Evaluation of the Combined Effect of *Heterodera glycines* and *Macrophomina phaseolina* on Soybean Yield in Naturally Infested Fields with Spatial Regression Analysis and in Greenhouse Studies

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ABSTRACT

Heterodera glycines, the soybean cyst nematode, and *Macrophomina phaseolina*, causal agent of charcoal rot, are economically important soybean pathogens. The impact and effect of these pathogens on soybean yield in coinfecting fields in the Midwest production region is not known. Both pathogens are soilborne, with spatially aggregated distribution and effects. Spatial regression analysis, therefore, is an appropriate method to account for the spatial dependency in either the dependent variable or regression error term from data produced in fields naturally infested with *H. glycines* and *M. phaseolina*. The objectives of this study were twofold: to evaluate the combined effect of *H. glycines* and *M. phaseolina* on soybean yield in naturally infested commercial fields with ordinary least squares and spatial regression models; and to evaluate, under environmentally controlled conditions, the combined effect of *H. glycines* and *M. phaseolina* through nematode reproduction and plant tissue fungal colonization. Six trials were conducted in fields naturally infested with *H. glycines* and *M. phaseolina* in Ohio. Systematic-grid sampling was used to determine the population densities of *H. glycines* and *M. phaseolina*, and soybean yield estimates. Though not used in any statistical analysis, *M. phaseolina* colony forming units from plant tissue,

charcoal rot severity, and *H. glycines* type were also recorded and summarized. In two greenhouse experiments, treatments consisted of *H. glycines* alone, *M. phaseolina* alone, and coinfection of soybean with both pathogens. Moran's *I* test indicated that the yield from five fields was spatially correlated ($P < 0.05$) and aggregated. In these fields, to account for spatial dependence, spatial regression models were fitted to the data. Spatial regression analyses revealed a significant interaction effect between *H. glycines* and *M. phaseolina* on soybean yield for fields with high initial population densities of both pathogens. In the greenhouse experiments, *H. glycines* reproduction was significantly ($P < 0.05$) reduced in the presence of *M. phaseolina*; however, soybean tissue fungal colonization was not affected by the presence of *H. glycines*. The direct mechanisms by which *H. glycines* and *M. phaseolina* interact were not demonstrated in this study. Future studies must be conducted in the field and greenhouse to better understand this interaction effect.

Keywords: charcoal rot, ecology and epidemiology, nematology, soybean cyst nematode, spatial analysis

The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is the most economically important pathogen of soybean in the United States (Allen et al. 2017; Koenning and Wrather 2010). Reduction in soybean (*Glycine max*) yield by *H. glycines* can range from 30 to 50%, and may occur in the absence of visible symptoms (Chen et al. 2001; Niblack et al. 1992; Wang et al. 2003; Young 1996). Another important pathogen of soybean is the fungus *Macrophomina phaseolina* (Tassi) Goid., causal agent of charcoal rot (Allen et al. 2017; Mengistu et al. 2015). This fungus is reported to affect more than 500 plant species, including soybean and corn (*Zea mays*), which are commonly used in crop rotation practices (Mengistu et al. 2015; Mihail and Taylor 1995; Su et al. 2001).

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Under conducive (hot and dry) environmental conditions, charcoal rot can cause significant soybean yield reduction and is a common disease in the southern United States (Allen et al. 2017; Koenning and Wrather 2010; Mengistu et al. 2015). However, charcoal rot has recently become more regularly observed in the north-central region of the United States (Bradley and del Río 2003; Cummings and Bergstrom 2013; ElAraby et al. 2003; Gearhart et al. 2010; Smith et al. 2014; Yang and Navi 2005).

Both *H. glycines* and *M. phaseolina* are soilborne root pathogens with the ability to survive long term as eggs (commonly enclosed in cysts) and microsclerotia, respectively. Similar to most soilborne pathogens, their distribution in a field can be influenced by several abiotic and biotic factors. Previous studies have reported that the spatial distribution of both *H. glycines* and *M. phaseolina* is aggregated in the soil (Campbell and van der Gaag 1993; Franci 1986; Gavassoni et al. 2001, 2007; Mihail 1989; Mihail and Alcorn 1987; Olanya and Campbell 1988). Consequently, the damage that these pathogens cause to soybean may not be spatially independent in infested fields but, rather, spatially correlated (Gavassoni et al. 2007; Melakeberhan et al. 2004).

More fields in the Midwest production region have become infested with *H. glycines* since it was first reported in the United States (Tylka and Marett 2014), and the almost ubiquitous presence of *M. phaseolina* results in high numbers of fields concomitantly infested with both pathogens (Lopez-Nicora et al. 2016). The impact and effect of *H. glycines* and *M. phaseolina* on soybean yield in coinfecting fields from this region is not known (Romero Luna

et al. 2017). Todd et al. (1987) evaluated *H. glycines*-resistant and -susceptible soybean cultivars in fields infested with both *H. glycines* and *M. phaseolina* and reported a positive correlation between fungal colonization of root tissue and *H. glycines* root densities. Greater yield losses were observed when soybean plants were concomitantly infected by *H. glycines* and *M. phaseolina* than by either one alone. Winkler et al. (1994) reported that *H. glycines*-susceptible soybean cultivars had greater density of *M. phaseolina* in roots when plants were infected with *H. glycines* compared with resistant cultivars. In contrast, there was no interaction but, rather, an additive effect on soybean yield when *H. glycines* was quantified from soil samples obtained at planting and harvest and *M. phaseolina* from soybean roots at harvest (Todd 1993). Similarly, there was no interaction on soybean yield when population densities of both pathogens were measured in soil (Francel et al. 1988).

To evaluate the effects of both *H. glycines* and *M. phaseolina* on soybean production, observational studies in fields concomitantly infested with both pathogens would be valuable. In observational studies, investigators observe, measure, and record variables of interest (e.g., crop yield, pathogen population densities, climatic factors, and so on) instead of assigning treatments to experimental units. In this type of study, therefore, researchers are not in control of where and how these variables of interest occur (Kutner et al. 2004; Montgomery 2013; Schabenberger and Pierce 2002). A common technique used to evaluate data generated from observational studies is ordinary least squares (OLS) linear multiple-regression analysis (Kutner et al. 2004). Multiple-regression models allow for tests of interaction between independent variables (e.g., soilborne pathogens) and their effect on the response variable (e.g., crop yield).

In OLS linear multiple regression, parameter estimates and standard errors are unbiased and efficient as long as the required assumptions (e.g., independent observations, homogeneity of error variance, and normally distributed residuals) are met (Kutner et al. 2004; Liu et al. 2014, 2015). The use of an OLS regression model to assess data from observational studies under violation of statistical assumptions may lead to erroneous conclusions (Hughes 1988; Liu et al. 2014, 2015). If the dependent variable or the OLS regression model residuals are spatially autocorrelated, then the independence assumption is violated, and the OLS regression model will produce biased or inefficient parameter estimates. Spatial dependence can arise in the dependent variable (e.g., soybean yield), the error term (e.g., OLS residuals), or exogenous variables (Anselin et al. 2004; Liu et al. 2014, 2015; Waller and Gotway 2004). Spatial regression analysis (Anselin et al. 2004; Bivand et al. 2013; Liu et al. 2014, 2015; Waller and Gotway 2004; Ward and Gleditsch 2008), therefore, would be an appropriate approach to account for the spatial dependency in either the response variable (e.g., soybean yield) or regression error term from data produced in fields naturally infested with *H. glycines* and *M. phaseolina*.

Therefore, the objectives of this study were to (i) evaluate the combined effect of *H. glycines* and *M. phaseolina* on soybean yield in naturally infested commercial fields with OLS regression and spatial regression models and (ii) evaluate, through nematode reproduction and plant tissue fungal colonization, the combined effect of *H. glycines* and *M. phaseolina* infecting soybean under environmentally controlled conditions in greenhouse experiments.

MATERIALS AND METHODS

Field experiments: trial locations and soybean cultivars.

In total, six different field trials (hereafter referred to as fields 1 to 6) were established during 2012, 2013, and 2014 (two fields each year). Trials were located in fields naturally infested with *H. glycines* and *M. phaseolina*. Five trials (fields 1 to 5) were conducted in commercial soybean fields in Pike and Brown County in southern Ohio, whereas the sixth trial was conducted at the

Waterman Agricultural and Natural Resources Laboratory on the Columbus campus of The Ohio State University. Soybean cultivars, relative maturities, and their responses to *H. glycines* were recorded. Soybean crops were planted and managed with standard, commercial production practices in all trials (Barker et al. 2017).

Field sampling methods. Systematic grid sampling was used to determine the population densities of *H. glycines* and *M. phaseolina*, and soybean yield estimates. A 10-by-10 grid of contiguous quadrats (7.62 by 7.62 m each) was used in five trials (Fig. 1). In field 6, a 7-by-15 grid of contiguous quadrats (7.62 by 7.62 m each) was used due to space limitations. Wire flags were placed in the center of each quadrat between two soybean rows and represented the middle point of a 2-m-long sampling distance from which soil and plant samples were collected. Plant and soil samples were collected at 100 sites in fields 1 to 5 and at 105 sites in field 6. Quadrats, therefore, were represented by samples (and data) collected from the area designated by wire flags. The spatial relation of each sampling point within the quadrat grids was recorded. Quadrats from left to right were alphabetically labeled, and those from top to bottom were numerically labeled (Fig. 1). Thus, the spatial position of each sampling point relative to the others was maintained.

Quadrat soil and plant sampling. Soil was sampled within 3 to 4 weeks of planting and at soybean growth stage R8 (Fehr et al. 1971) to determine the initial (Pi) and final (Pf) population densities of *H. glycines* eggs and *M. phaseolina* colony forming units (CFU). In the center of each quadrat, a composite sample of 15 to 20 soil cores was collected with a 2.54-cm-diameter cylindrical soil probe (Model LS; Oakfield Apparatus, Fond du Lac, WI, U.S.A.) within a 2-m-long distance between two soybean rows to a depth of 15 to 25 cm. Soil cores were combined in labeled sample bags and placed in insulated containers during transport to the laboratory. Cores in each composite sample were thoroughly mixed and samples were stored in a cold room at 4°C for approximately 2 weeks until processed. Additionally, soybean plants were arbitrarily collected from the two soybean rows within the 2-m-long sampling distance described above. These plants were used to quantify *M. phaseolina* in soybean tissue, charcoal rot severity, and soybean yield, as described below.

Data collection from field experiments. Weather data. Monthly ambient temperature (minimum, maximum, and mean) and precipitation data were collected from May to October for fields 1 to 6 from the Parameter-Elevation Regressions on Independent Slopes Model (PRISM) Climate Group (<http://www.prism.oregonstate.edu/>).

***H. glycines* and *M. phaseolina* quantification from soil.** Two subsamples of 100 cm³ and 1 g were obtained from each composite soil sample to determine the population densities of *H. glycines* and *M. phaseolina*, respectively. *H. glycines* eggs were extracted and quantified as the number of eggs per 100 cm³ of soil according to a standard procedure (Faghihi and Ferris 2000). *M. phaseolina* CFU were quantified from each 1-g subsample of air-dried soil, as described by Mengistu et al. (2009). Details of the *H. glycines* and *M. phaseolina* quantification methods are given in Supplementary File S1.

***M. phaseolina* quantification from plant tissue and charcoal rot severity.** A subsample of 5 plants from 25 plants collected for yield analysis (described below) was uprooted and the lower portion of the stems and roots were removed, placed in sample bags, and used to assess *M. phaseolina* colonization and to quantify the density of *M. phaseolina* per gram of dry tissue. The upper portion of these five plants were returned to the bundle of plants for yield analysis.

Lower stems and roots from plants collected at R8 were air dried and cross sectioned. Intensity of *M. phaseolina* colonization was assessed on a 1-to-5 scale, as described by Mengistu et al. (2007). After the visual assessment of *M. phaseolina* colonization in root tissue (a proxy for charcoal rot severity), stem and root sections were pulverized through a 600-µm-aperture screen using a Wiley mill (Model 3379-K05; Thomas Scientific, Swedesboro, NJ, U.S.A.), and CFU of *M. phaseolina* were determined per gram of

plant tissue (Mengistu et al. 2007). To quantify CFU of *M. phaseolina* from plant tissue rather than 1 g of soil, a 0.05-g subsample of ground soybean tissue was used as starting material and final CFU counts were reported per gram of soybean tissue. In 2012 and 2013, *M. phaseolina* colonization was visually assessed from plants on a 1-to-5 scale on samples from 10 quadrats (the ones constituting the diagonal) within each field. In 2014, samples from all quadrats within each field were assessed for *M. phaseolina* colonization. In 2012, *M. phaseolina* was not quantified in plant tissue. In 2013 and 2014, a subsample of 5 plants from 25 plants collected for yield analysis, as described above, was used to quantify *M. phaseolina*.

H. glycines virulence profile. Subsamples of soil from each quadrat within a field were combined to form one composite sample for each trial location. *H. glycines* type (HG type) tests (Niblack et al. 2002) were conducted to determine the virulence profile of the *H. glycines* populations from each field. Details for the HG type protocol are presented in Supplementary File S1.

The HG type of each population was assigned based on the calculated female index (FI). An indicator line with an FI ≥ 10 would be considered a compatible host for that particular population (Niblack et al. 2002; Tylka 2016). For example, if an *H. glycines* population produces FI ≥ 10 on PI 88788, PI 209332, and PI 548316, then it is an HG type 2.5.7 (Niblack et al. 2002). An HG type 0, on the other hand, is one that produces no FI ≥ 10 .

H. glycines and *M. phaseolina* Pf in soil, *M. phaseolina* CFU from plant tissue, charcoal rot severity (evaluated with a 1-to-5 ordinal scale), and HG type were not used in any statistical analysis. These

variables were collected, summarized, and used as internal controls to better understand results from the statistical analyses.

Soybean harvest and yield data. Twenty-five soybean plants were collected from each sampling quadrat (plot) and hand threshed with a static thresher (LD 350; Wintersteiger Inc., Salt Lake City, UT, U.S.A.). Soybean seed weight was determined and seed moisture content was measured with a portable moisture tester (Harvest Hand; Dickey-John Corporation, Auburn, IL, U.S.A.). Final weight was adjusted to 13% moisture and yield estimated as grams per plant.

Field data analysis. Standard OLS, spatial error, and spatial lag multiple-regression models were fitted to data from each trial. Soybean yield (grams per plant) from each quadrat was the dependent (or response) variable, and the independent (or explanatory) variables were *H. glycines* Pi (eggs per 100 cm³ of soil), *M. phaseolina* Pi (CFU per gram soil), and the cross-product term *H. glycines* $\times$ *M. phaseolina* for interaction effect. *H. glycines* eggs and *M. phaseolina* CFU were transformed to natural log values prior to analysis to stabilize variance. Natural log-transformed data were subsequently mean-centered to alleviate multicollinearity generated when the cross-product term (*H. glycines* $\times$ *M. phaseolina*) was included in the model for the interaction effect (Kutner et al. 2004; Vittinghoff et al. 2012). Regression models from fields in which a significant interaction was not detected were fitted to data excluding the interaction coefficient. *H. glycines* eggs and *M. phaseolina* CFU were natural log-transformed prior to analysis to stabilize variance; however, in the absence of the interaction term, variables were not mean-centered.

Fig. 1. Layout of a 10-by-10 grid (enlarged in the inset) of contiguous quadrats (7.62 by 7.62 m) in field 4. Spatial relation between each quadrat was recorded by labeling files and ranks alphabetically (A to J) and numerically (1 to 10), respectively. Samples were collected from the center of each quadrat (red dot).

Assessing model assumptions. Residuals from the fitted models were plotted against the predicted values to assess homoscedasticity. Normal distribution of residuals was assessed with Shapiro-Wilk test and normal probability plots. Variance inflation factor (VIF) was used to check for multicollinearity (Kutner et al. 2004). Maximum VIF values exceeding 10 or mean VIF values significantly greater than one were considered indications of collinearity between the independent variables (Kutner et al. 2004).

Independence in the observations and the residuals was also assessed. Moran's *I* test for spatial autocorrelation was applied to both the dependent variable (i.e., yield) and the OLS multiple-regression residuals. Significant values of Moran's *I* ($P < 0.05$) on the OLS residuals or the response variable were evidence of spatial autocorrelation and justified the implementation of spatial rather than OLS regression models to data with spatial structure (i.e., lack of independence). Furthermore, Moran's *I* test was applied to the independent variables (i.e., *H. glycines* and *M. phaseolina* Pi in soil) to examine the spatial pattern of these pathogens in each field.

Model selection. Akaike's information criterion (AIC) was obtained after fitting OLS, spatial error, and spatial lag regression models, and used to select the model that most efficiently summarized the data. Therefore, three AIC values were produced for each field, each corresponding to one of the three regression models. Subsequently, the regression model with the lowest AIC was considered the most appropriate for the conditions of that particular field (Kutner et al. 2004).

Details of standard OLS, spatial error, and spatial lag regression models are provided in Supplementary File S1. Likewise, details of the spatial weight matrix *W* used to obtain Moran's *I* and to fit the spatial regression models mentioned above are also provided in Supplementary File S1.

Computation methodology. Geographic representation of the field quadrats was constructed with the *Create Fishnet* tool in ArcGIS 10.2 (ArcGIS; ESRI, Redlands, CA, U.S.A.). The resulting shapefile was read into R, version 3.5.1 (R Core Team 2018), via the *read.shapefile* and *readShapePoly* functions from R packages 'shapefiles' (Stabler 2013) and 'mapproj' (Bivand and Lewin-Koh 2018), respectively. All spatial analyses, including construction of queen contiguity spatial weight matrix *W* (*poly2nb* and *nb2listw*), Moran's *I* analysis (*moran.test* and *lm.morantest*), and computation of spatial error and lag models (*errorsarlm* and *lagsarlm*) were done via functions available in the R package 'spdep' (Bivand et al. 2013). The package 'stats' (R Core Team 2018) was used to fit

standard OLS regression model using the *lm* command and to assess regression diagnostics using the R commands *qqplot* (to evaluate normal distribution of the residuals), *shapiro.test* (to test normality), and *AIC* (to obtain the AIC). From the package 'faraway' (Faraway 2016), the *vif* command was used to obtain the VIF and, from the package 'graphics' (R Core Team 2018), the command *plot* was used to plot residuals against predicted variables.

Greenhouse experiments. Two greenhouse experiments were conducted in October (experiment 1) and December (experiment 2) 2018. *H. glycines*- and *M. phaseolina*-susceptible soybean cultivar Williams 82 was used in both experiments. Constant conditions were maintained in the greenhouse and ambient temperature and humidity did not vary greatly with the external conditions. Most importantly, water tables were used in the greenhouse to maintain the plant rhizosphere at a constant temperature. Water tables are wooden boxes with a pond liner, filled with water and kept at a constant temperature ($27^{\circ}\text{C} \pm 1^{\circ}\text{C}$) using a refrigerating and heating system. Water in the greenhouse was also set at a constant temperature (27°C for this study) and used to water the plants when needed.

***H. glycines* inoculum.** An *H. glycines* population (designated WM001-SCN), HG type 2.5.7. (Table 1), was obtained from field 6 at the Waterman Agricultural and Natural Resources Laboratory of The Ohio State University in Columbus, OH and used as inoculum in the greenhouse experiments. Population WM001-SCN was increased and maintained in the greenhouse at 27°C for 30 days with a 16-h photoperiod on susceptible soybean cultivar Williams 82. After 30 days, *H. glycines* eggs were collected as previously described, and diluted in sterile water to approximately 2,500 eggs/ml.

***M. phaseolina* inoculum.** A single *M. phaseolina* isolate (designated Br007-Mp) was obtained from a symptomatic soybean plant collected from field 4 in Brown County, OH. Pure Br007-Mp isolates were used to produce *M. phaseolina*-infested Japanese millet seed (*Echinochloa frumentaceae*), as described by Mengistu et al. (2007). Details for the production of *M. phaseolina*-infested Japanese millet seed are given in Supplementary File S1.

A sieve (250- μm aperture) containing 15 g of *M. phaseolina*-infested millet seed was placed on top of nested 150- μm - over 45- μm -aperture sieves. Microsclerotia were dislodged from colonized millet seed with high-pressure water spray (Model B-0100-32H; T&S Brass, Travelers Rest, SC, U.S.A.) and collected on the 45- μm -aperture sieve. Washed millet seed and debris retained on the

TABLE 1. *Heterodera glycines* and *Macrophomina phaseolina* population densities, *H. glycines* reproduction factor (RF), charcoal rot severity, soybean yield, and *H. glycines* virulence profile for each field

Field ^c	<i>H. glycines</i> ^a		<i>M. phaseolina</i> ^b				Yield ^d	HG type ^e
	Pi	RF	Pi	Pf	Root	Severity		
2012								
1	48.8	15.1	37.7	25.2	...	3.3	10.5	2.5.7
2	607.8	6.7	37.0	34.1	...	2.0	10.1	2.5.7
2013								
3	965.2	6.4	20.0	14.1	12,164.0	1.3	13.0	2.5.7
4	2,984.4	2.6	53.6	39.0	56,553.0	2.1	11.1	5.7
2014								
5	3,622.0	1.9	61.2	44.6	168.0	1.0	14.0	5.7
6	2,713.9	6.8	45.2	36.3	2,161.2	2.5	10.2	2.5.7

^a Pi = mean of *Heterodera glycines* initial population (Pi) in soil reported in eggs per 100 cm³ of soil. *H. glycines* RF = ratio between *H. glycines* final population (Pf) to Pi in soil (Pi/Pf).

^b Pi and Pf = mean of *M. phaseolina* Pi and Pf, respectively, in soil reported in CFU per gram of soil. Root = mean of *M. phaseolina* in soybean lower stem and root reported in CFU per gram of soybean tissue. No roots were processed from fields 1 and 2 in 2012. Severity = charcoal rot severity rating using a 1-to-5 rating scale (Mengistu et al. 2007), where 1 = no microsclerotia visible in tissue, 2 = few visible microsclerotia in tissue, 3 = partial discoloration of vascular tissue with few visible microsclerotia, 4 = discoloration of vascular tissue with microsclerotia embedded in tissue, and 5 = vascular tissue darkened due to high numbers of microsclerotia both inside and outside of the stem and root tissues.

^c Year and field trial.

^d Soybean yield estimates in grams per plant.

^e HG type = *H. glycines* virulence profile from each field; 2 indicates >10% reproduction on PI 88788, 5 indicates >10% reproduction on PI 209332, and 7 indicates >10% reproduction on PI 548316.

250- μ m- and 150- μ m-aperture sieves were discarded. Microsclerotia were combined with 100 ml of 0.5% sodium hypochlorite (i.e., 10% bleach) and agitated in an orbital shaker at 200 rpm for 1 min. The mixture was then poured over a 45- μ m-aperture sieve and rinsed well with distilled water. Finally, *M. phaseolina* microsclerotia were diluted in sterile water at an inoculum density of approximately 10,000 microsclerotia/ml. To confirm the viability and purity of the inoculum, 1 ml of the solution containing *M. phaseolina* microsclerotia was delivered onto Petri dishes containing potato dextrose agar and placed in an incubator at 27°C with no light for 2 to 3 days.

Greenhouse experimental design, soil infestation, and data collection. The experimental design consisted of four treatments arranged in a completely randomized design with 10 replicates. Treatments were soil infested with *H. glycines*, soil infested with *M. phaseolina*, soil coinfecting with both pathogens, and an uninoculated control. An experimental unit was a single PVC tube (3 cm in diameter by 15 cm in length) filled with 100 cm³ of pasteurized soil substrate. A mixture (3:1 vol/vol) of pasteurized sand-Turface MVP (Turface Athletics Inc., Buffalo Grove, IL, U.S.A.) was used as soil substrate in both experiments. For each tube, a hole was produced in the soil into which the treatment was delivered and a uniform-sized 2-day-old seedling of Williams 82 was planted. Subsequently, tubes were randomized in plastic containers and placed in water tables for 30 days at 27°C with a 16-h photoperiod. The water table served as a water bath to keep the rhizosphere at a constant temperature. Plants were watered every day to keep the soil moist while avoiding saturation.

The *H. glycines* treatment received 1 ml of the WM001-SCN inoculum suspension (containing approximately 2,500 *H. glycines* eggs) per tube. Subsequently, single soybean seedlings were transplanted into each tube. For the *M. phaseolina* treatment, 1 ml of the Br007-Mp inoculum suspension (containing approximately 10,000 *M. phaseolina* microsclerotia) was delivered to each tube. The *H. glycines* and *M. phaseolina* coinfection treatment consisted of both inoculation methods described above applied to the same experimental unit (i.e., 2,500 *H. glycines* eggs and 10,000 *M. phaseolina* microsclerotia delivered per tube). Finally, the uninoculated control consisted of tubes filled with pasteurized soil, no pathogen added, and soybean seedlings planted.

Plants in each tube were removed and soil was gently washed from roots. Each plant was held by the shoots and the root portion was placed on nested 850- μ m- over 250- μ m-aperture sieves. High-pressure water was applied to the roots to dislodge *H. glycines* females, which were collected in the 250- μ m-aperture sieve, counted, and reported as *H. glycines* females per plant (i.e., experimental unit).

Washed plants were then immersed in 0.5% sodium hypochlorite for 1 min, rinsed in distilled water for 3 min, and dried with paper towels. Surface-sterilized plants were placed in labeled paper bags and air dried. Once completely dried, plants were weighed and ground, and 0.1 g of dried tissue was plated to determine *M. phaseolina* CFU per gram of dry tissue. Each datum was standardized using its corresponding plant dry weight, and *M. phaseolina* CFU per plant (i.e., experimental unit) was reported.

Greenhouse data analysis. Two dependent variables were obtained from each experiment and analyzed separately. Number of *H. glycines* females per plant was one dependent variable with three treatments: *H. glycines* alone, *H. glycines*-*M. phaseolina* coinfection, and control. The other dependent variable was *M. phaseolina* CFU per plant, also with three treatments: *M. phaseolina* alone, *H. glycines*-*M. phaseolina* coinfection, and control. *H. glycines* females and *M. phaseolina* CFU per plant were analyzed separately with a generalized linear mixed model (GLMM) in R, version 3.5.1 (R Core Team 2018).

H. glycines female and *M. phaseolina* CFU counts were assumed to follow a Poisson distribution and were each analyzed using a Poisson GLMM, with treatments as fixed effect and experiment

runs as random effect. The assumed Poisson distribution was tested and, under violation of the mean-variance equality assumption, the model was refitted using an alternative distribution, the negative binomial, with an extra parameter to account for overdispersion.

Based on results from initial analyses, when *H. glycines* female count was considered the dependent variable, data from each experiment were analyzed separately. On the other hand, when *M. phaseolina* CFU count was considered the dependent variable, data from each experiment were pooled and analyzed together.

Likelihood ratio and goodness-of-fit tests as well as AIC values were used to assess the fit of each model. Significant differences were declared at an α level of 0.05. R packages used in the analyses included 'stats' (R Core Team 2018), 'MASS' (Venables and Ripley 2002), 'lme4' (Bates et al. 2015), 'lmerTest' (Zeileis and Hothorn 2002), 'ggplot2' (Wickham 2009), and 'tidyverse' (Wickham 2016).

RESULTS

Soilborne pathogen population densities and soybean response. *Environmental conditions and soybean yield.* In this study, monthly average ambient temperature did not exceed 27°C in any of the fields (Supplementary Fig. S1). Moreover, maximum temperatures never exceeded 33°C in any field. Rainfall was lowest in 2012; however, total precipitation per month exceeded 36 mm in each field over the course of the study. Average soybean yield per field ranged from 10 to 14 g/plant (Table 1).

Soilborne pathogen population densities. Overall, Pi densities of *H. glycines* were <1,000 eggs per 100 cm³ of soil in fields 1 to 3 and >2,000 eggs per 100 cm³ of soil in fields 4 to 6 (Table 1). With the exception of fields 1 and 6, *H. glycines* was detected in all quadrats within each field at the beginning of the season. In field 1, *H. glycines* was not detected in 51% of the quadrats, while 17% of the quadrats in field 6 had no detectable *H. glycines*.

The nematode virulence profile for these fields was either HG type 2.5.7 or HG type 5.7 (Table 1). Reproduction factor (RF), a ratio of Pf to Pi density (Pf/Pi), indicated that the *H. glycines* population increased in each field approximately 2- to 15-fold at the end of the season. Soybean cultivars planted in each field were labeled resistant to *H. glycines* (Supplementary Table S1). The *H. glycines* resistance response was specific to HG type 0 (race 3) and 1.3- (race 14).

M. phaseolina Pi densities in these fields ranged from approximately 20 to 60 CFU/g of soil at the first sampling and declined at the end of the season (Table 1). At harvest, charcoal rot symptoms were observed on stems in fields 1 and 6 (Table 1). In field 1, symptoms were more prevalent and evident (severity ratings ranging from 1 to 5) whereas, in field 6, symptoms were more sporadic and less severe, and only evident under close inspection of lower stems. In fields 3 and 4, *M. phaseolina* CFU per gram of soybean root tissue was greater from plants with low charcoal rot severity ratings (Table 1). Roots were not processed for *M. phaseolina* quantification in soybean tissue at harvest for fields 1 and 2, the first year of the study.

Regression model, spatial dependence, and interaction effect. *OLS multiple-regression model assumptions.* After each model was fit, the residual's normal distribution and homoscedasticity assumptions were met. Similarly, a lack of multicollinearity in the independent variables was verified based on VIF values. Before mean-centering the independent variables (*H. glycines* and *M. phaseolina*) and before including the interaction term (*H. glycines* $\times$ *M. phaseolina*), the mean VIF values for each model fitted to data from each field did not exceed 1.09, indicating that *H. glycines* and *M. phaseolina* Pi were not linearly related. When the interaction term was included, the mean VIF values for the six regression models ranged between 21.75 and 141.47. After mean-centering *H. glycines* and *M. phaseolina* Pi, and including the interaction term of mean-centered variables, the mean VIF values

for the six regression models corresponding to each field ranged between 1.01 and 1.63.

Moran's I values for spatial autocorrelation test on soybean yield, OLS residuals, and *H. glycines* and *M. phaseolina* Pi in soil are presented in Supplementary Table S2. Significant spatial autocorrelation in both the dependent variable and OLS residuals was observed in all fields, except field 3. In field 2, for example, a strong positive spatial autocorrelation (i.e., clustering of similar values) in both yield response ($I=0.480$, $P<0.001$) and OLS residuals ($I=0.454$, $P<0.001$)

was observed (Fig. 2). Moran's I values did not differ from zero for yield ($P = 0.058$) and OLS residuals ($P = 0.166$) in field 3; therefore, observations from this field were spatially independent and arranged at random (Fig. 2). The spatial distribution of *H. glycines* and *M. phaseolina* Pi densities in soil was also examined. Significant Moran's I values for both soilborne pathogens were positive, indicating aggregation or clustering of the observations in the field (Fig. 3).

Model selection. The OLS and the two spatial regression models were compared based on AIC values. The standard OLS regression

Fig. 2. Choropleth map of **A**, soybean yield and **B**, ordinary least squares (OLS) regression residuals for each field. Soybean yield and OLS residuals are in grams per soybean plant. NA represents missing values. Figure not drawn to scale.

model had the greatest AIC values for all fields, except field 3, in which its value was the lowest (Table 2). Model comparison based on AIC suggested that the best fit to data from field 3 was the OLS regression model. Likewise, based on comparison of AIC values, a spatial error regression model was the best fit to data from fields 1, 4, and 5, and the spatial lag model performed best at fitting data from fields 2 and 6 (Table 2). Moreover, spatial coefficient parameters from the spatial error (λ) and spatial lag (ρ) models were significant ($P < 0.05$) after these models were fitted to data

from all fields, except field 3 (Table 2). In field 3, spatial coefficient parameters from the spatial error models ($\lambda = 0.18$, $P = 0.290$) and spatial lag ($\rho = 0.21$, $P = 0.196$) were not significant (Table 2).

Effect of H. glycines and M. phaseolina on soybean yield. Spatially correlated error terms in the standard OLS regression model were motivation for employing spatial regression models. When the spatial error and spatial lag models provided indistinguishable AIC values (Table 2), the spatial error model was

Fig. 3. Choropleth map for **A**, *Heterodera glycines* and **B**, *Macrophomina phaseolina* initial population (P_i) densities in soil from each field. *H. glycines* P_i are reported in eggs per 100 cm³ of soil and *M. phaseolina* P_i in colony forming units per gram of soil. NA represents missing values. Figure not drawn to scale.

preferred to model the spatial structure in the error term (Bivand et al. 2013; Waller and Gotway 2004).

The main objective of the field study was to determine the presence (or not) of a significant interaction effect between *H. glycines* and *M. phaseolina* on soybean yield through linear multiple-regression models. A significant interaction effect between *H. glycines* and *M. phaseolina* on soybean yield was observed in fields 4 and 5, which were among fields that had, on average, Pi densities of *H. glycines* and *M. phaseolina* >2,000 eggs per 100 cm³ of soil and >45 CFU/g of soil, respectively (Tables 1 and 2). The spatial error model presented stronger evidence for the interaction effect than the OLS model; furthermore, significant interaction effect coefficients from field 4 and 5 were positive

(Table 2). At an α level of 0.05, there was not sufficient evidence to conclude a significant interaction effect between *H. glycines* and *M. phaseolina* on soybean yield in fields 1, 2, 3, and 6 (Table 2).

Regression models from fields in which a significant interaction was not detected were fitted to data excluding the interaction coefficient, and results are presented in Supplementary Table S3 and Supplementary Figure S2. Spatial regression models were most appropriate to fit data from fields 1, 2, and 6, while the OLS regression model was the best fit for data from field 3. Coefficients for each soilborne pathogen were not significant ($P > 0.05$) in fields with low inoculum density (fields 1, 2, and 3) (Table 1). In field 1, the *H. glycines* coefficient was significant in the OLS model;

TABLE 2. Coefficients (Coef) and standard errors (SE) for the ordinary least squares (OLS), spatial error (SEM), and spatial lag (SLM) multiple-regression models and associated probability (P) values for soybean yield regressed against initial population (Pi) of *Heterodera glycines*, *Macrophomina phaseolina*, and the interaction effect between these pathogens in each field^a

Field, variables ^b	OLS				SEM				SLM			
	Coef	SE	P	Model	Coef	SE	P	Model	Coef	SE	P	Model
2012												
Field 1												
Intercept	10.42	0.28	<0.001	...	10.38	0.73	<0.001	...	3.27	0.98	<0.001	...
SCN_Pi	-0.28	0.12	0.025	...	-0.14	0.10	0.151	...	-0.17	0.10	0.080	...
CFU_Pi	-0.26	0.42	0.540	...	-0.56	0.35	0.107	...	-0.41	0.33	0.218	...
SCN×CFU	0.23	0.19	0.232	...	0.22	0.14	0.120	...	0.24	0.15	0.123	...
ρ	...	...	...	...	...	...	...	...	0.68	...	<0.001	...
λ	...	...	...	...	0.70	...	<0.001	...	...	...	...	...
AIC	...	...	...	491.58	...	...	...	458.63	...	...	...	459.14
Field 2												
Intercept	10.11	0.31	<0.001	...	10.22	0.94	<0.001	...	2.43	0.83	0.003	...
SCN_Pi	0.52	0.33	0.123	...	0.22	0.26	0.399	...	0.28	0.24	0.241	...
CFU_Pi	0.78	0.76	0.306	...	-0.26	0.52	0.611	...	0.03	0.55	0.949	...
SCN×CFU	0.45	0.99	0.651	...	0.78	0.70	0.259	...	0.66	0.71	0.352	...
ρ	...	...	...	...	...	...	...	...	0.76	...	<0.001	...
λ	...	...	...	...	0.77	...	<0.001	...	...	...	...	...
AIC	...	...	...	492.71	...	...	...	447.25	...	...	...	447.06
2013												
Field 3												
Intercept	12.96	0.23	<0.001	...	12.96	0.27	<0.001	...	10.26	2.10	<0.001	...
SCN_Pi	-0.35	0.24	0.160	...	-0.27	0.23	0.253	...	-0.30	0.24	0.197	...
CFU_Pi	0.04	0.30	0.889	...	0.09	0.31	0.772	...	0.07	0.29	0.808	...
SCN×CFU	0.31	0.34	0.364	...	0.27	0.33	0.419	...	0.29	0.33	0.386	...
ρ	...	...	...	...	...	...	...	...	0.21	...	0.196	...
λ	...	...	...	...	0.18	...	0.290	...	...	...	...	...
AIC	...	...	...	455.31	...	...	...	456.19	...	...	...	455.63
Field 4												
Intercept	11.12	0.17	<0.001	...	11.17	0.26	<0.001	...	7.21	1.55	<0.001	...
SCN_Pi	-0.19	0.27	0.478	...	-0.07	0.26	0.775	...	-0.12	0.25	0.620	...
CFU_Pi	-0.68	0.34	0.047	...	-0.77	0.34	0.023	...	-0.63	0.32	0.052	...
SCN×CFU	1.26	0.53	0.019	...	1.32	0.49	0.008	...	1.23	0.50	0.014	...
ρ	...	...	...	...	...	...	...	...	0.35	...	0.029	...
λ	...	...	...	...	0.40	...	0.013	...	...	...	...	...
AIC	...	...	...	394.54	...	...	...	390.38	...	...	...	391.77
2014												
Field 5												
Intercept	13.81	0.33	<0.001	...	13.82	0.59	<0.001	...	6.59	1.73	<0.001	...
SCN_Pi	-1.09	0.45	0.017	...	-0.89	0.45	0.046	...	-0.81	0.40	0.044	...
CFU_Pi	0.41	0.53	0.444	...	0.45	0.47	0.337	...	0.41	0.47	0.387	...
SCN×CFU	1.08	0.63	0.091	...	1.11	0.56	0.048	...	1.01	0.56	0.073	...
ρ	...	...	...	...	...	...	...	...	0.52	...	<0.001	...
λ	...	...	...	...	0.53	...	<0.001	...	...	...	...	...
AIC	...	...	...	517.83	...	...	...	505.32	...	...	...	505.39
Field 6												
Intercept	10.32	0.28	<0.001	...	10.10	0.63	<0.001	...	3.95	1.01	<0.001	...
SCN_Pi	0.04	0.09	0.703	...	-0.01	0.09	0.921	...	0.02	0.08	0.845	...
CFU_Pi	-2.71	0.59	<0.001	...	-1.68	0.55	0.002	...	-1.82	0.51	<0.001	...
SCN×CFU	-0.36	0.17	0.042	...	-0.23	0.15	0.122	...	-0.25	0.15	0.084	...
ρ	...	...	...	...	...	...	...	...	0.62	...	<0.001	...
λ	...	...	...	...	0.63	...	<0.001	...	...	...	...	...
AIC	...	...	...	517.37	...	...	...	499.15	...	...	...	495.43

^a OLS, SEM, and SLM multiple-regression models were used to fit data from each field.

^b Field represents the year and field trial. SCN_Pi and CFU_Pi = natural log-transformed and mean centered *H. glycines* and *M. phaseolina* Pi, respectively, in soil; SCN×CFU = multiplicative interaction effect between the natural log-transformed and mean-centered SCN_Pi and CFU_Pi; ρ = spatial parameter for the SLM; and λ = spatial parameter for the SEM. Akaike information criterion (AIC) values were produced for OLS, SEM, and SLM. The lower the AIC value, the better the model.

however, after accounting for spatial autocorrelation, the main effect for the nematode was no longer significant ($P > 0.05$) for either of the spatial regression models. Despite the high population densities of *H. glycines* in field 6 (Table 1; Fig. 3), its coefficient was not significant in the regression model. *M. phaseolina* Pi densities, however, significantly ($P < 0.05$) reduced soybean yield in field 6.

Greenhouse experiments. The objective of the greenhouse study was to evaluate, through nematode reproduction and plant tissue fungal colonization, the combined effect of *H. glycines* and *M. phaseolina* infecting soybean under environmentally controlled conditions and to better understand the mechanism of the interaction effect detected in the field study.

For the greenhouse experiment with *H. glycines* females per plant as dependent variable, the Poisson generalized linear model (GLM) was the best fit to data from experiment 1, while a negative binomial GLM best fit the data from experiment 2.

The presence of *M. phaseolina* inoculum in coinfecting treatments significantly ($P < 0.05$) reduced *H. glycines* females per plant (Fig. 4). In the *H. glycines*-alone treatment, on average, over 100 females/plant was recorded (Niblack et al. 2002); there were 111 and 127 *H. glycines* females/plant in experiments 1 and 2, respectively. In combination with the fungus, a mean of 66 and 86 *H. glycines* females/plant was recorded in experiments 1 and 2, respectively.

In the greenhouse study with *M. phaseolina* CFU per plant as dependent variable, negative binomial GLMM fit pooled data from experiments 1 and 2 the best. Counts of *M. phaseolina* CFU per plant in the presence of the nematode was not significantly different ($P = 0.223$) from the treatment with the fungus alone (data not shown). *H. glycines* and *M. phaseolina* were not recovered from uninoculated control pots.

DISCUSSION

The spatial distribution of soilborne pathogens may affect the spatial pattern of their damage (e.g., yield losses) on the host

Fig. 4. Boxplot diagrams summarizing the distribution of *Heterodera glycines* female counts per plants as a function of treatments from two greenhouse experiments. Horizontal black lines inside the boxes represent the median value of *H. glycines* female counts per plants for each treatment: SCN = *H. glycines* alone and SCN_Mp = soil coinfecting with *H. glycines* and *Macrospora phaseolina*. The top and bottom of the boxes are the 25th and 75th percentiles, respectively. Reference red line indicates 100 *H. glycines* females/plant. Within each experiment, boxes with different letters differed significantly based on the Poisson (experiment 1) and negative binomial (experiment 2) generalized linear model at $\alpha = 0.05$. Statistical analyses were performed on the log link scale; however, data are displayed on the original scale.

(Gavassoni et al. 2007; Hughes 1988). The spatial distribution of *H. glycines* (Francel 1986; Gavassoni et al. 2001, 2007) and *M. phaseolina* (Mihail 1989; Mihail and Alcorn 1987; Olanya and Campbell 1988, 1989) were previously described as aggregated. In all of the fields sampled in this study, both or either *H. glycines* and *M. phaseolina* Pi densities in soil were significantly aggregated. The spatial pattern of soybean yield and OLS residuals in each field were evaluated by means of Moran's *I*. When spatial dependence was significant, Moran's *I* values were positive, indicating aggregation of the observations. Spatial aggregation in yield data has been previously reported and our results were similar (Kulkarni et al. 2008a,b; Liu et al. 2014, 2015). The use of spatial regression models allowed accounting for spatial autocorrelation whenever this was detected and more precisely testing the interaction effect between *H. glycines* and *M. phaseolina* on soybean yield.

In this study, a significant interaction effect between *H. glycines* and *M. phaseolina* on soybean yield was measured in two fields with high Pi densities of both pathogens. Under a significant interaction, interpretations of the main effects (*H. glycines* and *M. phaseolina* Pi densities) could be misleading, because the effect of one of the pathogens on soybean yield is dependent on the population density of the other pathogen. Even if the effect of one of the main terms is not significant in the regression model, under a significant interaction effect, the main terms are a linear function of each other (Kutner et al. 2004). Therefore, the main effects are inextricably linked under a significant interaction (Wallace 1983).

The significant interaction coefficients (in fields 4 and 5) were positive, suggesting that less soybean yield reduction took place under a significant interaction between *H. glycines* and *M. phaseolina* Pi. We hypothesized that the positive interaction coefficient resulted from competition between these pathogens for similar ecological niches, a common phenomenon in disease complexes (Eisenback 1993; Lamichhane and Venturi 2015). To further evaluate this hypothesis, we conducted a greenhouse study in which *H. glycines* females per soybean plant were significantly reduced in the presence of *M. phaseolina*. Similar results have been previously reported in greenhouse interaction studies between *Fusarium virguliforme* (causal agent of sudden death syndrome of soybean) and *H. glycines* (Gao et al. 2006; McLean and Lawrence 1993). Similarly, Marburger et al. (2014) observed a negative association between *H. glycines* and *F. virguliforme* in commercial soybean fields in Wisconsin. However, the mechanisms by which *H. glycines* females per soybean plant were reduced in the presence of *M. phaseolina* were not evaluated in our greenhouse experiments and are yet to be investigated.

Many studies that evaluated soybean yield loss as an effect of *H. glycines* Pi identified fields with a wide range of population densities or artificially generated them (Chen et al. 2001; De Bruin and Pedersen 2008; Koenning 2000; MacGuidwin et al. 1995; Todd et al. 1995; Wang et al. 2000, 2003). In field 6, for example, an interaction effect between *H. glycines* and *M. phaseolina* was not significant. Similarly, the effect of *H. glycines* on yield was not significant despite the high Pi density. Close examination of the choropleth map for *H. glycines* Pi distribution in field 6 indicated a lack of range in the population density, which can affect the significance of a regression coefficient. Smrtnik et al. (2016) also observed an even distribution of *H. glycines* density in some fields with high populations from Ohio. The use of resistant and susceptible cultivars or a more unevenly distributed *H. glycines* Pi may have revealed yield reduction due to the nematode.

The choropleth map for *M. phaseolina* Pi in field 6, however, revealed a wide range. Reportedly, under hot (>28 to 30°C) and dry environmental conditions, *M. phaseolina* can cause charcoal rot and significantly reduce soybean yield (Gupta et al. 2012; Mengistu et al. 2015; Mihail 1989; Pearson et al. 1984). *M. phaseolina* Pi in field 6 significantly reduced soybean yield even when the environmental conditions typically reported for charcoal rot were not prevalent. Similar findings related to environment have been reported (Francel

et al. 1988; Kendig et al. 2000; Mengistu et al. 2011) and suggest that there may be additional factors or a wider range of environmental conditions that favor charcoal rot.

In addition to evaluating the effects of *H. glycines* and *M. phaseolina* on soybean yield, we also described other aspects of the field biology of these soilborne pathogens. The RF (RF = Pf/Pi) is an indicator of the host's ability to support nematode reproduction and this ratio is commonly used to describe host status (Chen et al. 2001; De Bruin and Pedersen 2008; Koenning and Sipes 1998). In this study, soybean cultivars were labeled "resistant to *H. glycines* race 3", which frequently indicates that the plants have the PI 88788 source of resistance (Mitchum 2016). Fields infested with HG type 2.5.7, which by definition will reproduce on PI 88788 (Niblack et al. 2002), had greater RF compared with fields infested with HG type 5.7 (fields 4 and 5). Other studies have reported RF > 1 on soybean cultivars with the PI 88788 source of resistance grown in fields infested with either HG type 0 or 2.5.7 (Chen et al. 2001; De Bruin and Pedersen 2008). Our results highlight the importance of determining the *H. glycines* virulence profile in a field and understanding the HG type test results, which can improve management strategies that rely on the use of soybean cultivars with resistance to *H. glycines* (Niblack 2005; Niblack et al. 2006, 2008; Tylka 2016).

Another element that may affect the RF is the nematode Pi density in soil, which is inversely related to the RF (Alston and Schmitt 1987; Bonner and Schmitt 1985; Chen et al. 2001; De Bruin and Pedersen 2008; Francl and Dropkin 1986; MacGuidwin et al. 1995). In the current study, the field with the lowest *H. glycines* Pi produced the largest RF, reflecting a 15-fold increase in nematode population densities at the end of the season. Disease management to keep *H. glycines* population densities low and avoid the rapid increase that could easily reach damaging levels in one growing season must be made (De Bruin and Pedersen 2008). Extrinsic abiotic or biotic elements that may enhance or diminish the *H. glycines* life (and disease) cycle could positively or negatively affect the RF. In the current study, two fields (fields 4 and 5) with the lowest *H. glycines* RF were fields with the highest *M. phaseolina* Pi in soil. Several studies that evaluated interactions between cyst nematodes and fungal soilborne pathogens reported a reduction in nematode RF in the presence of the fungus (Gao et al. 2006; Hillnhütter et al. 2011; McLean and Lawrence 1993). This hypothesis was confirmed by results from our greenhouse study.

In all of the field trials in this study, *M. phaseolina* population densities in soil decreased at the end of the season. Previous studies reported similar results (Campbell and van der Gaag 1993; Francl et al. 1988; Zveibil et al. 2012). *M. phaseolina* survival in soil is affected by several abiotic and biotic factors such that this fungus is a poor soil competitor (Dhingra and Sinclair 1978; Elad et al. 1986; Zveibil et al. 2012). Therefore, sampling at the end of the season may underestimate *M. phaseolina* population densities in soil. On average, the CFU per gram of soil detected in this study fall in the ranges of previously reported *M. phaseolina* population densities (Francl et al. 1988; Kendig et al. 2000; Lopez-Nicora et al. 2016; Mengistu et al. 2009; Wrather et al. 1998, 2007).

Similarly, CFU per gram of tissue observed in this study were within the range of *M. phaseolina* propagule densities in soybean tissue previously reported (Mengistu et al. 2011, 2013; Smith and Carvil 1997; Wrather et al. 2008). In our study, quantification of the fungus from soybean tissue was done from plants collected at harvest (i.e., soybean growth stage R8). At soybean growth stage R8, plants are senesced and saprophytic growth of *M. phaseolina* may affect the association measured between fungus population densities in tissue and soybean yield response (Mengistu et al. 2011). Additionally, in this study, high counts of CFU per gram of tissue were recorded in fields with low disease severity (fields 3 and 4). Previous studies have observed that lack of visible charcoal rot symptoms and vascular discoloration does not

mean absence of *M. phaseolina* infection or reduction in yield due to the fungus (Kendig et al. 2000; Mengistu et al. 2007, 2013; Wrather et al. 2008).

In soybean commercial fields in Ohio, a significant interaction effect between *H. glycines* and *M. phaseolina* was detected via spatial regression analysis. The statistical interaction effect did not explain the mechanism by which these pathogens interact. Wallace (1983) recommended that, after significant interaction effects are detected in statistical models, further physiological studies be conducted. Additionally, regression analysis does not imply causation but relationship or association between the independent and the dependent variables (Kutner et al. 2004). Results from the greenhouse experiment suggested an explanation for the positive coefficient for the interaction term in the spatial regression models from the field study. The direct mechanisms by which *H. glycines* and *M. phaseolina* interact were not demonstrated in this study. Future studies must be conducted in the field and greenhouse, where experimental designs with appropriate treatments can be controlled to better understand the interaction between *H. glycines* and *M. phaseolina* on soybean production.

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