15-ADON			X
Gz112VA10	--	NIV			X
Fusarium cerealis					
Fc1134NY13	*Hordeum vulgare*	(NIV)			X

[a] Isolate names are formatted as 'Species-Collection Number-State-Year'
[b] Determined by PCR based methods[33,34] prior to selection for use in root rot assay and after use in overwintering and spore production assays.

Reproduction

Spore production per dry gram of host tissue was quantified in a laboratory experiment adapted from Pereyra & Dill-Macky[5]. Six grasses and each of the 10 isolates plus a sterile water control treatment were arranged in a random complete block design with three replicates per 'isolate x grass' combination. The moisture content of host tissue was determined with an OHAUS MB25 moisture meter (Parsippany, NJ) so spore counts could later be converted to a per dry gram basis. An equal wet mass of each grass (0.25-0.30 g) was placed into 60 mm glass petri dishes containing 10 mL of sterile sand, which was included to maintain moist conditions. Dishes were inoculated with 5 mL of spore suspension from single isolates or sterile water and kept at room temperature under 12 hr black light for two weeks, when the contents of each plate were moved to 15 mL tubes and mixed with 10 mL of sterile water and one drop of 0.01% (v/v) Tween 20. Samples were mixed well and left to release spores for 12 hr. After being vortexed for two min, 1 mL subsamples were aliquoted from these tubes and both conidia and ascospores were counted on a hemocytometer. The experiment was performed twice.

Root rot assay

Susceptibility to Fusarium root rot was assayed at the seedling growth stage. Seeds of each host were surface sterilized in 1.65% NaOCl for 60 s, rinsed in sterile water for 30 s, and soaked in the inoculum from one of five isolates for 15 min. Sterile water served as a control treatment and two replicates of five seeds were germinated for each of the 'host x isolate' combinations. Inoculated seeds were incubated in moist paper

towels at room temperature under 12 hr white light in partially closed plastic bags for two weeks. The number of seeds germinated and number of seedlings exhibiting symptoms of Fusarium root rot, browning of root tissue or water soaking at the root-shoot juncture, were recorded. The roots of five healthy and five symptomatic seedlings from each grass were then randomly selected, surface sterilized as described above and incubated on PDA to test for *F. graminearum* colonization. The experiment was performed twice.

Data analysis

All analyses were performed in RStudio Version 1.1.453 using linear models[24]. Experimental repeats and blocks were included in all models as fixed effects. The recovery of overwintered *F. graminearum* from debris was analyzed with a binomial model using grass and year of recovery as fixed effects. Model estimates (log-odds ratios) were compared with Tukey adjusted pairwise contrasts to compare grasses[25]. Spore counts were converted to a dry gram basis and $\log_{10}$ transformed to fit the assumption of a normal distribution before analysis. These data were modeled with a fixed effect for grass and a random effect for isolate. Least-squares means and 95% confidence intervals were then produced to compare hosts. The effect of *Fusarium* inoculum on germination in the seedling assay was modeled on a binomial distribution using fixed effects for grass species and a factor with two levels, inoculum and water control. Since pathogen inoculum did not reduce germination rate and mock inoculated seeds did not display disease symptoms, data points for non-germinated plants and water controls were removed before further analysis. The presence or

absence of disease symptoms on seedlings was used in a binomial model and subjected to a two-way analysis of variance using grass species and isolate as interacting terms.

RESULTS AND DISCUSSION

Overwintering

Artificially infested grass debris only retained *F. graminearum* at a high rate (25-100%) in the first year after colonization (Figure 4.1). A significant effect on overwintering was detected for grass species ($X_5 = 57.05$, $P < 0.001$), and *L. perenne* was less likely to be infested than all the other grasses ($P \leq 0.001$). Pathogen retention after two years was 0-40%, and year was a significant predictor of infestation ($X_1 = 190.51$, $P < 0.001$). This lower pathogen recovery rate coincided with physical deterioration of host tissue. The rate of pathogen recovery over two years confirms previous findings on wheat[26,27] but adds to our understanding of how well wild host species might enable *F. graminearum* to persist in field margins or fallow fields between cropping cycles.

Reproduction

Inoculum production was compared on gramineous species under controlled conditions in two experiments. The number of both ascospores and conidia produced on grass stem tissues were significantly affected by host species ($F_{5,8} = 14.30$, $P < 0.001$ and $F_{5,8} = 43.45$, $P < 0.001$, respectively). *Dactylis glomerata* was the least suitable grass for spore production, though all grasses supported over 1×10^5

ascospores or conidia per dry gram of tissue (Figure 4.2). The variation in spore production recorded across hosts may be related to nutrient composition or physiological structure. The ratio of C:N has been suggested as a relevant factor[26,28,29], as have silica containing cells[30,31].

Root rot assay

Inoculation did not have a significant effect on germination rate for any grass ($F_{1,717} = 2.20$, $P = 0.138$), and seeds soaked in sterile water exhibited no symptoms of disease. Wheat was the most susceptible host while *P. pratense* (no observed infections) and *P. arundinacea* (a single observed infection) were the least susceptible (Figure 4.3). Grass species was a significant predictor of disease incidence ($F_{5,419} = 91.04$, $P \leq 0.001$), and the 'grass x isolate' interaction was significant ($F_{20,419} = 3.01$, $P < 0.001$). No *F. graminearum* was recovered from asymptomatic or water control treated plants, and symptomatic plants contained no fungi other than *F. graminearum*. *Fusarium graminearum* has been recovered from the root or crown tissues of many grasses under natural conditions[8,11,32]. Because root and crown rot infections may lead to the colonization of grass residues and eventual inoculum production, grasses not readily colonized in this manner may be less competent as sites for pathogen survival and reproduction. The variable interactions seen between grass species and isolate indicates that pathogen fitness may be optimized for one host over another.

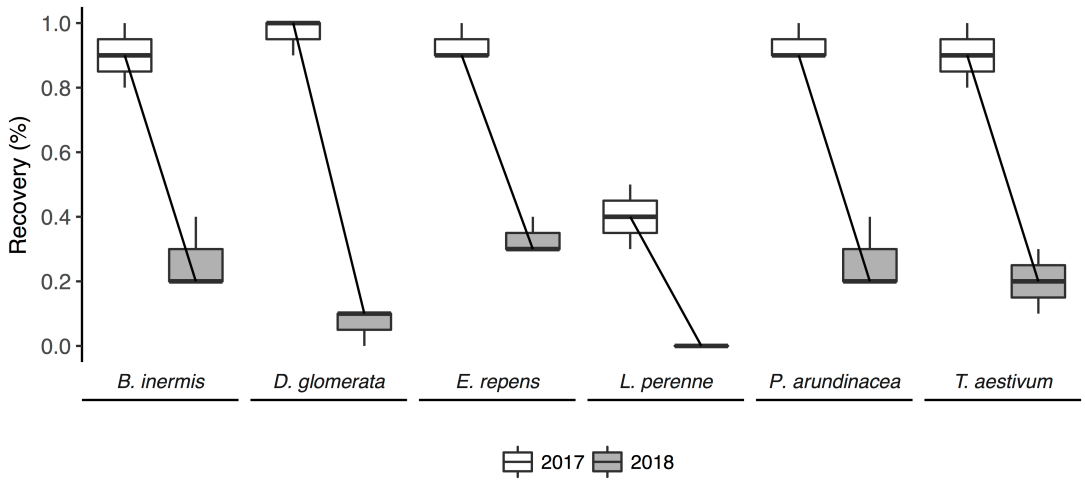

Figure 4.1. Survival of *Fusarium graminearum* in grass stems over two winters. Bars show percent pathogen recovery from stems after one and two years of overwintering in artificially infested debris deposited outside in fall 2016. Whiskers represent standard deviation of three replicates, each containing 10 stem segments. *Lolium perenne* was significantly less likely to be colonized than other hosts ($P \leq 0.001$), and by year two all the grasses had decreased to less than 40% infestation rates ($P < 0.001$).

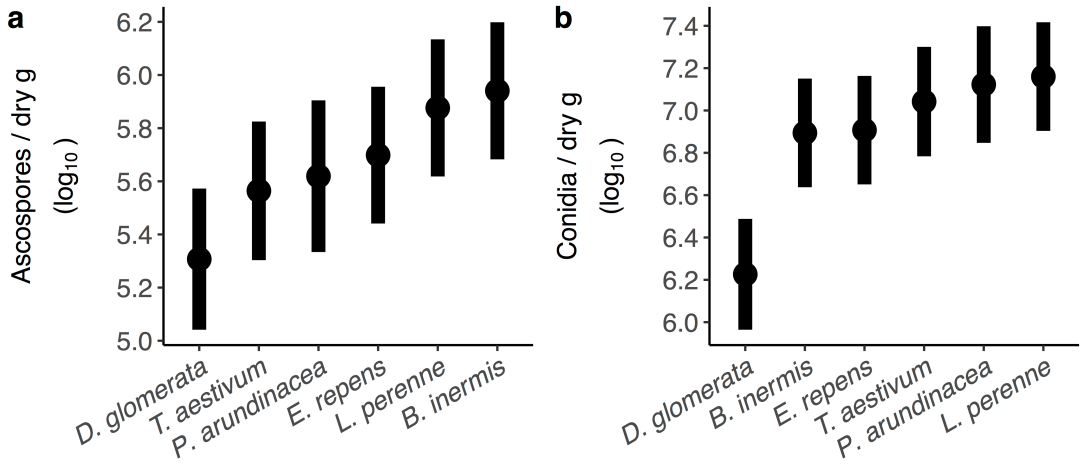

Figure 4.2. *Fusarium graminearum* spore production on six different grasses. Bars are confidence intervals (95%) around least-squares means from a linear mixed model of (a) ascospore and (b) conidia production on grass stems averaged across 10 isolates of *F. graminearum*. The number of spores produced was significantly affected by host in both cases ($F_{5,8} = 14.30$, $P < 0.001$ and $F_{5,8} = 43.45$, $P < 0.001$, respectively).

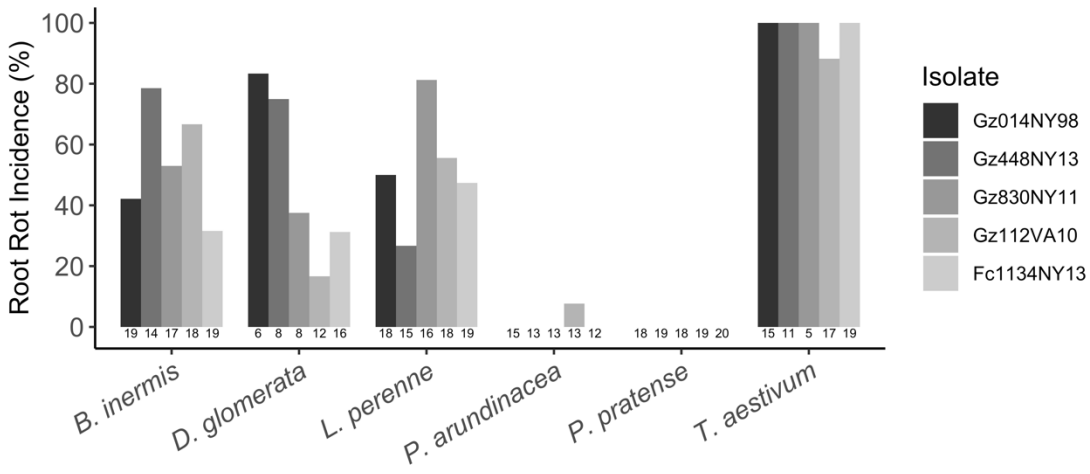

Figure 4.3. Incidence of root rot caused by five isolates of *Fusarium* on the seedlings of six grass species. Host species had a significant effect on disease incidence ($F_{5,419} = 91.04$, $P \leq 0.001$), and there was a significant interaction between host and isolate ($F_{20,419} = 3.01$, $P \leq 0.001$). Sample sizes are included below each bar.

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CHAPTER 5

FUSARIUM GRAMINEARUM ISOLATES FROM WHEAT AND NON-CULTIVATED GRASS SHOW SIGNS OF PHENOTYPIC DIFFERENTIATION AND TRADE-OFF BETWEEN GROWTH AND ASEXUAL REPRODUCTION

ABSTRACT

Fusarium graminearum, causal agent of Fusarium head blight in small grain cereals, is able to colonize a wide range of both cultivated and non-cultivated host plants. To better understand the role of non-cultivated grasses in the evolution of this important crop pathogen, isolates were collected from wheat and grasses at a single field site for phenotypic and genotypic comparison. Genotyping was performed at two toxin related loci to predict the production of mycotoxin variants and at eight microsatellite loci to determine relatedness and population structure. Phenotyping experiments were conducted for ten traits related to four fitness attributes: asexual reproduction, sexual reproduction, mycelial growth rate, and virulence on crops. A significant difference was found between the mean conidia production and mean conidia length of grass and wheat derived isolates ($P \leq 0.036$). Individuals taken from wheat had a greater value for these asexual reproductive traits, which were negatively correlated with mycelial growth rate ($P = 0.040$), suggesting a trade-off. A comparison of phenotypic and genetic differentiation indicated differences in conidia length were due to directional selection, rather than random genetic drift. Together, these results provide a preliminary assessment of the different selective pressures non-cultivated and crop hosts exert on *F. graminearum*.

INTRODUCTION

The role of non-cultivated hosts in the evolution of the cereal crop pathogen *Fusarium graminearum* is unknown. Numerous wild grasses have been recorded as asymptomatic hosts[1–3] and are often found in close proximity to agricultural hosts[4]. Non-cultivated grass hosts rarely develop the symptoms characteristic of Fusarium head blight (FHB) in crops and, under natural conditions, do not appear to accumulate the mycotoxins normally associated with disease[5]. This inconsistency in host interactions suggests different adaptations may be required for the colonization of crops and grasses.

The potential exists for *F. graminearum* to adapt to wild hosts at the cost of virulence or reproductive ability on crop species, like wheat and corn. While isolates collected from non-cultivated hosts are known to be pathogenic on wheat seedlings and inflorescences[6,7], the phenotypic variation of other important traits, like mycotoxin production or reproduction, has not been measured. Isolates collected from regions with low crop density or isolated host communities, where adaptation to one category of hosts may be encouraged by physical separation, are of particular relevance.

The northeastern region of New York, USA contains a diverse population of *F. graminearum*, with a mixture of three sympatric North American populations and a uniquely high frequency of isolates expected to produce the recently discovered mycotoxin NX-2[8–10]. The agricultural landscape in this region contains a small, fragmented acreage of small grain cereals, and the geography is dominated by the Adirondack Mountain wilderness. Together, the genetic diversity of the pathogen

population and the isolated nature of host populations make isolates collected from this region ideal for use in studies on evolution or adaptation to non-crop hosts.

This study had three objectives, (i) to contrast the relative fitness of isolates from winter wheat and non-cultivated grass hosts found in the same environment, (ii) to assess evidence of life-history trade-offs based on those traits, and (iii) to establish whether fitness related traits differed enough between host sources to suggest host dependent selection. Because environmental conditions were controlled for with use of a single site, any differences in mean trait values were expected to relate to the host of origin. Negative correlations between traits were expected if trade-offs in fitness attributes are present. Using neutral genetic markers to measure population differentiation, significantly greater phenotypic differentiation would be attributed to selection.

MATERIALS AND METHODS

Isolates and genotyping

Single conidia derived isolates were taken from a previously reported collection (Fulcher, in review). Isolates collected from symptomatic wheat (n = 12) and three asymptomatic grass hosts (*Phalaris arundinacea* n = 4, *Bromus inermis* n = 4, *Dactylis glomerata* n = 4) collected at a single field site were chosen at random without prior knowledge about phenotypes or genotypes. Species identity was confirmed by sequencing a portion of the translation elongation factor 1-alpha (*TEF1-*α) and comparison to GenBank accessions. Multiplex PCR reactions and allele sizing by gel electrophoresis were used to differentiate 15-ADON, 3-ADON and NIV

genotypes at the *TRI12* locus, and a restriction enzyme digest following PCR of a portion of the *TRI1* gene was used to identify potential NX-2 producers[11,12]. Eight microsatellite loci were amplified from a subset of 16 isolates (Supplemental Table 1)[13–15]. Fluorescently labelled microsatellite primers (Applied Biosystems G5 dye set) were split evenly between two multiplex reactions. The QIAGEN Multiplex PCR Plus Kit (Holden, Germany) was used, and reaction mixtures were 25 µl with 0.2 µM primer concentrations. The same cycling conditions were used for both reactions: 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 58°C for 1 min 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 10 min. Amplified products were diluted 1:10 in water, and 1 µl of diluted product was mixed with 10 µl of HiDi Formamide and 0.2 µl of GeneScan 500 LIZ size standard (Life Technologies, Woolston, United Kingdom). Fragments were separated on an ABI 3730xl DNA Analyzer at the Cornell Biotechnology Resource Center, and alleles were sized in Geneious Prime version 2019.0.4 (Biomatters, Auckland New Zealand) using the Microsatellite Analysis plugin version 1.4.6.

Growth rate

Radial growth (mean mm/day) was measured on potato dextrose agar (PDA). An electronic pipette was used to dispense 10 mL of growth media into 60 mm plates. Three plugs of each isolate were taken from the actively growing edge of young mycelia and transferred to these plates using a 7 mm diameter cork-borer. Cultures were then incubated at 22°C in darkness for five days. Isolates were grown on two plates each, for a total of six colonies measured per isolate. The assay was performed

twice.

Conidia production

Conidia production per mm^2 of PDA was quantified. Plates were prepared as above, and cultures were grown for seven days under 12 hr fluorescent light at 22.5±1°C. Two agar plugs were taken from the same points near the center of each plate and placed into 1 mL of sterile water. Samples were vortexed for 60 sec to suspend conidia, and spore counts were taken twice using a hemocytometer. Two replicate plates were used for each isolate and the experiment was conducted twice. Conidia produced by each isolate and washed from these plates were also photographed and measured (n = 50 spores divided between replicates) using ImageJ version 1.49b[16,17].

Perithecia formation

Perithecia formation was measured on four substrates: ¼ strength PDA, corn stalks, wheat stems and reed canarygrass (*P. arundinacea*) stems. All plant material was taken from 2 – 3 month-old plants grown in a greenhouse. Corn stalks with a diameter of 2.5 – 3.0 cm were trimmed to 9 cm in length and split lengthwise so they would fit into petri dishes. Wheat and *P. arundinaceae* stems were trimmed to 3 cm in length. Two pieces of host stem were placed into a glass petri dish lined with a single filter paper and autoclaved on three consecutive days. Stems were then inoculated with 2 mL of a 10^4 conidia/mL suspension. Inoculated plant tissues were incubated at 25°C under 12 hr fluorescent lights for 28 days. At 7, 14, and 21 dpi, 1 mL of sterile water

was added to the samples in order to maintain moist conditions. At 28 dpi, the percent of plant tissue covered in mature, dark-blue to black perithecia was estimated visually using a key for wheat stem rust severity[18]. Perithecia formation was similarly estimated on PDA after 28 days of colony growth from plugs under 12 hr black light at 22.5±1°C. The synthetic media assay was performed independently from the plant tissue assay. Each 'isolate-by-substrate' treatment was replicated three times, and sterile water controls were included. Both experiments were performed twice.

Seedling assay

Aggressiveness was recorded on maize seedlings (of an unknown feed variety). Seed was surface disinfected in 1.65% NaOH for one minute, rinsed in sterile water, and soaked in a 10^4 conidia/mL suspension for 5 min. Inoculated seeds were then germinated in moist paper towels. After 21 days under 12 hr white light at 22.5°C, infection was rated on an ordinal scale of 1(no infection)-6(complete necrosis). Five kernels were treated with each isolate, and sterile water served as a control. The experiment was performed twice.

Greenhouse assay

Aggressiveness and mycotoxin production were recorded in spring wheat (cv. 'Norm') grown in a greenhouse in a complete random block design experiment. Three replicate pots of wheat were grown for each treatment, and plants were trimmed to single tillers before inoculation. At anthesis (Feekes' 10.5), 10 µl of a 10^4 conidia/mL inoculum suspension or a sterile water control were applied to single spikelets using a

micropipette. Spikes were kept under plastic bags for 48 hrs to maintain humidity during the infection period. Disease severity ratings (percent of spike showing symptoms of scab) were taken after three weeks. Grain was bulked from replicate pots and milled to a fine flour before deoxynivalenol (DON) content was quantified using GC/MS at the University of Minnesota[19]. The experiment was performed twice, but toxin analysis was completed only for samples from the first experiment.

Data analysis

All data analyses were performed in RStudio version 1.1.453[20]. Chemotype allele frequencies in grass and wheat derived populations were compared with a X^2 test. Genetic distances between isolates and differentiation by isolate source were determined with microsatellites markers using Bruvo's distance and Nei's G_{st} as implemented the 'poppr' package[21].

Phenotypic data did not differ significantly between experimental replications, so all data were combined. Values were adjusted relative to the average of all isolates before analysis in order to compare traits measured on different scales. Following Shapiro-Wilk tests for normality, radial growth, conidial length, conidia production, disease severity on wheat spikes and total DON production were contrasted using linear models and analysis of variance with chemotype and host of origin as predictors. Chemotype has been associated with differences in pathogenicity, toxin production and in some studies disease severity[22–26], so was included as a predictor in these analyses. In the analysis of DON production, NX-2 and NIV producing isolates were dropped since they do not produce DON, and because these isolates were evenly

divided between host sources sample sizes remained similar. Perithecia production and maize seedling blight severity were compared using non-parametric Mann-Whitney tests.

A principle components analysis and Pearson correlations of phenotypic traits found to vary between isolate sources were used to assess evidence of life-history trade-offs. A series of Mantel tests were used to look for correlation between matrices of genetic distance and phenotypic distance using the 'vegan' package[27]. A universal test was conducted, as well as individual tests for each phenotype measurement, and each test was run with 1000 random permutations. Phenotypic differentiation (P_{st}) between hosts of origin was compared to genetic differentiation (G_{st}). These two measures are analogous, both comparing within group variability to between group variability[28]. Using the subset of isolates (n = 16) for which microsatellite and phenotype data were available, 95% confidence intervals for P_{st} and G_{st} were generated by bootstrapping procedures, each using 1000 repetitions[29,30]. These confidence intervals were compared to test the hypothesis that differentiation in phenotypic traits could not be explained by random drift inferred from neutral genetic markers.

RESULTS

Genotyping

Morphological assignment of isolates to *Fusarium graminearum* sensu stricto was confirmed by comparing partial *TEF1*-α sequences (NCBI Genbank Accession: NA) to known specimens using the Nucleotide Basic Alignment Search Tool and reference

sequences deposited to the National Center for Biotechnology Information (NCBI, www.ncbi.nih.gov). All isolates had a 99-100% identity match to *F. graminearum*. Chemotype was predicted for all 24 isolates based on *TRI12* alleles, and of the 12 3-ADON genotypes found, six were determined to have the NX-2 associated allele at the *TRI1* locus (Table 1). Chemotypes were not found at different frequencies in wheat and grass derived isolates ($X^2_3 = 5.939$, $P = 0.115$). Sixteen isolates were genotyped at eight microsatellite loci. No clones were identified and differentiation between isolate sources was low, $G_{st} = 0.057$. A minimum spanning tree built from Bruvo's genetic distances showed the isolates in this study were not closely related and did not form clusters related to host of origin (Figure 1).

Phenotyping

Ten traits were evaluated in four categories of fitness: sexual reproduction, virulence on crops, growth, and asexual reproduction. The mean relative values for all recorded traits in grass and wheat derived populations is displayed in Figure 2. Significant differences in relative conidia production and conidia length were found based on isolate source ($F_{2,19} = 4.92$, $P = 0.039$ and $F_{2,19} = 19.97$, $P < 0.001$, respectively) (Figure 3). No other traits were found to vary significantly. Mycotoxin test results showed that samples from non-DON producing isolates contained a background level of 0.01 – 0.20 ppm DON, interpretable as cross-contamination during sample processing, but determined insignificant as samples from true DON producers contained 12 – 55 ppm DON.

Table 5.1. Isolates used in this study, host of origin, predicted chemotypes, use in full genotyping study and Genbank deposit number.

		Predicted Chemotype		
Host	Isolate	*TRI12*	*TRI1*	SSRs
Triticum aestivum	Gz6463NY17	3-ADON	3-ADON	X
	Gz6467NY17	15-ADON	-	
	Gz6470NY17	15-ADON	-	X
	Gz6472NY17	3-ADON	NX-2	X
	Gz6475NY17	3-ADON	3-ADON	X
	Gz6477NY17	3-ADON	NX-2	X
	Gz6479NY17	3-ADON	3-ADON	X
	Gz6481NY17	15-ADON	-	X
	Gz6491NY17	3-ADON	3-ADON	
	Gz6492NY17	NIV	-	
	Gz6494NY17	3-ADON	NX-2	
	Gz6495NY17	3-ADON	3-ADON	
Bromus inermis	Gz6497NY17	3-ADON	NX-2	X
	Gz6499NY17	3-ADON	3-ADON	X
	Gz6546NY17	15-ADON	-	X
	Gz6575NY17	15-ADON	-	
Dactylis glomerata	Gz6512NY17	15-ADON	-	
	Gz6519NY17	15-ADON	-	X
	Gz6538NY17	3-ADON	NX-2	X
	Gz6560NY17	15-ADON	-	X
Phalaris arundinacea	Gz6532NY17	15-ADON	-	
	Gz6556NY17	15-ADON	-	X
	Gz6583NY17	3-ADON	NX-2	X
	Gz6584NY17	15-ADON	-	X

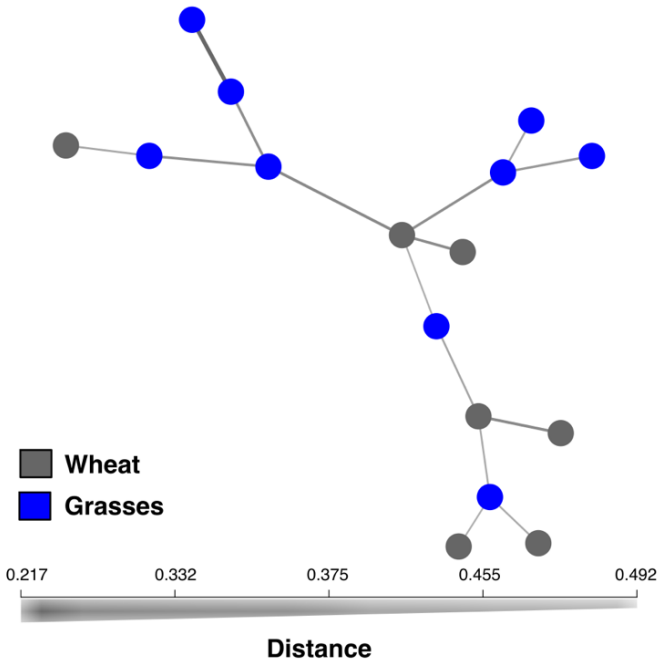

Figure 5.1. A minimum spanning tree based on Bruvo's distance calculated with eight microsatellite markers shows no clear clustering of wheat or grass derived isolates. Isolates were not closely related despite being collected from a single, isolated field site.

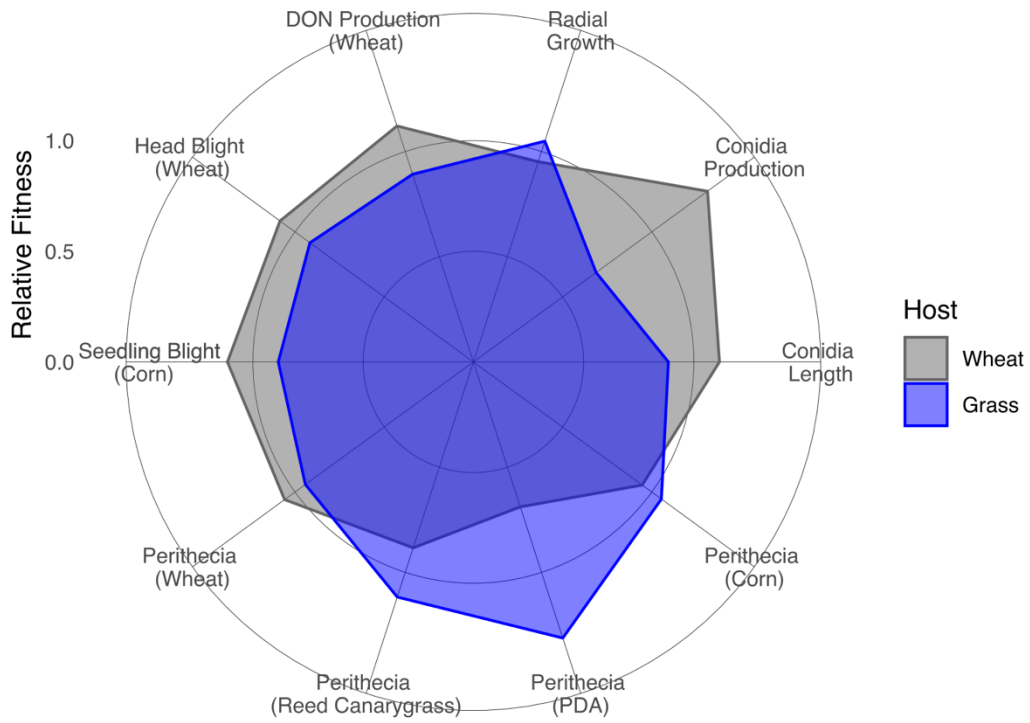

Figure 5.2. A radar plot of mean relative phenotype values for 10 traits related to isolate fitness shows a qualitative difference in phenotypes between isolates from wheat and grass.

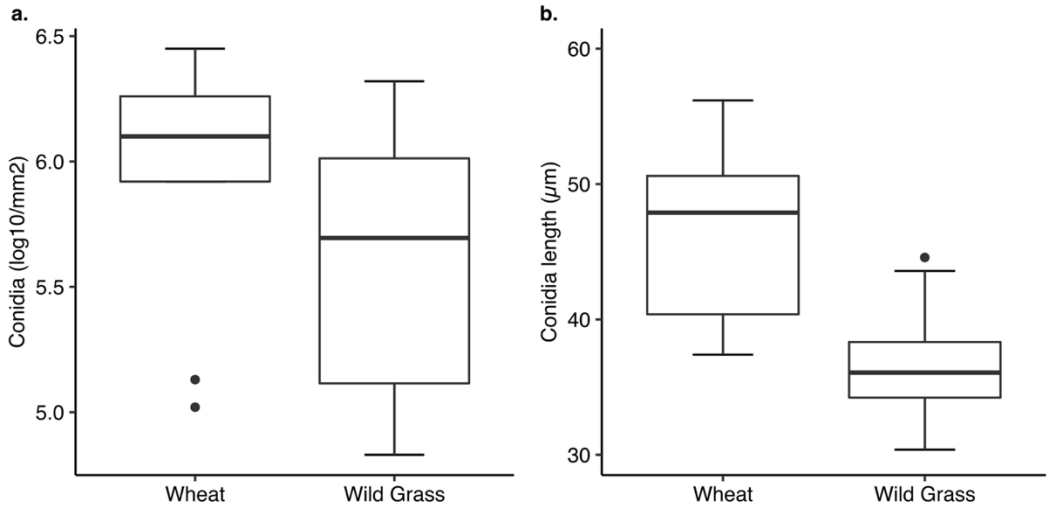

Figure 5.3. Wheat derived isolates had a higher mean asexual reproductive ability, measured as both (a) conidia length and (b) conidia production on synthetic growth media, than grass derived isolates.

Trade-offs

A principle components analysis was run on a correlation matrix of phenotypic data. The first two components accounted for 32.6 and 20.3 % of variance (Figure 4). The production of perithecia appeared positively correlated on all four substrates assayed while asexual reproduction and radial growth appeared to have a negative correlation. The latter was confirmed with Pearson correlations (Table 2). Radial growth and conidia length had a significant negative correlation after applying a false discovery rate correction to p-values ($r = -0.59$, $P = 0.038$).

Phenotype-Genotype Relationship

There was no significant correlation between phenotypic and genotypic distances according to the universal Mantel test ($r = -0.004$, $P = 0.478$). Mantel tests were also conducted for individual phenotypic traits, and conidia production was modestly but significantly correlated with genetic distances after correction for false discovery rates ($r = 0.30$, $P = 0.040$) (Table 3). Differentiation of phenotypes (P_{st}) was greater than genetic differentiation (G_{st}) only in the case of conidia length. Confidence intervals for the total P_{st} and individual traits are plotted along with G_{st} in Figure 5. This method has been applied to a number of organisms, including at least one plant pathogenic fungus[31], but has been criticized, so interpretation must be cautious[32,33].

Table 5.2. Pearson correlations of asexual reproductive traits and other fitness related phenotypes. Bold indicates a significant correlation ($P \leq 0.05$).

Fitness Category	Phenotype	Conidia Length	Conidia Production
Asexual Reproduction	Conidial Length	1.00	**0.76**
	Conidia Production	**0.76**	1.00
Sexual Reproduction	Perithecia - Corn	-0.21	0.03
	Perithecia - PDA	-0.24	0.02
	Perithecia - Canarygrass	-0.04	0.23
	Perithecia - Wheat	-0.30	0.27
Crop Virulence	Maize Seedling Blight	0.24	-0.11
	Fusarium Head Blight	0.21	-0.17
	DON Production in Wheat	0.25	0.34
Growth	Radial Growth	**-0.59**	-0.34

Table 5.3. Significance tests for correlation between phenotypic distance and genetic distance**.**

Fitness Category	Phenotype	Mantel r (*P*-value)
Asexual Reproduction	Conidial Length	0.11 (0.603)
	Conidia Production	**0.28 (0.044)**
Sexual Reproduction	Perithecia - Corn	-0.04 (0.677)
	Perithecia - PDA	-0.03 (0.764)
	Perithecia - Canarygrass	-0.02 (0.677)
	Perithecia - Wheat	-0.08 (0.688)
Crop Virulence	Maize Seedling Blight	0.03 (0.684)
	Fusarium Head Blight	0.22 (0.275)
	DON Production in Wheat	0.28 (0.154)
Growth	Radial Growth	0.01 (0.677)

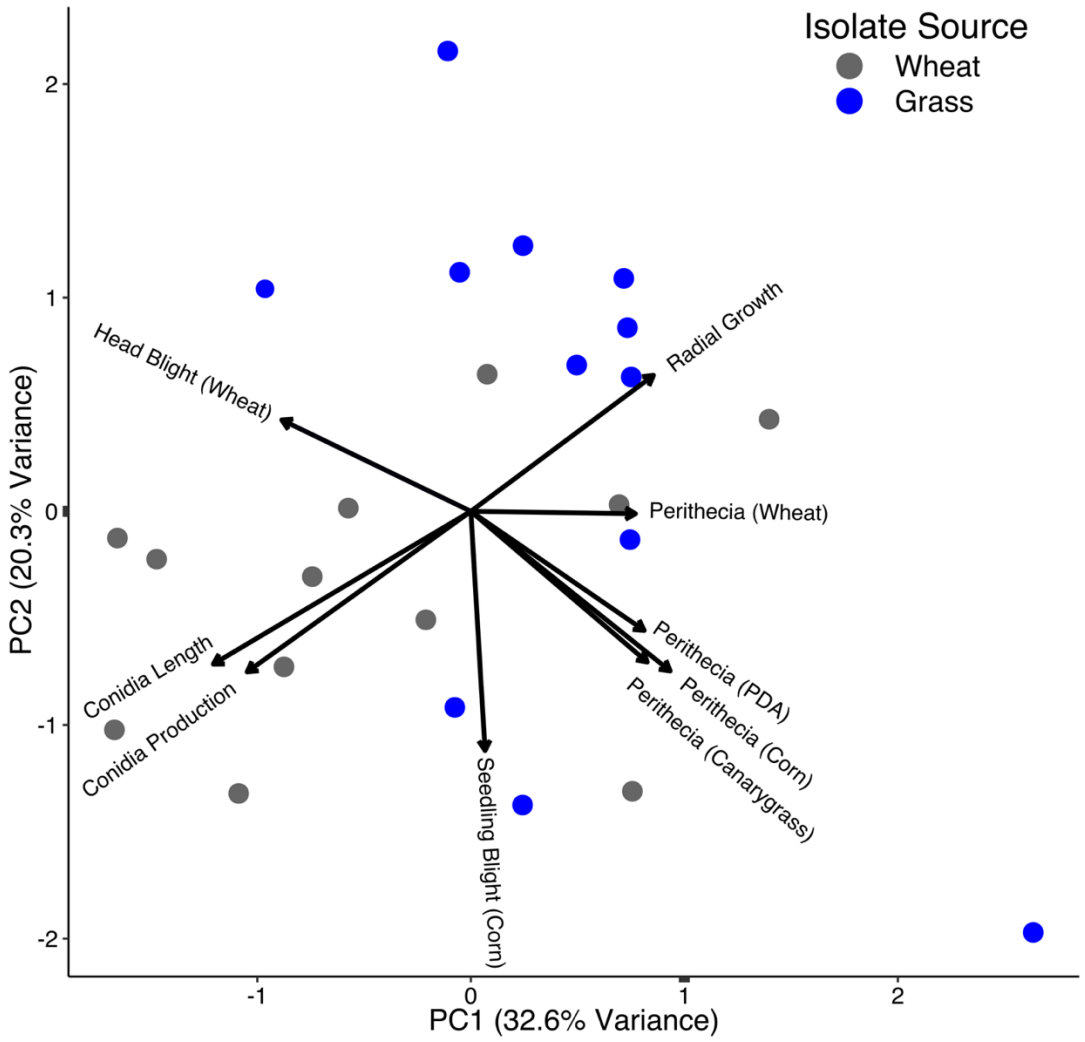

Figure 5.4. A principle components analysis of fitness related phenotype data from 24 isolates indicated postive correlation between sexual reproduction on various substrates as well as negative correlation between asexual reproduction and radial growth. Some grouping of isolates is apparent based on host of origin, indicating differences in overall phenotypic variation.

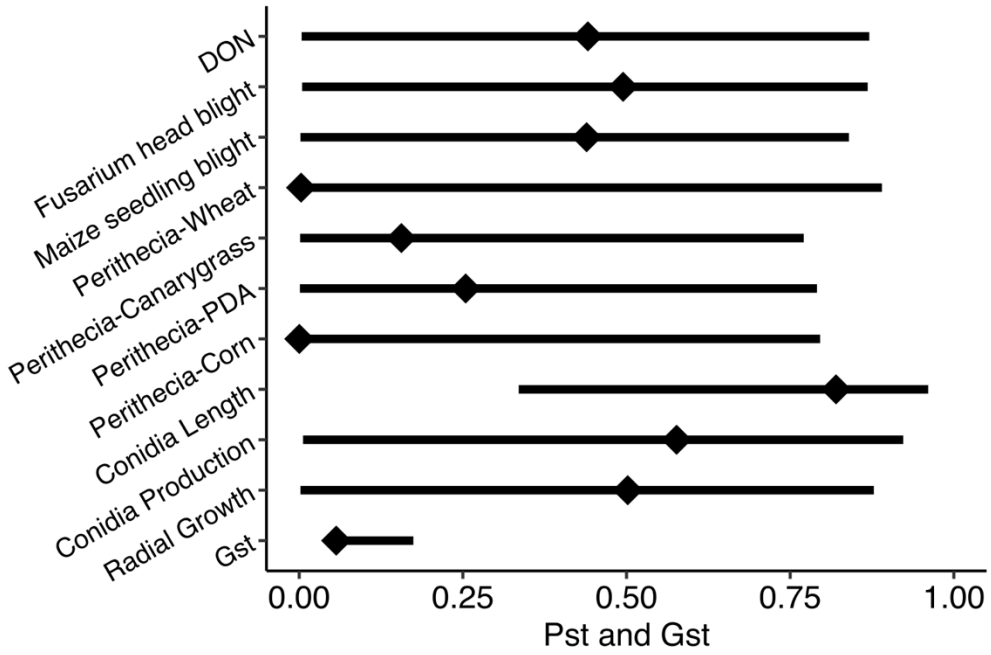

Figure 5.5. A comparison of phenotypic (Pst) and genetic (Gst) differentiation between isolates derived from wheat and grass spikes shows only the difference in conidia length could be ascribed to directional selection with greater certainty than to random genetic drift. Lines plotted here represent 95 % confidence intervals found with 1000 random samplings of both data sets.

DISCUSSION

This study is the first to measure and compare multiple fitness related traits in *F. graminearum* collected from wheat and non-cultivated grasses at a single field site. A significant difference in asexual reproduction phenotypes was observed between isolate sources, and a negative correlation between one of these traits with mycelial growth rate suggests that a life-history trade-off could have occurred. A high phenotypic differentiation (P_{st}) in conidia length further suggests that the trait is undergoing positive selection. Taken together, these results provide preliminary evidence that wild grasses and crop hosts select for a different *F. graminearum* phenotype and that this selection may result in evolutionary trade-offs.

The differences observed between grass and wheat derived isolates were amount of sporulation and spore size. Both of these traits were significantly greater in the wheat derived population. Rain- and wind-dispersed conidia are expected to play a role in secondary infections occurring in crop fields[34,35]. Uniform host communities, such as wheat fields, could benefit the spread of *F. graminearum* individuals with a greater chance of starting secondary infection cycles. Longer spores tend to have more septa and may contain greater nutrient reserves, which would allow for more germ tubes or longer germ tubes that increase infection success. Length also alters the aerodynamics of a spore, and could influence patterns of conidia dispersal or deposition[36–38].

The potential for a trade-off in the evolution of fitness related traits has implications for the maintenance of agriculturally relevant phenotypes. For example, fungicides are widely used to control FHB in the United States, but resistance or

tolerance to commonly applied chemicals is not widespread. One possible explanation is that wild grass communities serve as refugia from fungicide-induced selection and help maintain high levels of sensitivity in populations[39].

Looking beyond differences in mean phenotype values can yield insight into the causes of phenotypic variation. Comparisons of phenotypic and genotypic differentiation are common in some disciplines but have not been used widely in plant pathology[40,41]. In the present study, this approach revealed a higher divergence in conidia length between groups of isolates than could be readily explained by random drift in neutral genetic markers. This apparent sign of selection reinforces the assumption that conidia length is meaningful in a biological context. Differences in conidia length were also correlated with genetic distance between isolates, indicating that genetically diverse populations may have diverse phenotypes for this trait. Characterizing the phenotypes of diverse isolates, particularly when individuals appear to be genetic outliers, may reveal valuable information about the range of phenotypic values maintained within the pathogen population.

A focus on traits deemed important based on an understanding of pathogens in an agricultural context limits our ability to compare isolates from diverse hosts, which are often asymptomatic and do not accumulate the mycotoxins important to crop infection. It is possible that traits relevant to the colonization of wild hosts are yet to be discovered, so go uncaptured in routine phenotyping experiments. A more focused examination of the plant-fungal interactions that occur in wild grasses could determine what other traits are important in a non-agricultural context, and whether selection on these traits entails a trade-off in traits of agricultural interest, like mycotoxin

production or fungicide resistance.

Importantly, as others have also noted[6,7], isolates collected from wild grass hosts are capable of causing disease in crops. In addition to this, we observed perithecia formation on both agricultural and non-agricultural grass stems, indicating that wild grasses could serve as a substrate for ascospore production and contribute inoculum to disease epidemics in proximate crops. Overall, the results from this study should encourage further research on the phenotypic diversity of *F. graminearum* recovered from remote regions or non-agricultural hosts. These plants likely play a role in the evolution of *F. graminearum*, and better understanding their interactions may ultimately benefit plant disease management.

Table 5.S1. Microsatellite loci used in genotyping

Microsatellite	Primers	Reference
Multiplex 1		
HK1073	TATGATGCAGCGAATGCAAC	Suga 2004
	TAGAGACCTGGCCCATACCA	
HK913	GCAGGACCTGGATGATGAA	Suga
	ATGTGTGCAGCCATGAGATT	
HK1043	ACAGGCATCCAAGGACATTT	Suga
	GTTTGATGGCGCATTCAAAG	
msFG60	GAGCCATTCACATGTACCCC	Naef 2006
	TCCTCTCGCAAGTGTTGTTG	
Multiplex 2		
HK977	AAACGTAAACGGATCAACGG	Suga
	AGATTCGCAACTTTGTGCTG	
HK917	ATCTCCCAAGCTGGCTAATT	Suga
	AGAACCGGCAAAGTTCGATT	
HK957	TCCGAAGGTAGAAGCGTTGT	Suga
	TCAAGCCCATCTATGCTGTT	
FusSSR19	AGCCGGACATGAGACAAAGTAG	Vogelgsang 2009
	TGTTGTTCCCTCCAGTACTCG	

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EPILOGUE

Based on the findings detailed in this dissertation, there are four areas of *Fusarium graminearum* biology research that may benefit from consideration of wild grass hosts. These include (1) Epidemiology, (2) Evolution, (3) Fungus-Host Interactions, and (4) Fungus-Fungus Interactions.

EPIDEMIOLOGY

It is apparent from present and past work that *F. graminearum* is commonly found in wild grasses, that overwintering and sexual reproduction is possible, that grass-derived isolates are virulent on crops, and that grasses both near and remote from agricultural fields can support pathogen populations indistinguishable from those in crop fields. Understanding the significance of inoculum rising from wild grasses compared to that from corn stubble is required before any management practices can be justified. Field-scale modeling and estimation of spore production is already possible, having been performed with maize alone[1]. With preliminary estimates newly provided for pathogen infestation, survival, and sporulation in wild grass stems, it may be appropriate to evaluate the relative contribution of inoculum from grass debris to overall disease pressure.

While the importance of large grasslands to regional disease risk is of interest, the management of in-field grasses to reduce local inoculum production may be particularly effective as a disease control practice in regions with low agricultural acreage and low levels of regional inoculum. MacInnes and Fogelman said as much in 1923, though no experiment has been conducted to test this[2]. Cultural practices like

weed management along crop field margins could be especially useful in organic production systems, where fungicides are unavailable for disease control.

EVOLUTION

Fusarium graminearum is a diverse pathogen adapted to diverse host species. We have shown a simple example of phenotypic differentiation between isolates from wheat and grass that suggests these hosts select for different traits. Understanding what selective pressures non-cultivated grass hosts exert may help explain how agriculturally relevant phenotypes are maintained or altered, particularly those related to toxin production and fungicide resistance.

It was only recently that apparently atoxigenic isolates of *F. graminearum* were discovered to produce the novel NX-2 toxin, which is now recognized as a marker for a unique North American population of the fungus[3]. Available evidence indicates this phenotype has existed for a long period of time in the northeastern US and parts of Canada[4]. The true extent of diversity in *F. graminearum* is likely undiscovered and may be found in part by searching for remote and isolated populations associated with non-cultivated hosts. Because grass debris is left in situ, there are many opportunities for recombination to produce new mixtures of virulence traits, adding agricultural relevance to this question.

The ability of grass communities to harbor fungicide sensitive individuals is another appropriate avenue of research. A fitness cost to carrying resistance may be apparent during wild grass colonization, or the recombination of sensitive and resistant isolates may occur. The theory underpinning the latter can be described as the refugia concept.

While this was developed for diploid insect pests that are required to outcross when reproducing[5], a similar process could maintain fungicide sensitivity in *F. graminearum*. While some fungicide resistances in *F. graminearum* are attributed to single allele mutations[6,7], sensitivity to the widely used triazoles is almost definitely a polygenic trait[8]. In the case of this haploid fungus, rather than preserving homozygous and heterozygous sensitive alleles for a single locus, recombination occurring in wild grasses could slow the development of quantitative resistance by pairing a sensitivity allele at one locus with a resistance allele at another locus.

More generally, genomic studies are greatly increasing the rate at which important pathogen genes are found and monitored. A number of virulence related genes and genomic regions under selection have been identified[9,10], and some of these could be related to the maintenance of a broad host range. The inclusion of isolates collected from non-crop hosts is warranted in future genomic analyses.

PLANT-FUNGAL INTERACTIONS

The colonization of wild grass spikes does not entail the necrosis of host tissue or the toxin accumulation associated with disease in crops, and we have found significant variation in host-pathogen interactions between grass hosts. The question of whether this is a pathogenic or endophytic relationship deserves continued study. The effects of natural spike colonization on seed germination, vigor and survival should be a subject for further experiments. This would help clarify the nature of *F. graminearum* – wild grass relationships and is also an important step in determining whether increased host acreage from expanded crop cultivated could lead to adverse impacts in natural host

communities.

Perhaps more informative would be using 'omics' approaches to understand exactly how interactions in grasses differ from what is known in crops. Though a stumbling block will be the lack of genetic resources for non-cultivated grasses, the use of one non-crop organism, *Brachypodium distachyon*, is a start[11]. This relates to maybe the most exciting opportunity these grasses present, the potential discovery of novel disease resistance genes or deoxynivalenol detoxification pathways. Current breeding efforts are focused on Type I and Type II resistance, corresponding to initial infection and intraspike spread. When these terms were originally coined, three others less often discussed today were included also, Type III-V[12,13]. Type V, the detoxification of trichothecenes like DON or the ability to avoid triggering production of these compounds, may very well exist in some wild grass that shows no necrotic symptoms and does not accumulate toxin. Identifying such a mechanism could be a significant advantage for plant breeders in the continued effort to generate tolerant or resistant crop varieties.

FUNGUS-FUNGUS INTERACTIONS

Most of the intragenic ecology studies involving *F. graminearum* have been primarily concerned with toxin production during multiple pathogen infections in crop hosts. Using biomass and toxin accumulation as metrics, the findings have been mixed and shed little light on how complex, natural *Fusarium* communities interact with crops[14]. Our work on the composition of *Fusarium* communities and their relationship to environmental variability lays a foundation for further study of *Fusarium*

community dynamics. The change in these communities over time is a relevant topic in light of how climate change or trends in crop production may drive pathogen species diversity. A focus on the long-term interactions between *Fusarium* species, rather than short-term, single plant experiments is justified. Significant changes in the dominant FHB causing species have been recorded, notably in Europe in the last two decades[15]. A continued focus on within plant competition could also be directed away from cereal spikes and towards maize stalk rot. How species interact to change colonization patterns might influence *F. graminearum* overwintering and dispersal from corn debris.

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