# Relationship Between *Fusarium virguliforme* and *Heterodera glycines* in Commercial Soybean Fields in Wisconsin

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## ABSTRACT

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*Fusarium virguliforme* (syn. *Fusarium solani* f. sp. *glycines*), the causal agent of sudden death syndrome, and *Heterodera glycines*, soybean cyst nematode (SCN), are economically important pathogens of soybean (*Glycine max*) in the Midwestern United States, including Wisconsin. In 2011 and 2012, samples submitted to a SCN detection program were assayed for SCN using a sieving/centrifugation method and for *F. virguliforme* using a real-time quantitative polymerase chain reaction (RT-qPCR) protocol. In 2011, 135 soil samples were submitted and *H. glycines* was detected in 56 samples, while 10 samples were positive for *F. virguliforme*. In 2012, 64 of 318 samples tested positive for *H. glycines* and 13 tested positive for *F. virguliforme.* The relationship between the occurrence of *H. glycines* and *F. virguliforme* was examined further for samples that were positive for *H. glycines* and/or *F. virguliforme*. Kendall's tau rank correlation coefficient was –0.59 ($P < 0.01$), indicating a negative association. Furthermore, the best-fitting logistic regression model that described the probability of detecting *H. glycines* in a soil sample based on detecting *F. virguliforme* confirmed the negative correlation. This result suggests that SCN and *F. virguliforme* do not rely on each other to colonize fields, indicating that fields heavily infested with SCN are not necessarily at greater risk of *F. virguliforme* colonization.

## INTRODUCTION

*Fusarium virguliforme* (syn. *Fusarium solani* f. sp. *glycines*), the causal agent of sudden death syndrome (SDS), and *Heterodera glycines*, soybean cyst nematode (SCN), are two of the most economically important diseases of soybean in the United States (14). The first report of SCN in Wisconsin was in Racine County in 1981, and as of 2012, the presence of SCN had been confirmed in 50 counties, representing 92% of the arable soybean acreage in the state (10). Compared to other states in the north-central region of the United States, incidence of SCN is much lower in Wisconsin. Nonetheless, once a field is infested with SCN, it remains infested and the development of a SCN management plan is required. In comparison, SDS was first confirmed in Wisconsin in 2006 (1). To improve disease management recommendations, new knowledge is needed about the distribution and prevalence of this fungus, about the prevalence of SDS in Wisconsin, and whether there is an association between SDS and SCN in soybean fields.

Studies elucidating the relationship between *F. virguliforme* and *H. glycines* have been conducted for nearly 30 years, yet the relationship is not fully understood. Several studies have shown positive associations between the two pathogens. For example, it was observed early after the discovery of SDS that both pathogens often occur in the same field. A survey by Hirrel (4) found SCN associated with 70 to 80% of SDS infected plants in 30 fields across four states. Most reports since those early studies have shown a positive correlation between populations of SCN in soil and SDS foliar symptoms (8,9,11,13,15). In field microplots, McLean and Lawrence (8) showed SDS symptoms occurred 3 to 7 days earlier and were more severe in plots infested with *H. glycines* and *F. virguliforme* than plots infested only with *F. virguliforme*. Similarly, in a greenhouse study by Roy et al. (11) soybeans inoculated with both pathogens expressed more foliar symptoms than soybeans inoculated with *F. virguliforme* alone.

While there is a body of literature indicating positive associations between the two pathogens, several published studies have indicated weak to no correlation (2,12,13). Furthermore, while cross-correlation analysis conducted by Scherm et al. (13) showed consistent association between *F. virguliforme* and *H. glycines* population densities, the cross-correlation coefficients were not always significant. Gao et al. (2) reported that the presence of both pathogens reduced soybean growth; however, *H. glycines* did not increase SDS symptoms.

These inconsistent results regarding the relationship between *H. glycines* and *F. virguliforme* further demonstrate the need for more research to elucidate potential interactions in order to provide more effective management recommendations to growers. Recently, a rapid real-time quantitative polymerase chain reaction (RT-qPCR) technique was developed to detect and quantify *F. virguliforme* in soil samples (7). By combining this novel detection technique with existing SCN testing programs, researchers are provided with an expanded set of tools to investigate the relationship between the incidence and population density of *H. glycines* and *F. virguliforme* in commercial soybean fields. Therefore, the objectives of this study were to: (i) determine the incidence of *H. glycines* and *F. virguliforme* in commercial soybean fields in Wisconsin; and to (ii) compare the distribution and population densities of *H. glycines* and *F. virguliforme* to determine if establishment of these pathogens is interrelated.

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## STUDYING THE RELATIONSHIP BETWEEN SDS AND SCN IN WISCONSIN

**Sample Collection.** As part of a Wisconsin Soybean Marketing Board program that offers free SCN soil testing to stakeholders, soil samples were voluntarily submitted from commercial soybean fields throughout Wisconsin by growers, crop consultants, and extension personnel in the 2011 and 2012 field seasons, following a standard protocol outlined in the program instructions (Tables 1 and 2). All soil samples were submitted to the University of Wisconsin Plant Diagnostic Laboratory for SCN analysis, from which soil samples were obtained for testing for both pathogens as outlined below.

**Soil Sample Preparation for SCN and DNA Extraction.** Upon arrival at the laboratory, a 100-cc subsample of soil was removed from the sample for wet-sieving and centrifugal-flotation and extraction of SCN cysts and subsequent egg counts following a modification of Jenkins (6) procedure. A separate 500-mg subsample of soil was also removed for bulk DNA extraction (see below).

**DNA Extraction.** Total DNA from the 500-mg soil subsamples was extracted using the PowerSoil-htp 96 Well Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA), following the standard protocol included in the kit. Overall quality and quantity of each DNA sample was checked with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). Absorbance at 260 nm was used as a measure of DNA quantity, and quality was checked using the 260:280 absorbance ratio. Extracted DNA was then used as template in a RT-qPCR assay to detect *F. virguliforme*.

**RT-qPCR Primer/Probe Optimization.** Primers and probe sequences were adapted from Mbofung et al. (7) based on their specificity to *F. virguliforme*. These oligonucleotides target the *FvTox1* gene, which has been shown to contribute to the leaf scorching symptoms of SDS caused by *F. virguliforme*. A pure culture of *F. virguliforme* (isolate 20485) was obtained from Kansas State University. This isolate was used because a pure isolate from Wisconsin was not available at the time of protocol optimization. DNA from the *F. virguliforme* isolate grown on potato dextrose agar (PDA) for 7 days at 23 ± 2°C was extracted using the FastDNA Spin Kit (MP Biomedicals, Santa Ana, CA). The resulting DNA was used to optimize the primer/probe set. Reaction volume, primer/probe concentrations, annealing temperature, and primer efficiency were tested. Optimized reactions were carried out in a total reaction volume of 25 µL, containing 1 µL of DNA template, 12.5 µL of PerfeCTa qPCR SuperMix (Quanta Biosciences Inc., Gaithersburg, MD), 0.5 µL of each primer (10 µM each), 0.5 µL of TaqMan probe (10 µM), and 10.0 µL of Ambion DEPC-treated water (Life Technologies Corp., Carlsbad, CA). Thermocycling conditions consisted of initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 20 sec, and 68°C for 30 sec. The threshold value of the qPCR machine was manually set at 25. Specificity of the protocol was tested in preliminary experiments (*data not shown*), and no cross reactivity to other *Fusarium* species was found. This is consistent with results of Mbofung et al. (7).

**TABLE 1**
**Number of samples received, number of samples in which either pathogen was detected, and propagule population densities for *Heterodera glycines* and *Fusarium virguliforme* in soil samples collected from soybean fields in Wisconsin counties, 2011.**

| | | *H. glycines* | | *F. virguliforme* | |
|---|---|---|---|---|---|
| County[a] | No. of Samples | No. Detected | Population[b] | No. Detected | Population[c] |
| Barron | 10 | 0 | 0 | N/A | N/A |
| Buffalo | 1 | 1 | 4,550 | 0 | 0 |
| Dane | 10 | 3 | 900–2,450 | 1 | D |
| Dodge | 9 | 8 | 150–10,050 | 1 | D |
| Dunn | 12 | 7 | 75–1,375 | 0 | 0 |
| Eau Claire | 1 | 1 | 5 | 0 | 0 |
| Fond du Lac | 3 | 0 | 0 | N/A | N/A |
| Grant | 5 | 2 | 125–1,775 | 0 | 0 |
| Iowa | 5 | 1 | 6 | 1 | D |
| Jefferson | 18 | 11 | 50–9,300 | 3 | 32,140–401,252 |
| Lafayette | 3 | 0 | 0 | N/A | N/A |
| Outagamie | 3 | 3 | 975–4,000 | 2 | D–15,761 |
| Polk | 4 | 0 | 0 | N/A | N/A |
| Rock | 17 | 6 | 100–1,250 | 2 | D |
| Sauk | 2 | 0 | 0 | N/A | N/A |
| Sheboygan | 3 | 0 | 0 | N/A | N/A |
| St Croix | 6 | 0 | 0 | N/A | N/A |
| Trempealeau | 4 | 0 | 0 | N/A | N/A |
| Walworth | 6 | 3 | 100–7,475 | 0 | 0 |
| Washington | 3 | 3 | 5 | 0 | 0 |
| Waukesha | 3 | 0 | 0 | N/A | N/A |
| Unknown[d] | 7 | 7 | 5–1,050 | 0 | 0 |
| Totals | 135 | 56 | 0–10,050 | 10 | 0–401,252 |

[a] Samples were not received from the remaining Wisconsin counties (Adams, Ashland, Bayfield, Brown, Burnett, Calumet, Chippewa, Clark, Columbia, Crawford, Door, Douglas, Florence, Forest, Green, Green Lake, Iron, Jackson, Juneau, Kenosha, Kewaunee, La Crosse, Langlade, Lincoln, Manitowoc, Marathon, Marinette, Marquette, Menominee, Milwaukee, Monroe, Oconto, Oneida, Ozaukee, Pepin, Pierce, Portage, Price, Racine, Richland, Rusk, Sawyer, Shawano, Taylor, Vernon, Vilas, Washburn, Waupaca, Waushara, Winnebago, and Wood).

[b] County-wide range in number of eggs/100 cc soil from samples where *H. glycines* was detected.

[c] County-wide range of estimated number of spores/g soil from detected samples. D = detected but not quantifiable; N/A = sample not screened for *F. virguliforme.*

[d] Soil samples submitted to the laboratory without site information.

**TABLE 2**
**Number of samples received, number of samples in which either pathogen was detected, and propagule population densities for *Heterodera glycines* and *Fusarium virguliforme* in soil samples collected from soybean fields in Wisconsin counties, 2012.**

| | | *H. glycines* | | *F. virguliforme* | |
|---|---|---|---|---|---|
| County[a] | No. of Samples | No. Detected | Population[b] | No. Detected | Population[c] |
| Adams | 2 | 2 | 4,450–15,000 | 0 | 0 |
| Barron | 1 | 0 | 0 | 0 | 0 |
| Brown | 45 | 14 | 5–20,500 | 3 | D–10,778 |
| Calumet | 7 | 1 | 10 | 0 | 0 |
| Chippewa | 11 | 1 | 5 | 0 | 0 |
| Clark | 1 | 0 | 0 | 0 | 0 |
| Columbia | 3 | 0 | 0 | 0 | 0 |
| Dane | 2 | 0 | 0 | 0 | 0 |
| Dodge | 34 | 6 | 5–3,650 | 0 | 0 |
| Dunn | 6 | 4 | 5–275 | 0 | 0 |
| Eau Claire | 5 | 0 | 0 | 0 | 0 |
| Fond du Lac | 36 | 12 | 5–37,200 | 0 | 0 |
| Grant | 5 | 1 | 5,025 | 0 | 0 |
| Green | 14 | 5 | 5–3,375 | 2 | D |
| Green Lake | 3 | 0 | 0 | 0 | 0 |
| Jackson | 8 | 1 | 5 | 0 | 0 |
| Jefferson | 6 | 2 | 5–150 | 0 | 0 |
| La Crosse | 1 | 1 | 1,275 | 0 | 0 |
| Lafayette | 1 | 0 | 0 | 0 | 0 |
| Manitowoc | 21 | 0 | 0 | 1 | D |
| Oconto | 4 | 1 | 5 | 0 | 0 |
| Outagamie | 11 | 3 | 5–75 | 0 | 0 |
| Pepin | 3 | 0 | 0 | 0 | 0 |
| Pierce | 5 | 0 | 0 | 0 | 0 |
| Richland | 2 | 0 | 0 | 0 | 0 |
| Rock | 27 | 4 | 5–825 | 4 | D–11,226 |
| Sheboygan | 22 | 1 | 5 | 1 | D |
| St Croix | 4 | 0 | 0 | 0 | 0 |
| Trempealeau | 8 | 1 | 200 | 0 | 0 |
| Vernon | 2 | 0 | 0 | 0 | 0 |
| Walworth | 6 | 3 | 5–3,250 | 1 | 10,705 |
| Waupaca | 8 | 0 | 0 | 1 | D |
| Winnebago | 4 | 1 | 200 | 0 | 0 |
| Totals | 318 | 64 | 0–37,200 | 13 | 0–11,226 |

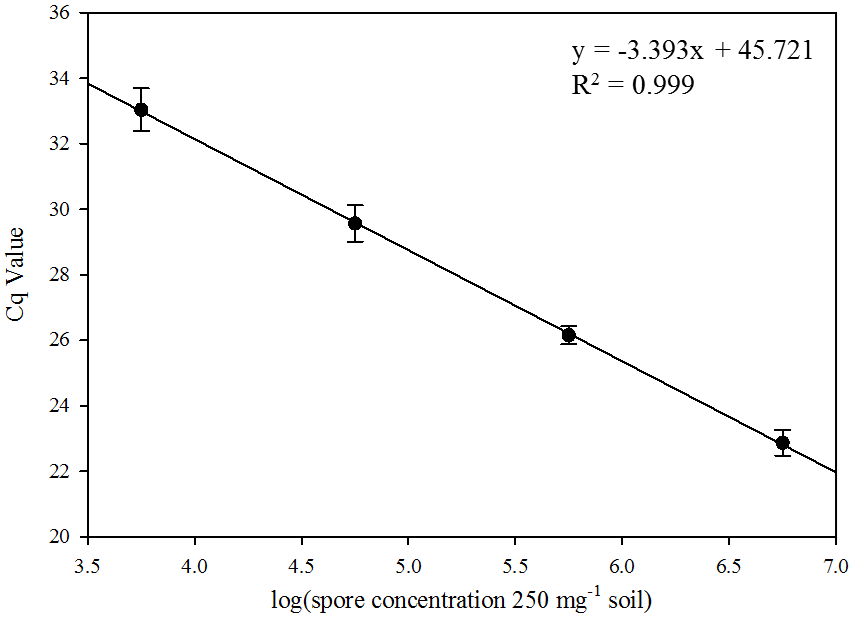
[a] Samples were not received from the remaining Wisconsin counties (Ashland, Bayfield, Buffalo, Burnett, Crawford, Door, Douglas, Florence, Forest, Iowa, Iron, Juneau, Kenosha, Kewaunee, Langlade, Lincoln, Marathon, Marinette, Marquette, Menominee, Milwaukee, Monroe, Oneida, Ozaukee, Polk, Portage, Price, Racine, Rusk, Sauk, Sawyer, Shawano, Taylor, Vilas, Washburn, Washington, Waukesha, Waushara, and Wood).

[b] County-wide range in number of eggs/100 cc soil from samples where *H. glycines* was detected.

[c] County-wide range of estimated number of spores/g soil from detected samples. D = detected but not quantifiable.

**FIGURE 1**

Standard curve for quantification of *Fusarium virguliforme*. Error bars are ± one standard error of the mean (n = 9).



**RT-qPCR Standard Curve Development.** Spores (macro- and microconidia) from several 7-day-old plates of the same isolate of *F. virguliforme* used above were harvested by flooding the plates with autoclaved Milli-Q purified water. Spore concentrations (spores/mL) were determined using a compound microscope Bright-Line hemacytometer (Hausser Scientific, Horsham, PA). Ten-fold serial dilutions of the spores were prepared. A standard curve (Fig. 1) for quantification of spore concentration (spores/g soil) was developed according to the protocol outlined by Mbofung et al. (7). Soil was autoclaved for 60 min at 121°C for two consecutive days. Spore suspensions (2 mL each of $2.22 \times 10^7$, $2.22 \times 10^6$, $2.22 \times 10^5$, $2.22 \times 10^4$, $2.22 \times 10^3$, 220, 22, and 2 spores/mL) were added to 2 g of autoclaved soil in 15 mL Falcon tubes and mixed. A control consisting of 2 mL autoclaved Milli-Q water mixed with 2 g of autoclaved soil was included. The suspensions were lyophilized for 12 hours. Three 250-mg samples from each suspension were weighed. Total soil DNA from each sample was isolated using the MoBio UltraClean Soil DNA isolation kit (MoBio Laboratories, Carlsbad, CA). The resulting DNA was used for quantification using the optimized RT-qPCR assay protocol. A linear regression equation was obtained from the average quantification of each dilution. At $10^4$ spores/g soil, *F. virguliforme* was detected in all of the replicates (n = 9), whereas at $10^3$ spores/g soil the fungus was only detected in six out of nine replicates. Thus, the limit of quantification was defined as $10^4$ spores/g soil, corresponding to a quantification cycle (Cq) value (i.e., the number of PCR cycles at which the target amplicon was quantified based on the RT-qPCR machine threshold) of 33.17, and the limit of detection was defined as Cq values between 33.17 and 35 for all subsequent reactions.

**RT-qPCR Analysis of Soil from Commercial Soybean Fields.** All reactions were carried out in 96-well plates with a BioRad CFX96 Touch Real-Time PCR System using the procedure outlined above. A water negative control and positive control using *F. virguliforme* pure-culture DNA were also included. Analysis of each submitted soil sample included three replicates. The average Cq value for samples ≤ 33.17 was inserted into the standard curve equation to obtain a population:

$$\text{Spores per g soil} = [10^{(\text{Cq value} - 45.721) / -3.393}] * 2$$

Cq values for samples ranging from 33.18 to 35 were considered "detected" but not quantifiable. Samples with Cq values over 35 were not detectable or quantifiable. It is possible that DNA of *F. virguliforme* detected in commercial samples might be from conidia and/or mycelium. Therefore, Cq values from field samples were "spore equivalents" and were considered a composite of DNA extracted from mycelium and spores in the same sample.

**Statistical Analysis.** To investigate the relationship between the presence and absence of *H. glycines* and *F. virguliforme*, samples were divided into two sets where one set contained samples where no *H. glycines* or *F. virguliforme* were detected (311 out of 435 samples) and one set contained samples where at least one or both pathogens were detected (124 out of 435 samples). For the set where *H. glycines* and/or *F. virguliforme* were detected, two new variables (FV and HG) were generated. FV and HG were binomial variables for each pathogen where a positive detection of each pathogen was coded as 1 and all other events as 0. The association between the presence of *H. glycines* and *F. virguliforme* was measured using the Kendall tau rank correlation coefficient (PROC CORR Kendall) in SAS (v. 9.3, SAS Institute Inc., Cary, NC). Subsequent logistic regression was used to describe the relationship between the probability of finding *H. glycines* (dependent variable) in a given soil sample based on detecting *F. virguliforme* (independent variable) in the same sample. Models were developed using PROC LOGISTIC in SAS (5). The FIRTH option was included in the model statement to reduce bias in the maximum likelihood estimates because of quasi-complete separation of data points (3). The best model was chosen based on the highest max-rescaled $R^2$ estimate and the area under the receiver operator curve (ROC) estimate.

## RESULTS SUGGEST A NEGATIVE CORRELATION

Voluntary soil sample submission was higher in 2012 than in 2011 in Wisconsin (Tables 1 and 2). In 2011, 56 of 135 (41.5%) samples were positive for *H. glycines* while 10 of 135 (7.4%) samples were positive for *F. virguliforme*. In 2012, 64 of 318 (20.1%) samples tested positive for *H. glycines* while 13 of 318 (4.1%) tested positive for *F. virguliforme*. Wisconsin counties testing positive for *H. glycines* in 2011 and 2012 were representative of the confirmed SCN-positive region of the state (Figs. 2 and 3). Oconto was the most northern county where SCN was detected in 2011 and 2012 samples. *F. virguliforme* detection was concentrated in counties in southern and eastern Wisconsin, and indicated a range that was greater than the original surveys of Bernstein et al (1), but who had also suggested that the distribution of *F. virguliforme* was more ubiquitous than originally thought.

Counties where both *H. glycines* and *F. virguliforme* were found in the same samples occurred infrequently (Table 1 and 2). Kendall's tau rank correlation coefficient was –0.59 ($P < 0.01$; n = 124), indicating a moderate negative association between *F. virguliforme* and *H. glycines*. The negative correlation indicated that the presence of *F. virguliforme* most often corresponds with absence of *H. glycines* in the same sample, and vice versa. The best fitting logistic regression model to describe the probability of detecting *H. glycines* in a soil sample based on detecting *F. virguliforme* was: $\hat{g} = 5.31 - 4.89x$ (max-rescaled $R^2 = 0.56$; area under the receiver operator curve, or ROC = 0.94; n = 124), where $\hat{g}$ is the logit of probability of *H. glycines* detected and $x$ = the presence (coded as 1) or absence (coded as 0) of *F. virguliforme* in the soil sample. To estimate the probability of detection, the following equation was used: estimated probability = $\exp^{\hat{g}} / 1 + \exp^{\hat{g}}$ (Fig. 4). The negative correlation suggests that SCN and *F. virguliforme* do not rely on each other to colonize fields. Therefore, fields with heavy SCN pressure are not at greater risk for colonization by *F. virguliforme*. The negative correlation and relationship in the probability of finding *H. glycines* and *F. virguliforme* in the same soil sample is counter to previous reports indicating that the presence of SCN can exacerbate sudden death syndrome of soybean (8,9,11,13,15). However, in controlled inoculations, Gao et al. (2) found that when *F. virguliforme* inoculum was applied at high levels to greenhouse grown plants, *H. glycines* reproduction was reduced. They speculated that reproduction was reduced because the fungus limited root mass availability for the biotrophic nematode.

**FIGURE 2**

Wisconsin counties where soil samples were submitted from soybean fields that tested positive for *Heterodera glycines* (orange shading), both *H. glycines* and *Fusarium virguliforme* (orange and yellow shading), did not test positive for either pathogen (green shading), or were not sampled (light gray) in 2011.

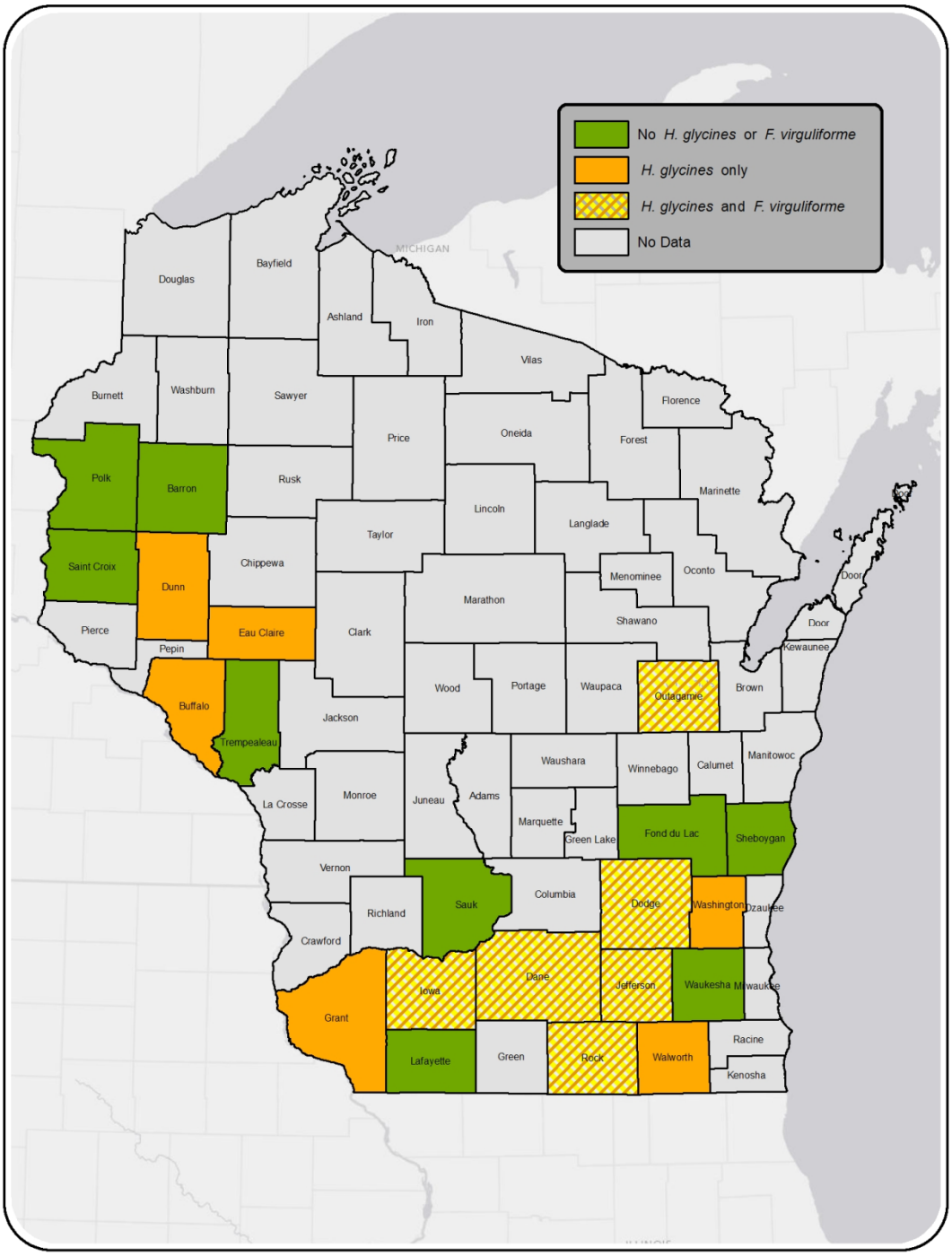
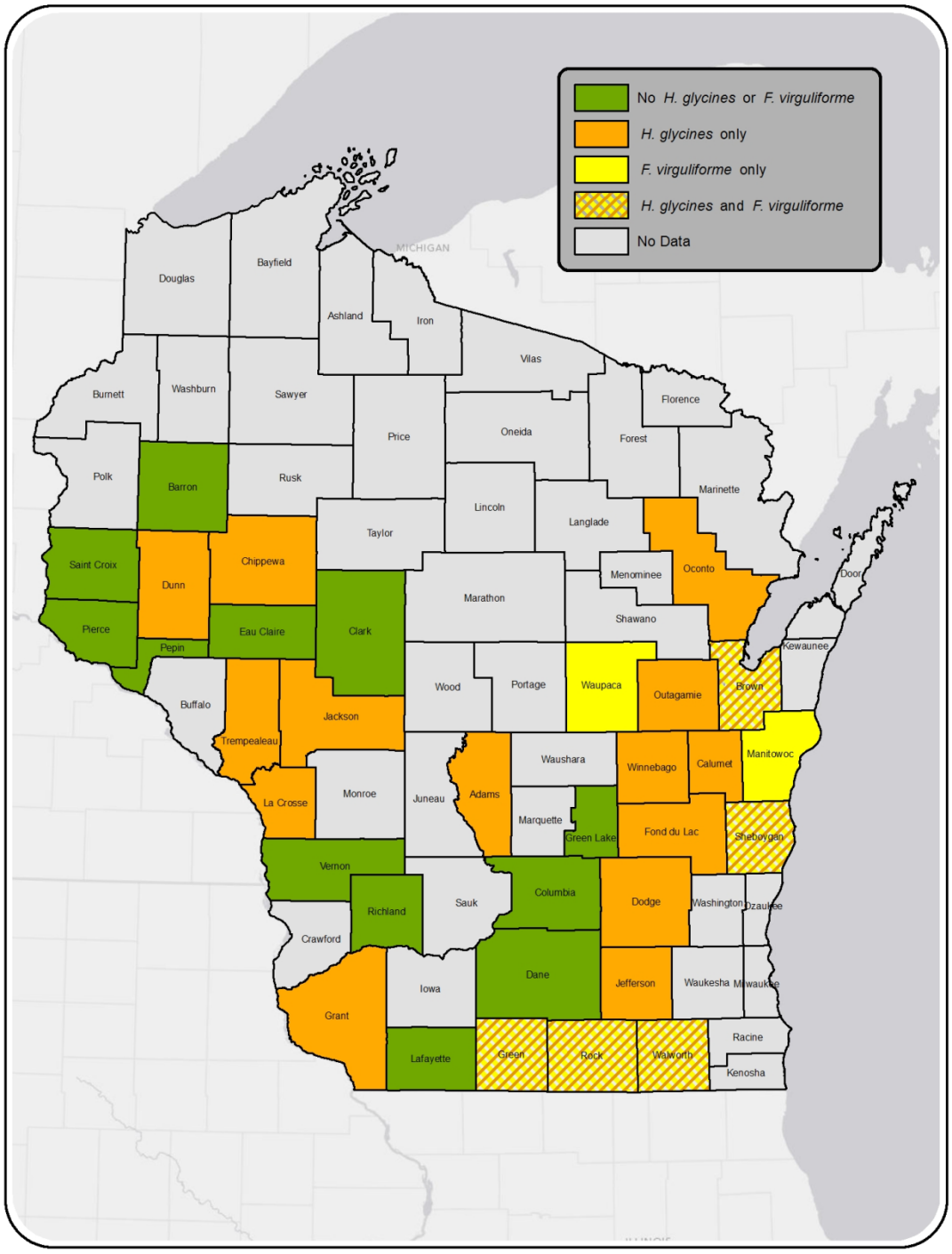


**FIGURE 3**

Wisconsin counties where soil samples were submitted from soybean fields that tested positive for *Heterodera glycines* (orange shading), *Fusarium virguliforme* (yellow shading), both *H. glycines* and *F. virguliforme* (orange and yellow shading), did not test positive for either pathogen (green shading) or were not sampled (light gray) in 2012.



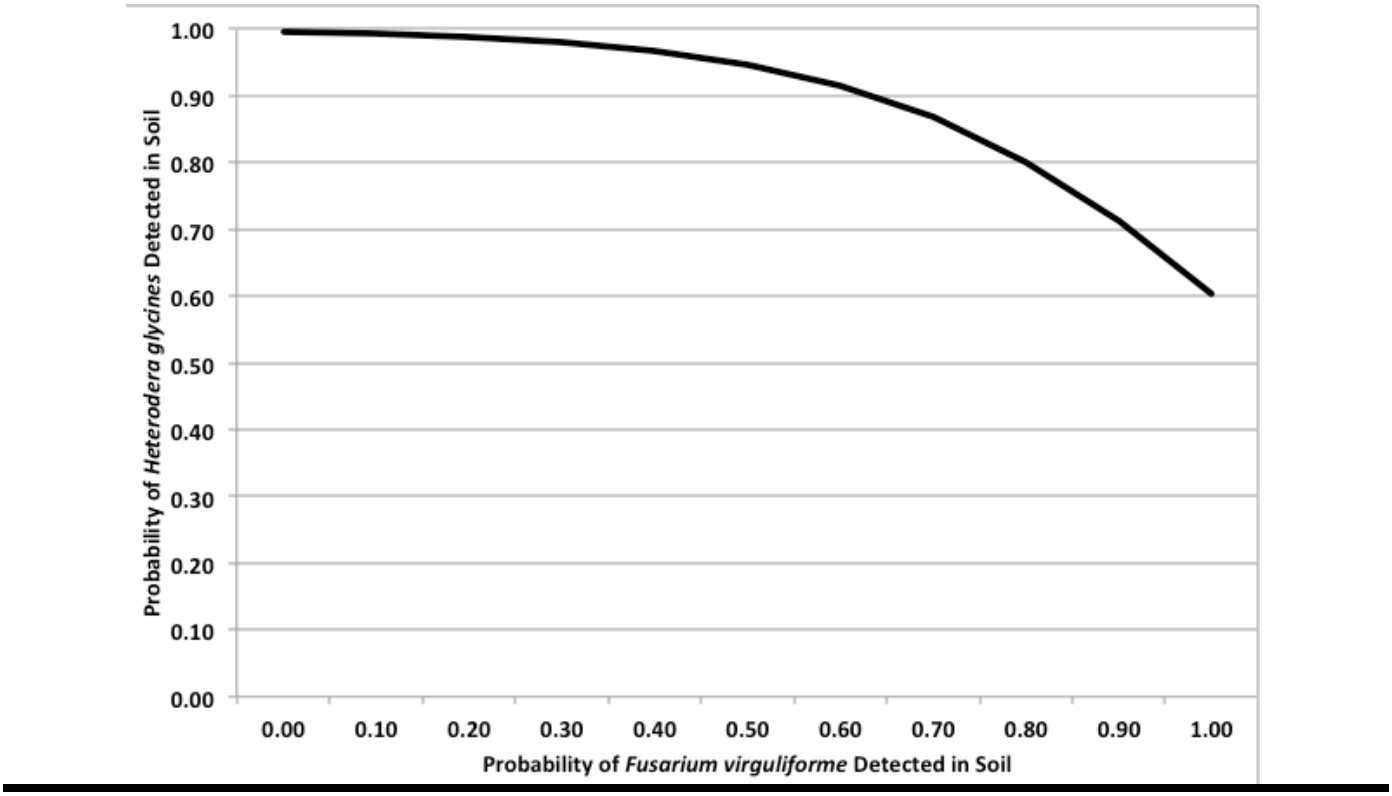



**FIGURE 4**

Predicted probability of soil samples testing positive for *Heterodera glycines* based on the probability of the same sample testing positive for *Fusarium virguliforme*. Predicted probabilities are based on the logistic model: Probability of *H. glycines* in a 100 cc soil sample = exp (5.31 – 4.89 *F. virguliforme*) / [1 + exp (5.31 – 4.89 *F. virguliforme*)]; Max-rescaled $R^2$ = 0.56; area under the receiver operator curve (ROC) = 0.94. Model build was based on samples that tested positive for *H. glycines* or *F. virguliforme* or both (n = 124).

This reasoning might also explain why we saw a negative correlation in the presence of *H. glycines* and *F. virguliforme* in grower-submitted samples. However, Kendall's tau rank correlation was non-significant ($r$ = 0.01; $P$ = 0.87) to examine the association between *H. glycines* egg population densities and *F. virguliforme* spore population, indicating that actual propagule population densities of the pathogens were not associated. This is not surprising however, as pathogen propagule population densities can fluctuate greatly based on sampling timing. Soil samples in the testing program were submitted between March and October in both years, and *H. glycines* egg population densities are known to vary widely across the season because of continuous production and hatching of eggs. In another study, *F. virguliforme* was isolated from at least one cyst in 71% of fields that also tested positive for *H. glycines*, *F. virguliforme* was only isolated from an average of 9% of cysts examined, further suggesting that there is a mutually exclusive relationship between the pathogens (12).

Quantification of *F. virguliforme* spores was possible at ≥ 10,002 spores/g soil, which is 10 times higher than the level of spore detection reported by Mbofung et al. (7). Reduced sensitivity in detection could be due to any number of variables including, but not limited to soil type, reagent variation, thermocycler variation, and sample handling. The reduced sensitivity may have resulted in false negatives for detection of *F. virguliforme* in some soil samples, and also may partially explain the negative correlation between *H. glycines* and *F. virguliforme*.

To our knowledge, this is the first statewide field application of the RT-qPCR assay to detect *F. virguliforme* from soil, as developed by Mbofung et al. (7). This is also one of the only statewide sampling efforts to detect *H. glycines* and *F. virguliforme* in commercial soil samples. Future testing will focus on improving sensitivity of the procedures described above and will continue to focus on improving the understanding of the relationship between *H. glycines* and *F. virguliforme* in soybean fields in Wisconsin.

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