ENHANCING YIELD AND QUALITY IN SILAGE AND GRAIN CORN THROUGH IMPROVED UNDERSTANDING OF PLANT-PATHOGEN INTERACTIONS

By

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ABSTRACT

Corn (*Zea mays* L.) in Michigan and the Great Lakes Region is prone to biotic stresses resulting in unavoidable yield and quality losses. Infections by ascomycete fungi causing ear or stalk rots and foliar diseases (such as tar spot) have become a major concern for corn growers in recent years. Ear and stalk rots in Michigan corn are primarily a result of *Fusarium graminearum* and *F. verticillioides* infections. These fungi not only impact the yield but also produce toxic compounds (mycotoxins), rendering corn unsafe for livestock consumption. Therefore, one of the major goals of this dissertation was to evaluate mycotoxin occurrence in silage corn (whole-plant corn fed to cattle) and explore integrated management strategies such as hybrid selection, fungicide application, planting date, and seeding rate. Samples from growers across Michigan revealed that deoxynivalenol, zearalenone, and fumonisins are the most commonly occurring mycotoxins in Michigan silage corn. Multi-location field trials for silage corn from 2019-2022 showed that the use of effective Bt proteins (such as Vip3A) in hybrid trait selection prevent corn ear injury due to Lepidopteran insects (primarily western bean cutworm), limiting access to ear rot causing fungi, eventually reducing mycotoxin accumulation. Fungicide application reduced ear rot incidence only at two out of eleven site-years and had limited benefit when disease incidence was driven by insect injury. Planting silage corn in late-April to early May and avoiding very high seeding rate (>100,000 seeds ha^{-1}) helped in escaping environmental conditions favorable to insect injury and fungal infections from coinciding with corn silking. Post-harvest ensiling studies showed that an infected silage corn is more prone to continued mycotoxin accumulation especially under low compaction density. Additionally, field trials were conducted for quantifying losses in forage or grain yield and nutritive value as impacted by foliar stresses (primarily tar spot). Evaluations of photosynthetic capacity showed that tar spot has an

impact that is proportionally greater than the observed disease symptoms. Variability observed in photosynthetic response to tar spot severity among current hybrids would be useful in future germplasm selection for disease resistance. Overall, this dissertation emphasizes the use of in-field integrated management such as hybrid Bt traits, disease resistance, fungicide application, optimal planting date, and seeding rate as potential solutions to minimize corn yield and quality losses due to multiple biotic stresses.

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To my grandparents. Thank you, nana daddy ji, nani ma, daddy ji and daadi ma for your blessings.

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LIST OF ABBREVIATIONS

ADF, acid detergent fiber;

Bt *Bacillus thuringiensis;*

Bt_E, Bt hybrids with protection against European corn borer;

Bt_{EW}, Bt hybrids with protection against both European corn borer and western bean cutworm;

CP, crude protein;

DM, dry matter;

DON, deoxynivalenol;

ECB, European corn borer;

ERI, ear rot incidence;

ERS, ear rot severity;

FB, Fumonisins;

GDD, Growing Degree Days;

IFI, insect feeding incidence;

IFS, insect feeding severity;

IVTD, in-vitro digestible dry matter;

LOD, Limit of Detection;

LOQ, Limit of Quantification;

NDF, neutral detergent fiber;

NDFD, NDF digestibility;

WBC, western bean cutworm;

ZON, zearalenone.

CHAPTER 1: OCCURRENCE AND ASSOCIATED AGRONOMIC FACTORS OF MYCOTOXIN CONTAMINATION IN SILAGE MAIZE IN THE GREAT LAKES REGION OF UNITED STATES

1.1 ABSTRACT

Silage maize in Michigan and the Great Lakes region is exposed to in-field ear and stalk rot fungal infections by *Fusarium spp.* which may result in production of toxic secondary metabolites called mycotoxins. These toxins can cause severe health complications in livestock but might remain unidentified as most silage maize is fed on-farm and not sold in formal markets. This study was conducted to quantify the status of mycotoxins and the agronomic management practices impacting their concentration in silage maize across Michigan farms. Samples (n= 122) were collected from across the state for three years (2019-21). Results show that 100% of the samples tested positive for deoxynivalenol (DON) at detectable levels. Other mycotoxins that occurred frequently were zearalenone (ZEN), fumonisins, enniatins, and beauvericin (BEA). Mycotoxin concentration was found to vary across regions due to differences in weather parameters such as temperature and humidity, driven partly by the proximity of some regions to the Great Lakes. Mycotoxins were also found to co-occur, with an average of 13 mycotoxins in each sample. Strong correlations were observed between DON, ZEN, and BEA ($r > 0.40$). Crop rotation and planting date explained 91 and 68% variability in DON and fumonisin, respectively. Deoxynivalenol and fumonisin concentration was 20 and 67% higher in silage maize following a host crop of *Fusarium spp*. than a non-host crop. Planting silage maize between May 10 and May 30 increased the mycotoxin concentration by at least 50% than outside this window. However, tillage did not significantly impact mycotoxin occurrence and concentration. Overall, multiple mycotoxins were found in silage maize across the region and knowledge of their presence and contributing factors can help growers develop integrated management strategies to mitigate mycotoxin accumulation.

1.2 INTRODUCTION

Silage maize (*Zea mays* L.) is harvested around 650 mg/g moisture and is a primary forage for livestock in the United States Corn Belt (Ferraretto *et al.*, 2018). It is a high energy and low protein forage, generally yielding higher than other common forages such as red clover (*Trifolium pratense* L.) and alfalfa (*Medicago sativa* L.) (Allen *et al*., 2003). Since silage maize contributes a major portion of fiber to the diet, it forms bulk of ration for livestock species such as dairy cattle. Also, it is considered as one of the most convenient forage crops as unlike other contemporary forages, it does not require multiple cuttings (Jones *et al*., 2004). Growing good quality silage maize for livestock involves a multistep decision-making process, beginning from in-field cultivation (e.g., crop rotation, hybrid selection, planting date, harvest timings) to the ensiling process (packaging densities, timings etc.). In-field agronomic management decisions determine the exposure of the crop to biotic and abiotic stresses and how well it can cope with them (Médiène *et al*., 2011).

One of the major biotic stresses that impacts maize quality in the Great Lakes region of North America is infection caused by ear and stalk rot fungi which result in production of toxic secondary metabolites (mycotoxins). The most commonly occurring ear rots in this region are pink ear rot, caused by *Giberrella zeae* (Schweinitz) Petch (teleomorph or sexual stage of *F. graminearum* Schwabe) and white or Fusarium ear rot caused by *F. verticilloides* (Saccardo) Nirenberg (Munkvold, 2003). Maize ear rot infections caused by *F. graminearum* generally occur under cool and humid conditions whereas *F. verticilloides* prefer warm and dry conditions for infection (Munkvold, 2003). The most common mycotoxins produced due to these infections are deoxynivalenol (DON), zearalenone (ZEN) produced by *F. graminearum*, and fumonisin produced by *F. verticilloides* (Reid *et al*., 1999). These toxins enhance the survival of fungi in the host plant as they prevent the fungal spore melanin degradation and also act as virulent factors which are crucial for pathogenesis in host plant (Venkatesh and Keller, 2019; Bennet and Klich, 2003; Ogunade *et al*., 2018). Mycotoxin production generally begins in the field and may continue in storage for silage maize, especially during the aerobic phase of ensiling (Xia *et al*., 2023). This makes field management of silage maize critical for minimizing mycotoxin production and accumulation.

Elevated concentrations of mycotoxins make silage maize unfit for animal consumption (Munkvold *et al*., 2019). For instance, DON causes feed refusals, vomiting, ketosis, reduced growth, and reduced milk production. Contamination by ZEN results in, hormonal imbalance causing issues with fertility premature udder development, swollen vulvas, and general oedema. Fumonisin accumulation could cause kidney failure and liver inflammation (Munkvold *et al*., 2019). Other emerging mycotoxins such as beauvericin (BEA), moniliformin (MON), and ennaitins have been found to cause endocrine disruptions, cytotoxic and genotoxic complications in monogastric animals while the data on ruminants is limited (Kˇrížová *et al*., 2021). In the United States, there are currently no established mycotoxin thresholds in silage maize. The guidelines provided for animal feed by both the U.S. Food and Drug Administration and European Union are either for grain maize or for maize feed and byproducts without providing a distinct threshold for silage maize (USDA 2023; Pinotti *et al.,* 2016). Nonetheless, research and reports compiled by Adams *et al*. (1993) and Goeser (2015) indicated that health issues tended to be more frequent and severe in dairy cattle when the concentration of DON, ZEN, or fumonisins exceeded 1.0, 0.4, and 2.0 µg/g, respectively when silage maize constitutes 50% of the total diet. These thresholds are mycotoxin concentrations above which the dairy cattle fed should be kept under observation and checked often to note any signs of performance decline. The feed should be limited or stopped at any sign of reduced performance in cattle. In general, dairy cattle can detoxify mycotoxins to some extent due to their rumen microbiota; however, the end products may be less or more toxic than the parent toxin depending on its nature (Fink-Gremmels, 2008). Moreover, mycotoxins do not occur in isolation and are often found to co-occur in plant biomass, making it difficult to understand their effect on livestock health. It also creates a concern about whether the impact will be additive, antagonistic, or synergistic (Alassane-Kpembi *et al.*, 2017; Assunção *et al.*, 2016).

Field management decisions, such as crop rotation, tillage, hybrid selection, planting date, irrigation, pesticide application, and harvest timing play an important role in infections causing ear rot disease and mycotoxins (Hooker and Schaafsma, 2005; Eli *et al.*, 2022). For instance, residues of host crops such as maize, wheat (*Triticum aestivum* L.), and other cereals are major sources of inoculum, critical in epidemics caused by various *Fusarium* species (Sutton, 1982). However, Miller *et al*. (1998) and Schaafsma *et al*. (2001) showed that amount of residue did not impact the mycotoxin concentration in the following crop, even under favourable environmental conditions. It is speculated that the type of residue from the previous crop may be of more importance than the amount of residue in the field (Hooker and Schaafsma, 2005).

Studies by Parsons and Munkvold (2012) showed that planting date influenced mycotoxin accumulation in grain maize, as it impacts crop phenological development and determines the growth stage that will be exposed to an environment favourable to fungal infections. Besides that, husk wounds caused by ear damaging insects, such as corn earworm (*Helicoverpa zea* Bod.), European corn borer (*Ostrinia nubilalis* Hub.), fall armyworm (*Spodoptera frugiperda* Smith), and western bean cutworm (*Striacosta albicosta* Smith), facilitates the entry of ear rot fungi into the maize (Parker *et al*., 2017; Smith *et al*., 2018; Farhan *et al.*, 2020). Therefore, spraying insecticides or planting hybrids expressing Bt toxins – both of which reduce insect damage, can play a critical role in reducing fungal infections and mycotoxins in both grain and silage maize (Kaur *et al.*, 2023; Singh *et al.*, 2023). Mycotoxin accumulation in fields is often variable and regional due to surrounding weather conditions, stresses, and agronomic management as well as various mycotoxin producing fungal species favoring different environmental conditions (Sutton, 1982, Reid *et al.*, 1999; Munkvold, 2003).

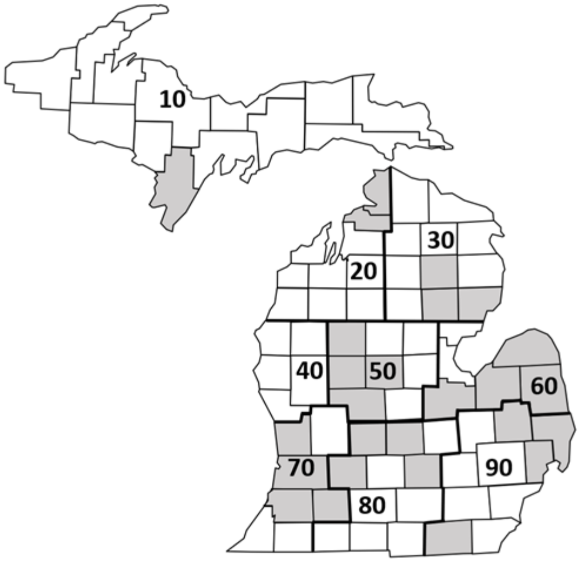
Silage maize is rarely sold in local markets; it is mostly fed on-farm rather than going to commercial facilities where mycotoxin testing is routine. Furthermore, easy 'quick-tests' are not available for growers to regularly do their own testing on-farm. Most contamination therefore goes unidentified. Livestock and silage maize producers would benefit from knowing the status of the mycotoxins in their area and the factors contributing to their occurrence. Most of the literature available on mycotoxin concentrations in the Great Lakes region is from small-plot trials at research locations and mostly focuses on grain maize and wheat with little information on silage maize. Also, the on-farm mycotoxin survey studies conducted in the United States had focused mostly on DON and Fumonisins, resulting in a lack of information on other modified and emerging mycotoxins (Eli *et al.,* 2022). Moreover, the weather conditions and variations observed in the Great Lakes region are unique due to the proximity to freshwater lakes. Therefore, this study was conducted with objectives of (i) developing a database for occurrence of various mycotoxins (regulated, modified, and emerging) in silage maize across Michigan farms by collecting silage maize samples from grower fields and (ii) quantifying the impact of agronomic factors on mycotoxin concentration.

1.3 MATERIALS AND METHODS

1.3.1 Sample Collection and Testing

Samples from silage fields across Michigan were collected for three years (2019, 2020, and 2021), by growers and extension educators. In the summer prior to silage harvest, a one-page request with instructions for silage collection was advertised through field days, meetings, and other extension venues. Cooperators were asked to collect and air-dry a one-pound fresh silage sample from each field (collected at harvest with target moisture of 650 mg/g). It was recommended to collect combine samples from at least five random spots in the field, mix them and draw one pound of composite sample out of the pile. After air-drying, samples were shipped or driven to the Michigan State University Agronomy Laboratory in East Lansing, MI. Accompanying each silage sample, growers submitted a survey sheet with information on field history and current-season management practices. Field history questions asked about three-year crop rotation, cover crops, tillage practices, irrigation, drainage, and previous mycotoxin issues. Management questions focused on planting date, hybrid, harvest date and moisture, pesticide applications, and any pest issues (e.g., ear-feeding insects, ear or stalk rots).

The counties from which samples were collected were grouped into regions based on United States Department of Agriculture crop reporting zones (Figure 1). These zones are based on differences in geography (soil type, terrain, elevation), climate (mean temperature, annual precipitation, growing season length), and cropping practices.



Crop reporting zone	Regions
Zone 10	Upper Peninsula
Zone 20	North-west Michigan
Zone 30	North-east Michigan
Zone 40	Western Michigan
Zone 50	Central Michigan
Zone 60	East Michigan
Zone 70	South-west Michigan
Zone 80	South-central Michigan
Zone 90	South-east Michigan

Figure 1. Michigan counties from which silage samples were submitted for analysis (shaded) in 2019-2021. Counties were grouped into zones (separated by bold lines) based on United States Department of Agriculture National Agricultural Statistics Service (USDA-NASS) crop reporting zones. (www.nass.usda.gov/Charts_and_Maps/Crops_County/boundary_maps/indexgif.php)

Once received, samples were dried in a hot-air dryer at 60°C for 72 hours (Eckard *et al*., 2011). Dried samples were ground to 1 mm sieve size and shipped to the University of Guelph Ridgetown Campus (Ontario, Canada) for mycotoxin detection and quantification. Mycotoxins were analysed using an Ionics EP 10+ modified API365 triple quadrupole mass spectrometer (LC-MS/MS) system equipped with an electrospray ionization source, in positive and negative polarity using protocol by Limay-Rios and Schaafsma (2021). Briefly, specific parameters for each mycotoxin were derived by directly infusing the analytical standard (6 nanograms per microliter in a solution of eluent A/B in a 50:50 ratio by volume) into the ESI-MS/MS system. This infusion was performed at a rate of 10 microliters per minute using a Fusion 100 infusion pump (Chemyx Inc, Stafford, TX) equipped with a 500-microliter syringe (Gastight 1750, Hamilton, Reno, NV). Every compound was analysed in either positive or negative ion polarity mode employing a multiple-reaction monitoring technique having one precursor ion and two product ions. The quantification relied on the most prominent peak in two product ions (quantifier ion), while the second peak served for confirmation (qualifier ion). Samples were tested for 26 different mycotoxins (Table 1). Cooperators were provided with a detailed mycotoxin report based on their sample to keep them informed and encourage them to participate again the following season.

1.3.2 Data analyses

Mycotoxin data was interpreted as percentage of samples testing positive and the number and frequency of mycotoxins. Deoxynivalenol, ZEN, and fumonisin levels were compared with the levels previously reported by Adams et al. (1993) and Goeser (2015) as increasing health issues in dairy cattle. To evaluate the occurrence of mycotoxins in specific zones across the state, data obtained from coordinates of all collected samples were interpolated using kriging in R studio. The R packages used for this analysis were sp, rgdal, splancs, maps, gstats, and RColorBrewer (RStudio Team, 2020).

To quantify the co-occurrence of mycotoxins, Pearson correlation co-efficient (r) for various toxins were obtained using PROC CORR in SAS 9.4 software (SAS Institute Inc.). Data were also analysed using PROC GLM with 0.10 as level of significance to determine the impact of agronomic factor on mycotoxin concentration across years. Additionally, PROC LOGISTIC was used for estimating Nagelkerke's R-squared values in order to determine the percent variability in mycotoxin concentration associated with various agronomic factors. Logistic regression is a multivariate nonlinear statistical analysis procedure used for categorical data variables in a non-normally distributed data set (Jiang *et al*., 2019). Since Zone 60 and Zone 90 contributed about 55% of the samples, they were analysed separately as well. Field history and management details for each sample were categorized into multiple classes for analyses. Categories were as follows: Planting date as early (plantings from April 20 to May 10), mid-

(from May 11 to May 30), and late season (from May 31 to June 20); crop rotation with previous crop as a host (maize or another host crop for *Fusarium spp*.) or a non-host; tillage as tilled (before planting) or no-till.

1.4 RESULTS AND DISCUSSION

1.4.1 Mycotoxins in silage maize

A total of 122 silage maize samples were submitted from 20 counties in Michigan over three seasons (n = 34 in 2019, 51 in 2020, and 37 in 2021). About 70% of the cooperating growers reported no history or knowledge of mycotoxin occurrence in their fields prior to this survey. However, all samples (n = 122) obtained from the grower fields tested positive for at least one mycotoxin at detectable concentrations, with an average concentration of 1.25 µg/g DON. This indicated that the mycotoxin issue had probably stayed unidentified in these silage maize fields due to lack of testing.

Deoxynivalenol was detected in all samples with an average of 1.39 µg/g. Other toxins that occurred in most samples (>95%) were enniatins (0.03 µg/g) and BEA (0.34 µg/g), although their concentrations were very low (< 1 µg/g) (Table 1). At least 60% samples in 2021 and 50% in 2019 had DON concentrations greater than 1 µg/g (concentration above which health impact on dairy cattle is severe), whereas in 2020 only 12% of samples had DON concentration above this level (Table 2). Other frequently occurring mycotoxins were ZEN, fumonisins, and MON with an average value of 0.12 µg/g, 0.21 µg/g, and 0.02 µg/g, respectively. However, only 26% samples were reported to have ZEN higher than 0.4 µg/g (concentration above which health impact on dairy cattle is severe) in 2019 while fumonisins were greater than 2 µg/g (concentration above which health impact on dairy cattle is severe) in 5% and 16% of samples only in 2019 and 2020, respectively.

Aflatoxins were not detected in any of the samples (Table 1), as the environmental conditions in the Great Lakes region are typically not conducive to the growth of *Aspergillus flavus*, which proliferates under hot and dry conditions (Payne and Widstrom, 1992). However, as temperatures rise and the probability of drought increases in the future with climate change, aflatoxins may become a problem in coming decades (Wu *et al*., 2011).

Table 1. Mean and maximum mycotoxin concentrations (µg/g), standard error of the mean (± 1), and percentage of positive silage maize samples collected across Michigan farms from 2019-21.

Mycotoxin	**Mean (µg/g)**	**Maximum (µg/g)**	**Standard Error (µg/g)**	**Samples positive (%)**	**Thresholds for dairy cattle (µg/g)[1]**	**LOD[3] (µg/kg)**	**LOQ[4] (µg/kg)**
Deoxynivalenol	1.40	18.47	0.20922	100.0	1.00	71.9	196.5
3-Acetyl-Deoxynivalenol	0.13	1.31	0.02096	71.9	nd[2]	6.5	15.9
15-Acetyl-Deoxynivalenol	0.17	1.59	0.02391	61.2	nd	31.2	59.3
Deoxynivalenol 3-β-D-glucoside	0.06	2.45	0.02920	15.7	nd	32.8	68.4
Culmorin	0.05	0.54	0.00950	50.4	nd	6.1	12.0
Zearalenone	0.12	2.69	0.02899	62.8	0.40	8.9	13.3
T-2 toxin	0.02	0.40	0.00530	53.7	0.10	1.1	2.6
HT-2 toxin	0.09	0.69	0.01157	75.2	2.00	11.9	25.4
Diacetoxyscirpenol	0.00	0.01	0.00019	21.5	1.00	1.1	2.5
Fumonisin B1	0.43	10.67	0.12819	71.1	2.00	1.5	3.5
Fumonisin B2	0.11	3.05	0.03408	91.7	nd	1.0	2.1
Fumonisin B3	0.13	3.01	0.03367	82.6	nd	1.5	3.4
Moniliformin	0.03	0.33	0.00450	71.9	0.10	1.2	2.2
Enniatin A	0.02	0.18	0.00263	97.6	nd	0.0	0.1
Enniatin A1	0.03	0.80	0.00876	98.3	nd	0.0	0.0
Enniatin B	0.05	1.86	0.01670	99.2	nd	0.0	0.0
Enniatin B1	0.03	1.88	0.01673	95.9	nd	0.0	0.0
Beauvericin	0.34	5.81	0.07485	99.2	nd	0.2	0.4
Roquefortine C	0.01	0.27	0.00256	59.5	nd	0.3	0.7
Penitrem A	0.00	0.02	0.00015	1.7	nd	1.5	3.5
Alternariolmethylether	0.00	0.08	0.00073	66.1	nd	2.3	4.6
Alternariol	0.01	0.18	0.00302	23.1	nd	8.8	11.9
Neosolaniol	0.00	0.03	0.00041	21.5	nd	0.7	1.7
Sterigmatocystin	0.00	0.002	0.00003	21.5	nd	0.2	0.5
Aflatoxin B1	0.00	0.00	0.00000	0.0	0.002		

Table 1 (cont'd)
[1] These thresholds are mycotoxin concentrations beyond which the dairy cattle fed should be kept under observation and checked often to note any signs of performance decline based on reports by Adams *et al.*, 1993 and Goeser 2015. The feed should be limited or stopped at any sign of reduced performance in cattle.
[2] (Not defined). These mycotoxins do not have severe concentrations defined for dairy cattle.
[3]LOD: Limit of detection.
[4]LOQ: Limit of Quantification.

Table 2. Percent of silage maize samples positive (out of n=122) with mycotoxins concentrations.

Toxin	2019	2020	2021
Presence of >1 co-occurring mycotoxins	100	100	100
Presence of >10 co-occurring mycotoxins	100	92	96
Deoxynivalenol, detectable[1]	100	100	100
Deoxynivalenol, >1 µg/g[2]	50	12	60
Zearalenone, detectable	100	35	100
Zearalenone, >0.4 µg/g	26	0	0
Fumonisins[3], detectable	95	96	100
Fumonisins, >2 µg/g	5	16	0
Moniliformin, detectable	62	56	100
Moniliformin >0.1 µg/g	0	0	3
Enniatins[4], detectable	100	100	100
Enniatins, >1 µg/g	0	3.92	2.94
Beauvericin, detectable	100	100	97.3
Beauvericin, >1 µg/g	5.88	13.7	0

[1] Detectable refers to the minimum concentration at which mycotoxins are detected.
[2] These thresholds are mycotoxin concentrations beyond which the dairy cattle fed should be kept under observation and checked often to note any signs of performance decline based on reports by Adams *et al.*, 1993 and Goeser 2015. The feed should be limited or stopped at any sign of reduced performance in cattle.
[3]Fumonisin refers to all its derivatives (B_1, B_2, and B_3).
[4]Enniatins include all its derivatives (A, A_1, B, B_1).

Mycotoxin concentration and occurrence in grower samples was variable across years probably due to differences in average daily temperatures and precipitation, especially around the time of maize silking (Table 3). In 2020, the growing season was drier, and rainfall was more sporadic (only 24.2 mm average rainfall across the state) during maize silking compared to 2019 (33.2 mm) and 2021 (60 mm), resulting in lower frequency and concentration of mycotoxins.

Also, the highest DON and ZEN concentration detected among all samples was lower in 2020 (1.4 and 0.07 µg/g) compared to 2019 (5.7 and 2.5 µg/g) and 2021 (18.4 and 0.23 µg/g, respectively). Humid conditions around silking time when maize is in most susceptible stage is optimal for *F. graminearum* infection (Sutton, 1982; Munkvold, 2003), leading to greater accumulation of DON and ZEN in 2019 and 2021 than 2020. The only toxin that occurred in higher concentration in 2020 than in 2019 and 2021 was fumonisin (highest concentration of 10.6 µg/g). This is probably because the fungus *F. verticilloides* which is responsible for fumonisin production is favored when the environment is warm and dry around silking (Munkvold 2003).

Table 3. Mean temperature, total precipitation, and relative humidity during the silking window (+/- 5 days around silking) in crop reporting zones[2] across Michigan.

Crop reporting zone [1]	Temperature (°C)[2]			Total Precipitation (mm) [2]			Relative Humidity (%)[2]		
	2019	2020	2021	2019	2020	2021	2019	2020	2021
Zone 10	20.2	19.8	20.1	13.7	1.0	26.4	77.2	71.4	81.1
Zone 20	20.1	20.5	20.7	48.1	6.6	99.8	70.0	73.7	76.7
Zone 30	19.0	20.7	19.8	44.9	40.9	76.5	69.3	69.1	71.3
Zone 40	21.7	21.5	21.6	38.4	32.3	33.8	72.8	68.1	71.6
Zone 50	21.0	22.4	21.7	12.0	19.2	39.0	70.6	70.2	70.7
Zone 60	20.8	20.7	20.7	46.3	56.9	33.8	72.5	66.2	70.5
Zone 70	22.4	22.6	23.0	27.7	20.6	58.9	70.1	69.2	70.4
Zone 80	22.2	23.3	21.7	38.1	16.4	95.8	70.7	68.1	73.5
Zone 90	22.2	24.3	21.5	29.7	24.1	67.6	67.1	65.3	68.5

[1] Crop reporting zones as defined by United States Department of Agriculture National Agricultural Statistics Service (USDA-NASS).
[2] Temperature, precipitation and relative humidity in a crop reporting zone were determined by averaging data collected from all weather stations in that zone (established by the Michigan State University Enviro-weather https://enviroweather.msu.edu/).

1.4.2 Co-occurrence of mycotoxins

The occurrence of more than one mycotoxin was observed in all samples with significant Pearson co-efficient across all the years (Figure 2). At least seven mycotoxins occurred in every sample. By year, co-occurring toxins averaged 15, 7, and 10 per sample in 2019, 2020, and 2021, respectively. The highest number of toxins in an individual sample was 13 in 2021 and 20 in 2020, but co-occurrence was most pronounced in 2019 with a maximum of 24 mycotoxins detected in a single sample. Co-occurrence for mycotoxins was also reported by Weaver *et al*., (2021) and Fusilier *et al*., (2022) in grain maize in the US and by Vandicke *et al*., (2019) in silage maize in Belgium.

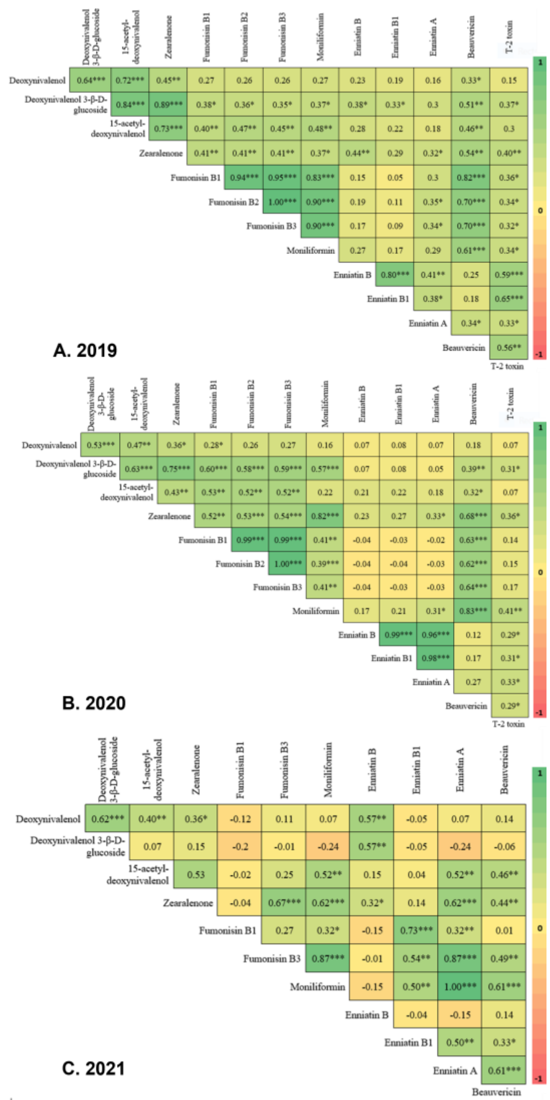


Figure 2. Pearson correlations for mycotoxin concentrations in silage maize samples collected in Michigan from 2019 to 2021. Color gradient represents a change in Pearson co-efficient from negative (red) to positive (green). * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.0001.

Strong correlations were observed between DON, deoxynivalenol 3-β-D-glucoside (D3G) and 15-acetyl-deoxynivalenol (ADON-15) with r = 0.64 and 0.72; 0.53 and 0.47; 0.62 and 0.40 respectively in 2019, 2020, and 2021 (Figure 2). This is probably because these mycotoxins are derivatives of DON. Furthermore, DON was also found to be significantly correlated with ZEN in all three years and with BEA in 2019 indicating that the same ear rot fungi (*F. graminearum*) is responsible for production of these toxins.

Fumonisins (FB1, FB2, and FB3) were only weakly correlated with DON and ZEN in all three years, primarily due to the different fungal species contributing to their production and also their preference for different environmental conditions. Other set of mycotoxins that were found to be strongly correlated were fumonisins (FB_1, FB_2, and FB_3), MON and BEA which is probably because these toxins are produced by closely related *F. verticillioides*, and *F. moniliforme*. Beauvericin was also correlated with ZEN likely because the former can be produced by multiple ear rot fungi. Similar observations were also reported by Gallo *et al.* (2021) in maize grain.

Co-occurrence of mycotoxins can be leveraged in developing low-cost mycotoxin testing for silage maize, by using presence of one mycotoxin as an indicator for existence and/or concentration levels of other mycotoxins. However, an extensive database across a wider geographical area will be needed for validation and commercial deployment of such technologies.

1.4.3 Mycotoxin variability across zones

There was a trend in mycotoxin detection across the state (Figure 3), Zones 60 and 90 had the highest occurrence and concentration, followed by Zones 70 and 80. The average concentration of DON was 1.68, 1.58, 1.36, and 0.35 µg/g in samples collected from Zones 60, 90, 70, and 80, respectively (Figure 3A). High DON concentration in Zone 60 and Zone 90 was likely due to cool and humid weather around maize silking (Table 3), and consistently higher ear-feeding insect injury (primarily WBC) in these zones as reported by Kaur *et al.* (2023). This may have contributed to ear wounds, resulting in more ear rot infections, and eventually higher mycotoxin concentrations. Weather data during the silking window also shows that Zone 60 received more than 120 mm of rainfall in 2021 and 2019 while it was more than 50 mm in 2020 (Table 3), resulting in a favorable environment leading to higher ear rot infections and mycotoxins as compared to other zones. Moreover, Zones 60 and 70 lie near the coast of Lake Huron and Lake Michigan (two of the Great Lakes), respectively, which results in relatively higher humidity and moderate temperatures. This provides a highly favorable environment for fungal infections and continued mycotoxin accumulation. We anticipate that other silage maize production regions with similar topographical features as the Great Lakes region might also experience higher mycotoxin accumulations.

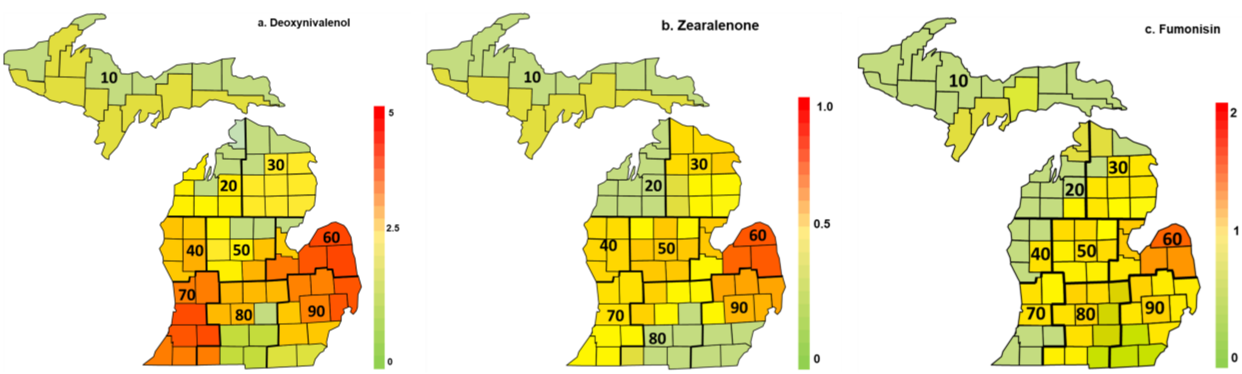


Figure 3. Average mycotoxin concentrations from silage maize samples collected in Michigan from 2019 to 2021, by crop reporting zones (as defined by United States Department of Agriculture National Agricultural Statistics Service). Colour gradient represents average concentrations from lower (green) to higher (red).

Fumonisin and ZEN were lower both in frequency and concentration across the state except Zone 80, where more than 96% samples tested positive for Fumonisin with an average concentration of 1.16 μg/g (Figure 3B and 3C). This is primarily due to drier conditions observed in Zone 80 around silking (precipitation less than 40 mm). Highest frequency of positive fumonisins samples was observed in Zone 60, however average concentration was less than 0.5 μg/g across the state (Figure 3C).

1.4.4 Impact of agronomic factors on mycotoxins

Our study showed that mycotoxin concentration in collected samples was impacted by field history and management decisions during the growing season. The multivariate nonlinear logistic regression showed that crop rotation and planting date were the most influential agronomic factors, collectively explaining 91% variability in DON and 68% in Fumonisin (Table 4). However, tillage explained only one percent variability in DON and three percent in Fumonisin. For ZEN, the variability explained by crop rotation, planting date, and tillage was collectively less than one percent (Table 4). Furthermore, it showed that skewness in factor responses resulted in regression estimates explaining 5% or less variability in DON, ZEN, and Fumonisins due to hybrid selection and pesticide application. The variability due to seeding rate, drainage and harvest moisture could not be determined. The low Nagelkerke's R-squared values on these agronomic practices (hybrid selection, seeding rate, pesticide, irrigation, field drainage, and harvest moisture) is primarily because more than 95% of the samples were collected from fields with same magnitude of these factors. For example, most of the fields where sampling occurred used a Bt hybrid with no pesticide application, were non-irrigated, well drained, and harvested around 650 mg/g moisture. Studies by Eli *et al*. (2022) and Abbas *et al*. (2012) also showed variable impact of population density on mycotoxins in grain maize. Irrigation and drainage did not show any impact on mycotoxin accumulation (Abbas *et al*., 2012). However, use of disease resistant and Bt hybrids reduced insect injury, disease, and mycotoxins (Parsons and Munkvold, 2010; Eli *et al*. 2022; Singh *et al*. 2023).

Table 4. P-values and Nagelkerke's R-squared estimates for multivariate nonlinear regression analysis on crop rotation, Planting Date, and Tillage to explain the extent variations in DON (Deoxynivalenol), ZEN (Zearalenone), and Fumonisin concentrations in the maize silage samples collected.

Agronomic Factor	DON		ZEN		Fumonisin	
	p-value	Nagelkerke's R-squared	p-value	Nagelkerke's R-squared	p-value	Nagelkerke's R-squared
Crop Rotation	0.07	0.42	0.21	0.003	0.04	0.37
Planting Date	0.03	0.49	0.42	<0.001	0.08	0.31
Tillage	0.23	0.01	0.38	<0.001	0.20	0.03

Since crop rotation and planting date had the highest influence on mycotoxin concentration (Table 4), they were explored in further detail. Silage maize that followed a *Fusarium* host crop [maize, wheat (*Triticum aestivum* L.), or barley (*Hordeum vulgare* L.)] had 20 and 67% higher DON and fumonisins, respectively, than when it followed non-host crops such as alfalfa, red clover, soybean (*Glycine max* L.), dry beans (*Phaseolus vulgaris* L.) or sugar beets (*Beta vulgaris* L.) (Figure 4). Zearalenone did not differ significantly between the host and non-host crops, however, fields with a history of host crops showed a numerical trend towards higher ZEN concentration as compared to non-host crops (p-value = 0.21).

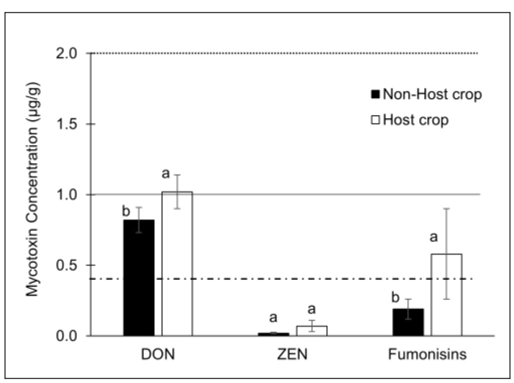


Figure 4. Impact of crop history on DON (deoxynivalenol), ZEN (zearalenone), and Fumonisin concentration in silage maize samples collected in Michigan from 2019 to 2021. The horizontal dashed line, bold line, and dotted line represent the DON, ZEN, and Fumonisin concentration beyond which the dairy cattle fed should be kept under observation and checked often to note

Figure 4 (cont'd)
any signs of performance decline and the feed should be limited or stopped at any sign of reduced performance in cattle. Bars with same letter do not differ from each other significantly (p-value < 0.10). Vertical lines on each bar represent ± 1 standard error.

Zone 60 and Zone 90 (regions with highest number of samples) showed similar trends between fields rotated with host and non-host crops (Table 5). Fumonisin accumulation was 80-85% higher in the fields for Zones 60 and 90 with silage maize following a host crop compared to a non-host crop. For DON, higher concentration was observed in fields with a history of host crops than in fields with non-host crops only in Zone 60. Hooker *et al*. (2005) and Munkvold (2014) also reported a higher mycotoxin concentration in fields where grain maize follows another host crop as *Fusarium* overwinters in plant debris. Our data indicate that silage maize growers could prevent fungal infections and benefit from planting their crop in fields where the previous crop was not a *Fusarium* host.

Table 5. Crop history impact on DON (deoxynivalenol), ZEN (zearalenone), and Fumonisins concentration in Zone 60 (East Michigan) and Zone 90 (South-east Michigan).

Crop Reporting Zones[1]	Crop history	DON	ZEN	Fumonisins
Zone 60	Non-host	0.94 (±0.21) b [2]	0.01 (±0.004) a	0.19 (±0.09) b
	Host	1.85 (±0.58) a	0.15 (±0.11) a	1.29 (±0.94) a
	p-value	0.04	0.23	0.06
Zone 90	Non-host	1.04 (±0.14) a	0.02 (±0.01) a	0.05 (±0.03) b
	Host	0.87 (±0.28) a	0.09 (±0.09) a	0.31 (±0.11) a
	p-value	0.12	0.34	0.07

[1] Crop reporting zones as defined by United States Department of Agriculture National Agricultural Statistics Service (USDA-NASS).
[2] Letters with same letter do not differ from each other significantly within a zone (p-value < 0.10). Values in parenthesis represent ± 1 standard error.

The other factor that had the highest impact on mycotoxin concentration was the planting date (Figure 5). Mid-season (between May 10 and May) planted silage maize had 69 and 65% higher DON concentration than early season (before May10 planting) and late season (after May 30) plantings. Fumonisin concentration was the lowest for late season planting but was not different for early and mid-season planting. Samples from Zones 60 and 90 also had the highest mycotoxin concentration for mid-season planting as compared to early and late season planting (Table 6). Mid-season planting in these zones also resulted in at least 30% higher DON concentration than planting outside of this window.

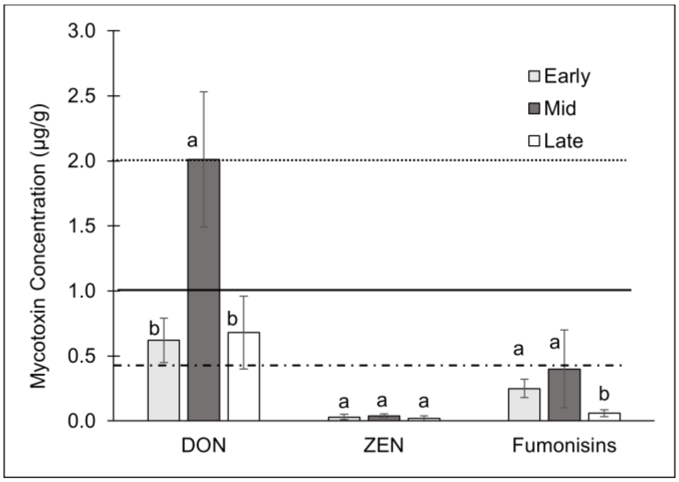


Figure 5. Impact of planting date on DON (deoxynivalenol), ZEN (zearalenone), and Fumonisins levels in silage maize samples collected in Michigan from 2019 to 2021. The horizontal dashed line, bold line, and dotted line represent the DON, ZEN, and Fumonisin beyond which the dairy cattle fed should be kept under observation and checked often to note any signs of performance decline and the feed should be limited or stopped at any sign of reduced performance in cattle. Bars with same letter do not differ from each other significantly (p-value < 0.10). Vertical lines on each bar represent ± 1 standard error.

Table 6. Planting date impact on DON (deoxynivalenol), ZEN (zearalenone), and Fumonisins levels in Zone 60 (East Michigan) and Zone 90 (South-east Michigan).

Crop Reporting Zone[1]	Planting Date	DON	ZEN	Fumonisin
	Early	0.78 (±0.31) b [2]	0.03 (±0.004) a	0.27 (±0.12) b
Zone 60	Mid	1.62 (±0.38) a	0.09 (±0.03) a	0.87 (±0.81) a
	Late	0.17 (±0.08) c	0.02 (±0.001) a	0.09 (±0.006) b
	p-value	<0.0001	0.82	0.02
	Early	0.80 (±0.11) b	0.002 (±0.0008) a	0.63 (±0.33) a
Zone 90	Mid	1.06 (±0.30) a	0.01 (±0.002) a	0.71 (±0.005) a
	Late	0.81 (±0.18) b	0.04 (±0.007) a	0.18 (±0.11) b
	p-value	0.04	0.25	0.07

[1] Crop reporting zones as defined by United States Department of Agriculture National Agricultural Statistics Service (USDA-NASS).
[2] Letters with same letter do not differ from each other significantly within a zone (p-value < 0.10). Values in parenthesis represent ± 1 standard error.

Planting time determines the silking window of maize plant, with delay in planting leading to later silking time. This eventually will affect the environmental conditions that the crop will be exposed to during its most susceptible stage to *Fusarium* infections. Generally, the mid-season planted maize silks during late July and early August in Michigan. Average temperature and precipitation during this period are 25.5°C and 83.1 mm, respectively, potentially exposing the plants to events of high humidity (>75%) and resulting in higher mycotoxin production. Similar observations were reported in grain maize by Eli *et al*. (2022) in Ontario, Canada. Research by Damianidis *et al*. (2018) in Alabama, United States also showed that the exposure of silage maize around silking to conditions optimum for *Aspergillus* resulted in higher infections and aflatoxin levels. Our data suggests that Michigan growers can mitigate mycotoxin issues in their fields by prioritizing the planting of silage maize early in the growing season. Further research on optimal planting time for silage maize is warranted due to vast latitudinal differences in growing regions across Michigan and the Great Lakes region.

Tillage did not impact the mycotoxin concentration significantly in this study. However, DON and fumonisin concentration showed a numerical decline (p-value = 0.23, 0.20, respectively) in fields with conventional tillage (0.81 and 0.15 µg/g, respectively) as compared to no-tillage (0.96 and 0.40 µg/g, respectively). This lack of significant impact of tillage on mycotoxin concentration was also observed in grain maize by Hooker and Schaafsma, (2005). On the contrary, Pfordt *et al*. (2020) reported reductions in *Fusarium* infections for grain maize tilled fields in Germany.

Overall, data from our and other studies suggest that the response of fungal infection and variability in mycotoxin accumulation to agronomic factors is highly influenced by the surrounding environment, necessitating continued research on this issue at regional level.

Additionally, the low regression values obtained in the multivariate analysis for most agronomic factors emphasizes the importance of a more comprehensive sampling to ensure representative dataset of responses in each category for the agronomic factors.

1.5 CONCLUSION

This study aimed at creating a database for mycotoxins present in silage maize. We found that mycotoxins occurred frequently with 100% of samples having at least one mycotoxin. Mycotoxin concentration varied across regions due to differences in humidity, rainfall, and temperature conditions, driven partly by the proximity of certain regions to the Great Lakes. Multiple mycotoxins were found in all the samples, emphasizing the importance of grower awareness of this issue in silage maize and need for further research into their additive impacts on animal health. Crop rotation and planting date explained 91 and 68% variability in DON and fumonisin, respectively, in the samples collected in this study. Therefore, field management decisions such as crop rotation with non-host crops and timely planting can lower mycotoxin concentration in silage maize. However, in order to quantify the impact of other agronomic decisions on mycotoxins, future research with more comprehensive field sampling is required. Overall, our study shows that it is critical to develop a comprehensive understanding of mycotoxin occurrence in relation to field-specific environmental conditions and management decision to mitigate mycotoxin accumulation in silage maize.

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CHAPTER 2: HYBRID INSECT PROTECTION AND FUNGICIDE APPLICATION FOR MANAGING EAR ROTS AND MYCOTOXINS IN SILAGE CORN

2.1 ABSTRACT

Mycotoxins in silage corn (*Zea mays* L.) accumulate due to ear rot infections by certain ascomycete fungi and cause health issues in livestock. Fungal infections intensify with increase in ear-damaging insect injury and favorable environment in the US Great Lakes region. Therefore, evaluating strategies such as hybrid insect protection and fungicide decisions to manage insects, ear rots, mycotoxin, and silage quality is crucial. Field trials were conducted in randomized complete block design at 11 Michigan site-years across 2019-2021 using three hybrid insect protection levels under two levels of prothioconazole fungicide (treated and non-treated). Hybrids (two per level) included: conventional (non-Bt); Bt with Cry1F and Cry1Ab (Bt_{E}), protection only against European Corn Borer (ECB); and Bt with Cry1F and Vip3A (Bt_{EW}), protection against both ECB and Western Bean Cutworm (WBC). Insect injury and ear rot index were 80-90% and 75-90% lower, respectively, in Bt_{EW} hybrids than non-Bt hybrids. Strong correlation between insect injury and ear rot severity was observed when insect pressure was high. Mycotoxin levels, particularly deoxynivalenol (DON), was also lowest in Bt_{EW} hybrids. Weak correlations were observed between ear injury and mycotoxin concentration. Under low disease pressure, a 50-70% reduction was observed in fungicide treated plots for both ear rot incidence and DON concentration. However, fungicide application did not reduce ear rot infections driven by high insect injury. Treatments had negligible impact on forage quality. Overall, results indicated that incorporating insect protection in hybrid selection decisions reduced insect feeding, disease occurrence, and mycotoxins, especially under high insect pressure.

2.2 INTRODUCTION

Ear infections in corn (*Zea mays* L.) caused by ascomycete fungi, for example *Fusarium graminearum*, *Fusarium verticillioides*, *Aspergillus flavus*, *Stenocarpella maydis*, *Trichoderma viride,* and *Penicillium* spp., are an important issue for North American corn growers. In the Great Lakes region of United States, *F. graminearum* (cause Gibberella ear rot), and *F. verticillioides* (cause Fusarium ear rot), are particularly common. Gibberella ear rot infections result in pink to reddish-colored molds on ears and kernels, typically initiating from tip of the ear and spreading downward whereas Fusarium ear rot appears as a white mold growth (Marasas et al., 2000). Severe infections may cover the ear and husks may adhere to the kernels (Sutton, 1982). *Fusarium* spp. also infect stalks, reducing their strength and increasing lodging (Jackson-Ziems et al., 2014). These fungi when present in the plant biomass produce secondary metabolites called mycotoxins (Sutton, 1982).

Stalk rots are more problematic in silage corn than grain corn, since silage production involves harvesting the whole plant for forage. Also, silage is stored and fermented in bunker silos to ensure off-season availability of forage. During fermentation, it undergoes an aerobic phase followed by anaerobic phase. The former can lead to additional mold growth which may aggravate the mycotoxin issue. Although most of the fungal growth halts at the onset of anaerobic phase, certain *Penicillium* spp. continues to grow, leading to production of toxins (Pereyra et al., 2008; Gonzalez Pereyra et al., 2011; Ogunade et al., 2018). Under optimum conditions, infections can cause both significant yield and quality loss in silage corn (Munkvold, 2003).

Gibberella ear and stalk infections predominate when weather conditions are cool to moderate (24 to 28°C) and humid (high dew point and abundant rainfall) at corn silking

(Munkvold, 2003). Such conditions are common in the Great Lakes region in July and early August. However, if the growing season is warmer and dryer, Fusarium ear and stalk rot infections are more predominant (Marasas et al., 2000). Infections occur when ascospores or macroconidia germinate and infect through silk channels within the six days after silk emergence, or via husk and kernel wounds (Munkvold et al., 1997a; Reid & Hamilton 1996; Farhan et al., 2020). Systemic stalk infections occur when spores and mycelia from soil or crop residue enter through root wounds, leaves, or leaf sheath wounds. Dispersal of inoculum is mainly through wind, rain, and water splatter.

A distinct feature of ear and stalk rot fungi is that they produce multiple mycotoxins such as deoxynivalenol (DON), zearalenone (ZON), fumonisin, and aflatoxins in plant biomass (Sutton, 1982). Mycotoxins are produced by fungi to enhance their survival in the host (Ogunade et al., 2018). Toxin production begins in the field and may continue in storage for silage corn. Mycotoxin production by *Fusarium* spp. is favored under cool to moderate temperatures (20-28°C) and elevated moisture levels (Llorens et al., 2004; Munkvold et al., 1997b; Vigier et al., 2001), conditions common in the Great Lakes region.

High mycotoxin concentrations make corn unfit for animals by causing feed rejections, regurgitations, and complications such as hormonal imbalance and pulmonary edema in livestock (Munkvold et al., 2019). Most of mycotoxins are very stable in plant and animal tissue and therefore can pass through food chains, accumulating in higher trophic levels. Complications are especially severe in animals such as swine with monogastric digestive systems (Perlusky et al., 1994). In the U.S., there are no levels set as thresholds for mycotoxins in silage corn. However, reports by Adams et al. (1993) and Goeser (2015) suggest that health problems are more frequent and severe when DON, ZON, or fumonisins exceeds 1.0, 0.4 and 2.0 µg g^{-1} for dairy cattle, and

1.0, 0.3 and 10.0 µg g^{-1} for swine, respectively. For poultry, toxin concentrations above 2.0, 0.1 and 20 µg g^{-1}, for DON, ZON and fumonisins, respectively can cause health complications (Goeser, 2015).

Unlike grain corn, silage is generally fed on farm, and thus it is rarely taken to a local elevator where mycotoxin testing would occur. Hence toxins can go undetected in final feed. A recent grower survey conducted across Michigan corroborates the widespread presence of mycotoxins in silage corn (Kaur et al., 2022). More than 100 samples were collected in the survey and all of them tested positive for multiple mycotoxins with DON, ZON, fumonisins, and beauvericin being the most prominent. Also, it is almost impossible to remediate mycotoxins , once present in plant biomass, they remain even after fermentation. Thus, it is crucial to understand the factors contributing to their production in the field, determine the levels present in harvested silage, and identify in-field management strategies (such as hybrid insect protection and fungicide application) to reduce future contamination.

Physical damage to ears and stalks caused by animal and insect feeding can expose plant tissue to fungal infection. Injury from multiple species of ear-feeding caterpillars such as corn earworm (*Helicoverpa zea* Bod.), European corn borer (ECB, *Ostrinia nubilalis* Hub.), fall armyworm (*Spodoptera frugiperda* Smith), and western bean cutworm (WBC, *Striacosta albicosta* Smith), results in husk wounds which allow entry of ear rot fungi into corn (Munkvold, 2003; Farhan et al., 2020). In the Great Lakes region, the primary ear-feeding species are ECB and WBC, both of which overwinter locally. For the last 25 years, damage from ECB has been well-controlled by the widespread planting of Bt corn hybrids expressing combinations of the effective toxins Cry1F, Cry1Ab, and/or Cry2Ab proteins. This has resulted in significant decline in ECB across the region, even in non-Bt corn (Hutchison et al., 2010). Western bean cutworm is more recent invader, expanding into the region from the western U.S. in the mid-2000s to become a key ear-feeding pest (Smith et al., 2018). Only hybrids with the Vip3A Bt protein currently provide control of this species (DiFonzo and Porter, 2023). Even low infestations of WBC were reported to cause higher severity of ear rot infection in Ontario grain corn (Smith et al., 2018). Strong correlations were reported between insect injury and ear rot incidence (Parker et al., 2017; Smith et al., 2017; Farhan et al., 2020), but weak correlations between WBC injury and DON levels (Smith et al., 2017). Most of these studies have focused on grain corn with little attention to silage corn. Therefore, use of hybrid insect protection in order to decrease ear rot infection in preventing mycotoxin accumulation in silage corn needs further exploration.

Another important component of *Fusarium* and mycotoxin management in cereal crops is the use of broad spectrum triazole fungicides. Prothioconazole (2-[2-(1-Chlorocyclopropyl)-3-(2-chlorophenyl)-2-hydroxypropyl]-1,2-dihydro-3H-1,2,4-triazole-3-thione) and tebuconazole (alpha-[2-(4-chlorophenyl)ethyl]-alpha-(1,1-dimethylethyl)-1H-1,2,4-triazole-1-ethanol) based fungicides are used to manage *Fusarium* infections in wheat (*Triticum aestivum* L.) (Paul et al., 2019; Edwards & Godley, 2010), but results have not been as consistent in grain corn. In a study by Limay-Rios and Schaafsma (2018), fungicide reduced ear rot severity and DON by 50-60% in comparison to non-treated corn. Also, prothioconazole applications for ear rot management in grain corn were time sensitive with respect to silk emergence (Limay-Rios and Schaafsma, 2018), however, no significant yield differences were found. Similarly, Andriolli et al. (2016) reported a 50% reduction in Gibberella ear rot severity in grain corn after a fungicide application at silking (R1) but no improvement in yield. In the same study, fungicide efficiency decreased as the interval between silking and fungicide application increased. In contrast, studies by Anderson et al. (2017) and Fusilier (2019) reported inconsistent results of fungicides in grain corn where reduction in ear rot severity and DON concentration was observed only at some site-years and not others.

Most studies on ear rot and mycotoxin management have been conducted on grain corn, with less attention to corn grown for silage purposes. Yet, silage corn may be at greater risk for ear mold and mycotoxin issues, as whole plants are harvested at high moisture (~65%) compared to grain harvest (~20%). Furthermore, most studies focus on DON concentration, but other mycotoxins such as ZON, fumonisin, and beauvericin may be more frequent or important in silage corn. Therefore, to understand the qualitative and quantitative occurrence of mycotoxins for exploring in-field management strategies and options for ear rots and multiple mycotoxins in silage corn, this study was conducted to (i) explore the role of Bt insecticidal proteins in managing ear damaging insects and reduce ear rots and mycotoxin accumulation in silage corn, (ii) understand relations between insect injury and ear rot infections, (iii) evaluate effectiveness of fungicide application in reducing ear rot infections and mycotoxins, and (iv) quantify the impact of these management strategies on forage quality and milk yield potential of silage corn.

2.3 MATERIALS AND METHODS

2.3.1 Experimental Sites and Design

Field trials were conducted at multiple on-farm locations of Michigan Corn Performance Trials (Singh et at., 2021) from 2019-2021 for a total of 11 site-years (Table 7). Each location was set up in a randomized complete block design with five replications of six treatments. The treatments were combinations of three levels of protection from the most common ear-feeding species in Michigan (none, ECB protection, ECB + WBC protection) and two levels of fungicide application (sprayed or none). The three levels of insect protection were achieved by planting two hybrids (Supplemental Table S3) under each category: non-Bt hybrids, hybrids with Cry1Ab and Cry1F Bt proteins to control only ECB (denoted Bt_E), and hybrids combining Cry1Ab, Cry1A.105/Cry2Ab2 or Cry1F, plus Vip3A Bt protein to control both ECB and WBC (denoted Bt_{EW}). All the hybrids were of same brand and had comparable ear rot resistance ratings on the scale provided by seed company. All Bt hybrids had 5% refuge (i.e., non-Bt) seed for insect resistance management. The fungicide used was the triazole based prothioconazole (Proline 480 SC Bayer CropScience LP, St. Louis, MO) at label rate of 417 ml ha^{-1} applied at silking stage. Fungicide applications were done using a pressurized CO_2 high clearance backpack sprayer with a 3.05 m wide boom, fitted with TeeJet 8001 VS nozzles spaced 50.8 cm apart. Boom height was maintained at 30 cm above the tassel.

Table 7. Agronomic details for field trials in Michigan and Ohio in 2019, 2020, and 2021.

Year	**Location[a]**	**Previous crop**	**Planting date**	**Irrigation**	**Fungicide Application[b]**	**Harvest date**	**Insect pressure[c]**
2019	Ingham	Soybean	7 June	Rainfed	19 Aug.	3 Oct.	High
	Huron	Silage corn	9 June	Rainfed	28 Aug.	12 Oct.	Low
2020	Ingham	Soybean	7 May	Rainfed	23 July	31 Aug.	Low
	Ingham[I]	Soybean	8 May	Irrigated	23 July	15 Sept.	Low
	Huron	Wheat	27 May	Rainfed	5 Aug.	29 Sept.	High
	Branch[I]	Soybean	13 May	Irrigated	26 July	10 Sept.	Low
	Lenawee	Soybean	13 May	Rainfed	26 July	11 Sept.	Low
	Allegan[I]	Soybean	5 June	Irrigated	7 Aug.	2 Oct.	Low
	Wood (OH)	Soybean	2 June	Rainfed	10 Aug.	16 Sept.	High
2021	Ingham	Wheat	11 May	Rainfed	27 July	10 Sept.	Low
	Ingham[I]	Grain Corn	13 May	Irrigated	22 July	11 Sept.	Low

[a] Location represents county and state. All field were in Michigan except Wood County (Ohio). Ingham[I], Allegan[I], and Branch[I] were irrigated. all other site-years were rainfed.
[b] Fungicide was applied when at least 50% of the ears were at silking stage.
[c] Insect pressure was considered high or low when insect egg/larvae were present on >10% or <10% plants, respectively

Field trials were planted at 84,016 seeds ha^{-1} between early May to late June from 2019-2021 using Almaco precision planter (Almaco, Nevada, Iowa). Plots were 3.05 m (= four rows) wide, 6.71 m long, with a 76 cm (30 inch) row spacing. The middle two rows were used for final disease and insect ratings and yield data at harvest. The outer rows were used for in-season insect egg mass and larvae scouting. Plots were managed using standard grower practices for the area (Table 7).

Pheromone traps were set up at each location starting at five leaf collar stage (V5) to monitor flight of ECB (Hercon European Corn Borer IA lures, 10/CS, Great Lakes IPM^{TM}, Vestaburg, MI) and WBC (Trece pherocon WBC lures, 3/CS, Great Lakes IPM^{TM}, Vestaburg, MI). Locations were scouted for ECB and WBC egg masses at 12 or 13 leaf collar stage (V12-V13). At R2 (blister stage) insect larvae and ear rot pressure were monitored to determine actual infestation levels. For ear rot, five ears from both first and fourth rows of the plot were examined at R2.

At Ingham site-years (except Ingham 2021), *F. graminearum* infection was artificially enhanced at silking stage, with an inoculation of a conidia suspension of fungal strains Ph1 and C13 721. One ml of suspension was applied per ear (on the silks) using a squirt bottle in the middle two rows at a concentration of 5 x 10^5 spores ml^{-1}.

2.3.2 Data collection

Insect and disease injury ratings were done in the field on the day of harvest. Five consecutive ears from each of the middle two rows were examined for a total of 10 ears per plot. Disease ratings were taken as ear rot incidence (ERI, percentage of ears with ear rot symptoms) and ear rot severity (ERS, percentage of kernels per ear with ear rot symptoms - a measure of the intensity of infection). Insect ratings were taken as insect feeding incidence (IFI, percentage of ears with insect feeding) and insect feeding severity (IFS, percentage of kernels in an ear fed on by insects - a measure of the intensity of infestation). All ratings were quantified on a per plot basis. An ear rot index was calculated for each plot as the product of ERI and ERS divided by 100 to quantify cumulative impact of disease (Fusilier, 2019; Groth et al., 1999).

Silage corn was harvested with a target moisture of approximately 650 mg g^{-1} with C1200 Kemper forage harvester (Anker Machinery Company Ltd, Braishfield, Romsey, UK) with a rear mounted Haldrup M-63 weight system (Haldrup, Ossian, Indiana) to measure fresh biomass yield. This system collects and homogenizes harvested biomass from the entire length of middle two rows. A representative sample (about 500 g) was then collected from each plot. Each sample was dried for 72 hrs in a hot air dryer (60°C) to estimate moisture content and dry yield (Eckard et al., 2011). These samples were ground to a 1mm sieve size and analyzed for forage quality and mycotoxins. Near Infrared spectroscopy (NIRS DS2500, FOSS North America, Eden Prairie, MN) was used to analyze the following quality parameters: neutral detergent fiber (NDF), acid detergent fiber (ADF), in vitro true digestible dry matter (IVTD), neutral detergent fiber digestibility at 48 hrs of incubation period (NDFD), crude protein (CP) and starch. Milk yields (milk per hectare and milk per megagram) were calculated from dry yield, IVTD, NDF, and CP using Milk Equation 2006 (de Los Campos et al., 2006). Quality predictions were validated on a subset of samples using wet chemistry analysis.

Samples were analyzed for mycotoxin type and concentration at the University of Guelph Ridgetown (Ontario, Canada). Using the protocol developed by Limay-Rios & Schaafsma, (2021), samples were analyzed using an Ionics EP 10+ modified API365 triple quadrupole mass spectrometer (LC-MS/MS) system equipped with an electrospray ionization source, in positive and negative polarity. The samples were tested for 26 mycotoxins including DON, ZON, fumonisin, and beauvericin (Fusilier et al., 2022). Samples from the site-years where ear rots were not very severe were sent to University of Minnesota (St. Paul, MN) for DON analysis using gas chromatography and mass spectroscopy (GC-MS) following protocols developed by Fuentes et. al, 2005.

2.3.3 Data Analyses

Response variables from 11 site-years were analyzed using ANOVA in SAS 9.4 software using PROC GLIMMIX with 0.10 as level of significance. Hybrid insect protection, fungicide application, and their interaction were considered as fixed factors and replication as random factor. Site-years were analyzed separately due to unique environmental conditions at every location. The interactions between hybrid insect protection and fungicide application at any of the site-years were not found to be significant. Data are presented by specific site-years only where significant differences between treatments were observed. For the remaining site-years, data are pooled and presented together.

Least square means were calculated using *lsmeans* statement and paired mean differences were calculated using Tukey's adjustment. Statement *dfmkr* was used to minimize the effect of heterogenicity in variances. Regression analysis was done to understand the relation between insect injury, disease damage, and mycotoxin concentration using statement PROC REG by pooling data across the treatments. Coefficient of determination (R^2) was reported to show how differences in one factor of the epidemiological triad affected the other variable.

2.4 RESULTS

2.4.1 Weather patterns across site-years

Silking dates averaged from mid to late August in 2019 due to delayed planting, whereas in 2020 and 2021 silking occurred from mid to late July. Gibberella ear rot infections are favored under cool and humid climate observed in 2019 and 2021 whereas Fusarium ear rot is favored under warmer and slightly dryer conditions, experienced in 2020. The lower peninsula of Michigan on average receives more than 60 mm rain during the months of July and August, with temperature ranges between 17 to 29°C; conditions conducive for fungal infections. However, rainfall was lower than average for 2019 and 2020 whereas it was closer to 30-yr average in 2021 during silking period (Table 8). An exception was Huron 2020, which received more than 120 mm of rainfall and a temperature range of 18 to 27°C around silking stage (Table 8). This resulted in higher ear rot pressure at Huron 2020 than at any other site-year.

Table 8. Monthly[a] and 30-year mean[b] temperature and precipitation for each site-year.

Location	Year	May	June	July	August	September
		Temperature (°C)				
Ingham	2019	13.7	18.9	23.7	21.2	18.9
	2020	13.6	20.1	23.4	21.2	15.6
	2021	13.6	21.1	21.3	20.8	17.6
	30-yr.	**14.6**	**19.6**	**21.8**	**20.7**	**16.4**
Huron	2019	11.4	17.3	21.8	19.6	17.7
	2020	12.3	19.6	23.1	20.9	15.8
	30-yr.	**13.9**	**18.9**	**21.4**	**20.2**	**15.9**
Branch	2020	13.0	20.4	23.2	20.8	15.8
	30-yr.	**14.6**	**19.6**	**21.8**	**20.7**	**16.4**
Lenawee	2020	13.8	15.4	24.4	21.6	16.9
	30-yr.	**14.6**	**20.0**	**22.4**	**21.3**	**17.1**
Allegan	2020	13.6	20.9	23.9	21.2	15.9
	30-yr.	**14.6**	**19.6**	**21.9**	**20.7**	**16.4**
Wood	2020	14.8	22.1	25.7	22.5	17.9
	30-yr.	**15.6**	**21.2**	**22.9**	**21.7**	**17.9**
		Precipitation (mm)				
Ingham	2019	66	189	69	39	87
	2020[c]	117	73	41	69	108
	2021[c]	24	176	93	96	74
	30-yr.	**80**	**93**	**79**	**93**	**91**
Huron	2019	87	94	47	37	97
	2020	83	49	70	102	56
	30-yr.	**71**	**81**	**71**	**85**	**96**

[a] Monthly precipitation data and monthly temperature data was collected from the nearest weather station in the MSU Enviro-weather.
[b] 30-year (1991-2020) mean temperature and precipitation data collected from the National Oceanic and Atmosphere Administration (https://www.ncdc.noaa.gov/cdo-web/datatools/normals).
[c] Ingham had two fields both in 2020 and 2021; one irrigated, one rainfed.
[d] Irrigated site-years. Precipitation data does not include irrigation amounts (data not available).

Table 8 (cont'd)

Location	Year	May	June	July	August	September
Branch	2020[d]	135	85	67	84	68
	30-yr.	**80**	**93**	**79**	**93**	**91**
Lenawee	2020	147	43	66	135	42
	30-yr.	**75**	**89**	**76**	**85**	**84**
Allegan	2020[d]	108	121	46	54	77
	30-yr.	**87**	**95**	**87**	**95**	**101**
Wood	2020	108	5.9	55	104	59
	30-yr.	**97**	**8.6**	**95**	**96**	**72**

[a] Monthly precipitation data and monthly temperature data was collected from the nearest weather station in the MSU Enviro-weather.
[b] 30-year (1991-2020) mean temperature and precipitation data collected from the National Oceanic and Atmosphere Administration (https://www.ncdc.noaa.gov/cdo-web/datatools/normals).
[c] Ingham had two fields both in 2020 and 2021; one irrigated, one rainfed.
[d] Irrigated site-years. Precipitation data does not include irrigation amounts (data not available).

2.4.2 Effect of hybrid insect protection levels

2.4.2.1 Insect Injury

Insect pressure, as measured by ear and kernel injury, was generally low at most site-years and hence there was a lack of significance of insect injury among hybrid insect protection levels. However, at five site-years (Ingham 2019, Huron 2020, Wood 2020, Ingham 2020, Ingham[I] 2020, Ingham[I] 2021), insect injury differed significantly by hybrid insect protection level (Table 9). Moderate (5-10%) to high injury (>10 %) was found at Ingham 2019 and 2021, Huron 2020, and Wood 2020 (Table 7); at each of these site-years, at least 14% of ears were injured in the non-Bt plots (Figure 6). Insect feeding severity was the greatest at Ingham 2019 and Huron 2020, with at least 12% of kernels injured in each ear in non-Bt plots (Figure 7).

Table 9. p-values for effect of hybrid insect protection for insect feeding incidence, insect feeding severity, ear rot incidence, ear rot severity, and deoxynivalenol concentration. p-values less than .10 show that response variable was impacted by insect protection level at that particular site-year.

Site-Year[a]	**Insect feeding incidence**	**Insect feeding severity**	**Ear rot incidence**	**Ear rot severity**	**Ear rot Index**	**Deoxynivalenol**
Ingham 2019	**<.001**	**.10**	.26	**.001**	**.01**	**.07**
Huron 2019	.14	.70	.22	.64	.32	.21
Ingham 2020	**.07**	**.10**	.23	.79	.12	.30
Ingham[I] 2020	**.05**	.64	.48	**.06**	**<.001**	**.03**
Huron 2020	**.07**	**.02**	.35	**.02**	**.01**	.44
Allegan[I] 2020	.22	**.09**	.90	**.09**	.13	.77
Branch[I] 2020	.12	.30	**.002**	.77	.23	.83
Lenawee 2020	.18	.18	.25	.36	.24	.67
Wood 2020	**<.001**	**.01**	**<.001**	**.05**	**.08**	**.06**
Ingham 2021	.12	.32	.14	.65	.33	.27
Ingham[I] 2021	**.01**	**.07**	.78	**.02**	**.02**	**.04**

[a] Ingham[I], Allegan[I], and Branch[I] were irrigated, all other site-years were rainfed

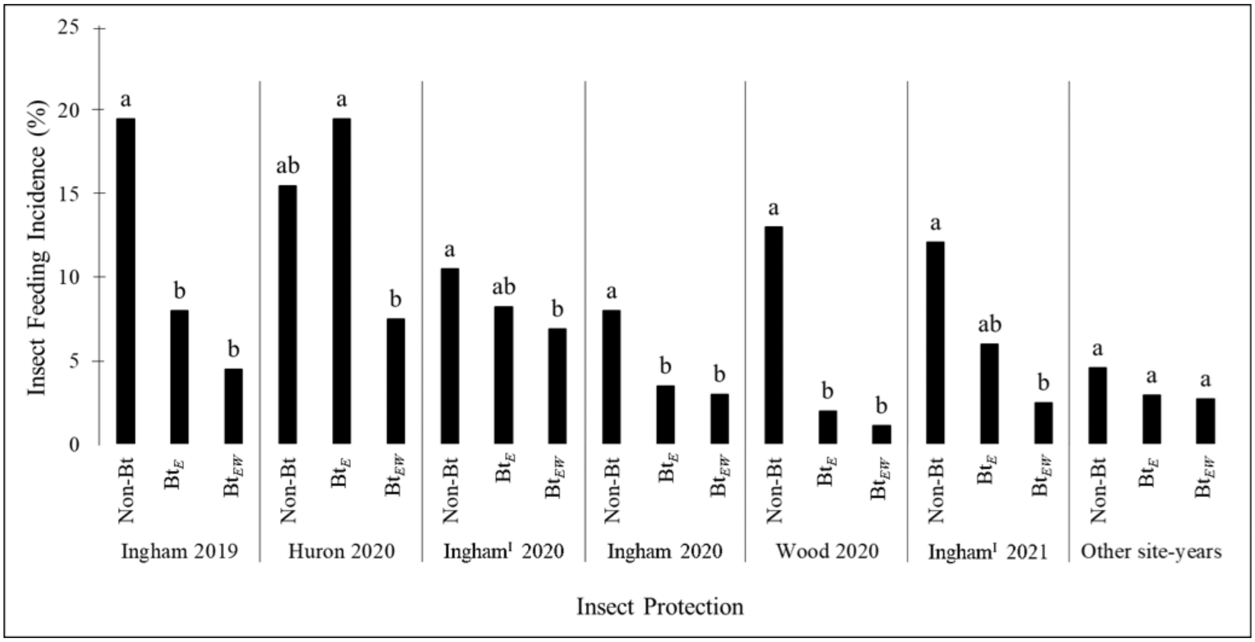

Figure 6. Mean insect feeding incidence (IFI) for hybrid insect protection levels. Other site-years represents data pooled from site-years where significant differences were not observed among the treatments. Non-Bt, No insect protection; Bt_E: protection only against ECB (European Corn Borer); Bt_{EW}, protection against both ECB and WBC (Western Bean Cutworm); Ingham[I] was irrigated. Bars with same letter within a site-year are not different (p-value < .10).

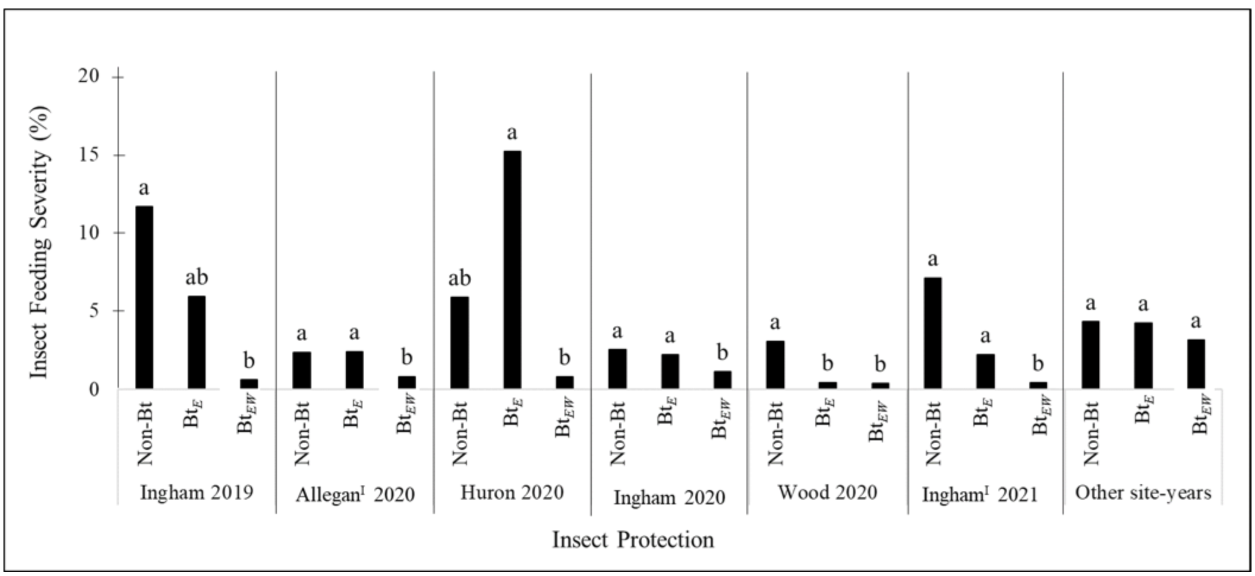

Figure 7. Mean insect feeding severity (IFS) across hybrid insect protection levels. Other site-years represents data pooled from site-years where significant differences were not observed among the treatments. Non-Bt, No insect protection; Bt_E: protection only against ECB (European Corn Borer); Bt_{EW}, protection against both ECB and WBC (Western Bean Cutworm); Allegan[I] and Ingham[I] were irrigated. Bars with same letter within a site-year are not different (p-value < .10).

Both IFI and IFS were lowest in Bt_{EW} hybrids (Figure 2-1 and 2-2). Overall, IFI was less than 10% and severity less than 1% in Bt_{EW} hybrids expressing Vip3A (Figures 1 and 2). At Wood 2020, Bt_{EW} hybrids and Bt_{E} hybrids had 90 and 84% lower IFI values, respectively and IFS values decreased by 88 and 84 % as compared to non-Bt hybrids. Similarly, at Ingham 2019, IFI for Bt_{EW} and Bt_{E} hybrids were 77 and 58 % lower and IFS were 95 and 50 % lower than non-Bt hybrids, respectively. Similar trends in reductions were observed at Ingham^{I} 2020 and Ingham2020. At Ingham^{I} 2021, lowest insect injury was observed in Bt_{EW} hybrids. However, for Huron 2020, only Bt_{EW} hybrids showed significant reduction for IFI and IFS and insect injury was not different between Bt_{E} hybrids and non-Bt hybrids (Figures 6 and 7).

2.4.2.2 Ear Rot Injury

Average ERI was low at most site-years, less than 5% and ERS less than 1%. Ear rot incidence values did not vary significantly across hybrid groups, except for Branch^{I} 2020 and Wood 2020 (Table 9). At both site-years, Bt_{EW} had lowest ERI, five and four percent, respectively. Ear rot incidence was 65.5 and 70 % lower for Bt_{EW} than non-Bt for these site-years (data not shown). The highest ERS values were recorded at Ingham 2019 and Ingham^{I} 2021 which were inoculated to increase disease pressure, and at Huron 2020 which had favorable environmental conditions for infection. At these three site-years and at Wood 2020 and Allegan^{I} 2020, Bt_{EW} hybrids had significantly lowest ERS (Figure 8), suggesting that insect feeding injury played a role in increasing ear rot in these plots.

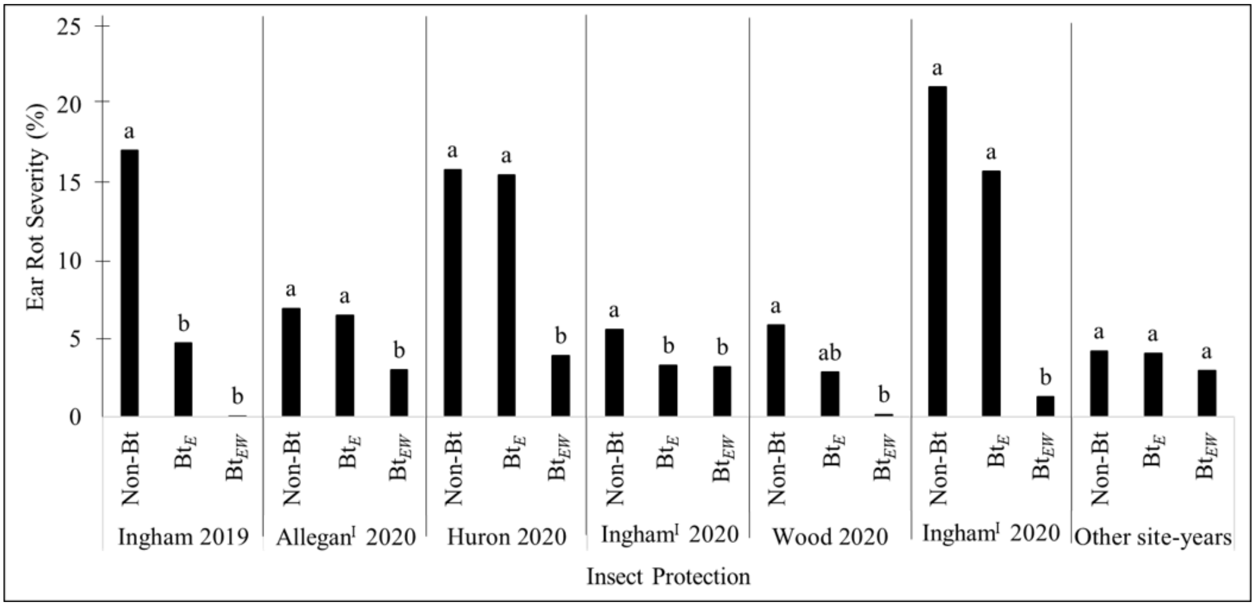

Figure 8. Mean percent ear rot severity (ERS) across hybrid insect protection levels. Other site-years represents data pooled from site-years where significant differences were not observed among the treatments. Non-Bt, No insect protection; Bt_E: protection only against ECB (European Corn Borer); Bt_{EW}, protection against both ECB and WBC (Western Bean Cutworm); Allegan^I and Ingham^I were irrigated. Bars with same letter within a site-year are not different (p-value < .10).

For all these site-years, lowest insect injury generally corresponded to lowest ear rot damage (Figure 8). Ear rot severity was observed to be 77 to 98 % lower for Bt_{EW} and Bt_E at Ingham 2019 than non-Bt, whereas at Huron 2020 and Ingham^I 2021 Bt_{EW} lowered ear rot severity values to 3.92 and 1.26%, respectively. Ear rot index values at Ingham 2019 and Ingham^I 2021 were lowest for Bt_{EW} (Figure 9). Similarly, at Huron 2020 and Wood 2020, the lowest ear rot index was seen for Bt_{EW}.

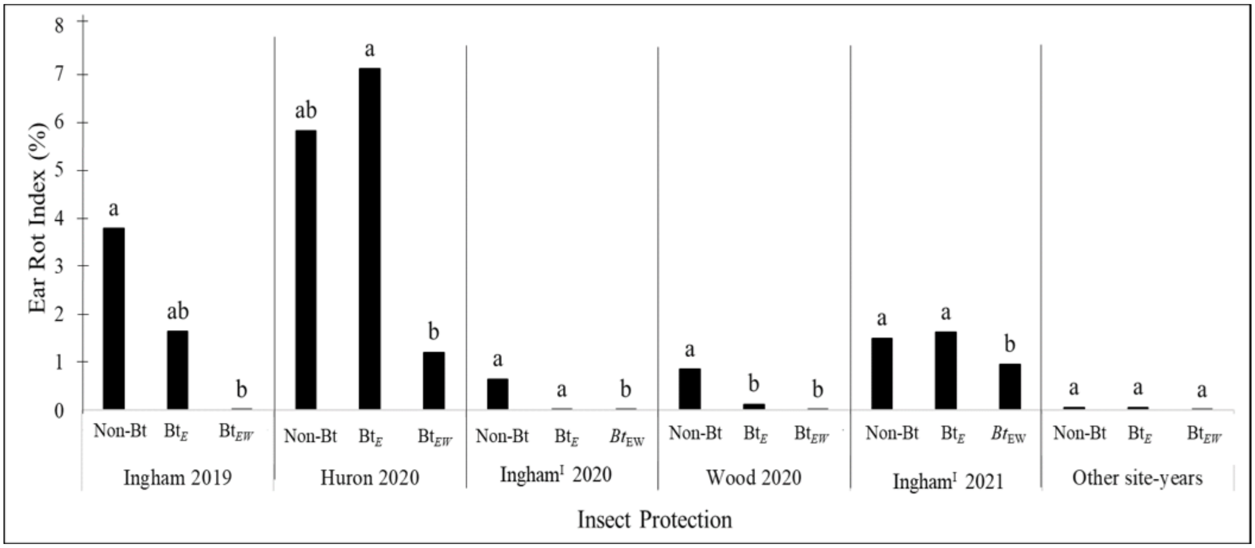

Figure 9. Mean percent ear rot index across hybrid insect protection levels. Other site-years represents data pooled from site-years where significant differences were not observed among the treatments. Non-Bt, No insect protection; Bt$_E$: protection only against ECB (European Corn Borer); Bt$_{EW}$, protection against both ECB and WBC (Western Bean Cutworm); Ingham[I] was irrigated. Bars with same letter within a site-year are not different (p-value < .10).

Ear rot incidence and severity were consistently the lowest in hybrids with insect protection against ECB and WBC and the highest in hybrids with no insect protection. Low insect and disease pressure caused a lack of significance among insect protection levels for IFI, IFS, ERI and ERS at several site-years (Table 9). Weak to no correlations between insect and disease damage were observed at most site-years (Table 10) except at Huron 2020 with high insect injury, where strong correlation was observed (Figure 10).

Table 10. p-values and R^2 values for relationship between insect feeding incidence (IFI) and ear rot incidence (ERI) at various site-years. P-values less than .10 show that the variation in ERI was impacted by variation in IFI.

Site-year[a]	**Ingham 2019**	**Huron 2019**	**Ingham 2020**	**Ingham[I] 2020**	**Huron 2020**	**Allegan[I] 2020**	**Branch[I] 2020**	**Lenawee 2020**	**Wood 2020**	**Ingham 2021**	**Ingham[I] 2021**
p-value	.07	.03	.06	<.001	<.0001	.73	.31	.002	<.001	.04	.08
R^2	0.06	.09	.12	.15	.54	.11	.003	.17	.07	.16	.12

[a] Ingham[I], Allegan[I], and Branch[I] were irrigated, all other site-years were rainfed.

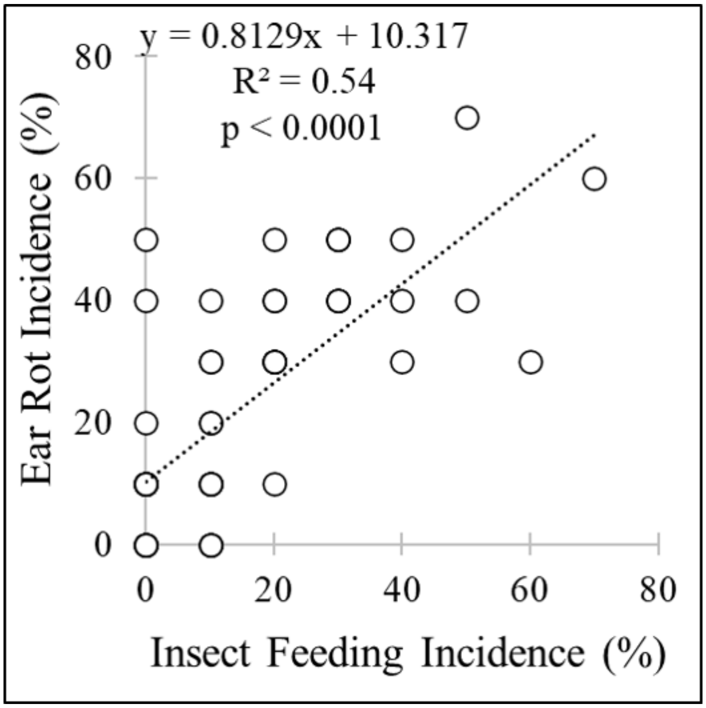

Figure 10. Correlation between ear rot incidence and insect feeding incidence at Huron 2020. Both ear rot and insect feeding incidence ratings were combined across hybrid types and fungicide treatments for the regression model.

2.4.2.3 Mycotoxin Concentration

Across all site-years, DON was detected in all samples tested. Inoculated trials at Ingham 2019, Ingham^I 2020, and Ingham^I 2021 had highest mycotoxin concentration, with DON levels ranging between 1.3 to 35 µg g^{-1} while naturally occurring DON concentration were highest at Huron 2019 (ranged from 0.7 to 4.5 µg g^{-1}). At Ingham 2019 under higher insect pressure, DON varied with hybrid insect protection. The lowest mean concentration of DON was in Bt_{EW} hybrids (2.7 µg g^{-1}), followed by Bt_E hybrids (6.8 µg g^{-1}) and non-Bt (7.2 µg g^{-1}) (Figure 11). Deoxynivalenol level was weakly correlated with IFS (but not with IFI) and with ear rot index, R^2 = .11 and .16, respectively (Figure 12). In 2020 and 2021, the only mycotoxin that occurred in significant concentration (>0.5 ppm) was DON, which was the lowest for Bt_{EW} hybrids with insect protection against both ECB and WBC at Ingham^I 2020, Wood 2020, and Ingham^I 2021 (Figure 13). At other site-years, DON concentrations were not different across hybrid insect protection levels (Table 9). Overall, mycotoxin concentrations were lower in 2020 and 2021 than in 2019.

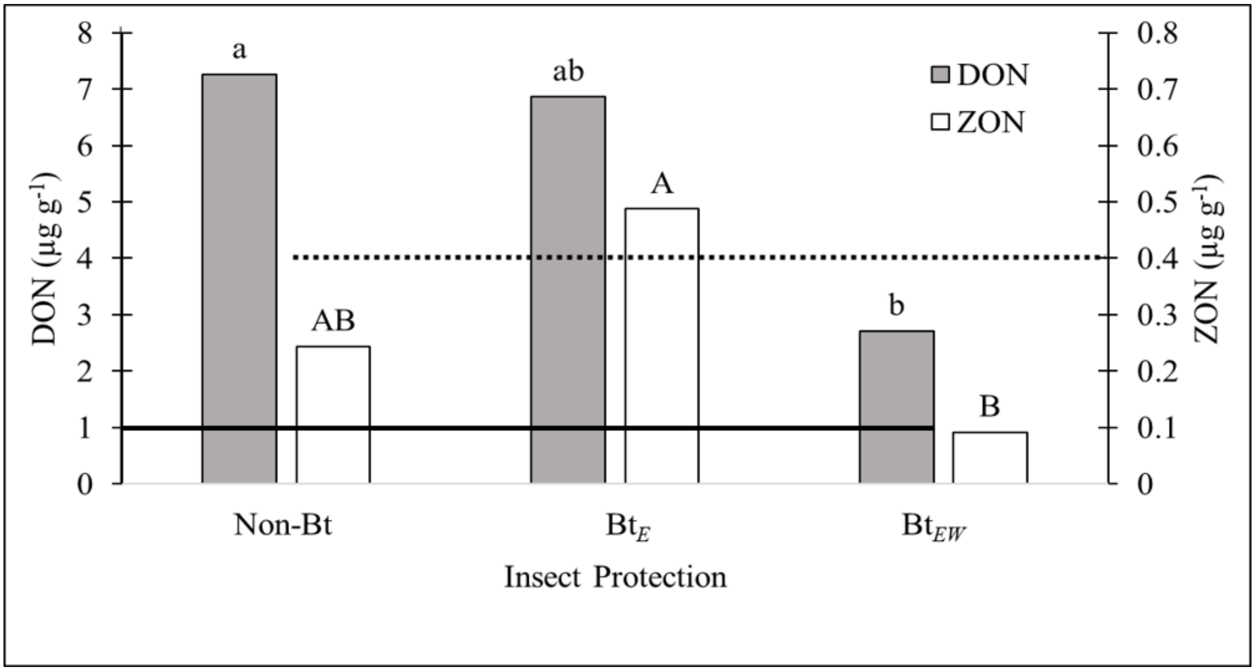

Figure 11. DON (deoxynivalenol) and ZON (zearalenone) levels at Ingham 2019 across three hybrid insect protection levels. Horizontal solid and dotted lines represent DON and ZON level, respectively, beyond which health problems are more frequent and severe in dairy cattle. Non-Bt, No insect protection; Bt_E: protection only against ECB (European Corn Borer); Bt_{EW}, protection against both ECB and WBC (Western Bean Cutworm). Bars with same capital or small letter do not differ from each other significantly (p-value < .10).

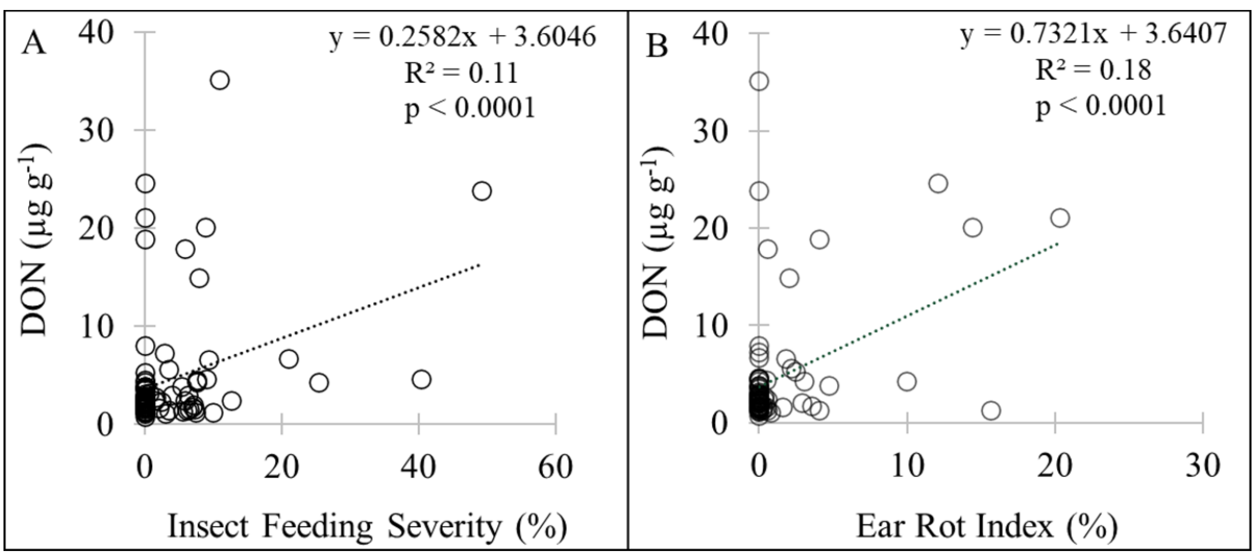

Figure 12. Correlation between deoxynivalenol (DON) and insect feeding severity (A) and between deoxynivalenol and ear rot severity (B) at Ingham 2019.

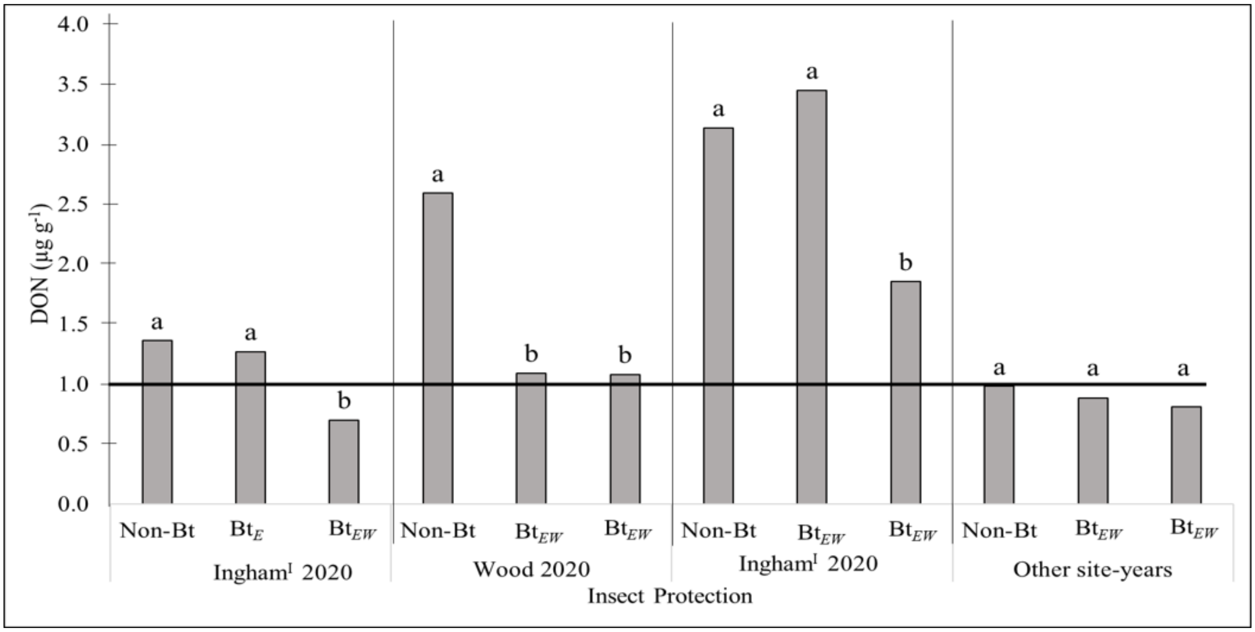

Figure 13. Deoxynivalenol (DON) levels at fields Ingham 2019, Ingham^{I} 2020, Wood 2020, and Ingham^{I} 2021. Other site-years represents data pooled from site-years where significant differences were not observed among the treatments. Horizontal solid line represents DON level beyond which health problems are more frequent and severe in dairy cattle. Non-Bt, No insect protection; Bt_{E}: protection only against ECB (European Corn Borer); Bt_{EW}, protection against both ECB and WBC (Western Bean Cutworm). Ingham^{I} was irrigated. Bars with same letter within a site-year do not differ from each other significantly (p-value < .10).

Other mycotoxins that occurred frequently were ZON and several fumonisins (FB1, FB2 and FB3). However, these were in very low concentrations and their frequency and level did not differ among hybrids with different insect protection levels except for ZON, which had the lowest mean concentration in Bt_{EW} hybrids at Ingham 2019 (Figure 2-6).

2.4.2.4 Yield and Forage Quality

Yield and forage quality parameters were not different (p-value > 0.10) among the hybrid groups with different levels of insect protection at any of the site-years except for Ingham 2019, where NDFD was higher for Bt_{EW} hybrids (571 g kg^{-1} of NDF) than Bt_{E} and non-Bt hybrids (545 and 532 g kg^{-1} of NDF, respectively), the latter two not different. Across all site-years, the average values for yield and quality parameters (on a dry matter basis) were: dry yield, 21.3 Mg ha^{-1}; NDF, 375 g kg^{-1}; ADF, 214 g kg^{-1}; IVTD, 801 g kg^{-1}; CP, 735 g kg^{-1}; and starch, 336 g kg^{-1}.

Average NDFD was 617 g kg^{-1} of NDF and milk per megagram and milk per hectare were 1447 kg Mg^{-1} of dry yield and 19.4 Mg ha^{-1}, respectively, across all site-years.

2.4.3 Effect of fungicide

The effect of fungicide application was inconsistent among site-years. Ear rot index, ERI and ERS were not significantly reduced by fungicide application at nine site-years except for ERI at Allegan[I] 2020 and Lenawee 2020 where the treated plots had 60 and 50 % less ERI than non-treated plots, respectively (Table 11). Differences were seen at the site-years where infections were not enhanced by insect feeding (i.e., insect pressure and injury were low), while fungicide application did not decrease ear rot infection for site-years where high insect injury may have enhanced infections.

Table 11. Impact of fungicide on ear rot incidence and deoxynivalenol (DON) concentration. Other site-years represents data pooled from site-years where significant differences were not observed among the treatments. Values followed by same letter within a site-year are not significantly different (p-value < .10).

Site-year[a]	**Fungicide treatment**	**Ear Rot Incidence (ERI) (%)**	**DON conc. (μg g^{-1})**
Allegan[I] 2020	Non-treated	16.3 a	1.50 a
	Treated	6.70 b	0.59 b
	p-value	.01	.02
Branch[I] 2020	Non-treated	10.3 a	1.94 a
	Treated	7.2 a	0.95 b
	p-value	.17	.03
Ingham[I] 2020	Non-treated	19.6 a	1.35 a
	Treated	23.3 a	0.83 b
	p-value	.52	.03
Lenawee 2020	Non-treated	20.0 a	1.64 a
	Treated	10.7 b	0.78 b
	p-value	.009	.08
Other site-years	Non-treated	10.5 a	2.07 a
	Treated	10.3 a	1.81 a
	p-value	.34	.22

[a] Allegan[I], Branch[I], and Ingham[I] were irrigated, all other site-years were rainfed.

Similarly, mycotoxin concentrations were not reduced by fungicide application at site-years with the highest insect injury. At Allegan[I] 2020 and Lenawee 2020; DON concentration was decreased by 60 and 50 %, respectively, in treated plots (Table 2-5). At Branch^{I} 2020 and Ingham^{I} 2020, though fungicide application did not reduce ERI (p = 0.17 and 0.52), it significantly decreased DON by 40-50% in fungicide treated plots (Table 2-5). Fungicide application did not increase yield or improve the forage quality parameters such as starch, crude protein, and digestibility at any site-years (data not shown).

2.5 DISCUSSION

This study quantified the relation between insect injury and ear fungal infections and evaluated management of mycotoxins using hybrid insect protection and fungicide application in silage corn. Our data shows that insect injury enables ear fungal infections and mycotoxin accumulation. Hybrid insect protection lowered the risk of these infections by preventing insect injury and prevent mycotoxin production. Fungicide application can be beneficial to lower the ear rot infections and mycotoxin concentration under conditions of low insect infestation, however, may not be a profitable investment when disease levels were high because of the insect injury.

At locations with high insect pressure, insect feeding injury varied significantly across hybrid insect protection levels. The lowest insect injury was observed in Bt$_{EW}$ hybrids that have protection against both WBC and ECB, as well as other secondary ear feeding caterpillars (Figure 2-1 and 2-2). Non-zero values for insect incidence for Bt$_{EW}$ may occur if insects colonized the non-Bt ‘refuge’ plants present at a rate of 5% in the field for resistance management. WBC larvae developing on non-Bt plants may stay on the refuge plant or, more likely move to and feed on a nearby Bt plant before being killed by the innate plant protection proteins in Bt hybrids (Smith et al., 2018). Also, lower insect injury values were observed in hybrids with Cry1F and Cry1Ab which provide protection against ECB as compared to the non-

Bt hybrids. Insect injury in these hybrids was mostly due to feeding of WBC. Similar reductions in insect feeding (incidence and severity) were observed by Smith et al., (2017). High insect flight at pre-tassel and favorable environmental conditions especially around silking increase insect injury which increases ear rot pressure and can eventually lead to higher mycotoxin accumulation in the plant biomass (Farhan et al., 2020). However, no differences in insect injury were seen across hybrids when insect pressure was less than 10% which was the case in many site-years of this study and would therefore seem to negate the need to plant Bt silage corn. However, WBC populations are unpredictable and vary greatly by location and season, and when outbreaks occur quality is affected. European corn borer and WBC can be managed with insecticides, but to effectively do so requires repeated and intensive scouting for egg masses and newly hatched larvae to time sprays. The time and capital investment needed for this level of management is difficult for the typical grower managing a livestock or dairy operation, which makes Bt corn a more viable option in the longer run.

Ear rot levels also show variation among hybrid groups with different insect protection levels. Lowest ERS and ear rot index was observed in Bt_{EW} hybrids when insect pressure was high (Figure 2-3 and 2-4). In site-years that were inoculated to increase disease pressure, ear rot index was low in Bt_{EW} hybrids. Although disease inoculum applied to each hybrid was similar in strength and amount, difference in disease levels across hybrid insect protection levels shows that since presence of Bt proteins help in lowering physical injury to ears and plant and therefore, limit the spread of fungus and hence lower disease incidence and severity. This was further affirmed by the trends seen in values for ear rot index and insect feeding (incidence and severity) injury across different hybrid groups. However, at all site-years significant but weak correlations between IFI and ERI were seen except for Huron 2020 which was the location with highest insect pressure across all site-years (Figure 2-5). Another crucial observation at Huron 2020 was IFI and IFS were lowered only in Bt_{EW} hybrids and were not different for non-Bt and Bt_{E} hybrids, indicating that most of the insect injury at this site-year occurred due to WBC. Corn earworm and fall armyworm, other ear-feeding species controlled by Vip3A, are uncommon in Michigan and were not present at the Huron site.

These data indicate that in conditions favoring higher insect pressure, use of hybrid insect protection can be a helpful tool to reduce ear rot infections. Hybrid insect protection also showed reduction in disease for the inoculated fields that were irrigated and had higher disease incidence and severity. Use of hybrids with Cry1F, Cry1Ab, Cry2Ab2, and Cry1A.105 help in managing ECB and under low infestations may lower WBC injury. However, development of resistance in WBC failed these hybrids in effectively managing the insect (Michel et al., 2010). This study provided evidence that use of hybrids with Vip3A can minimize ear injury from WBC and therefore, prevent easy entry of fungus, reducing ear rot infections and mycotoxin accumulation.

Mycotoxins were in general low at most site-years due to unfavorable environmental conditions, except at Ingham 2019 and $Ingham^{I}$ 2021 which were inoculated with *F. graminearum* and therefore, resulted in high levels of DON. The Bt_{EW} hybrids had comparatively lower DON concentration, and their use reduced mycotoxin levels below or closer to safe levels for dairy cattle at multiple site-years (Figures 2-6 and 2-7) compared to non-Bt hybrids. Deoxynivalenol and ZON values followed similar trend as observed in insect injury and ear rot index. Fumonisin levels did not show a significant difference across hybrids, probably due to their relatively low concentrations. Although pathogens were not isolated, high levels of DON detected indicates the abundance of *F. graminearum* in Michigan. This was also affirmed by higher number of pink and white ear molds observed during field scouting. Mycotoxin concentration was found to be weakly correlated with ear injury both in terms of insect and ear rots. Certain plots showed relatively higher concentration of mycotoxins even when symptoms of ear insect and disease injury were minimal or absent. This implies that mycotoxins might occur even in the absence of visual symptoms on ears or may be contributed by factors such as stalk rot infections. Further research is therefore, needed in understanding fraction of mycotoxins contributed by stalk and ear rot in silage corn.

A critical observation from the study was the inconsistency in the effect of fungicides on ear rot infections in silage corn. No impact of fungicide was seen on ear rot at site-years where ear rot incidence and severity were higher due to insect injury. Fungicide treatment reduced ERI only at site-years where disease pressure was in general low (<20% incidence). Fungicide did not reduce mycotoxin concentration at most of the site-years where ear rot fungal infections were enhanced by insect injury. This inconsistency in fungicide response was also seen in previous studies performed on grain corn (Fusilier, 2019; Limay-Rios & Schaafsma, 2018). Similar results were reported by Anderson et al., (2017), where fungicides reduced eat rot infections in two out of three site-years but no reduction in DON was observed. In wheat, prothioconazole based fungicides especially in combination with tebuconazole are effective in lowering DON levels and therefore, it is speculated that low fungicide efficiency in corn may be due to limited systemic movement in corn and also thick husk barrier on ears (Anderson et al., 2020 and Paul et al., 2019). Besides that, since fungicides were applied using a backpack sprayer in this study, there might be some coverage issues which may have impacted the fungicide performance.

No effect or interaction of hybrid insect protection and fungicide was seen on yield and silage corn feed quality parameters at any of the site-years (except for NDFD at one site-year).

Also, no correlation was observed between yield and mycotoxin concentrations. This implies that accumulation of mycotoxins due to ear rot infections had negligible impact on silage feed quality and dry yield. It also shows that incorporation of Bt proteins for insect resistance neither compromise nor improve yield and feed quality potential of hybrids.

2.6 CONCLUSION

This study evaluated the presence of mycotoxins in silage corn, contributing factors, and management strategies that can help mitigate the problem. Hybrid insect protection played a crucial role in preventing insect injury to corn ears which indirectly reduced subsequent fungal infections and mycotoxin accumulation. However, when insect pressure is low, insect injury, ear rots, and mycotoxins were found to be weakly related which indicates that benefits of insect management strategies in minimizing mycotoxins in silage corn might be limited to areas with high insect pressure. Fungicide application showed minimal impact on ear rots, mycotoxin accumulation, and silage corn forage quality for site-years with higher insect infestation. Data from this study and more research evaluating various insect and disease management strategies across wide environments in silage corn can provide opportunities to design more economic and sustainable production systems. Overall, an integrated in-field management approach to minimize mycotoxins in silage corn is essential with continued research at regional level on mycotoxins contributed by ear compared to stalk rots, harvest and plant timing, hybrid selection, crop rotation, and tillage practices.

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CHAPTER 3: PLANTING TIME AND SEEDING RATE IMPACT INSECT FEEDING, EAR ROTS, AND FORAGE NUTRITIVE VALUE IN SILAGE CORN

3.1 ABSTRACT

Planting date and seeding rate determine the microclimate within a crop field. They can influence lepidopteran insect infestation and fungal infections and are an important part of crop management strategy. Altering planting date and seeding rate can also influence yield and quality of silage corn (*Zea mays* L.). Therefore, this research was conducted to identify optimum planting time and seeding rate to minimize insect feeding, ear rot infections, and mycotoxin accumulation in silage corn without compromising yield and quality. Replicated field trials were conducted across multiple site-years in Michigan with three planting dates (early: April 25 to May 10; mid: May 11 to May 25; late: May 26 to June 10) and/or four seeding rates (ranging from 69,160 to 113,620 seed ha^{-1}, in increments of 14,800 seeds ha^{-1}). Mid-planting yielded 12 to 15% less forage than early and late plantings, partly due to greater insect feeding injury that incentivized more fungal infections observed in the former. Neutral detergent fiber digestibility, starch, and crude protein concentration were greatest for early planting. Greater predicted milk per hectare and milk per megagram for early planting also indicated superior silage quality. Increasing seeding rate increased insect feeding and ear rot injury only when severity was >5% and >15%, respectively. Impact of increasing seeding rate on dry forage yield was specific to each site-year. Overall, results showed that early planting of silage corn helps to escape both insect and disease pressure and provide better yield and quality, while seeding rate response is variable and dependent on field environment.

3.2 INTRODUCTION

Silage corn (*Zea mays* L.) is a high yielding forage crop and contributes a major portion of energy and fiber to the livestock diet (Allen et al., 2003) and is widely grown in the dairy production regions of United States (U.S.). It is considered one of the most convenient forage crops as unlike other contemporary forages, it does not require multiple cuttings. Since silage corn often forms the bulk of diet ration for dairy cattle, the forage nutrient concentration and digestibility play a crucial role in livestock productivity. Producing silage corn for livestock involves a multistep agronomic management process, from hybrid selection, planting date, seeding rate to harvest timings, and eventually packaging densities during the ensiling process (Ferreira and Brown, 2016). These management decisions determine the exposure of the plant to biotic and abiotic stresses and its potential to respond to them (Médiène *et al*., 2011).

Silage corn in the Great Lakes region of U.S. is prone to injury by ear damaging insects, mainly, corn earworm (*Helicoverpa zea* Bod.), European corn borer (*Ostrinia nubilalis* Hub.), and western bean cutworm, (WBC, *Striacosta albicosta* L.). Caterpillar injury may enhance ear rot infections caused by these ascomycete fungi (Smith *et al*., 2017). The most commonly occurring ear rots in the Great Lakes region are the pink ear rot, caused by *Giberrella zeae* (Schweinitz) Petch (teleomorph or sexual stage of *F. graminearum* Schwabe) and white or Fusarium ear rot caused by *F. verticilloides* (Saccardo) Nirenberg (Sutton, 1982; Munkvold, 2003). The injury due to ear damaging insects can considerably increase the ear rot severity (ERS) due to husk wounds and facilitated infection pathways for fungi (Singh *et al*., 2023; Kaur *et al*., 2023a; Farhan *et al*., 2020). Infections by these fungi can also result in production of mycotoxins (secondary toxic metabolites) which can make silage corn unfit for livestock consumption (Ogunade *et al*., 2018; Munkvold, 2019). Under favorable conditions, these infections can cause both significant loss of yield and quality in silage corn (Munkvold, 2003).

Out of various crop management decisions, planting date is critical as it influences the exposure of silage corn at a particular stage and the extent of its susceptibility to insect injury and fungal infections (Pfordt *et al*., 2020, Eli *et al*., 2022). Planting date determines the time of corn silking (most susceptible stage to stresses) and environmental conditions around it which influence the type and extent of ear infections and mycotoxin accumulation (Parsons and Munkvold, 2012, Krnjaja *et al*., 2022). A recent grower survey in Michigan showed that variability in planting time is a major factor impacting mycotoxin concentration (Kaur *et al*., 2023b). Grower fields where silage corn was planted between late April and early May had lower mycotoxin concentration than silage corn planted in late May. Infections under severe conditions may also lead to loss of dry forage yield and quality of silage corn (Haer *et al*., 2015). Additionally, as planting date impacts the harvesting time, it influences the overall environmental conditions during harvesting as well (Fairey, 1983).

Plant density, as determined by the seeding rate, influences the microclimate within a crop field and ability of plants to adapt to specific growing conditions, as well as the severity of insect injury and fungal infections (Eli *et al*., 2022). Plant densities have increased across the U.S., due to increasing stress tolerance in corn hybrids (Williams *et al*., 2021). Usually increases in plant densities for grain have coincided with greater grain yields, especially in high yielding environments (Assefa *et al*., 2016). For instance, agronomic optimum plant density ranged from 73,000 plants ha^{-1} in low yielding environments to 92,000 plants ha^{-1} in very high yielding environments in a meta-analysis from small-plot research (Assefa *et al*., 2016), while it was obtained at 79,410 plants ha^{-1} in an extensive field scale study (Nielsen et al., 2019). Even higher plant densities can be a desirable option for silage corn because they can produce greater biomass yields (Ferreira *et al*., 2014). However, higher plant densities can also increase ear mold infections and mycotoxin accumulation (Krnjaja *et al*., 2019) and cause loss of forage yield and quality (Ferreira and Teets, 2017). Silage corn at higher plant densities show a decline in CP, NDFD, and predicted milk per megagram whereas fiber content increased (Cusicanqui and Lauer, 1999; Ferreira and Teets, 2017). Furthermore, the change in the crop microclimate due to planting time and geographic location may also influence the response of the crop to stresses at higher plant densities. Therefore, it is critical to understand the implications of increasing plant densities not only on forage yield but also on nutritive value at a regional level.

Crop management practices for grain corn are generally well established, with importance of timely planting and establishing higher plant densities to ensure optimum grain yield and profits (Ferreira *et al*., 2014; Cusicanqui and Lauer, 1999). For instance, growers typically prioritize planting grain corn earlier than silage corn since the latter is harvested earlier than physiological maturity. These later plantings in silage corn might provide less time for the ear rot fungi to produce and accumulate mycotoxins. This may not be true if the delayed planting results in a favorable environment for fungal infection around silking (Eli *et al*., 2022). However, research is lacking regarding the impact of planting time and plant density on silage corn response to biotic stresses and eventually dry forage yield, nutrient concentration, and digestibility. Therefore, this research was conducted to (i) evaluate the role of planting date and seeding rate on crop exposure to lepidopteran ear feeding insects and ear rots, (ii) quantify planting date and seeding rate impacts on forage yield and nutrient value, and (iii) identify an agronomic optimum seeding rate to minimize the exposure to insect injury and ear rot infections and achieve optimum yield and quality. We hypothesized that planting corn early in the growing season would shift the highly susceptible corn stage (silking) away from the most favorable conditions for insect injury and consequently the development of disease and mycotoxins. Higher plant density would provide microclimate ideal for insect and disease development and negatively impact silage corn quality.

3.3 MATERIALS AND METHODS

3.3.1 Experiment Sites and Design

Silage corn field trials were conducted at Michigan State University Agronomy Farm in Ingham County, MI (MSU) in 2020 and 2022, and in commercial corn fields in Allegan County in 2020, Ottawa County in 2021, Huron County in 2020-2021, Lenawee County in 2020-2022, and Saginaw County in 2022 (Table 12). Trials at MSU location evaluated both planting date and seeding rate factors, whereas trials in commercial corn field locations only evaluated the impact of seeding rate. At MSU, treatments were set up in a randomized complete block design, organized in a split plot arrangement and replicated four times. Planting date (three levels) as a whole plot factor and seeding rate (four levels) as a subplot factor. Planting dates ranged from late April to early June and were divided into three categories based on typical planting times in central Michigan (Gammans and Singh, 2023): early (April 25 to May 10), mid (May 11 to May 25), and late (May 26 to June 10). Seeding rates ranged from 69,160 to 113,620 seeds ha^{-1} (28,000 to 46,000 seeds $acre^{-1}$), in increments of 14,800 seeds ha^{-1}. For all other site-years, seeding rate was the only factor tested using same rates as explained above and trials were conducted in a randomized complete block design with four replications.

Table 12. Agronomic details for silage corn field trials across various locations in Michigan from 2020-2022.

Year	Location[a]	Previous crop	Planting Dates	Irrigation	Silking Date	Harvest Date	Insect Pressure[c]
2020	MSU	Soybean	7 May	Rainfed	23 July	31 Aug.	Low
			22 May		4 Aug.	16 Sept.	High
			9 June		12 Aug.	4 Oct.	Low
	Huron	Wheat	27 May	Rainfed	5 Aug.	29 Sept.	High
	Lenawee	Soybean	13 May	Rainfed	26 July	11 Sept.	Low
	Allegan	Soybean	5 Jun	Irrigated	7 Aug.	2 Oct.	Low
2021	Huron	Soybean	18 May	Rainfed	3 Aug.	15 Sept.	High
	Lenawee	Soybean	13 May	Rainfed	24 July	7 Sept.	Low
	Ottawa	Soybean	25 May	Irrigated	4 Aug.	17 Sept.	Low
2022	MSU	Soybean	30 April	Rainfed	22 July	6 Sept.	Low
			20 May		1 Aug.	15 Sept.	High
			10 June		14 Aug.	29 Sept.	Low
	Saginaw	Soybean	25 May	Rainfed	7 Aug.	21 Sept.	Low
	Lenawee	Silage Corn	1 June	Rainfed	11 Aug.	20 Sept.	High
	Ingham	Soybean	11 May	Rainfed	1 Aug.	15 Sept.	High

[a] MSU refers to the location at Michigan State University Agronomy Farm in Ingham County. There were three planting times at this location during both 2020 and 2022, with dates ranging from end April to early June. All other locations had one planting time.
[b] Insect pressure was considered high or low when insect egg or larvae were present on >10% or <10% plants, respectively.

Fields were managed using general grower practices of the region. No fungicide or insecticide applications were made. All site-years were rainfed except Allegan 2020 and Ottawa 2021 which were irrigated as per the cooperating farmers' practice. Corn hybrid DS9508 (with relative maturity of 109 and growing degree units to silking of 1320) was planted at all site-years using Almaco corn planter (Almaco, Nevada, Iowa). Plots were 3.05 m (four rows spaced 76 cm apart) wide and 6.71 m long. The middle two rows of each plot were used to assess final disease and insect ratings and measuring dry forage yield during harvest. The outer rows were used for scouting insect egg masses (by examining upper leaf canopy before ear development) and larvae (by examining ears) throughout the growing season to determine insect pressure at each site-year (Table 12).

3.3.2 Data Collection

Plant counts were done at the six-leaf collar stage (V6) in the middle two rows in each plot. Percent emergence was calculated by dividing the plant counts (plant density) with the seeding rate. Phenology ratings were done weekly at the MSU location to record the silking date for each planting date.

Field evaluations for insect and ear rot injury were conducted on the day of harvest. A total of 10 ears per plot were examined, with five consecutive ears selected from each of the middle two rows. Disease ratings were recorded as ear rot incidence (ERI), which measures the percentage of ears exhibiting symptoms of ear rot, and ear rot severity (ERS), which measures the percentage of kernels on an ear affected by ear rot - an indicator of the extent of infection. Insect ratings were recorded as insect feeding incidence (IFI), representing the percentage of ears with insect feeding, and insect feeding severity (IFS), indicating the percentage of kernels on an ear which was fed on which is a measure of the severity of infestation. All ratings were quantified on a per plot basis (Kaur *et al*., 2023a).

Silage corn was harvested using a C1200 Kemper forage harvester (Anker Machinery Company Ltd, Braishfield, Romsey, UK), at a moisture content of approximately 650 mg g^{-1}. The forage harvester was equipped with a rear-mounted Haldrup M-63 weigh system (Haldrup USA, Ossian, Indiana) for measuring fresh biomass yield. This system collected and homogenized the harvested biomass from the entire length of the middle two rows. Subsequently, a representative sample of 500 g was collected from each plot. Samples were dried for 72-h in a hot air dryer at 60°C and then weighed to determine moisture content and dry forage yield (Eckard *et al*., 2011).

The dried samples were further processed by grinding to a particle sieve size of 1mm and analyzed for forage nutrient and mycotoxin concentrations. Near infrared reflectance spectroscopy (NIRS, Model DS2500, FOSS North America, Eden Praire, MN) was used to analyze forage nutrient concentrations and digestibilities including amylase neutral detergent fiber (NDF), acid detergent fiber (ADF), in vitro true digestible dry matter (IVTD), neutral detergent fiber digestibility after a 48-hour incubation period (NDFD), crude protein (CP), and starch, using unfermented silage corn calibration equations from the NIRS Feed and Forage Testing Consortium (NIRSC, Berea, KY) similar to the approaches followed by Bhattarai *et al*. (2020) and Agnew *et al*. (2022). Predicted milk yields were calculated as milk per hectare (Mg ha^{-1}) and milk per megagram (kg Mg^{-1}) based on the dry forage yield, IVTD, NDF, and CP values, utilizing the Milk Equation 2006 (de Los Campos *et al*., 2006).

Samples from MSU 2020 and 2022 were shipped to the University of Guelph Ridgetown (Ontario, Canada) for mycotoxin analysis. The analysis followed the methodology developed by

Limay-Rios and Schaafsma, (2021). An Ionics EP 10+ modified API365 triple quadrupole mass spectrometer (LC-MS/MS) system, equipped with an electrospray ionization source in both positive and negative polarity, was utilized for the analysis. The samples were screened for 26 mycotoxins, including deoxynivalenol (DON), zearalenone (ZON), fumonisin, and beauvericin. Samples from site-years with seeding rate trials were shipped to University of Minnesota (St. Paul, MN) for DON analysis following protocols by Fuentes *et al*. (2005) using gas chromatography and mass spectroscopy.

3.3.3 Data Analyses

A generalized linear mixed model (PROC GLIMMIX) was used to determine the effect of planting date and seeding rate on insect injury, ear rot injury, forage nutrient concentration and digestibility in silage corn using SAS 9.4 (SAS Institute Inc.). The level of significance (α) was 0.10 for insect and ear rot injury to account for the higher variability caused by variation in insect flight and disease inoculum at various site-years. For forage nutrient concentration and digestibility α was 0.05. For both trials (at MSU and commercial fields), data was analyzed by site-years due to the unique and variable environmental conditions present for each location and growing season. For trials at MSU, planting date and seeding rate were considered as fixed factors while replication as a random factor. No interaction was observed between planting date and seeding rate; therefore, data is shown only for the main effects of planting date and seeding rate. For the seeding rate trials at commercial fields, seeding rate was considered a fixed factor and replication a random factor.

Least square means were calculated using *lsmeans* statement and mean differences were calculated using Tukey's adjustment. Statement *dfmkr* was used to minimize the effect of heterogenicity in variances. Regression analysis was conducted in R studio (RStudio Team, 2020) using 'lm' function to compare linear and quadratic models and 'nls' function to run nonlinear plateau models to determine the impact of plant density on dry forage yield and predicted milk yield. The agronomic optimum plant density was then quantified using 'which.max' statement. Observed plant density was used instead of the average seeding rate for regression analyses to account for variations observed in emergence. A regression approach was used because ANOVA may not be useful to determine agronomic optimum plant density for dry forage yield and predicted milk yields as it mostly lies in between the tested seeding rates (Hammam *et al*., 2021). These data were plotted using the R package ggplot2.

3.4 RESULTS

3.4.1 Weather Data

Silking dates averaged from late July to mid-August (Table 12) across site-years depending largely on the planting time. The Lower Peninsula of Michigan on average receives more than 60 mm rain during the months of July and August, with temperature ranges between 17 to 29°C; conditions conducive for fungal infections. However, rainfall was lower than average in 2020 (12 mm) but was closer to 30-year average (24 mm) in 2021 (23 mm) and 2022 (22 mm) during the silking period (Table 13). An exception was Huron 2020, which received more than 50 mm of rainfall and had a mean temperature of 20.4°C (lower than 30-year average) around silking (Table 13).

Table 13. Mean temperature and total precipitation during the silking window (± 5 days around silking) at various site-years in Michigan.

Year	Location[a]	Temperature (°C)	Precipitation (mm)	Relative Humidity (%)
2020	MSU: Early	23.3	5	72.7
	MSU: Mid	21.1	25	78.2
	MSU: Late	21.8	3	66.4
	Huron	20.4	56	78.3
	Lenawee	24.1	2	73.1
	Allegan	20.9	23	70.6
2021	Huron	18.2	26	63.5
	Lenawee	22.5	22	73.7
	Ottawa	20.8	22	66.7
2022	MSU: Early	23.4	24	67.6
	MSU: Mid	22.6	31	71.8
	MSU: Late	19.3	11	69.7
	Saginaw	22.2	0	71.9
	Lenawee	22.2	0	67.7
	Ingham	22.6	31	71.8

[a] MSU 2020 and 2022 had three planting times. Early: planting between April 25 – May 10; Mid: planting between May 11- May 25; Late: planting between May 26 – June 10.
Note: Temperature and precipitation at all site-years were determined using data obtained from the weather stations closest to the field location (established by the Michigan State University Enviro-weather https://enviroweather.msu.edu/).

3.4.2 Effect of Planting Date

Planting date did not impact plant emergence at any of the site-years (Supplementary Table S4). Insect feeding incidence and severity varied across planting date in MSU 2020 (p = 0.07 and 0.02, respectively) but in MSU 2022, planting date impacted only IFS (p = 0.13 for IFI and p = 0.005 for IFS). Insect feeding incidence for the mid- planting was 60 and 59 % greater than early and late planting at MSU 2020, respectively (Figure 14a). In 2022, IFI was 43 and 37% greater in mid-planting than early and late planting, although the difference was not significant (p = 0.13). The mid-planting also had 84 and 85% greater IFS at MSU 2020 and 80 and 83 % greater IFS at MSU 2022 as compared to early and late planting, respectively (Figure 14a).

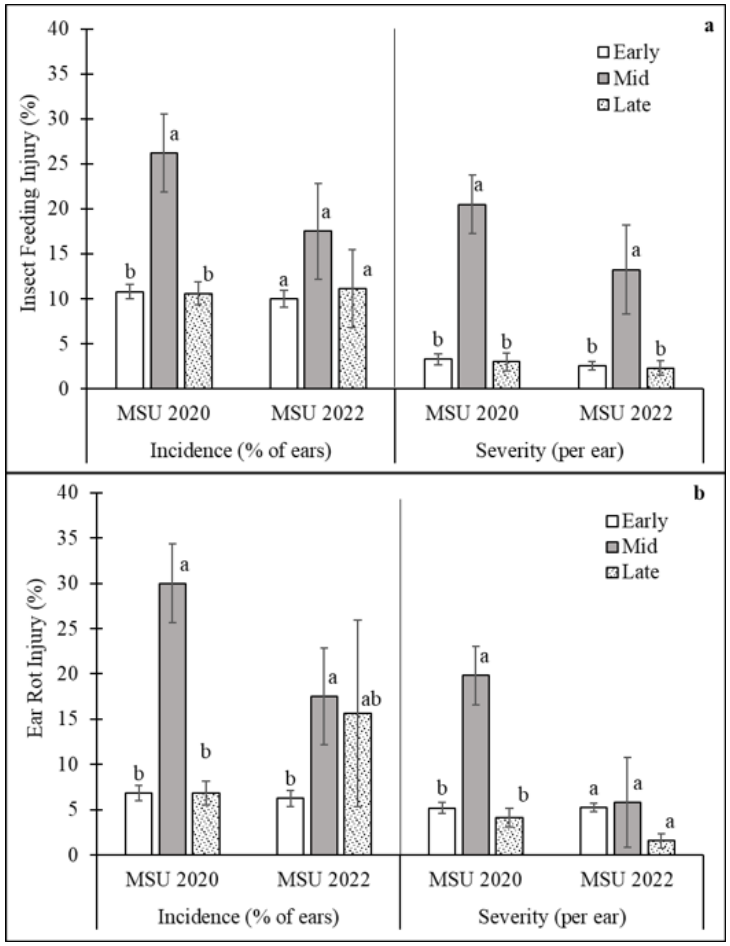

Figure 14. (a) Average insect feeding injury (incidence and severity) and (b) average ear rot injury (incidence and severity) for different planting dates at two site-years. Early: planting between April 25 – May 10; Mid: planting between May 11- May 25; Late: planting between May 26 – June 10. Bars with same letters within a site-year for a response variable are not different ($\alpha = 0.10$). Vertical lines on each bar represent ± 1 standard error.

In 2020, both ERI and ERS varied significantly by the planting date ($p = 0.03$ and $p = 0.04$, respectively) whereas in 2022, only ERI was significantly different ($p = 0.02$ for ERI and $p = 0.18$ for ERS). For mid-planting, ERI was 77% greater than early and late planting in 2020 and 64% greater than early planting in 2022 (Figure 14b). Ear rot severity was 70 and 79% greater for mid-planting compared to early and late planting and 64% greater than early planting in 2020 (Figure 14b). Ear rot severity did not show any differences among planting dates in 2022 (Figure 14b).

Mycotoxin concentrations were low at MSU in both 2020 and 2022, and planting date did not significantly impact the concentrations for any of the mycotoxins analyzed (data not shown). Overall, the mycotoxin that occurred most frequently and had the highest concentration was DON (positive in 58% samples) but only 27% of the samples had DON >1 $\mu g\ g^{-1}$. About 33% of samples from mid-planting had DON >1 $\mu g\ g^{-1}$ whereas only 15% samples from early and 21% samples from late planting had DON>1 $\mu g\ g^{-1}$. Other frequently occurring toxins were ZON, beauvericin, and fumonisins but all with concentration less than 1 $\mu g\ g^{-1}$. Weak to no correlations were observed between ear damage and mycotoxin concentration (data not shown).

Planting date impacted the dry forage yield and nutrient concentration and digestibility (Table 14). Mid-planting in MSU 2020 had 12 and 15% less dry forage yield than early and late planting ($p = 0.003$), respectively, and 24 and 30% less in MSU 2022 ($p = 0.07$), respectively. Crude protein and starch were greatest in early planting (Table 14). In 2020, ADF was the lowest in early planting as compared to mid and late planting, whereas in 2022, both ADF and NDF varied across the planting date (Table 14). Digestibility, both IVTD and NDFD, were 5-7% and 9-10% greater for early planting than mid and late planting, respectively. Milk per megagram was 20 and 29% greater in early planting than mid and late planting, respectively at MSU 2020, while at MSU 2022, it was 8% greater than mid-planting but was not different from late planting. Predicted milk yield per hectare was also greatest for early planting (70 and 36% greater than mid and late planting, respectively) at MSU 2020, whereas, at MSU 2022, it did not vary for early and late planting but was 18-20% less in mid-planting compared to other planting times (Table 14).

Table 14. Effect of planting date on dry forage yield, nutrient concentration, digestibility, and predicted milk yield of silage corn at two site-years.

Site-year	Planting Date[a]	Yield	ADF	NDF	Starch	CP	IVTD	NDFD	Milk per megagram	Milk per hectare
		Mg ha^{-1}			g kg^{-1} of DM			g kg^{-1} of NDF	kg Mg^{-1}	Mg ha^{-1}
MSU 2020	Early	16.9 a	153 b	200 a	463 a	76.7 a	881 a	605 a	1540 a	28.9 a
	Mid	14.8 b	198 a	209 a	409 b	70.5 b	843 b	552 b	1237 b	20.2 b
	Late	17.6 a	196 a	217 a	356 c	75.4 a	844 b	581 b	1090 b	21.4 b
	p-value	0.003	<0.001	0.18	<0.001	<0.001	0.001	<0.001	0.008	<0.001
MSU 2022	Early	17.9 a	161 b	264 a	459 a	74.3 a	905 a	636 a	1668 a	31.7 a
	Mid	13.6 b	172 a	258 a	406 b	54.2 b	864 b	570 b	1530 b	24.9 b
	Late	19.7 a	168 ab	221 b	412 b	76.9 a	863 b	589 b	1635 ab	31.2 a
	p-value	0.07	<0.001	0.002	0.002	<0.0001	0.02	0.01	0.02	0.005

[a]Early: Planting between April 25 to May 10, Mid: planting between May 11 to May 25, Late: planting between May 26 to June 20.
Note: ADF (acid detergent fiber), NDF (neutral detergent fiber), CP (crude protein), IVTD (in-vitro digestible dry matter), NDFD (NDF digestibility), DM (dry matter).
Values with same letters within a site-year and variable are not different (α = 0.10 for yield and 0.05 for forage nutrient concentration and digestibility and predicted milk yield).

3.4.3 Effect of Seeding Rate

Seeding rate did not impact insect feeding incidence at any site-year (including trials at MSU) except at Huron 2021 ($p = 0.01$) where IFI was 68% greater at the highest seeding rate (113,620 seeds ha^{-1}) as compared to the lowest seeding rate of 69,160 seeds ha^{-1} (data not shown). Insect feeding severity (Figure 15a) increased with an increase in seeding rate at Huron 2020, Huron 2021, and Lenawee 2022 ($p = 0.003$, 0.03, and 0.08, respectively). For all these site-years, IFS was the greatest in the plots planted at 113,620 seeds ha^{-1}. At all other site-years (both MSU and commercial fields), IFS was not significantly different across the seeding rate and had an average value of less than 5%.

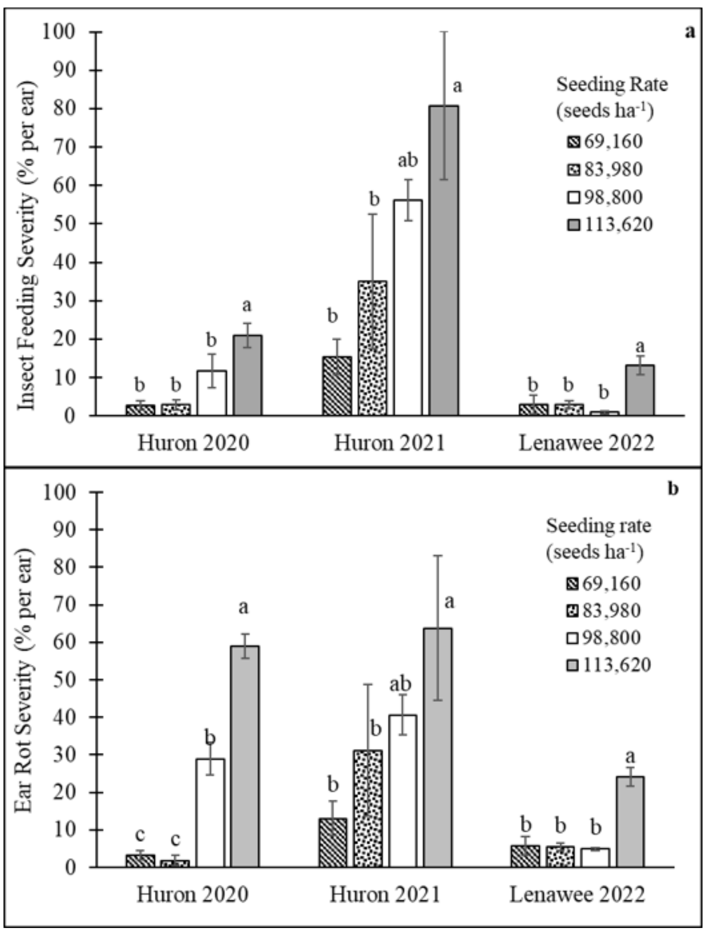

Figure 15. Average insect feeding severity (a) and ear rot severity (b) across various seeding rates (number of seeds ha^{-1}). Bars with same letters within a site-year and variable are not different ($\alpha = 0.10$). Vertical lines on each bar represent ± 1 standard error.

Ear rot incidence (Figure 15b) did not vary with seeding rate at any of the site-year (both MSU and commercial fields), but ERS increased with increasing seeding rate at Huron 2020, Huron 2021, and Lenawee 2022 (p = 0.04, 0.01, and 0.09, respectively), the same site-years with differences in IFS. Plots planted at 113,620 seeds ha^{-1} had greater ERS than those planted at lower seeding rates. At other site-years, the ERS was <15% and not different across various seeding rates. Most common mycotoxin was DON, occurring in 28% samples, but at a concentration < 1 µg g^{-1}. Deoxynivalenol concentration did not differ across the seeding rates at any of the site-years and did not show any trends (data not shown). Other mycotoxins that occurred in more than 10% of samples were ZON and fumonisins.

Plant density increased with seeding rate at all the site-years, however plant emergence did not vary significantly at most site-years (Supplementary Table S5). Dry forage yield was impacted by seeding rate at Allegan 2020, Huron 2020, Lenawee 2020, Huron 2021, Ottawa 2021, and Saginaw 2022 (Supplementary Table S6). A nonlinear regression showed that the quadratic model had greater adjusted R^2 value than linear model and plateau models and better explained the variability in dry forage yield due to plant density (Supplementary Table S6).

For the site-years where plant density impact was significant on dry forage yield, the adjusted R^2 values showed a large variation and greatest dry forage yield was not obtained consistently at the highest plant density (Figure 16). The agronomic optimum plant density at Allegan 2020, Huron 2020, and Saginaw 2022 (site-years with adjusted $R^2 > 0.40$) was 103,636, 109,832, and 89,799 plants ha^{-1}, respectively (Figure 16a, 16b, and 16f).

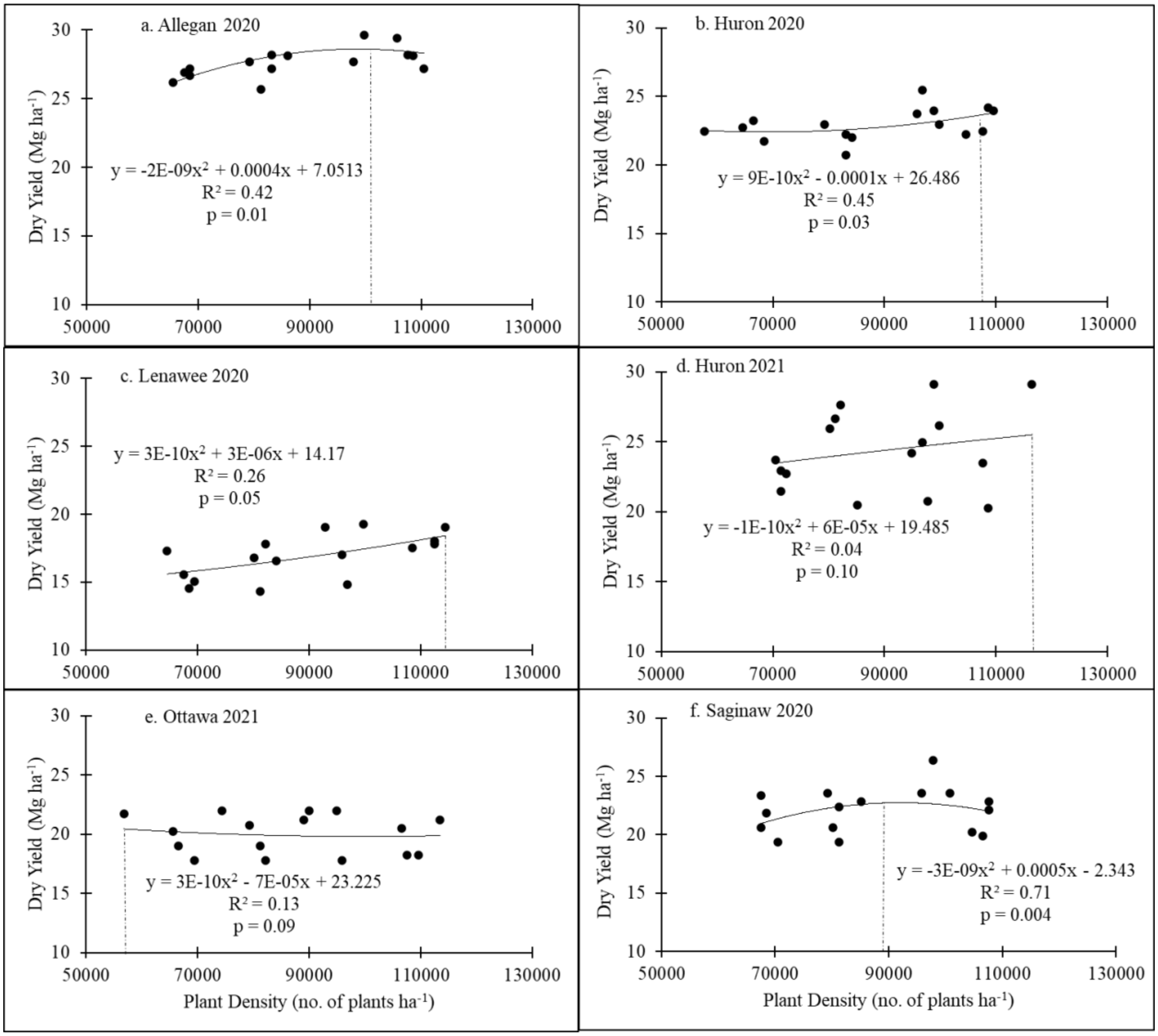

Figure 16. Response of silage corn dry forage yield to plant density at various site-years. The point of predicted agronomic optimum plant density for a given site-year on x-axis falls on the vertical dotted line that runs through the model line. The point of intersection is the greatest dry forage yield obtained.

Seeding rate did not impact CP concentration and predicted milk per hectare at any of the site-years. However, ADF, NDF, and IVTD at Huron 2021 and Lenawee 2022 were affected by variation in seeding rate (Table 15). Starch concentration was impacted by seeding rate only at Lenawee 2022 ($p = 0.05$). Both the ADF and NDF showed an increase with increasing seeding rates. Seeding rates above 98,800 seeds ha^{-1} had 10 to 35% greater ADF and 20 to 24% higher NDF than the lower seeding rates (Table 16). Starch concentration, however, was the lowest for the highest seeding rate (Table 16). Neutral detergent fiber digestibility was impacted by seeding rate at Huron 2021, Ottawa 2021, and Lenawee 2022 where it showed a decline with increase in seeding rate (Table 16). The only site-year that showed a response to predicted milk per megagram was Huron 2021 ($p = 0.04$). Lenawee 2022, although not significant showed a numeric trend ($p = 0.07$) and both followed a quadratic regression model (Figure 17). The greatest predicted milk per megagram was 1,577 kg Mg^{-1} and was obtained at the plant density of 79,462 plants ha^{-1} at Huron 2021, and 1,679 kg Mg^{-1} at plant density of 99,565 plants ha^{-1} at Lenawee 2022 (Figure 17).

Table 15. ANOVA p-values for the effect of seeding rate on dry forage yield, nutrient concentration, digestibility, and predicted milk yield of silage corn.

Site-year	ADF	NDF	Starch	CP	IVTD	NDFD
MSU 2020	0.24	0.17	0.53	0.20	0.41	0.13
MSU 2022	0.31	0.11	0.21	0.39	0.18	0.65
Allegan 2020	0.85	0.89	0.98	0.64	0.76	0.91
Huron 2020	0.2	0.14	0.16	0.14	0.47	0.45
Lenawee 2020	0.33	0.35	0.24	0.28	0.26	0.88
Huron 2021	**0.02**	**0.02**	0.07	0.32	**0.01**	**0.01**
Lenawee 2021	0.66	0.65	0.68	0.35	0.64	0.76
Ottawa 2021	0.33	0.26	0.20	0.97	0.28	**0.04**
Ingham 2022	0.95	0.56	0.90	0.87	0.48	0.44
Lenawee 2022	**0.02**	**0.02**	**0.05**	0.15	**0.008**	**0.01**
Saginaw 2022	0.86	0.77	0.88	0.28	0.94	0.14

Note: ADF (acid detergent fiber), NDF (neutral detergent fiber), CP (crude protein), IVTD (in-vitro digestible dry matter), NDFD (NDF digestibility).
p-values in bold denote significant values at $\alpha = 0.05$.

Table 16. Effect of seeding rate on dry forage yield, nutrient concentration, and digestibility of silage corn at Huron 2021 and Lenawee 2022.

Forage nutrients	Site-year	Seeding Rate (no. of seeds ha^{-1})			
		69,160	83,980	98,800	113,620
ADF (g kg^{-1} of DM)	Huron 2021	210 b	200 b	230 ab	272 a
	Lenawee 2022	174 b	184 ab	207 ab	223 a
NDF (g kg^{-1} of DM)	Huron 2021	373 bc	358 c	381 b	466 a
	Lenawee 2022	341 b	353 b	384 ab	407 a
Starch (g kg^{-1} of DM)	Huron 2021	378 a	405 a	397 a	298 a
	Lenawee 2022	372 ab	399 ab	415 a	339 b
IVTD (g kg^{-1} of DM)	Huron 2021	841 a	846 a	841 a	791 b
	Lenawee 2022	855 b	846 b	891 a	868 ab
NDFD (g kg^{-1} of NDF)	Huron 2021	585 a	571 ab	576 ab	552 b
	Ottawa 2021	603 a	607 a	595 b	594 b
	Lenawee 2022	682 a	625 b	625 b	626 b

Note: ADF (acid detergent fiber), NDF (neutral detergent fiber), IVTD (in-vitro digestible dry matter), NDFD (NDF digestibility), DM (dry matter).
Values with same letters for a variable within a site-year are not different ($\alpha = 0.05$).

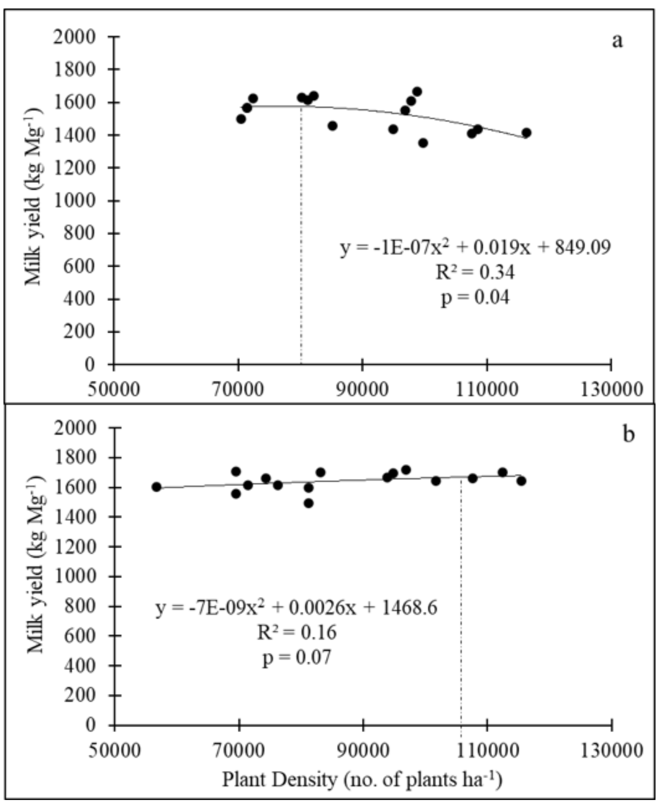

Figure 17. Response of predicted milk yield to plant density at Huron 2021 (a) and Lenawee 2022 (b). The point of predicted agronomic optimum plant density for a given site-year on x-axis falls on the vertical dotted line that runs through the model line. The point of intersection is the greatest milk yield obtained.

3.5 DISCUSSION

Our study evaluated the relationship of planting date and seeding rate to insect injury and ear rot fungi infections in silage corn and quantified its impact on dry forage yield and quality. Insect injury enables ear rot fungi infections and is highly dependent on the field microclimatic conditions (such as temperature and precipitation). Planting silage corn early in the planting window of the growing season lowered the risk of these infections and protected yield potential by averting insect injury (primarily by WBC) and preventing high mycotoxin concentration. Higher seeding rate did not always provide the greatest dry forage yield and coincided with a greater insect injury and a greater ear infection, indicating that agronomic optimum plant density may not be obtained at the highest seeding rate due to increased severity of biotic stresses with increase in seeding rate. In addition, predicted milk yield per megagram although not impacted at most site-years, showed a quadratic relation with increase in plant density.

In the U.S. Great Lakes dairy production regions, planting silage corn early is not considered as crucial as to grain corn since the former is harvested before physiological maturity. However, our study shows that silage corn planted early (last week of April to early May in central MI) or very late (first week of June or later) in the planting window can help avoid the environment conditions adequate for ear rot infections during silking, while having time to develop yield. In contrast, silage corn planted during mid-planting window silked during the first week of August and received >25 mm of rainfall resulting in favorable environment for infections. These observations were synchronous with a grower survey study conducted by Kaur *et al*., (2023b) in Michigan, which reported that growers who planted silage corn in early planting window had lower ear rot infections and mycotoxin than late planting. The same study also reported that the samples collected from the regions which received more rainfall during silking window had greater mycotoxin concentration. Studies by Eli *et al*., (2022) and Parsons and Munkvold, (2012) also corroborated the importance of varying planting dates to avoid conditions favorable for fungal infections during silking window. Moreover, in our study it was observed that the silage corn from mid-planting had a more severe insect feeding injury as compared to early and late planting. The insect injury further provided favorable infection pathway through husk wounds for ear rot infections. Research by Farhan *et al*., (2020), Singh *et al*., (2023), and Kaur *et al*., (2023a) have reported an increase in ear rot infections and mycotoxin accumulation due to insect injury by lepidopteran ear damaging insects.

Dry forage yield and predicted milk yield were also lowest in mid-planting as compared to the early and late planting, partly due to greater insect injury and ear rot infections. Forage nutritive value was also better both in terms of lower ADF and greater IVTD and NDFD for early planting than mid and late planting. A similar decline in NDFD of silage corn due to delayed planting date was observed by Kim *et al*., (2001), Graybill *et al*., (1991), and Fairey, (1983); the average NDFD was lower in these studies. Overall, the average NDF values observed for all planting dates in our study were lower than the lower limit of the average range (30-50 g kg^{-1}) for silage corn (Nestor, 2010 & Hoffmann *et al*., 2001).

For the seeding rate trials, insect injury (primarily by WBC) varied significantly across seeding rates at Huron 2020, Huron 2021, and Lenawee 2022. Insect feeding severity showed an increase with an increasing seeding rate, especially when seeding rate increases beyond 98,800 seeds ha^{-1}. This could be because increase in seeding rate leads to more surface for the insects to breed or provide easier movement to the insect larvae by virtue of which they can damage multiple ears. However, no differences were seen across treatments when less than 10% of the field was infested by ear damaging insects.

Ear rot severity was also greater in the higher seeding rates, probably due to an easier infection route for fungus through husk wounds caused by insect injury accompanied with a more humid environment and a greater plant-to-plant competition for resources impacting the overall plant health and defense (Rankin and Grau, 2002). Another reason for greater severity of ear rot infections under higher plant densities could be the presence of smaller ears. Eli *et al.* (2022), and Abbas *et al.* (2012) reported that for higher plant densities, ear rot infections were more severe under stress conditions in grain corn. However, the research for silage corn is sparse and may show a different relation as silage corn is harvested earlier than grain corn providing less time for ear molds to develop and accumulate mycotoxins. Furthermore, no interaction was observed for insect and disease injury between planting date and seeding rate in this study. This could be due to the limited number of site-years and replications for the trials looking at planting and seeding rate decisions. Future research with more site-years and replications to increase the power of test would help in exploring this issue further.

Our data also showed that the agronomic optimum plant density is not necessarily obtained at the highest seeding rates. It was true for both dry forage yield and the predicted milk yield where a quadratic relation explained the variability better in comparison to linear and plateau models. Based on site-years with adjusted R^2 >0.40, greatest yields were observed when plant density ranged between 89,799 and 103,636 plants ha^{-1}. This could partly be due to greater insect injury and ear rot infections at higher plant density, and partly due to increased plant-to-plant competition for resources at higher seeding rate (Assefa *et al.*, 2016) leading to lesser kernel number and smaller corn ears than lower seeding rate. These trends were similar to the relations obtained by Ferreira *et al.*, (2014), Ferreira and Teets, (2017), Stanton *et al.*, (2007) for silage corn and by Williams *et al.*, (2021) in grain corn. An exception was Huron 2020 in the current study, where the greatest dry forage yield was obtained at plant density of 109,832 plants ha^{-1}.

Forage predicted milk per megagram did not decline as drastically as expected with decline in dry yield due to increase in plant density. Also, it did not increase linearly and followed a quadratic relation. These observations were similar to Cusicanqui & Lauer, (1999), however, their seeding rate range (75,000 to 85,000 seeds ha^{-1}) to maximize predicted milk per megagram was lower than that observed in our study. Silage corn was also observed to have greater fiber content at higher seeding rates which was in contradiction to Graybill *et al*. (1991) which did not show any differences in either NDF or ADF across various seeding rates. Silage corn digestibility (both NDFD and IVTD) showed a decline at higher plant densities which was similar to Ferreira *et al*. (2014), and Ferreira and Teets (2017). However, the starch concentration showed differences only at Lenawee 2022 with a similar trend observed at Huron 202. At the highest seeding rate (113,620 seeds ha^{-1}) used in this study the starch content was 8 to 18% less than the lower seeding rates. We did not observe any significant differences in starch content at any of the site-years. This may be due to higher number of ears in the plots with higher seeding rate even when the ear size was smaller. A similar lack of differences in starch content was also reported by Diepersloot *et al*. (2021).

Overall, the greatest dry forage yield was obtained within 89,799 and 109,832 plants ha^{-1} and forage nutritive value was the greatest between 79,462 and 99,565 plants ha^{-1}. Also, an increased risk of insect injury was observed at seeding rates higher than 98,800 ha^{1}. Therefore, the most beneficial choice for both optimum dry forage yield and nutritive value could be plant densities of at least 90,000 ha^{-1} but staying below 100,000 plants ha^{-1}.

Mycotoxins detected in this study did not differ across the planting dates and seeding rates at any of the site-years, primarily because they were low in concentration. However, they might be a prime concern under very high plant densities if the environmental conditions are more conducive and there is greater inter-plant competition for resources. Also, we recognize that our study was conducted only on a limited site-years and the observations were variable and very location specific. Therefore, more extensive research studying the microclimate of the field by installing multiple in-field sensors measuring canopy air temperatures and moisture could provide more information that will be crucial to further support the findings of this research.

3.6 CONCLUSION

Overall, our results showed that planting silage corn early in the growing season in the U.S. Great Lakes Region can result in less insect injury and ear rot pressure at silking when corn is most prone to stress. Corn planted later in the planting season (late May onwards in central Michigan) had greater insect injury and ear rot infections. Planting later (June) in the season can help escape favorable conditions for insect injury and disease infection. However, late planting can cause a forage yield penalty, whereas the greatest forage yields, nutrient concentration, and predicted milk production were obtained in early planting (Mid-May or earlier in central Michigan). The impact of seeding rate on biotic stresses, yield, and nutritive value of silage corn was dependent on field microclimate and mostly showed a quadratic relation with plant density. Our results suggest that in order to achieve the optimum dry forage yield and maintain high quality, growers should aim for the agronomic optimum plant density to fall between 90,000 to 100,000 plants ha^{-1}. Future research with intensive data collection on various weather variables, insect, and disease pressure monitoring, along with targeting locations with known pest pressure can help refine integrated pest management strategies. Furthermore, exploring the impact of factors such as harvest timing and field management history that can impact crop’s exposure to the above-mentioned stresses will be critical for maximizing forage yield and quality in silage corn.

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CHAPTER 4: FOLIAR STRESSES IMPACT SILAGE CORN YIELD AND FORAGE NUTRITIVE VALUE

4.1 ABSTRACT

Silage corn (*Zea mays* L.) in Michigan and the Great Lakes region is prone to an emerging foliar disease called tar spot. When corn is infected with *Phyllochora maydis* (fungi causing tar spot), stromata develop on the leaves and ears resulting in early senescence and drying. Therefore, to understand the impact of tar spot on forage yield, nutritive value, and predicted milk yield, field trials were conducted at multiple Michigan locations from 2021-23. Field trials were arranged in randomized complete block design with four replications. Treatments included hybrid resistance (one susceptible and one partially resistant hybrid) and three fungicide treatments using Delaro 325 SC @ 8 oz acre^{-1} [non-treated, one application at silking (R1) and two applications (one at R1 and second at dough stage)]. Results show that tar spot severity increased over time in silage corn. Fungicide application in susceptible hybrid had the lowest tar spot severity across all hybrid and fungicide treatments. Hybrid disease resistance resulted in 50% reduction of tar spot severity and preserved dry yield by 27%. Reduction of disease severity due to hybrid disease resistance also minimizes decline in neutral fiber digestibility and predictive milk yield. Fungicide application reduced tar spot severity but did not impact dry yield and forage nutritive value. Overall, our study shows that tar spot reduces forage yield and nutritive value and requires an integrated approach to disease management.

4.2 INTRODUCTION

Silage corn (*Zea mays* L.) is a major part of diet ration for lactating cows in Europe, North America, and South America (FAO, 2023). In the United States (U.S.), approximately 129 million tons of silage corn was produced in 2022 (USDA, 2023). However, silage corn growers across the world frequently face infestations by ear feeding, stalk boring, leaf chewing insects, root nematodes, and fungal diseases such as rots, rusts, smuts, blights etc. (Farhan *et al*., 2017). These stresses under severe cases can impact the forage yield and nutritive value of silage corn. Moreover, many types of fungi degrade plant cells releasing toxins that kill plant tissues (Sexton and Howlett, 2006). Fungal infections can occur on above ground shoot (stalks, ear, and leaves) and in the roots. Many others appear as foliar lesions or stromata on the leaves and destroy the photosynthetic tissue. Fungi affect corn plants both in the field and post-harvest.

Most common foliar fungal pathogens observed in the Great Lakes Region of the U.S. are, *Cercospora zeae-maydis* (causes gray leaf spot), *Setosphaeria turcica* (causes northern leaf blight), *Puccinia sorghi* (causes common rust), and *Bipolaris zeicola* (causes northern leaf spot). These pathogens generally thrive in humid, and cool to mildly warm environments (65 to 80 °F); conditions which are common in the Great Lakes Region during the growing season and have been historically known to result in the high yield losses (0.05 to 1 million bushels, depending on severity) in corn (Mueller *et al*., 2016 and 2020). However, a foliar pathogen, native to Latin America, *Phyllachora maydis* which causes tar spot was reported in 2015 in Indiana and Illinois (Ruhl et al., 2016). It appears as oval to circular, small black stromata, or lesions on corn leaves. The lesions start as yellow and then quickly turn to brown and black surrounded by chlorotic ring (Liu 1973). Under severe conditions these stromata on leaves grow in size, coalesce and can also develop on ear husk. The disease usually starts on the lower leaves and progresses to the upper canopy (Bajet *et al*., 1994). Tar spot is an evolving threat to corn yield and quality in the Great Lakes Region. Researchers reported that tar spot contributed to a loss of 242.6 million bushels in the U.S. from 2018 to 2020 (Mueller *et al.*, 2020). In 2021 alone, tar spot resulted in an estimated loss of 205.4 million bushels of grain corn in the U.S. (Crop Protection Network, 2021).

Since tar spot is relatively new in the Great Lakes region, major knowledge gaps exist for appropriate management strategies. Research on grain corn shows that the use of hybrid disease resistance can be beneficial and area under disease progression curve was lower in partially resistant hybrids than susceptible hybrids (Ross *et al*., 2023b). However, existing hybrids only provide partial resistance against tar spot. Therefore, a combined strategy of field scouting and timely fungicide application is being explored extensively. Fungicide treated grain corn had lower tar spot severity as compared to non-treated corn (Telenko *et al.*, 2022a and 2022b). Yield benefits of fungicide treatments were inconsistent and only a three percent yield protection was observed only by Telenko *et al*., (2022b). Ross *et al*., (2023a) further showed that fungicide applications were economically benefit only under high disease severity.

Where the whole plant silage corn is concerned, tar spot not only can cause yield losses but also impact the fiber digestibility, crude protein and starch content, resulting in lower predicted milk yields. Furthermore, silage corn has a shorter growing season and is harvested about 3-4 weeks earlier than grain corn and the disease progression is different in magnitude when harvested earlier than later. Harvesting a grain crop for silage is a strategy that is sometimes followed by growers to avoid yield losses in grain corn under high disease environments (Silver, 2021). However, there is still possibility that with increase in tar spot severity, the forage nutritive value would decline regardless of harvest window. Moreover, fungicide applications which is one of the key management strategies for tar spot, can also impact the overall forage nutritive value (Reed *et al*., 2021; Cardoso *et al*., 2020; Kalebich *et al*., 2017).

Therefore, it is critical to understand the progress and ramifications of tar spot and the influence that management strategies can have on yield and nutritive value in silage corn. Most of the research being conducted on foliar diseases and tar spot are focused on grain corn. This study was conducted to explore the following specific objectives, (i) quantify decline in yield and forage nutritive value in silage corn due to tar spot, (ii) explore potential management strategies such hybrid disease resistance and fungicide application to manage tar spot in silage corn.

4.3 MATERIALS AND METHODS

4.3.1 Experiment Sites and Design

Silage corn field trials were conducted at six site-years from 2021-2023 in central and western Michigan in Ingham, Ottawa, Branch, and Barry Counties (Table 1). All the trials evaluated the role of hybrid disease resistance and fungicide application in reducing the tar spot and preserving the forage nutritive value. The hybrids with variable disease resistance and fungicide application were organized in a randomized complete block design with four replications. Two hybrids, one partially resistant and one susceptible to tar spot (Supplement Table 7), were used and fungicide application was done using a prothioconazole and trifloxystrobin based fungicide, Delaro 325 SC (Bayer CropScience, St. Louis, MO) @ 8 oz acre^{-1} (Telenko *et al*., 2022a). There were three fungicide treatments: non-treated, one application (at silking, R1), and two applications (at R1, and then at dough stage, R3) (Table 17).

Table 17. Agronomic details for field trials conducted across the years at various locations.

Year	**Location**	**Previous crop**	**Planting date**	**Irrigation**	**First fungicide application[a]**	**Second fungicide application[b]**	**Harvest date**
2021	Branch	Seed Corn	20 May	Irrigated	26 Jul	14 Aug.	9 Sept.
	Ottawa	Soybean	25 May	Irrigated	3 Aug.	23 Aug.	17 Sept.
2022	Ingham	Soybean	12 May	Rainfed	28 Jul	18 Aug	15 Sept.
	Ottawa	Grain Corn	17 May	Irrigated	4 Aug	24 Aug	19 Sept.
2023	Barry	Wheat	17 May	Rainfed	31 Jul	21 Aug	10 Sept.
	Ingham	Soybean	10 May	Rainfed	31 Jul	21 Aug	11 Sept.

Table 17 (cont'd)
[a] First fungicide application was done at the silking (R1) corn stage.
[b]Second fungicide application was done at dough stage (R3) of corn.

Trials were planted using Almaco corn planter (Almaco, Nevada, Iowa) in May in all three years using 34,000 seeds acre^{-1}. Plots were 5 feet (four rows spaced 30 inches apart) wide and 22 feet long, with a 3 feet alley between plots. Fungicide applications were done using a pressurized CO_2 high clearance backpack sprayer with a 10 feet wide broom, fitted with TeeJet 8001 VS nozzles spaced 20 inches apart. Boom height was maintained at 30 cm above the tassel. The middle two rows of each plot were used to assess final disease severity and measuring dry forage yield during harvest. Fields were managed based on general grower practices of the region. All site-years were rainfed except Branch and Ottawa which were irrigated as per the cooperating farmers' practice.

4.3.2 Data Collection

Field scouting was done to determine phenological stages and identify silking stage for timely fungicide applications. Field evaluations for tar spot began at the first incidence of disease or at R1 and were done at weekly to 10 days interval. A total of 20 ear leaves per plot (10 from each of the middle two rows) were examined and rated for tar spot using a standard scale of disease severity ratings provided by Telenko *et al.*, (2021). All the severity ratings were averaged across the plot to obtain one value for disease levels per plot. The number of times disease severity ratings were taken depended on the amount of disease on the leaf and the time of first disease appearance. Final disease evaluations were done at harvest and were used to study the relationship between foliar infection, yield and nutritive value.

Silage corn was harvested with a C1200 Kemper forage harvester (Anker Machinery Company Ltd, Braishfield, Romsey, UK) at an approximate moisture level of 65%. This harvester had a Haldrup M-63 weigh system (Haldrup USA, Ossian, Indiana) mounted at the rear to determine the fresh biomass yield. This system uniformly collected and mixed the harvested biomass from the central two rows. Afterwards, a representative sample (around 1 lb) was taken from each plot. These samples underwent a 72-hour drying process in a hot air dryer set at 140°F to establish both moisture content and dry forage yield (Eckard *et al.*, 2011).

The dried samples were subsequently ground to a particle size of 1mm (0.04 in) and analyzed for forage quality. Near-infrared reflectance spectroscopy (NIRS, Model DS2500, FOSS North America, Eden Praire, MN) was employed to assess quality parameters such as neutral detergent fiber (NDF), acid detergent fiber (ADF), in vitro true digestible dry matter (IVTD), neutral detergent fiber digestibility after a 48-hour incubation period (NDFD), crude protein (CP), and starch as followed by Bhattarai et al. (2020) and Agnew et al. (2022). This analysis utilized unfermented silage corn calibration equations from the NIRS Feed and Forage Testing Consortium (NIRSC, Berea, KY). Milk yields were computed in terms of milk per acre (lbs acre^{-1}) and milk per ton (lbs ton^{-1}) based on dry forage yield, IVTD, NDF, and CP values, employing the Milk Equation 2006 (de Los Campos *et al*., 2006).

4.3.3 Data Analysis

A generalized linear mixed model (PROC GLIMMIX) was used to determine the effect of hybrid resistance and fungicide application on tar spot disease severity, dry yield, and forage quality parameters ($\alpha = 0.05$) using SAS 9.4 (SAS Institute Inc.). Data was analyzed by site-years to account for the unique and variable environmental conditions present for each location and growing season. For all the site-years, hybrid resistance and fungicide applications and their interaction were considered as fixed factors while replication as a random factor.

Least square means were calculated using *lsmeans* statement and mean differences were calculated using Tukey's adjustment. Statement *dfmkr* was used to minimize the effect of heterogenicity in variances. Regression analysis was conducted in R studio (RStudio Team, 2020) using ‘lm’ function to compare linear and quadratic relations and ‘nls’ function to run nonlinear plateau relations to determine relation between the disease severity and dry yield, forage nutritive value, and predicted milk yield.

4.4 RESULTS AND DISCUSSION

4.4.1 Weather

The weather conditions in Michigan during the corn growing season are typically cool to mild warm (50 to 85 °F). Most of the corn around the state silks in the months of July and August. The lower peninsula of Michigan on average receives more than 2.4 inches rain during the months of July and August, with temperature ranges between 62 to 84°F. Foliar diseases such as tar spot typically prefer temperatures between 60 to 73°F, relative humidity more than 75% and leaf wetness of more than 7 hours (Valle-Torres *et al.*, 2020). Weather data showed that conditions were consistent with their 30-year averages, except Ingham 2023 and Barry 2023. These two site-years had the wettest July recorded in history followed by a dry September (Table 18). However, our field trials did not show very high disease severity. This is because at most site-years tar spot infections began later in August and silage harvest was done between early to mid-September, therefore, providing very little window for disease progression.

Table 18. Monthly[a] and 30-year mean[b] temperature and precipitation for each site-year.

Location	Year	July	August	September	July	August	September
		Temperature (°F)			Precipitation (inches)		
Branch	2021	70.7	72.9	64.5	4.6	4.8	2.7
	30-yr	71.3	72.9	64.5	4.2	4.4	3.4
Ottawa	2021	70.9	73.3	65.4	2.5	1.9	2.4
	2022	71.3	70.2	63.7	3.8	4.3	3.4
	30-yr	72.4	70.6	61.6	3.9	3.5	3.6

Table 18 (cont'd)

Location	Year	July	August	September	July	August	September
		Temperature (°F)			Precipitation (inches)		
Ingham	2022	73.5	72.1	64.3	2.3	5.9	2.2
	2023	71.4	67.7	63.5	6.8	4.2	1.9
	30-yr	71.3	69.5	62.1	2.9	3.5	3.1
Barry	2023	71.4	68.2	63.6	6.1	3.8	1.4
	30-yr	71.2	68.9	61.6	3.0	3.2	3.5

[a]Monthly precipitation data and monthly temperature data was collected from the nearest weather station in the MSU Enviro-weather.
[b] 30-year (1991-2020) mean temperature and precipitation data collected from the National Oceanic and Atmosphere Administration (https://www.ncdc.noaa.gov/cdo-web/datatools/normals).

4.4.2 Disease Occurrence

Although silage corn in Michigan is prone to numerous foliar diseases, the most frequently occurring foliar disease throughout our research trials was tar spot with disease severity between 0 to 15%. The only site-years which showed foliar disease were Branch 2021, Ottawa 2021, and Ottawa 2022. Disease severity on average was greater in 2021 than in 2022 whereas minimal disease was observed in 2023. As expected, disease severity increased over time for all the three site-years where disease was present. Results show that the area under the disease progress curve increased with time (Figure 18). It was higher at Branch 2021 (334.88) than at Ottawa 2021 (121.37). These values were 50-55% lower than those observed in grain corn studies being conducted in the Midwest U.S. around the same time. Disease progression in silage corn with time was similar in grain corn as explained by Bajet *et al.*, 1994 in corn which indicated that tar spot is generally first spotted 8-10 days around silking and reach to the highest severity after 8-10 weeks (around early dent or R5). Furthermore, since silage is harvested 7-8 weeks after silking, it also explains the lower disease severity observed in silage corn as compared to the grain corn. Therefore, if viable, an economic strategy for growers could be to decide harvesting for a silage crop instead of waiting for physiological maturity in case the disease severity is >50% around dent growth stage because severe yield losses are anticipated (Telenko *et al.*, 2021).

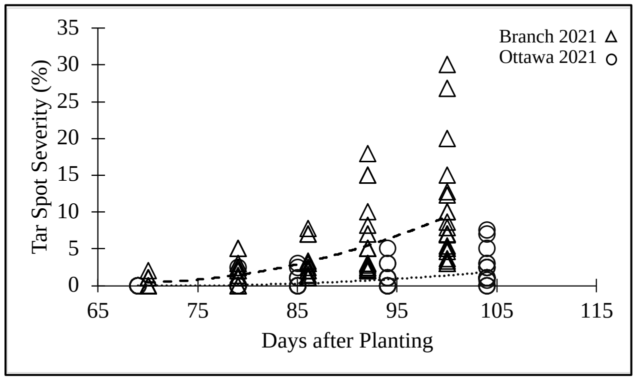

Figure 18. Tar spot severity progression with time across the partially resistant and susceptible hybrid at Branch 2021 (dashed line) and Ottawa 2021 (dotted line).

Hybrid resistance and fungicide application impacted the tar spot occurrence at Branch 2021 (p = 0.01 and 0.0.5, respectively) and Ottawa 2021 (p = 0.01 and 0.0003, respectively), whereas no effect was seen at Ottawa 2022 (p = 0.81) probably due to low tar spot severity. Significant interaction between hybrid resistance and fungicide application was observed only at Ottawa 2021 (p = 0.008), where the lowest tar spot severity was observed for the susceptible hybrids with two fungicide applications, 23% less than the partial resistant hybrids with two fungicide applications (Figure 19). Similar interaction trends between susceptible hybrids and fungicide applications were observed in a multistate grain corn study conducted by Ross *et al.*, (2023b).

Figure 19. Average tar spot severity across different fungicide treatments in resistant and susceptible hybrid at Ottawa 2021. Bars with the same letters within a site-year are not different ($\alpha = 0.05$).

Partially resistant hybrids reduced tar spot severity by 50% at both Branch 2021 and Ottawa 2021 (Figure 20), under low disease severity conditions. Partially resistant hybrids in grain corn also showed significantly lower disease severity, indicating that hybrid disease resistance is a promising management strategy (Ross *et al.*, 2023b). Therefore, exploration of disease resistance genes in corn parental inbred lines to develop resistant hybrids is an important vehicle for tar spot management (Singh *et al.*, 2023 and Yan *et al.*, 2021).

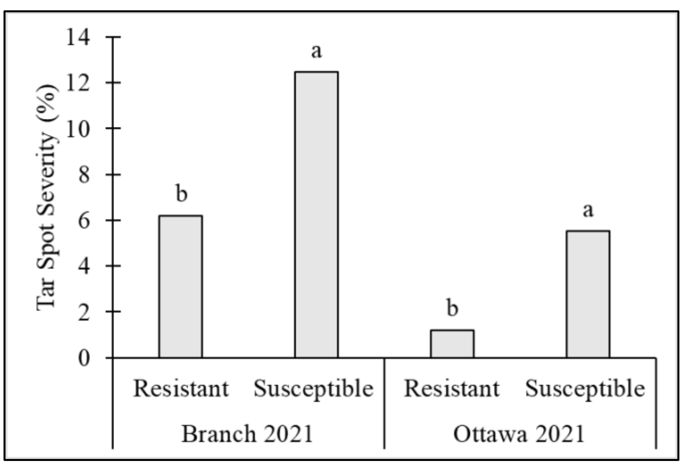

Figure 20. Average tar spot severity in resistant and susceptible hybrid at Branch 2021 and Ottawa 2021. Bars with the same letters within a site-year are not different ($\alpha = 0.05$).

Fungicide application also reduced tar spot severity by 26 to 34 % and 67% to 73%, than the non-treated plots at Branch 2021, and Ottawa 2021, respectively (Figure 21). However, no differences were observed between the plots that received one and two applications. Regardless of the absence of significant differences, the plots with two applications showed lower tar spot severity than in the plots with a single application (p = 0.15 at Branch 2021, and p = 0.22 at Ottawa 2021) (Figure 21). No differences were observed at other site-years probably due to low tar spot severity. Reductions in tar spot severity and an increased canopy greenness were also observed in grain corn due to fungicide treatments between tassel and dough stages (Telenko *et al.*, 2022a and 2022b). The lack of significant differences for fungicide treatment under lower disease severity was also observed by Ross *et al.*, (2023b) and Reed *et al.*, (2021).

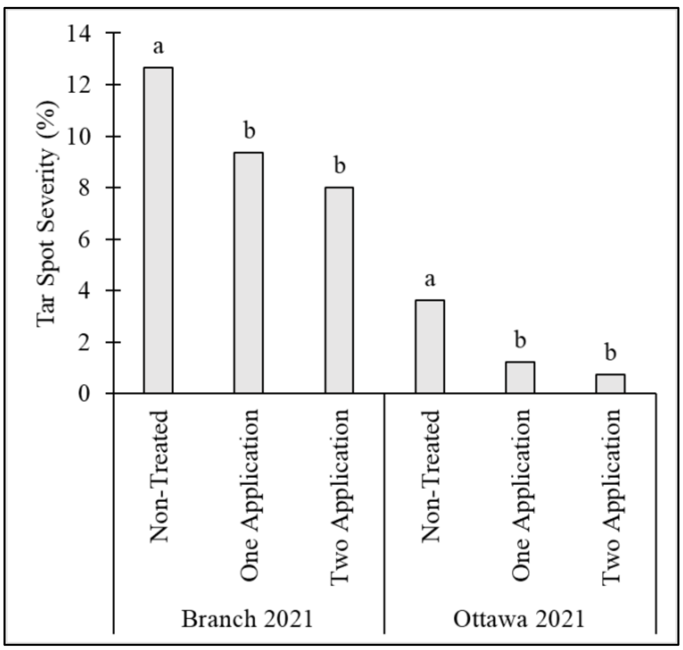

Figure 21. Average tar spot severity across different fungicide treatments at Branch 2021 and Ottawa 2021. Bars with the same letters within a site-year are not different (α = 0.05).

4.4.4 Dry Yield

Dry yield was influenced only by hybrid resistance at Branch 2021 (p = 0.02) and Ottawa (p <0.001) 2021, i.e., the site-years where >15% of tar spot severity were observed. No interactions were observed between hybrid resistance and fungicide applications for dry yield at any of the site-years. However, partially resistant hybrid had 22 and 20% higher dry yield than susceptible hybrids at Branch 2021 and Ottawa 2021, respectively (Table 19). This would probably be partially due to lower disease severity observed in partially resistant hybrid (six percent) than susceptible hybrid (13%) at these site-years. At Branch 2021, forage yield was lower in plots with tar spot severity in the range 10-20% (high) but was similar in plots with tar spot severity in range of 6-10% (medium) and 1-5% (low) (Figure 22). Therefore, the role of hybrid genotype becomes very critical while accessing yield differences. No differences were observed at any other site-years. Furthermore, dry yield loss showed a quadratic relation with increase in tar spot severity, only at Branch 2021 for partially resistant hybrid ($p = 0.01$, adjusted $R^2 = 0.27$). However, no such decline was observed for susceptible hybrid, further hinting at the role of hybrid genotype in determining dry yield. No other site-years show any significant relation between disease severity and dry yield (Figure 23). The lack of relationship can be attributed to lower disease severity. It is anticipated that under higher levels of disease severity, disease resistance can be beneficial in protecting the dry yield (Ross *et al.*, 2023b).

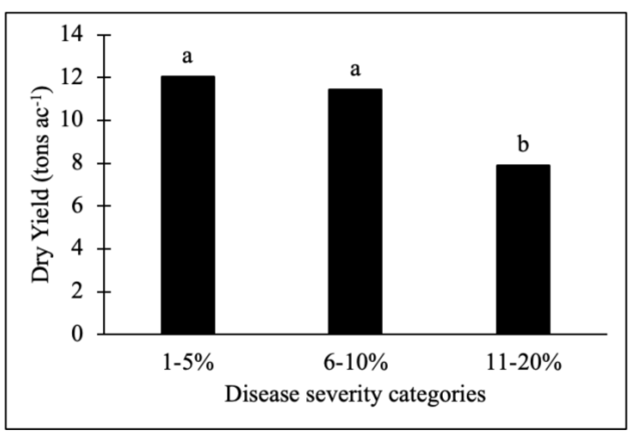

Figure 22. Dry yield across different disease categories at Branch 2021. Yield values are averaged across hybrids and fungicide treatments.

Table 19. Nutrient concentration, digestibility, and predicted milk yield of silage corn across the two hybrid types.

Site-year	Hybrid	Dry yield (tons ac^{-1})	ADF (% DM)	NDF (% DM)	Starch (% DM)	CP (% DM)	IVTD (% DM)	NDFD (% NDF)	Milk per ton (lbs ton^{-1})	Milk per acre (lbs acre^{-1})
Branch 2021	Susceptible	9.89 b	20.0 a	34.9 a	45.4 a	7.7 a	84.1 b	62.5 b	3650.3 a	36,083 b
	Partially Resistant	12.6 a	18.7 a	30.4 b	40.5 b	6.8 b	86.4 a	64.4 a	3558.6 a	44,991 a
	p-value	*0.02*	*0.13*	*0.01*	*0.05*	*<0.001*	*0.01*	*0.04*	*0.24*	*0.04*
Ottawa 2021	Susceptible	8.02 b	18.0 a	34.9 a	44.5 a	7.7 a	86.1 b	61.7 b	3634.1 b	29,084 b
	Partially Resistant	9.55 a	17.1 a	33.5 a	43.9 a	7.1 b	87.5 a	65.1 a	3734.3 a	35,619 a
	p-value	*<0.001*	*0.33*	*0.46*	*0.74*	*0.003*	*0.01*	*<0.001*	*0.01*	*<0.001*
Ingham 2022	Susceptible	8.28 a	18.9 a	36.7 a	36.1 a	6.8 a	87.4 a	69.4 a	3741.2 a	31,863 a
	Partially Resistant	8.73 a	19.0 a	38.3 a	36.4 a	6.6 a	88.5 a	72.5 a	3779.8 a	30,771 a
	p-value	*0.57*	*0.91*	*0.73*	*0.98*	*0.41*	*0.33*	*0.22*	*0.38*	*0.53*
Ottawa 2022	Susceptible	9.09 a	21.3 a	40.5 a	38.2 a	6.7 a	85.9 a	69.1 a	3636.1 a	36,221 a
	Partially Resistant	9.38 a	19.1 a	37.0 a	34.7 a	6.5 a	87.7 a	70.6 a	3738.3 a	34,145 a
	p-value	*0.23*	*0.13*	*0.18*	*0.19*	*0.42*	*0.16*	*0.3*	*0.17*	*0.26*
Ingham 2023	Susceptible	8.33 a	19.0 a	40.2 a	35.1 a	6.7 a	86.9 a	65.9 a	3673.2 a	30,125 a
	Partially Resistant	8.58 a	19.5 a	39.5 a	36.7 a	6.7 a	86.8 a	66.0 a	3683.1 a	31,028 a
	p-value	*0.59*	*0.81*	*0.67*	*0.32*	*0.92*	*0.90*	*0.90*	*0.81*	*0.50*
Barry 2023	Susceptible	8.21 a	22.9 a	45.5 a	32.7 a	6.9 a	82.9 a	62.5 b	3403.3 a	28,788 a
	Partially Resistant	8.42 a	24.1 a	44.0 a	32.0 a	6.8 a	84.6 a	65.1 a	3507.6 a	28,588 a
	p-value	*0.60*	*0.34*	*0.38*	*0.62*	*0.43*	*0.14*	*0.02*	*0.17*	*0.90*

Note: ADF (acid detergent fiber), NDF (neutral detergent fiber), CP (crude protein), IVTD (in-vitro digestible dry matter), NDFD (NDF digestibility), DM (dry matter).
Values with same letters within a site-year and variable are not different ($\alpha = 0.05$).

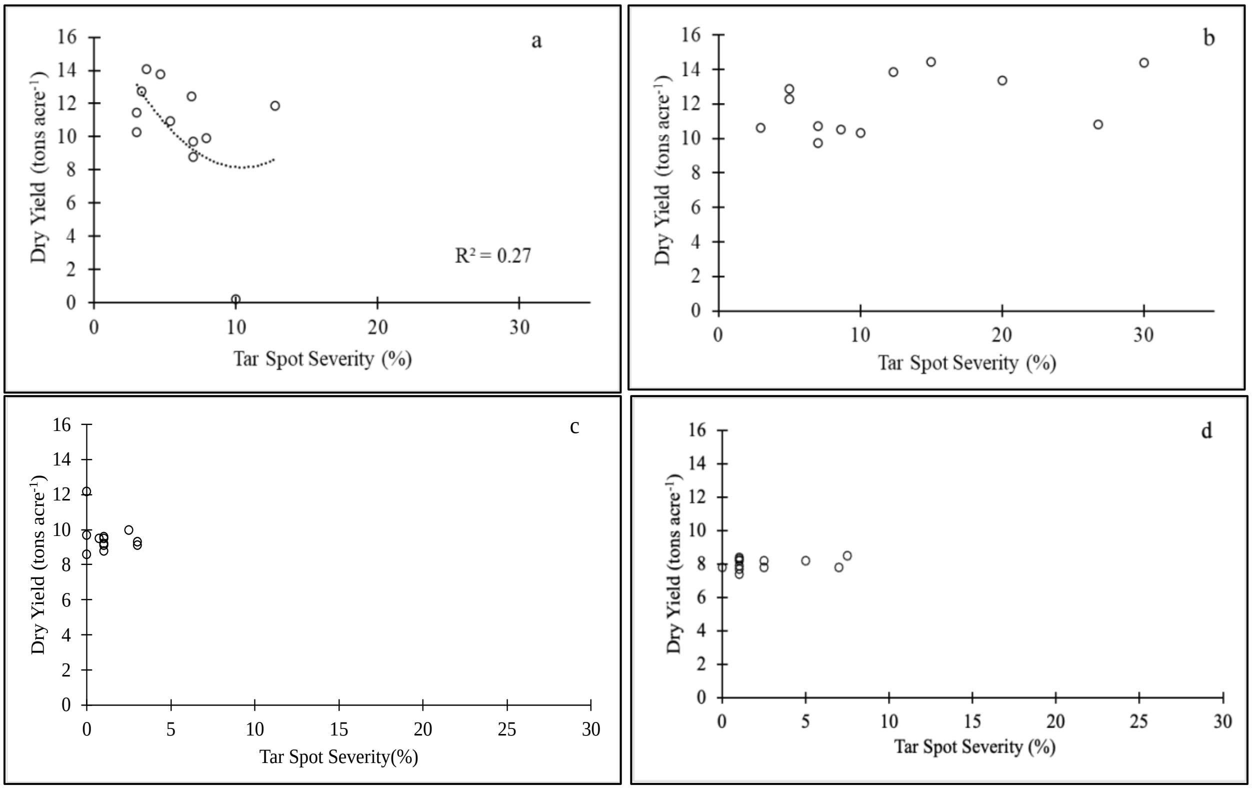

Figure 23. Relation between dry yield (tons acre^{-1}) and tar spot severity for Branch 2021: resistant hybrid (a), susceptible hybrid (b); Ottawa 2021: resistant hybrid (c), susceptible hybrid (d).

Fungicide applications did not improve or protect dry yield at any of the site-years. The disease severity was probably low (<15%) to not see the impact of fungicide on yield. This was similar to Ross *et al*., (2023a), where fungicide application was only economically impactful for yield protection only under high tar spot severity (>20%). Telenko *et al*., (2022a and 2022b) also show the inconsistent impact of fungicide application on dry yield in grain corn under low severity.

4.4.5 Forage Nutritive Value

Forage nutritive value was also impacted by the presence of foliar disease in silage corn. However, no interactions were observed between hybrid disease resistance and fungicide application for forage nutritive value. Neutral detergent fiber was higher in susceptible hybrid (average tar spot severity 13%) than in partially resistant hybrid (average tar spot severity 13%) (Table 19). Acid detergent fiber concentration did not vary at any of the site-years. However, a trend towards higher value of ADF concentration in susceptible hybrid than resistant hybrid at Branch 2021 and Ottawa 2022 ($p = 0.13$). Similar trends were also observed for NDF concentration at Branch 2021 ($p = 0.01$) and Ottawa 2022 ($p = 0.18$), with it being 15% greater in susceptible hybrid than resistant hybrid at the former site-year (Table 19). The differences observed could be due to both the genotypic trait of the hybrid, and the lower disease levels in partially resistant hybrid than susceptible hybrid. A research study with multiple hybrids in each category and in a year of very high disease severity would be helpful in resolving these ambiguities.

In-vitro digestible dry matter was three to five percent greater in partially resistant hybrid at Branch 2021, Ottawa 2021($p = 0.001$), and similar trends were observed at Ottawa 2022 ($p = 0.16$) and Barry 2023 ($p = 0.14$). The NDFD was two to six percent greater in partially resistant hybrid than susceptible hybrid at Branch 2021 ($p = 0.04$), Ottawa 2022 ($p < 0.001$), and Barry 2023 ($p = 0.02$), however, no differences or trends were observed at any other site-year (Table 19). These trends could also be the combined effect of genotypic traits and also the resultant low disease severity in partially resistant hybrid. Starch concentration varied only at Branch 2021 ($p = 0.05$), where susceptible hybrid had 12% higher value than resistant hybrid (Table 19). Crude protein concentrations were also 8 and 13% higher in susceptible hybrid as compared to resistant hybrid at Branch 2021 ($p < 0.001$) and Ottawa 2021 ($p = 0.003$), respectively.

The predicted milk yield, both milk per ton and milk per acre had a variable response at various site-years (Table 19). Milk ton^{-1} and milk $acre^{-1}$ was three percent and 18% greater in resistant hybrid than susceptible hybrid at Ottawa 2021 ($p = 0.01$, Table 2). Other site-years that showed a trend towards a greater milk ton^{-1} in resistant hybrids were Ottawa 2022 and Barry 2023 ($p = 0.17$ for both). At Branch 2021, resistant hybrid had 25% greater milk acre^{-1} than susceptible hybrid ($p = 0.04$, Table 19). However, it must be acknowledged that our study only had one hybrid in each category, therefore, the main effect of hybrid on dry yield, NDF digestibility, and predicted milk yield might not be coming entirely from disease levels but also from the hybrid genotypic traits.

Fungicide application did not impact forage nutritive value at any of the site-years, probably due to lower disease levels. Studies by Kalebich *et al*., (2017), and Hollis *et al*., (2019) indicated the role of fungicides in improved NDFD and predicted milk yields whereas Kaur *et al*., (2023) showed the lack of any impact. However, none of these studies focused primarily on foliar diseases. Therefore, it is critical to conduct further research under very high disease severity with multiple hybrids and fungicide treatments to explore the extent of impact of foliar diseases such as tar spot on silage corn dry yield and forage nutritive value.

4.5 CONCLUSION

This study was an important step towards understanding the foliar disease development and its impact on silage corn. Tar spot infections were not severe in this study and the yield losses are not as devastating, partly because it was a silage corn crop and harvested sooner, giving fungi less time to develop. Therefore, if the conditions are forecasting a bad year for tar spot infection, harvesting crop for silage rather than grain could be economically helpful, if it can be used on farm or sold in the market. Furthermore, use of hybrid resistance along with timely fungicide applications can be helpful especially under very high disease severity. Overall, at present the most practical approach to combat the tar spot issues is incorporating disease resistance, field scouting, and timely fungicide application in silage corn production.

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CHAPTER 5: QUANTIFYING PHOTOSYNTHESIS LOSSES DUE TO TAR SPOT IN CORN

5.1 ABSTRACT

Tar spot (caused by *Phyllachora maydis)* is an emerging foliar disease in corn (*Zea mays* L.) in Michigan and the Great Lakes Region of the U.S. The pathogen strains plant resources and can cause substantial yield losses. Understanding the interaction between this pathogen and corn plant is critical to mitigate yield losses. Field trials were conducted in a randomized complete block design with four replications at multiple Michigan locations in 2022-23. Treatments included three hybrids (partially resistant, tolerant, and susceptible to tar spot) and three fungicide treatments (non-treated, one application [at the silking stage, R1], and two applications [at R1 and at dough stage, R3]) using Delaro 325SC @ 584.62 mL ha^{-1}. Trials were scouted for tar spot starting R1 and leaf gas exchange was measured to estimate photosynthetic rate. Decline in CO_2 assimilation rate per unit increase in disease severity was estimated using a non-linear model, $y = (1 - x)^{\beta}$, where y is relative CO_2 assimilation rate, x is visual lesion area, and β represents the relationship between visually impacted leaf area and the impacted leaf area around the visual stromata (virtual lesion). Harvest yield data was collected at physiological maturity and stalk samples were collected to perform carbohydrate analysis. CO_2 assimilation rate declined linearly with plant age and disease severity. Rate of decline due to age did not differ between hybrids. However, β was greater in partially resistant hybrid than in susceptible and tolerant hybrids, suggesting a higher cost of resistance due to greater loss in photosynthetic rate per unit of disease. Total non-structural carbohydrates did not vary across different disease severities and no yield differences were observed. Overall, disease resistance minimized disease levels, but it had a greater decline in CO_2 assimilation rate and limited yield benefits. These

results emphasize the importance of weighing trade-offs when incorporating resistant traits in corn hybrids for managing tar spot.

5.2 INTRODUCTION

Tar spot is a fungal disease of corn caused by *Phyllachora maydis* and has been an emerging threat in Michigan and the Great Lakes Region. Its first appeared in the United States (U.S.) in 2015 in Indiana and Illinois (Ruhl *et al*., 2016). In 2018, a significant outbreak of the disease was documented in six Midwestern states, resulting in average yield loss of 3-5 metric tons acre^{-1} at a tar spot severity > 50% (Telenko *et al*., 2021). Total corn yield losses between 2018 to 2020 due to tar spot has estimated to be around 6 million metric tons in the U.S (Mueller *et al*., 2020). Currently tar spot is a major disease across the Corn Belt, encompassing, Indiana, Illinois, Michigan, Ohio, Wisconsin, Iowa, Pennsylvania, Missouri, Iowa, and also parts of Ontario, Canada (Valle Torres *et al*., 2020).

The fungus *P. maydis* is native to Latin America, where it forms the tar spot complex (TSC) in association with two other fungi, *Monographella maydis* and *Coniothyrium phyllachorae (*Valle-Torres, 2020). In the U.S., only the presence of *P. maydis* has been confirmed. However, the changing climate may create more favorable conditions for the pathogen's growth, and the introduction of the other TSC fungi to the U.S. could pose future challenges (Mottaleb *et al*., 2019). Tar spot is characterized by small, raised, black, and circular spots that form on leaf surfaces called stromata (Liu *et al*., 1973). As disease severity increases, stromata are surrounded by necrotic leaf tissue halos, which coalesce and cause leaf blight and death of plants (Liu *et al*., 1973; Bajet *et al*., 1994) The infection results in early and rapid senescence, reduced ear size, poor kernel fill, vivipary, and reduced stalk strength which can lead to lodging (Hock *et al*., 1995; Valle Torres *et al*., 2020).

Proposed disease management strategies for tar spot include hybrid resistance, crop rotation, and fungicides. Hybrid selection is being explored as an effective management tool, however, there is a lack of complete resistance in currently available hybrids (Singh *et al*., 2023). Nevertheless, partially resistant hybrids demonstrated a reduced area under the disease progression curve compared to susceptible hybrids, as reported by Ross *et al*., (2023). Consequently, a combined strategy of field scouting and timely fungicide application is being explored extensively. Corn treated with fungicides exhibited lower tar spot severity than non-treated corn (Telenko *et al*., 2022a and 2022b). Fungicide application has been effective but is highly time dependent and more research is warranted to determine best management practices (Kleczewski, 2019; Telenko *et al*., 2022a). Furthermore, the return on investment from fungicide application is dependent on timing and disease severity (Ross *et al*., 2023). Therefore, to address the knowledge gap in tar spot management an improved understanding of the plant-pathogen interactions is required.

There is an information deficit related to the plant-pathogen interactions of tar spot in corn. As an obligate biotroph, *P. maydis* requires plant metabolites (such as carbohydrates produced in photosynthesis) as a food source to grow and reproduce (Roco da silva *et al*., 2021). Moreover, the leaf lesions reduce healthy leaf surface area for photosynthesis impacting carbohydrate production, which may contribute to reduction in yield. The extent of loss in photosynthetic activity vary in different pathosystems; it can be proportional greater, lesser, or equal to the visible symptom (Shteinberg, 1992).

In certain pathogens of corn, for example, *Stenocarpella macrospora* and *Phaeosphaeria maydis* losses in photosynthetic performance of the infected leaves may extend beyond the visible lesions, i.e., exhibit virtual lesions (Bermúdez-Cardona *et al*., 2014; Godoy *et al*., 2001).

These relations can be explored by the model $y = (1 - x)^{\beta}$ given by Bastiaans, (1991); where y is the relative carbon dioxide assimilation rate (a measure of photosynthesis), x is the visual disease lesion, and β describes the relation between virtual and visual lesions. β is generally >1 for pathogens with biotrophic, hemibiotrophic, and necrotrophic feeding habit with values increasing in the same order (Shteinberg, 1992). Information on the proportional impact of tar spot lesion on photosynthesis in corn is lacking and is important to quantify eventual losses in yield. Additionally, understanding the rate of losses in net gaseous leaf exchange with increase in tar spot severity could aid with time-sensitive fungicide application.

Furthermore, natural plant metabolic defense pathways can be harnessed in applied management strategies to control or reduce disease incidence (Shumilak *et al.*, 2023; Gorman *et al.*, 2020), however, basic knowledge of plant defense for tar spot is lacking. Understanding the deviations in plant hormones such as salicylic acid and jasmonic acid could be helpful in exploring management decisions.

Therefore, this study was conducted to (i) evaluate the effects of tar spot severity on ear leaf carbon dioxide assimilation rate in corn hybrids with variable levels of resistance and fungicide applications, and (ii) to understand the impact of tar spot and fungicide applications on metabolic defense responses, grain yield, and quality. We hypothesized that the decline in leaf physiological functionality will be variable among the hybrids not only for leaf area at lesions but also for area around lesions. Tar spot infection will lead to a decline in corn yield and quality and plants may mount a defense response to the tar spot pathogen by stimulating defense hormones such as salicylic acid to a greater extent in the resistant compared to the susceptible hybrid.

5.3 MATERIALS AND METHODS

5.3.1 Experimental design and sites

Field trials were conducted at four site-years in Michigan in 2022 and 2023 (Table 20). The experiment included two factors, hybrid resistance and fungicide treatment, arranged in a randomized complete block design with four replications. Three hybrids (one partially resistant, one tolerant, and one susceptible to *P. maydis*) were used (Supplemental Table S8). Fungicide application was done using a prothioconazole and trifloxystrobin based fungicide, Delaro 325 SC (Bayer CropScience, St. Louis, MO) @ 584.62 mL ha^{-1} (Telenko *et al.*, 2022a). There were three fungicide treatments: non-treated, one application (at silking, R1), and two applications (one at R1, and second at dough stage, R3) (Table 20). This treatment arrangement helped to ensure the availability of asymptomatic and variable disease severity leaves throughout the season. Fields were managed based on general grower practices of the region. Field trials at Ingham were inoculated around V8 stage using plant litter from infected field (collected in fall of previous years), to increase disease pressure using plant debris (Check *et al.*, 2023).

Table 20. Agronomic details for field trials conducted across various years and locations.

Year	Location	Previous crop	Planting date	Irrigation	First Fungicide application[a]	Second Fungicide application[b]	Harvest date
2022	Ingham	Soybean	12 May	Rainfed	21 Jul.	12 Aug.	22 Oct.
	Ottawa	Grain Corn	17 May	Irrigated	28 Jul.	18 Aug.	11 Nov.
2023	Barry	Wheat	17 May	Rainfed	27 Jul.	16 Aug.	7 Nov.
	Ingham	Soybean	10 May	Rainfed	25 Jul.	16 Aug.	4 Nov.

[a] First fungicide application was done at the silking (R1) stage.
[b] Second fungicide application was done at dough stage (R3).

Field trials were planted at 84,016 seeds ha^{-1} using Almaco precision planter (Almaco, Nevada, Iowa). Plots were 3.05 m (= four rows) wide, 6.71 m long, with a 76 cm (30 inch) row spacing. The middle two rows were harvest using for Kincaid 8-XP (Kincaid equipment manufacturing, Haven, Kansas) to measure grain yield and its quality (moisture content and test weight). The outer two rows were used for destructive sampling of corn stalk for lab analysis of carbohydrates and plant stress metabolites.

A controlled trial was conducted in Michigan State University, plant biology research greenhouse during 2022 and 2023 following methods developed by Purdue growth facility (Eddy and Hahn, 2010). Hybrid resistance and fungicide treatment were set in randomized complete block design and five replications to quantify any improvements in CO_2 assimilation rate due to fungicide under controlled disease free environments. Treatments included two hybrids (partially resistant and susceptible, supplementary Table S8), and two fungicide treatments (non-treated and treated) using Delaro 325SC at the label rate at V8 stage. Corn trials were conducted using a 50:50 greenhouse media (SUREMIX, Michigan Grower Products, INC ™ Galesburg, MI) and sterilized field soil using 11.3 L nursery containers. The temperature of the growth chambers was maintained between 21 – 27°C with a 16–hour light period using high pressure sodium lamps.

5.3.2 Data Collection

5.3.2.1 Measuring carbon dioxide assimilation over time

Five corn ear leaves were selected at random in the middle two rows of each treatment combination (n=45) and tagged using fluorescent colored plastic tags at 75 and 78 days after planting (DAP) at Ingham 2022 and Ottawa 2022, respectively, and at 73 and 77 DAP at Barry 2023 and Ingham 2023. CO_2 assimilation rate was measured with a portable infrared gas analyzer (LICOR 6400XT, LI-COR Biosciences, Lincoln, NE) on 6 cm^2 leaf area (on the widest part of the leaf) on these tagged leaves at 10-12 d interval. Measurements were done between 1000 and 1400 h under sunny to partly cloudy days at 1800 μmol m^{-2} s^{-1} photosynthetic photon flux density using the 6400-02 light-emitting diode light source (LI-COR). The CO_2

concentration in sample chamber was set at 400 μmol CO_2 mol^{-1} air, and the air flow rate through the sample chamber was maintained at 500 μmol s^{-1}. Temperature was maintained at 24 ± 1°C and relative humidity was maintained between 60 and 70%, mirroring the surrounding environmental conditions. Assessments of foliar stresses, particularly tar spot, was done at each measurement interval on all the tagged leaves using a standardized scale of disease severity ratings outlined by Telenko *et al.* (2021).

5.3.2.2 Relations between photosynthesis and disease severity

To quantify the effects of disease severity on photosynthesis while minimizing the impact of ear leaf age, an additional set of photosynthetic measurements were collected on diseased and asymptomatic ear leaves of similar age. On each day of data collection, measurements were conducted from the middle two rows from each of the hybrid with visual disease symptoms. Symptomatic ear leaves with differential disease severity were selected from non-sprayed and fungicide treated plots with one application for hybrids with variable disease resistance to determine the reduction of photosynthesis at a given disease severity, whereas asymptomatic leaves were selected from fungicide treated plots with two applications on the same day to determine photosynthetic rates under disease free conditions. Ear leaves were selected across a range of tar spot severities (most commonly occurring foliar disease during this study) to quantify the relationship between photosynthesis efficiency and disease severity. Relative leaf CO_2 assimilation rate and stomatal conductance was calculated by comparing it with the average of asymptomatic ear leaves, collected on the same day.

Photosynthetic measurements for the greenhouse study also included leaf gas exchange with a LICOR gas analyzer. These measurements were performed approximately 7-10 days after the fungicide application to ensure that the differences observed between diseased and asymptomatic leaves was not a function of fungicide application.

5.3.2.3 Relations between carbohydrate accumulation, phytohormones, and disease severity

At Ottawa 2022 and Ingham 2023, plant tissue (leaf and stalk) was collected at physiological maturity from different levels of disease severity and preserved on liquid nitrogen and stored at -80 ºC. Samples were categorized as asymptomatic, low disease severity (1-5%), medium disease severity (5-10%), and high disease severity (10-20%), based on the disease severity ratings observed in this study. It was ensured to have at least 10-15 samples in each category. These were analyzed for quantifying total non-structural carbohydrate content, salicylic acid and jasmonic acid hormone profiles, based on the methods of Liu *et al.*, (2018).

To extract total non-structural carbohydrates (glucose, fructose, and sucrose), a 50 mg portion of dried and pulverized tissue samples underwent homogenization in a centrifuge tube containing 1 mL of 92% ethanol, at a speed of 14000 rpm for 10 minutes. Subsequently, the supernatant was transferred to a 15 ml clean tube, and this process was repeated two additional times. The combined supernatants were then mixed with deionized water to achieve a total volume of 10 ml. This resulting extract was utilized for the determination of reducing sugars (glucose and fructose) and sucrose, while the residue was preserved for starch determination. The quantification of reducing sugars involved a reaction with 2N sulfuric acid and arsenomolybdate solution, followed by spectrophotometric assessment using Geneysus 10S UV-VIS Spectophotometer (Thermo Electron Scientific Instruments LLC, Madison, WI, USA). Sucrose levels were determined by reacting with sodium hydroxide and alkaline ferricyanide, followed by spectrophotometer measurements at 515 nm. Starch content was determined using the remaining pellet after hydrolysis with α-amylase and amyloglucosidase. The total storage carbohydrate content was then calculated by using the extraction solution from starch analysis, involving a reaction with 1N sulfuric acid and subsequent spectrophotometric determination.

Extracts of plant tissue were analyzed for salicylic acid and jasmonic acid by high performance liquid chromatography (HPLC) analysis. Tissue samples for HPLC were extracted using butylated hydroxytoluene, methanol, and formic acid. These were then homogenized with internal standards for salicylic acid (Sigma-Aldrich, Cat#247588; MW = 138.12 g mol^{-1}) and jasmonic acid (Sigma-Aldrich, Cat#J2500, Cayman Chemical, 88300); MW = 210.3 $gmol^{-1}$) and sent to Michigan State University metabolomics core facility (East Lansing, MI, USA).

5.3.3 Data Analyses

The decline of CO_2 assimilation rate over time series was analyzed using nonlinear regression 'nls' in R studio (Rstudio Team, 2020). The differences between the rate of decline across hybrid resistance and fungicide treatments were analyzed using analysis of variance with an inbuilt function 'lm' in R studio. During analysis, hybrid resistance, fungicide treatment and their interactions as fixed factors and replications as a random factor. Data was analyzed separately for each site-year to account for unique environmental conditions and disease pressure at each location.

Relations between CO_2 assimilation rate and disease severity were analyzed using a nonlinear model, $y = (1 - x)^{\beta}$, as described by Bastiaans (1991). In this model, y represents the relative CO_2 assimilation rate of a diseased leaf compared to that of an asymptomatic leaf, while x represents the visually observed lesion area. The relative CO_2 assimilation rate was estimated for diseased leaves in relation to asymptomatic leaves. The parameter β characterizes the relationship between the virtual and visual lesion areas. The virtual area denotes the reduction in photosynthetic capacity extending beyond the visual lesion area. Therefore, β indicates whether the impact of the disease on photosynthesis is greater ($\beta > 1$), lesser ($\beta < 1$), or equivalent ($\beta = 1$) to the influence accounted for by the visually measured lesion area. The nonlinear mixed effects (NLME) in R studio were used to estimate β and assess significant differences ($\alpha = 0.05$) in β values between hybrid disease resistance levels.

On a given sampling date, treatment differences for disease severity and CO_2 assimilation rate on tagged ear leaves were analyzed using analysis of variance by 'lm' function in R studio. Non-structural carbohydrates, phytohormone, yield, and quality (moisture content and test weight) over different levels of disease severity were also analyzed using 'lm' function in R studio, by categorizing samples in to asymptomatic, low disease severity (1-5%), medium disease severity (5-10%), and high disease severity (10-20%), based on the disease severity ratings observed in this study.

Leaf gas exchange data for photosynthesis from the greenhouse trial was analyzed using analysis of variance by 'lm' function in R studio, with hybrid resistance, fungicide treatment and their interactions as fixed factors and replications as a random factor. Data from both the trials were analyzed separately because the light sources available in both trials were different.

5.4 RESULTS

5.4.1 Disease Severity and carbon dioxide assimilation rate over time

Tar spot symptoms were observed only in 2022 but not in 2023. Disease lesions were first observed in the field on corn ear leaves around 130 and 105 DAP at Ingham 2022 and Ottawa 2022, respectively (Figure 24). Disease severity increased over time for all the hybrids. However, we did not see any interaction between hybrid disease resistance and fungicide application. The highest tar spot severity observed in susceptible hybrid was 18%, while it was 16% for tolerant, and only eight percent for partially resistant hybrid at Ottawa 2022 (Figure

24a). In general, disease severity ratings were significantly lower in partially resistant hybrid ($p = 0.02$), while they did not differ for susceptible and tolerant hybrids. At Ingham 2022, the disease severity was not different across hybrids ($p = 0.\ 18$). The greatest disease severity observed in susceptible hybrid was only 10%, followed by eight percent in tolerant, and five percent in partially resistant hybrid (Figure 24b). Disease severity was lower in significantly lower in the plots with two fungicide applications and majority of asymptomatic leaves came from these.

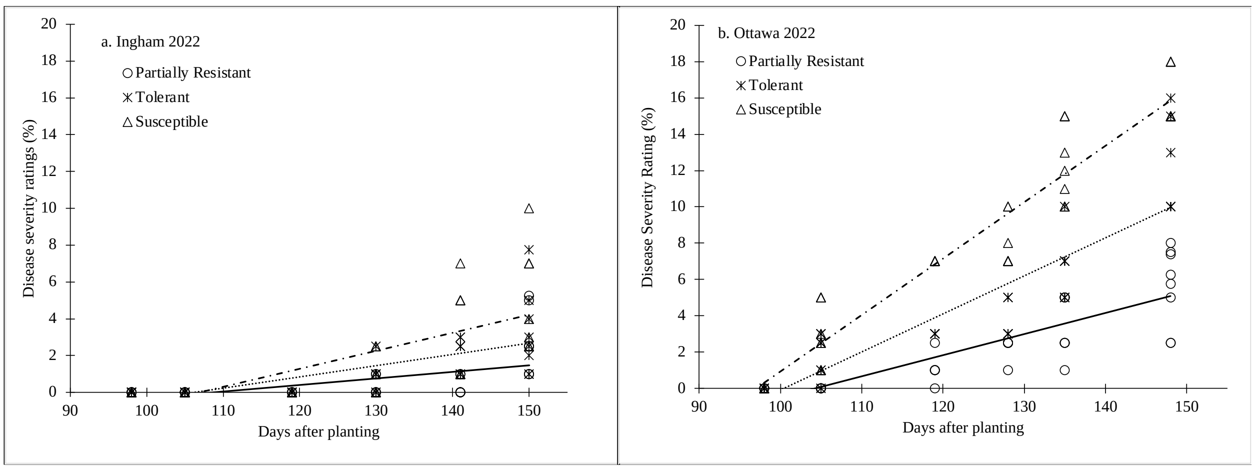

Figure 24. Disease progression at Ingham 2022 (a) and Ottawa 2022 (b) for three hybrids with different levels of resistance averaged across fungicide treatments. First disease symptoms were spotted at 130 DAP at Ingham 2022 and at 105 DAP at Ottawa 2022.

The mean CO_2 assimilation rate did not vary across hybrids or fungicide treatments and their interactions at any of the site-years. On average it was 42.3, 44.7, 45.1, and 42.8 $\mu mol\ m^{-2}\ s^{-1}$ at Ingham 2022, Ottawa 2022, Ingham 2023, and Barry 2023, respectively on the first day of measurements. Also, as expected the CO_2 assimilation rate for the tagged leaves declined with increase in ear leaf age (Figure 25). However, the rate of decline was not different across the hybrids at any day of measurement. On the last day of measurement, the mean CO_2 assimilation rate across the hybrids was 28.3, 22.4, 26.3, and 23.8 $\mu mol\ m^{-2}\ s^{-1}$ at Ingham 2022, Ottawa 2022, Ingham 2023, and Barry 2023, respectively. Furthermore, mean CO_2 assimilation rate did not differ between the hybrids on any given day of measurement, even when there were differences in disease severity among them.

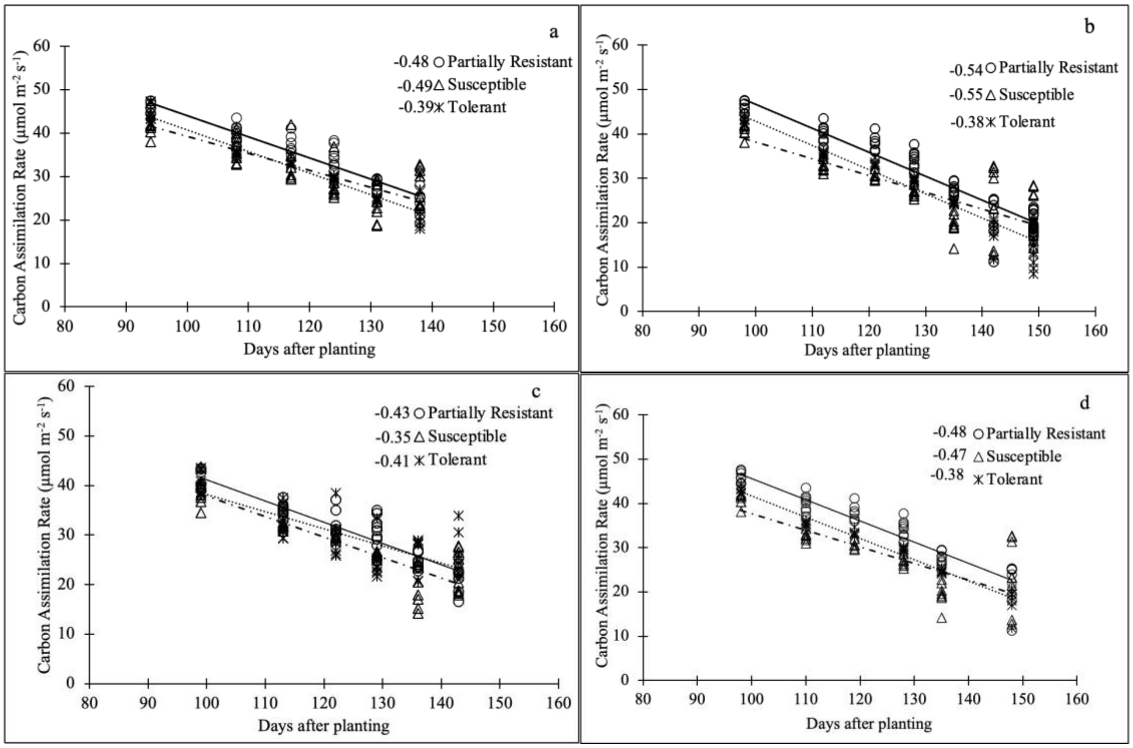

Figure 25. Carbon dioxide assimilation rate overtime for each individual hybrid at Ingham 2022 (a); Ottawa 2022 (b); Ingham 2023 (c); Barry 2023 (d). The values on regression model lines show the rate of decline (slope) in carbon dioxide assimilation rate with time. Bold line (partially resistant hybrid), dotted line (tolerant hybrid), and dashed line (susceptible hybrid).

5.4.2 Carbon dioxide assimilation rate and disease severity relations

At Ingham 2022, and Ottawa 2022, the mean CO_2 assimilation rate of asymptomatic leaves for the growing season averaged at 32.1 and 29.8 µmol m^{-2} s^{-1}, respectively and did not differ across hybrids. For all the three hybrid disease resistance levels, relative CO_2 assimilation rate had a negative relation with increasing disease severity (Figure 26). The β values for all three hybrid categories at DAP > 140 was >1.0, indicating that fungi impact the photosynthetic leaf area beyond the visible necrotic lesion area in 2022. However, β values for 2023 could not be determined due to lack of any observed disease. At Ottawa 2022, the β value for partially resistant hybrid (β = 2.26) was significantly greater (p = 0.05) than the β value for susceptible (β

= 1.61) and tolerant hybrid (β = 1.74) (Figure 26). This indicated a 30-40% greater reduction in CO_2 assimilation rate at a given disease severity for partially resistant hybrids as compared tolerant and susceptible hybrids, respectively (Figure 26). For instance, relative CO_2 assimilation rate observed for partially resistant hybrids at disease severity between 3-8% is similar to the values observed when disease severity was >10% for tolerant and susceptible hybrids. At Ingham 2022, β values were not significantly different, however, they were numerically greater (p = 0.18) in partially resistant hybrid (β = 1.63) than susceptible (β = 1.32) and tolerant (β = 1.37) hybrid.

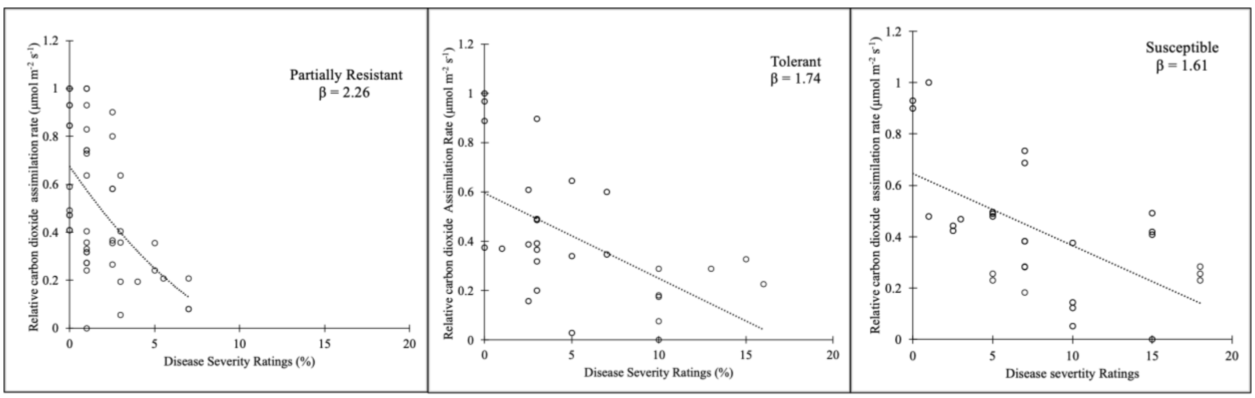

Figure 26. Relation between relative carbon dioxide assimilation rate and disease severity ratings for individual hybrids with different levels of hybrid disease resistance at Ottawa 2022. Β values represent the rate in decline in relative carbon dioxide assimilation rate with a unit increase in disease severity.

CO_2 assimilation rate measurements in the greenhouse trials showed no improvement due to fungicide application under controlled no disease environment (Figure 27). This indicated that the differences observed between asymptomatic and diseased leaves in the field trials were a function of disease severity and not fungicide applications in the plots from where CO_2 assimilation rate for asymptomatic leaves were measured.

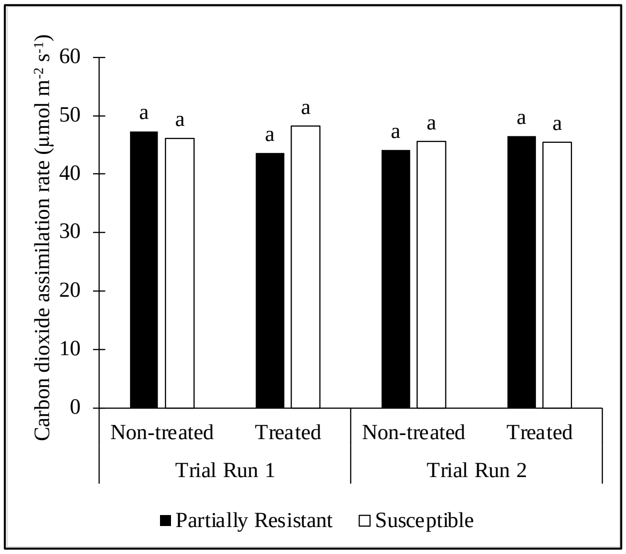

Figure 27. Carbon dioxide assimilation rate across hybrids and fungicide treatments observed in greenhouse trials conducted at Michigan State University, plant biology research greenhouses.

5.4.3 Non-structural carbohydrates and phytohormone responses

The non-structural carbohydrates (reducing sugars and starch) were not different across hybrids, fungicide application, and their interactions. A closer look at trends between hybrids show that partially resistant hybrid had lower accumulation of reducing sugars (86.5 mg g^{-1}) and starch (115.3 mg g^{-1}) both in the leaf and stalk tissue as compared to tolerant (84.8 mg g^{-1} and 113.8 mg g^{-1}) and susceptible hybrid (84.6 mg g^{-1} and 114.9 mg g^{-1}) at Ottawa 2022 ($p = 0.18$ and 0.14, respectively). Non-structural carbohydrates also did not vary across different disease severities. However, leaf samples from plants with lower and medium disease severity had marginally lower loss of reducing sugars than under high disease levels ($p = 0.16$). Furthermore, salicylic acid and jasmonic acid content at Ottawa 2022 was not different between asymptomatic and diseased plant tissue. At Ingham 2023, in the absence of disease, no differences were observed among hybrids and fungicide treatments for non-structural carbohydrates and defense hormone levels.

Average yield observed in our study in 2022 and 2023 was 11.8 metric tons ha^{-1} and 12.08 metric tons ha^{-1} the moisture content at harvest was around 150 µg g^{-1} in both years. The

100-seed weights were in range of 45 – 55g. However, we did not observe any significant differences in yield, moisture content, and 100-seed weight across hybrids and fungicide applications at any of the site-years.

5.5 DISCUSSION

Our study focused on evaluating losses in rate of CO_2 assimilation due to increase in severity of a tar spot in corn. Data showed that the photosynthesis activity decline due to leaf age and disease. Furthermore, the area of leaf that suffers from photosynthetic losses extends beyond the area under observed lesion, i.e., a virtual lesion is present (Figure 28) and possibly with a higher rate of loss in partially resistant hybrid than tolerant and susceptible hybrid. These higher losses in photosynthetic rate can explain the comparative accumulation of non-structural carbohydrates in hybrids with lower disease resistance and higher disease severity than partially resistant hybrid.

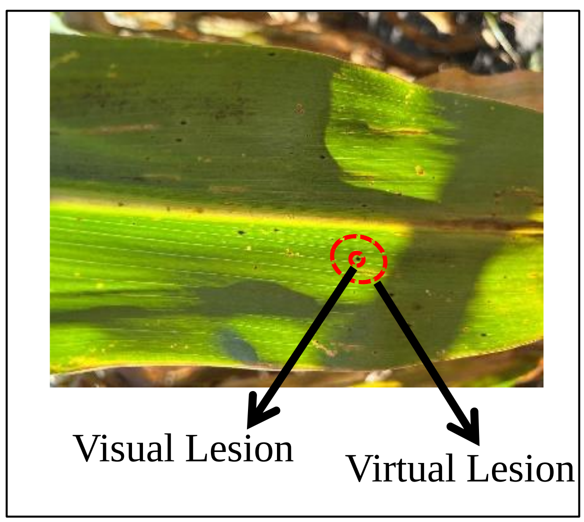

Figure 28. Decline in CO_2 assimilation rate is proportionally greater than the visual lesion area.

Tar spot severity on corn ear leaves increased with time (i.e. leaf age) in all hybrids and the greatest severities were recorded for susceptible hybrid (Figure 24). However, we did not see any interactions between fungicide treatment and hybrid disease resistance, and there were no significant differences between fungicide treatments. Our site-years did not show a very high tar spot severity (greatest disease severity was 18%) which can explain a lack of difference among treatments. The disease progression and the variability in the hybrids with variable resistance was similar to Telenko *et al*., (2022a) and the lack of fungicide treatment under low disease severity was also reported by Ross *et al*., (2023a).

The CO_2 assimilation rate in all the three hybrids declined with time was due to both increase in the age of plant leaf tissue (Figure 25). Decline in net assimilation rate in corn leaf due to age post tasseling had been documented well by Settimi and Maranville, (1998); Dwyer and Stewart, (1986); Thiagarajah *et al*., (1981). The rate of decline over the time series was not different among hybrids of variable disease resistant and fungicide treatment, indicating that any decline observed is only due to age and tar spot severity. However, since fungicide treatment under greater levels of disease severity is found to be more beneficial (Ross *et al*., 2023), lower decline in CO_2 assimilation rate under fungicide might be reported under high disease pressure conditions. Therefore, continued research on leaf gas exchange under higher disease severity (>30%) is warranted to further understand the role of fungicides.

A negative relation between relative CO_2 assimilation rate and tar spot severity, shows that the net assimilation rate decreases due to loss of functional leaf area because of tar spot lesions. Silveira *et al*., (2019) and Bermúdez-Cardona *et al*., (2014) reported similar relation between CO_2 assimilation rate and disease severity for macrospora leaf spot and northern leaf blight, respectively. Furthermore, our study showed that tar spot not only have photosynthetic ramifications on visually impaired area but also the area that extends beyond the lesion ($\beta > 1$, Figure 25). This indicates that loss of photosynthetic rate is not directly proportional to disease severity. Other foliar pathogen that has been reported to exhibited similar behavior in corn and has a proportionally higher impact than the visible disease symptom is *Stenocarpella* *macrospora* that causes Macrospora leaf spot (Bermúdez-Cardona *et al*., 2014) *Phaeosphaeria maydis* that causes Phaeosphaeria leaf spot (Godoy *et al*., 2001). These reductions might come from a decline in carboxylation efficiency, or a reduced chlorophyll content as observed by Bermúdez-Cardona *et al*., (2014), in corn and Singh *et al*., (2011) in peanuts (*Arachis hypogea* L.). Notably, the β values in these pathogens were greater than observed in our study, possibly due to their necrotrophic feeding habit, as opposed to an obligate biotroph *P. maydis*.

Additionally, β was reported to be greatest in partially resistant hybrid than tolerant and susceptible hybrid (Figure 26). This indicates a higher loss in relative assimilation rate in partially resistant hybrid than in tolerant and susceptible hybrid. Although the greatest tar spot severity was only 8% in partially resistant hybrid, the corresponding relative net assimilation rate is similar in magnitude to that observed in tolerant and susceptible hybrid when severity was >10%. Similar behavior of disease resistance in cultivars was reported by Singh *et al*., (2011) in peanut due to late leaf spot infections. These observations indicate that the cost of resistance may be paid by a higher overall loss in photosynthetic rate for a given disease level. It may indicate that incorporation of resistant traits to prevent infections may come at an additional cost and might not impact overall CO_2 assimilation compared to other hybrids. This may also be the reason behind inconsistent yield benefits observed in hybrids partially resistant to tar spot. Moreover, our data showed a steeper decline in photosynthesis rate even at a lower disease severity in comparison to other studied pathogens. This may explain the complete plant shut down and extensive losses under tar spot as observed during the 2018 growing season (Telenko *et al*., 2021; Mueller *et al*., 2021). However, further research in a year of high disease severity is warranted to draw concrete conclusions.

Lack of differences between asymptomatic and diseased plant tissue in non-structural carbohydrate accumulation and phytohormone hormone (salicylic and jasmonic acid) content probably came from lack of high levels of disease. Furthermore, higher reduction in CO_2 assimilation rate in resistant hybrids may have contributed to lower carbohydrate accumulation than tolerant and susceptible hybrids, thereby, diminishing differences in overall corn yield. However, this theory needs continued investigation using multiple hybrids and preferably under higher tar spot severities. Elevated levels of salicylic acid have a critical role in corn defense against wilt diseases (Shumilak *et al*., 2023), and jasmonic acid is a key player in defense against anthracnose stalk rots (Gorman *et al*., 2020), therefore, their role in tar spot defense to support timely fungicide management decisions need to be explored. Repeated measures with at multiple sampling stages could provide helpful insights.

5.6 CONCLUSION

Overall, this study evaluated the variability in photosynthetic response to tar spot severity. The CO_2 assimilation rate declined with time, plant age and increase in disease severity. Partially resistant hybrid showed a greater decline in photosynthetic performance at a given disease severity than susceptible and tolerant hybrids, indicating a more significant photosynthetic impairment. Therefore, partially resistant hybrid that helps to limit disease severity might only provide a limited yield benefit due to greater loss in photosynthetic rate per unit of disease. Future research in germplasm screening should thereby emphasize on evaluating additional photosynthetic parameters and improving resistant traits in corn hybrids for managing tar spot. Furthermore, less labor-intensive techniques such as spectral estimated of chlorophyll for estimating losses related to photosynthesis should be explored to further understand the nature of losses. Detailed insights in losses of photosynthetic ability of the plant due to tar spot would eventually help in generating informed management strategies for the growers.

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APPENDIX A: ENSILING CONDITIONS IMPACT MYCOTOXIN CONCENTRATIONS AND FORAGE NUTRITIVE VALUE IN CORN SILAGE

INTRODUCTION

Corn silage is obtained by harvesting the whole corn plant followed by controlled fermentation under anaerobic conditions (i.e., ensiling process) and ensures off-season availability of forage (Borreani *et al*., 2018). The overall quality of corn silage depends on nutritive value of whole-plant corn and any in-field ear and stalk rot infections at the time of harvest, harvest decisions (e.g., timing, particle size of chopped corn), and conditions during ensiling (removal of air from the silo, efficient compaction) and at the feed-out phase (Borreani *et al*., 2018). Specifically, in-field ear and stalk rot infections caused by mycotoxin producing fungi are critical in determining the safety of the feed for livestock (Ogunade *et al*., 2018). Mycotoxins are toxic secondary metabolites produced by ascomycete fungi such as *Aspergillus* spp., *Pennicilium* spp., and *Fusarium* spp. Commonly produced mycotoxins include aflatoxins, deoxynivalenol (DON), zearalenone (ZON), fumonisins, ochratoxins, and several other minor or emerging toxins (Munkvold *et al*., 2019). Feeding contaminated corn silage to livestock causes various acute and chronic metabolic disorders and diseases (Gerlach *et al.*, 2013). An already contaminated whole-plant corn from field is prone to continued accumulation of mycotoxins during and after ensiling. A short aerobic phase occurs during the ensiling process before forage enters the anaerobic phase, and can lead to additional mold growth and intensify the mycotoxin issue (Gonzalez Pereyra *et al.*, 2011; Ogunade *et al*., 2018).

Additionally, nutritive value and additional mycotoxin accumulation during ensiling is determined by particle size and compaction density in the silos. Compaction is done to eliminate the amount of air, and consequently oxygen, as much as possible and allow anaerobic fermentation (Toledo *et al*., 2020). The compaction density determines how effectively the air is eliminated from the silo. Lower compaction density can cause higher porosity and allow more movement of air, making mycotoxin accumulation and loss of nutritive value a continuing problem during ensiling.

Therefore, it is important to evaluate (i) the impact of ensiling process on mycotoxin concentration and forage nutritive value of pre-infected silage corn and (ii) continued mycotoxin production under variable compaction density in silos.

MATERIALS AND METHODS

Fresh whole-plant corn samples were collected from field trials with two non-Bt hybrids in Ingham (2020-2021) and Huron (2020) County, MI. Soon after harvest, replicated (n=4 each) samples from each hybrid and site-year were either dried in a hot air oven (72 hrs at 140°F) or sealed using a food grade vacuum sealer (LEM MaxVac 500 Vacuum Sealer, West Chester, Ohio) for 105 days. The latter ensiled samples were then opened after 105 days and dried in similar way as the fresh samples. All samples were ground to 1mm sieve size and analyzed for forage nutritive value and mycotoxin concentration.

Additionally, bulk corn was collected from a field trial at Ingham, MI during 2020-2021. It was divided into three different compaction densities with four replications. The polyvinyl chloride pipes with airtight rubber caps at each end (mini-silos, Figure S1) were used to pack fresh corn and simulate silo like conditions (Anesio *et al*., 2017). Variable compaction densities were achieved by filling mini-silos with chopped whole-plant corn (Table S1). These were ensiled for 105 d and then dried as explained above.

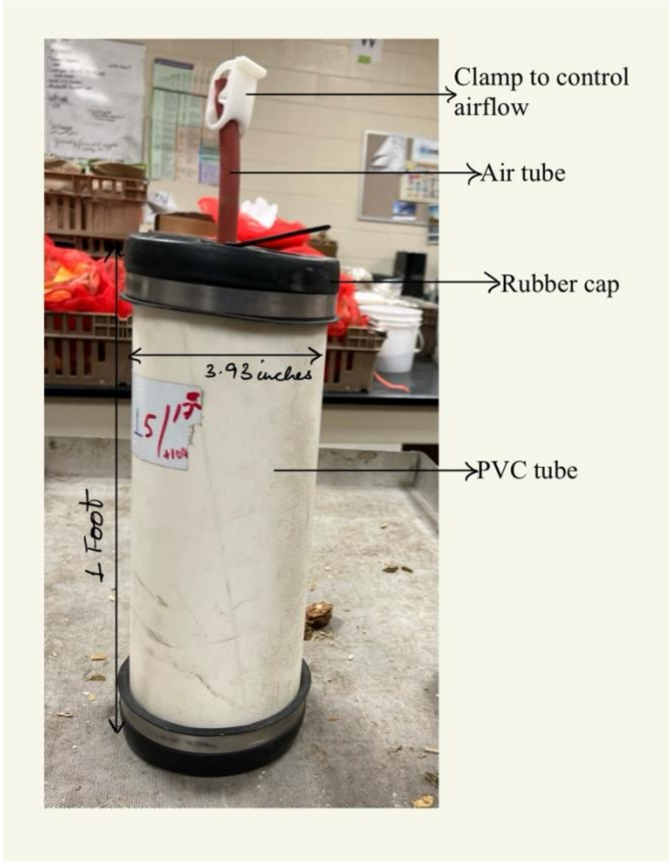

Figure S1. Mini-silo (made from polyvinyl chloride (PVC) pipe) used to study different compaction density.

Table S1. Amount of corn required to achieve various compaction densities using mini-silo (volume of 0.08 ft^3).

Compaction density ($lbs\ ft^{-3}$)	Amount of chopped whole-plant corn used (lbs)	Compaction Level
15.0	1.22	Low
30.0[a]	2.44	Medium
50.0	4.06	High

[a]Compaction density most commonly used in commercial silos.

The dried samples were pulverized to a particle size of 1mm and analyzed for forage nutritive value using near-infrared reflectance spectroscopy (NIRS, Model DS2500, FOSS North America, Eden Praire, MN) as done by Bhattarai et al. (2020) and Agnew et al. (2022). Calibration equations for unfermented and fermented silage corn from the NIRS Feed and Forage Testing Consortium (NIRSC, Berea, KY) were used. Predicted milk yields were calculated as milk per acre (lbs acre^{-1}) and milk per ton (lbs ton^{-1}), using the Milk Equation 2006 (de Los Campos et al., 2006).

Samples were analyzed for mycotoxins at the University of Guelph Ridgetown (Ontario, Canada), following the protocol by Limay-Rios & Schaafsma (2021), using mass spectrometer and liquid chromatography equipped with an electrospray ionization source, in positive and negative polarity.

Mycotoxin concentration and forage nutritive value for pre- and post-ensiling (fixed factors) and variable compaction densities (fixed factors) were analyzed in SAS 9.4 using analysis of variance with PROC GLIMMIX (a = 0.05). Replications were considered a random effect and data were pooled across the hybrids for each site-year. Least square means were calculated using *lsmeans* statement and mean differences were determined using Tukey's adjustment. Statement *dfmkr* was used to minimize the effect of heterogenicity in variances.

RESULTS AND DISCUSSION

The most commonly occurring mycotoxin in our study was DON (range 0.1 to 4 ppm), followed by ZON (0 to 1 ppm), and fumonisins (0.1 to 0.5 ppm). At Ingham 2020 and 2021, DON increased by 33% and 35%, respectively post-ensiling ($p = 0.03$ and 0.05, respectively), while ZON increased by 10% only at Ingham 2021 ($p = 0.03$) (Figure S2). An increase in fungal strains producing aflatoxin, DON, and ZON was observed post-ensiling by Gonz´alez Pereyra *et al*., (2011) and Pereyra *et al*., (2008), indicating continued growth of fungi in pre-contaminated whole-plant corn. Fumonisin concentration did not show any differences for any collected samples (data not shown).

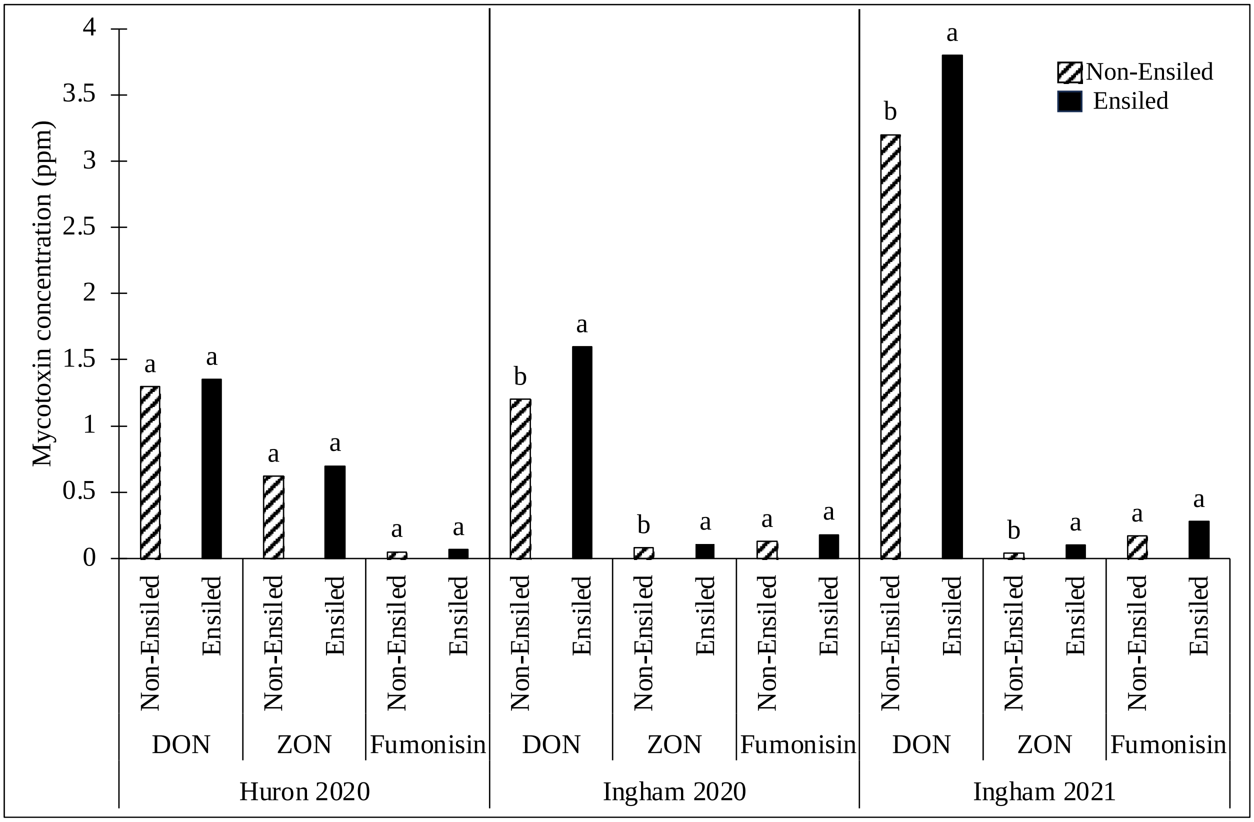

Figure S2. Average mycotoxin concentration in non-ensiled and ensiled samples across hybrids collected from various site-years. Bars with same letters for a mycotoxin type in a site-year do not differ (α = 0.05). DON, deoxynivalenol; ZON, zearalenone.

Another critical observation was the presence of mycotoxin penitrem A and roquefortine post-ensiling at Ingham 2020 and 2021 at concentrations < 1ppm which were not present pre-ensiling. They are produced by *Penicillium commune* and *P. roqueforti* which can survive under both aerobic and anaerobic conditions (Wagner *et al*., 2017). The detection of these toxins post-ensiling can be related to the presence of the pathogen as indicated by Penicillium ear rot reported during our field scouting.

Average DON, ZON, and fumonisin concentrations in the non-ensiled bulk sample were 1.09, 0.03, and 0.51 ppm in 2020, and 0.78, 0.21, and 0.15 ppm in 2021, respectively. Post-ensiling, a greater magnitude of increase in concentration of DON (only in 2020) and ZON (only in 2021) was observed under low compared to typical or high level of compaction (Figure S3).

Fumonisin concentration did not show any differences across compaction density. The reason for higher mycotoxin concentration under lower compaction density might be the inability to push the air out effectively and ensure anaerobic conditions, resulting in continued production of mycotoxins during early phase of ensiling (Kung Jr., *et al*., 2021; Boreni *et al*., 2017). On the contrary, Toledo *et al*., (2020) did not show any significant differences with different compaction density, however, the number of samples with positive mycotoxins was higher under lower compaction.

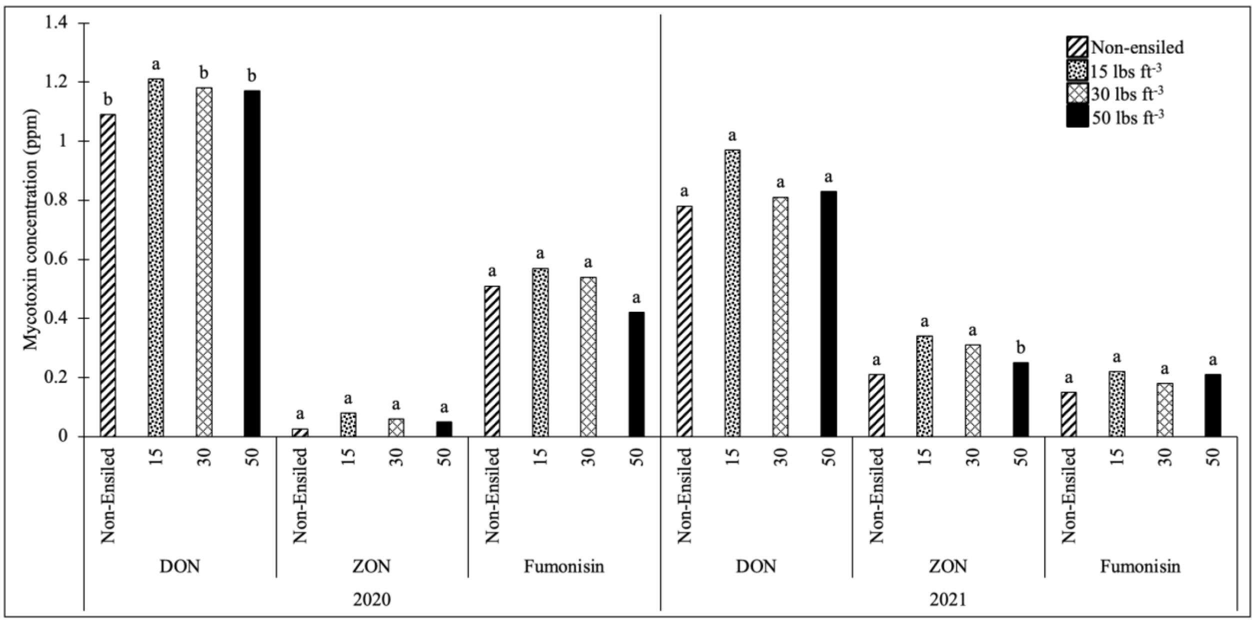

Figure S3. Average mycotoxin concentration across different compaction densities. Bars with same letters for compaction level in a site-year do not differ (a = 0.05). DON, deoxynivalenol; ZON, zearalenone.

For forage nutritive value, crude protein content increased by 5-10%, and starch content declined by 4-8% post-ensiling at Huron 2020 and Ingham 2020 (Table S2). The neutral detergent fiber declined by 15-28% post-ensiling while no difference was seen in acidic detergent fiber. The in-vitro true digestible dry matter increased by five to eight percent and neutral detergent fiber digestibility increased by three to eight percent post-ensiling (Table S2).

Similar post-ensiling observations of decline in fiber and starch content and an increase in fiber digestibility were reported by Hristov *et al*., (2020).

Table S2. Nutrient concentration and digestibility of non-ensiled and ensiled samples across hybrids at various site-years.

	Huron 2020			Ingham 2020			Ingham 2021		
	Non-Ensiled	Ensiled	p-value	Non-Ensiled	Ensiled	p-value	Non-Ensiled	Ensiled	p-value
ADF (%DM)	22.3 a	21.9 a	0.36	19.8 a	19.5 a	0.26	20.3 a	20.1 a	0.48
NDF (%DM)	38.3 a	32.6 b	0.009	33.7 a	28.4 b	0.03	36.7 a	36.9 a	0.32
Starch (%DM)	44.3 a	42.5 b	0.05	43.8 a	39.9 b	0.03	43.3 a	42.8 a	0.24
CP (%DM)	6.48 b	6.81 a	0.04	6.49 b	7.12 a	0.05	6.73 a	6.66 a	0.51
IVTD (%DM)	83.8 b	87.9 a	0.05	82.3 b	88.4 a	0.002	81.5 a	83.4 a	0.16
NDFD (%NDF)	58.33 a	60.1 a	0.03	60.3 b	64.3 b	0.004	65.7 a	64.9 a	0.22

Note: ADF (acid detergent fiber), NDF (neutral detergent fiber), CP (crude protein), IVTD (in-vitro digestible dry matter), NDFD (NDF digestibility), DM (dry matter).

CONCLUSION

Our study showed that mycotoxins concentration increased post-ensiling. Also, some mycotoxins (e.g., penitrem and roquefortine) that were not present in the fresh silage were detected post-ensiling. This emphasizes the need of using corn free from ear rots and molds in silos for ensiling. Furthermore, lower compaction density caused the greatest increase in mycotoxins and deterioration fiber digestibility of feed. Therefore, packaging corn with higher compaction density is critical to minimize airflow and prevent additional mycotoxin accumulation and loss of feed digestibility.

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APPENDIX B: CHAPTER 2 SUPPLEMENTAL TABLES

Table S3. Bt hybrids trait packages used with the lepidopteran-active insecticidal proteins and hybrid notations.

Trait Package	**Lepidopteran-active Insecticidal Proteins**	**Hybrid Name**	**Hybrid Group Notation**
Conventional	none	G12W66 G10T63	Non-Bt
Agrisure 3120	Cry1Ab, Cry1F	G10T63-3120	Bt_E
Agrisure 3122	Cry1Ab, Cry1F	G12W66-3122	
Agrisure Viptera 3330	Cry1Ab, Vip3A, Cry1A.105/Cry2Ab2	G09A86-3330	Bt_{EW}
Agrisure Viptera 3220	Cry1Ab, Vip3A, Cry1F	G09Y24-3220A	

APPENDIX C: CHAPTER 3 SUPPLEMENTAL TABLES

Table S4. Effect of planting time on plant density and emergence at two site-years in Michigan.

Site-year	Planting time[a]	Plant Density (plants ha^{-1})	Plant Emergence (%)
MSU 2020	Early	90,025	98.5
	Mid	87,774	96.1
	Late	89,144	97.6
	p-value	0.25	0.78
MSU 2022	Early	88,655	97.0
	Mid	89,438	97.9
	Late	88,851	97.0
	p-value	0.31	0.85

[a]Early: planting between April 25 - May 10; Mid: planting between May 11- May 25; Late: planting between May 26 - June 10.

Table S5. Effect of seeding rate on plant density and emergence rates at various site-years.

Site-years	Seeds ha^{-1}	Plant Density (plants ha^{-1})	Plant Emergence (%)
MSU 2020	69,160	67,519 c	95.8
	83,980	83,176 bc	95.5
	98,800	96,875 b	95.2
	113,620	109,596 a	93.3
	p-value	<0.0001	0.13
MSU 2022	69,160	65,562 d	93.1
	83,980	80,240 c	92.1
	98,800	92,961 b	91.3
	113,620	106,660 a	90.8
	p-value	0.02	0.18
Allegan 2020	69,160	67,519 c	95.9
	83,980	83,176 bc	95.5
	98,800	96,875 b	95.2
	113,620	109,596 a	93.2
	p-value	0.001	0.14
Huron 2020	69,160	65,563 d	93.2
	83,980	80,244 c	92.8
	98,800	92,966 b	91.8
	113,620	106,667 a	91.2
	p-value	0.02	0.23

Note: Data for MSU 2020 and MSU 2022 were pooled across all planting dates.
Values with same letters within a site-year and variable are not different ($\alpha = 0.10$).

Table S5 (cont'd)

Site-years	Seeds ha^{-1}	Plant Density (plants ha^{-1})	Plant Emergence (%)
Lenawee 2020	69,160	67,813 d	96.3
	83,980	83,958 c	96.4
	98,800	97,854 b	96.2
	113,620	112,532 a	95.8
	p-value	0.005	0.25
Huron 2021	69,160	69,476 c	98.6 a
	83,980	85,915 b	98.7 a
	98,800	99,811 ab	98.1 a
	113,620	104,703 a	89.2 b
	p-value	0.002	0.03
Lenawee 2021	69,160	69,280 d	98.3 a
	83,980	86,111 c	98.9 a
	98,800	98,538 b	96.8 b
	113,620	107,639 a	91.7 c
	p-value	0.007	0.09
Ottawa 2021	69,160	65,562 d	93.1
	83,980	85,915 c	98.7
	98,800	96,875 b	95.2
	113,620	112,532 a	95.8
	p-value	0.0002	0.16
Ingham 2022	69,160	65,562 d	93.1
	83,980	80,240 c	92.1
	98,800	94,918 b	93.3
	113,620	109,596 a	93.3
	p-value	0.01	0.26
Lenawee 2022	69,160	67,813 d	96.3
	83,980	84,252 c	96.7
	98,800	96,875 b	95.2
	113,620	112,532 a	95.8
	p-value	0.008	0.18
Saginaw 2022	69,160	69,476 c	98.6
	83,980	85,916 bc	98.7
	98,800	100,789 ab	99.0
	113,620	115,663 a	98.5
	p-value	0.004	0.27

Table S5 (cont'd)
Note: Data for MSU 2020 and MSU 2022 were pooled across all planting dates.Values with same letters within a site-year and variable are not different (α = 0.10).

Table S6. p-values and adjusted R^2 values for linear and quadratic relations between plant density of silage corn and dry forage yield.

Site-year	p-value (Linear model)	Adjusted R^2 (Linear model)	p-value (Quadratic model)	Adjusted R^2 (Quadratic model)
MSU 2020	0.34	0.01	0.18	0.05
MSU 2022	0.25	0.09	0.43	0.06
Allegan 2020	**0.01**	0.33	**0.01**	0.42
Huron 2020	**0.04**	0.11	**0.03**	0.45
Lenawee 2020	**0.01**	0.33	**0.05**	0.26
Huron 2021	0.40	0.02	0.10	0.04
Lenawee 2021	0.97	0.09	0.93	0.14
Ottawa 2021	**0.001**	0.67	**0.09**	0.13
Ingham 2022	0.41	0.05	0.50	0.09
Lenawee 2022	0.97	0.07	0.93	0.09
Saginaw 2022	**0.001**	0.65	**0.004**	0.71

Note: Data for MSU 2020 and MSU 2022 were pooled across all planting dates.
p-values in bold denote significant regression models (α = 0.10).

APPENDIX D: CHAPTER 4 SUPPLEMENTAL TABLES

Table S7. Hybrid details used in the field trial in three years of the study.

Growing Year	Susceptibility to tar spot	Hybrid	Silking GDD	Maturity GDD
2021	Susceptible	G09Y24-5222A	1420	2570
	Partially Resistant	G07F23-3111	1375	2570
2022 and 2023	Susceptible	G09Y24-5222A	1420	2570
	Partially Resistant	G12S75-5122	1430	2630

APPENDIX E: CHAPTER 5 SUPPLEMENTAL TABLES

Table S8. Information on hybrids (as provided by the seed company) used in the field trials across all site-years.

Disease Resistance	**Hybrid**	**Tar spot resistance rating**	**Silking GDD[a]**	**Maturity GDD**
Susceptible	G98M44-5122	5	1310	2410
Partially Resistant	G00H12-5122	2	1315	2420
Tolerant	G02K39-5122	3	1335	2475

[a]GDD: Growing Degree Day.