A Risk Assessment Model for Bacterial Leaf Spot of Pepper (*Capsicum annuum*), Caused by *Xanthomonas euvesicatoria*, Based on Concentrations of Macronutrients, Micronutrients, and Micronutrient Ratios

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ABSTRACT

The phytopathogenic bacterium *Xanthomonas euvesicatoria* causes bacterial leaf spot (BLS) of pepper and has a worldwide distribution. BLS is difficult to control and an integrated management strategy that incorporates crop rotation, use of clean seed and clean plants, weed control, resistant varieties, applications of bactericides, biocontrol agents, and systemic acquired resistance (SAR) inducers is generally recommended. However, even with that arsenal of weapons, BLS can still be responsible for severe losses under favorable environmental conditions. Thus, additional tools need to be added to an overall integrated management strategy to combat BLS. In this article, we developed several models from 2012 to 2014 that were based on how macronutrients, micronutrients, and micronutrient ratios affect BLS severity. Factors used to select a model for validation included highly significant *P* values, high adjusted *R*² values, low variance inflation factor values (<5), root mean square error, Mallow's Cp, and high Akaike's information criterion correction values. In addition, salicylic acid (SA) concentrations and relative expression of nonexpresser pathogenesis-related gene1 (*NPR1*) and pathogenesis-related protein 1 (*PR1*) in pepper tissues were also

considered in model selection. A model (ECGA1) consisting of concentrations of copper, manganese, potassium, and the iron/zinc ratio as independent variables was used for validation in three different commercial pepper fields in Georgia: Colquitt County and Worth County in 2015 and Tift County in 2016. When area under the disease progress curve (AUDPC) values for two field sites (Colquitt and Worth Counties) in 2015 were pulled together and plotted against ECGA1-predicted values for both sites, the resulting relationship was highly significant (*P* = 0.0001) with an *R*² value of 0.92. A significant relationship between observed AUDPC versus predicted values was also observed in Tift County in 2016 (*P* < 0.001; adjusted *R*² = 0.98). Relative gene expression of both *NPR1* and *PR1* genes was significantly (*P* < 0.01) higher in pepper grown in predicted low-risk sites compared with pepper from high-risk sites in Colquitt, Worth, and Tift Counties. Although BLS severity will fluctuate depending on environmental conditions, the data indicate that the level of risk at a particular location may be influenced by how macronutrient and micronutrient concentrations affect plant disease resistance genes in the SAR pathway.

Bacterial leaf spot (BLS), caused by *Xanthomonas euvesicatoria*, is a serious disease of pepper (*Capsicum annuum* L.) that is responsible for moderate to severe losses annually in Georgia (Gitaitis et al. 1991; McInnes et al. 1988; Sijam et al. 1991) and elsewhere (Bouzar et al. 1999; Jones et al. 1986; Ritchie and Dittapongpitch 1991; Sahin and Miller 1996). Primary inoculum can come from a variety of sources, including seed, weeds, volunteer plants, and even short-term carryover from plant debris in soil (Jones et al. 1986). An integrated management strategy that includes crop rotation, use of clean seed, clean plants, weed control, and sprays with labeled bactericides is generally recommended. In Georgia, growers currently apply multiple applications of copper (Cu) and mancozeb throughout the season, which can be as often as twice a week. In some instances, novel approaches using applications of bacteriophages with specific virulence to *X. euvesicatoria* (Jones et al. 2002) or sprays with nanoparticles doped with elements such as silver (Ochoy et al. 2013) have been used. However, most strategies fail to control BLS when

environmental conditions are optimum for disease development. Furthermore, control is hampered by the development of Cu-tolerant bacterial strains (Marco and Stall 1983; Jones et al. 1991; Ritchie and Dittapongpitch 1991). Although resistant varieties against different races of this pathogen are available, development of new races often renders this management strategy ineffective (Kousik and Ritchie 1996; Pohronezny et al. 1992). Exclusion of initial inoculum by eliminating infected seedlots through the use of seed health assays is effective to some degree but no test is 100% effective due to the inherent sampling and detection problems associated with seed testing (Dutta et al. 2013, 2014b; Gitaitis and Walcott 2007).

Another strategy to control bacterial diseases in vegetables has been the use of compounds that stimulate systemic acquired resistance (SAR) in the plant (Buonaurio et al. 2002; Huang et al. 2012; Louws et al. 2001; Obradovic et al. 2002). A permanent label is available for acibenzolar-S-methyl use for nonbell, chili, and hot pepper in Georgia along with a section 24 (c) for its use on bell pepper until the end of 2017. Although there has been some success with using SAR-inducing compounds, further research is needed on how to induce SAR and to enhance the activity of SAR-inducing compounds without negatively affecting plant health. Despite the best efforts of the industry, BLS continues to be a serious disease causing losses in most years. It is currently assumed that BLS outbreaks in the field are due to either the introduction of inoculum

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*The e-Xtra logo stands for "electronic extra" and indicates that three supplementary figures are published online.

through infested pepper seed or seedlings or locally survived inoculum on weeds and volunteers. Thus, after seed testing, management is primarily a combination of planting resistant varieties and application of bactericides. Cultural practices, although a vital factor, are an often forgotten cog in the overall strategy to manage BLS of pepper.

Macronutrient and micronutrient concentrations in soil and plant tissue has been associated with plant diseases (Datnoff et al. 2007). In preliminary studies, it was observed that nutrients such as Cu, iron (Fe), manganese (Mn), potassium (K), and zinc (Zn) in soil and tissue consistently occurred as independent variables in multiple regression models, which significantly correlated with levels of sour skin (Selph et al. 2014) and tomato spotted wilt (Rooks et al. 2015) in onion and tobacco, respectively. Results from these preliminary studies support the metal defense hypothesis of Poschenrieder et al. (2006), who contend that metal compounds employed as biocides may also be absorbed by plants as a self-defense mechanism. Furthermore, on a physiological level, metal defense against biotic stress suggests shared or corresponding pathways of signal perception, transduction, and metabolic events downstream. These commonalities are a reflection of cross talk between plants and pathogens as they evolved in environments where metal acquisition and ion homeostasis were necessary for survival (Poschenrieder et al. 2006).

In our preliminary tests, Selph et al. (2014) developed models from both soil and tissue analyses that significantly correlated with incidence of sour skin of onion in multiple years. Those models consistently comprised Cu, Fe, Mn, and Zn and, interestingly, the Cu-to-Fe ratio (Cu:Fe) alone was significantly correlated with sour skin incidence. Onion bulbs sampled from areas with high and low soil values for Cu:Fe that also had low and high levels of incidence of sour skin were used for transcriptome analysis. The plant defense gene pathogenesis-related protein 1 (*PR1*), an indication of SAR and the salicylic acid (SA) pathway (Durrant and Dong 2004), was upregulated >5,000 fold in onion tissues with a high Cu:Fe compared with onion tissues with a low Cu:Fe (Watson-Selph et al. 2016).

In another field study with tomato spotted wilt (TSW) of tobacco, caused by *Tomato spotted wilt virus*, Cu:Fe values in the soil were significantly correlated with TSW incidence and location in the field (Gitaitis et al. 2009, 2010) and resulted in a significant disease gradient. A risk model was developed from the data and successful predictions of TSW risk prior to planting were made in 2014 and 2015 (Rooks et al. 2015). Furthermore, gene expression analysis indicated a 650-fold increase in nonexpressor pathogenesis-related gene 1 (*NPRI*), a plant disease defense gene, in healthy tissue samples from plants in the predicted low-risk zone compared with those in the high-risk zone (Rooks et al. 2015).

In this article, we developed and validated a model based on nutrient concentrations in soil and pepper tissues that predicts risk of BLS. In addition, we also report that the plant resistance genes *NPRI* and *PR1* and superoxide dismutase (SOD) genes (*MnSOD* and *FeSOD*) were significantly upregulated in pepper tissues from predicted low-BLS-risk compared with high-BLS-risk sites.

MATERIALS AND METHODS

Model development. Two experimental sites with a field history of BLS outbreaks in pepper were selected for this study. In previous years, *X. euvesicatoria* was identified from symptomatic foliage from these sites. The pepper plots were established at the University of Georgia Blackshank Research Farm in 2012 to 2014 and on a commercial farm in Echols County in 2014. A commercial pepper variety 'Aristotle' were transplanted on 6, 8, and 6 August at the Blackshank Farm in 2012, 2013 and 2014, respectively. The pepper seedlings 'Aristotle' were transplanted in Echols County on 15 July 2014. The soil type at these locations was predominantly sandy loam. The soil series at the Blackshank Farm was fine-loamy,

kaolinitic, thermic plinthic kandiodults whereas the soil series at the Echols County location was siliceous, thermic psammentic paleudults. At each location, 40 raised bed plots were prepared with black polythene mulch and a bed dimension of 13.70 by 3.05 m at each experimental site. Each bed was separated with a spacing of 1.5 m. The pepper plots at all locations had overhead irrigation and the plants were irrigated every day for 30 min. The insecticide, fertility, weed, and disease management (with no Cu spray) were conducted according to the University of Georgia Cooperative Extension recommendations for all sites (Boyhan et al. 2009). Natural outbreak of BLS occurred on 2 October 2012, 10 October 2013, and 3 October 2014 in plots at the Blackshank Farm. The disease outbreak in Echols County occurred on 20 September 2014. A whole-plot assessment of BLS severity was conducted using a Horsfall and Barratt scale of 0 to 100 (Horsfall and Barratt 1945). The final disease assessments were made at the time of harvest or when crop reached harvest maturity: 15 October 2012, 18 October 2013, and 16 October 2014 for the University of Georgia Blackshank Research Farm and 27 September 2014 for Echols County. Tissue samples (n = three fruit/plot/sample site) were collected from healthy-appearing pepper and mineral contents were analyzed by the Agricultural and Environmental Services Laboratory, University of Georgia (AES-UGA), Athens, GA. Fruit tissue samples (50 g each) were analyzed for their nutrient concentrations using a P1 test package of the AES-UGA. The analysis included determining concentrations of the nutrients B, Ca, Cu, Fe, K, Mg, Mn, P, and Zn, where tissue samples were acid treated and subjected to microwave digestion. The digested materials were analyzed by the inductively coupled plasma-optical emission spectroscopy (ICP-OES) method using an ICP-OES 7400 radial view spectrometer with nebulizer MFC gas system (Thermo iCAP, West Palm Beach, FL). Total N was analyzed using the dry combustion method with an Elementar Vario Max CNS Analyzer (Elementar Americas, Inc., Mt. Laurel, NJ).

Using an approach similar to the crop optimization production system (COPS; National Onion Labs Inc., Collins, GA), concentrations (in parts per million) of tissue nutrients as well as ratios of nutrients were used to develop a predictive model for BLS. BLS severity ratings for each plot were regressed with concentrations (in parts per million) of tissue nutrients as well as ratios of nutrients. Several models were generated using stepwise regression and maximum R^2 improvement regression (SAS, version 9.4; SAS Institute, Cary, NC), with BLS ratings as the dependent variable and concentrations (in parts per million) of nutrients and ratios of nutrients as independent variables. Models were selected for further evaluation based on consistent occurrence of nutrients or their ratios in the model, R^2 values, P values, variance inflation factor (VIF), root mean square error, Mallow's C_p , and Akaike's information criterion correction (AICc) values.

Because initial statistical analysis indicated that model ECGA1 would be a best fit, fruit samples from plots with highest (70%) and lowest (10%) disease severity were collected and assayed for expression of a defense gene and SA concentrations. Three plots each displayed BLS ratings of 10 and 70% were selected. Healthy-appearing fruit (5 fruit/plot/severity ratings) from plots with lowest (10%) and highest (70%) BLS severity at harvest were used for determining relative expression of *NPRI* and *PR1* genes using a reverse-transcription quantitative polymerase chain reaction (qPCR) assay, as described below. The fruit samples were analyzed for gene expression individually and mean expression levels from plots with 10 and 70% BLS severity were compared statistically using Fischer's least significant difference (LSD) at $P < 0.05$.

Furthermore, SA concentrations (in parts per million) were determined for healthy-appearing fruit samples from plots with lowest (10%) and highest (70%) disease severity at harvest. Healthy-appearing pepper fruit (5 fruit/plot/severity ratings) were harvested and transported to the BioProfile Testing Laboratories,

LLC (Minneapolis, MN) in a -20°C cooler. The samples were analyzed for SA concentrations (in parts per million) using a high-performance liquid chromatography technique. The fruit samples were analyzed for SA concentrations individually and mean tissue concentration from plots with 10 and 70% BLS severity were compared statistically using Fischer's LSD at $P < 0.05$.

Model validation. The ECGA1 model ($\text{BLS} = 14.08 \text{ Fe:Zn} - 2.33 \text{ Mn} + 0.23 \text{ Cu} - 0.0064 \text{ K} + 208.37$) was selected for validation (Table 1) and was evaluated in commercial pepper production plantings in Colquitt and Worth in 2015. The model was also validated in a commercial pepper field in Tift County, GA in 2016. The commercial field plots (area = $8,093 \text{ m}^2$) at each site were used for soil sampling and BLS prediction. The soil type at these locations was sandy loam, with a soil series that consisted of fine-loamy, kaolinitic, thermic plinthic kandiodults. In total, 24 raised beds were prepared with a bed dimension of 13.70 by 3.05 m at each field site. Each bed was separated with a spacing of 1.5 m . An area of $39 \text{ m}^2/\text{plot}$ was sampled in an "X" pattern by taking 20 soil cores at a depth of approximately 15.25 cm . The soil cores from each plot were combined for a composite soil sample of that area. In total, 60 soil samples were taken from Colquitt ($n = 18$), Worth ($n = 18$), and Tift counties ($n = 24$). These field sites had a history of BLS outbreaks over the last 6 years.

Soil samples were sent to the AES-UGA for analysis. Soils were subjected to Mehlich 1 extraction using dilute hydrochloric and sulfuric acid. Extracts were analyzed for Ca, Cu, Fe, K, Mg, Mn, P, and Zn using ICP-OES, as above. Soil nutrient concentrations (in parts per million) from each plot were substituted into the ECGA1 model to generate predicted BLS severity levels (SAS, version 9.4; SAS Institute) for the different sample sites. Based on the predicted BLS severity values for each county site, sample sites or plots were ranked from highest risk to lowest risk. Four plots with high- and low-risk values in Colquitt and Worth Counties in 2015 were predicted and chosen for further validation. In Tift County, three plots each with low- and high-risk values were predicted using the ECGA1 model. Other sites or plots in three locations whose BLS severity values were intermediate were used as reference plots for gene expression studies.

Pepper seedlings 'Aristotle' were transplanted in the Colquitt County and Worth County sites on 12 and 14 August 2015, respectively. The pepper plots at all locations had overhead irrigation and the plants were irrigated every day for 30 min. The insecticide, fertility, weed, and disease management (with no Cu spray) were conducted according to the University of Georgia Cooperative Extension recommendations for all sites (Boyhan et al. 2009). Natural outbreak of BLS occurred on 8 and 12 September 2015 in Colquitt and Worth Counties, respectively. A whole-plot assessment of BLS severity was conducted using a Horsfall and Barratt scale of 0 to 100 (Horsfall and Barratt 1945). Plants ratings were taken on 15, 22, and 29 September and 6 and 13 October. The

final dates of harvest were 15 and 12 October for Colquitt County and Worth County, respectively.

The commercial pepper plots in Tift County was transplanted on 10 August 2016. The first appearance of BLS in Tift County was observed on 23 September 2016. BLS ratings were taken on a scale of 0 to 100 on 26 September and 3, 10, 14, and 19 October. The final harvest was on 21 October. Final BLS ratings for each site from high- and low-risk plots were statistically compared using Fisher's LSD test at $P = 0.05$. BLS levels for plots for multiple assessment periods were used to calculate area under the disease progress curve (AUDPC) for each field site as $\sum_{i=1}^n [(Y_i + Y_{i+1})/2] [X_{i+1} - X_i]$, where Y_i = BLS severity at the i th observation, X_i = time in days at the i th observation, and n = the total number of observations. The AUDPC values for each risk site were compared with values predicted by the model for each field site in 2015 and 2016 using a regression analysis in SAS, as described above. In order to confirm that symptoms observed were due to *X. euvesicatoria* infections, isolations were made from at least three symptomatic seedlings per plot. Small pieces (approximately 4 mm^2) of leaf tissue along lesion margins were excised and chopped in $100 \mu\text{l}$ of phosphate-buffered saline. Then, $10 \mu\text{l}$ of the resulting suspensions were streaked on yeast extract-dextrose- CaCO_3 (YDC) medium. After 3 days of incubation at 28°C , yellow-colored colonies were tentatively identified as *X. euvesicatoria* colonies. Identities were confirmed by assaying three random colonies per plate using a real-time PCR assay, as described by Dutta et al. (2014a).

In addition, healthy-appearing fruit (5 fruit/plot) were taken from high- and low-risk plots in each field (2015 and 2016). Fruit samples ($n = 5$ each from three plots) were also taken from field plots with a medium level of predicted BLS risk according to the ECGA1 model. Total plant RNA was extracted (RNeasy Plant Mini Kit, St. Louis) and relative expression levels of *NPR1* and *PR1* genes were calculated. The detailed protocol on gene expression study using qPCR is stated below.

Gene expression of *NPR1*, *PR1*, and *SOD* (*CuZnSOD*, *FeSOD*, and *MnSOD*) genes. Relative expressions of *NPR1*, *PR1*, and *SOD* (*CuZnSOD*, *FeSOD*, and *MnSOD*) genes were determined in pepper fruit collected from different field samples: Echols County in 2014, Worth and Colquitt Counties in 2015, and Tift County in 2016. Pepper fruit were collected at harvest and transferred to the Coastal Plain Experimental Station (CPES), UGA, Tifton Laboratory in a cooler (approximately 5°C). Fruit were then stored in the freezer at -20°C until they were used for further analysis. For analysis, surfaces of the fruit were sterilized by spraying them with 70% ethanol, followed by wiping with a sterilized paper towel. Fruit were further sprayed with sterile water and rewiped with a sterile paper towel. Later, fruit tissue (50 mg) was excised with a sterile scalpel and used for RNA extraction using the manufacturer's protocol (RNeasy Plant Mini Kit). RNA ($2 \mu\text{l}$) was converted to cDNA using the iScript cDNA synthesis kit

TABLE 1. Predictive risk models developed from stepwise and maximum R^2 for bacterial leaf spot (BLS) of pepper (*Capsicum annuum*) from 2012 to 2014 using concentrations (in parts per million) of macronutrients and micronutrients as well as ratios of key nutrients in pepper fruit as independent variables

Year	Model name ^a	Model	P value	Adj. R^2 ^b	VIF ^c	Mallow's Cp	RMSE ^d	AICc ^e
2012	TIFGA1	$\text{BLS} = 5.99 \text{ Fe:Zn} - 0.11 \text{ Mn} + 12834 \text{ Cu:P} + 0.88$	0.0008	0.39	1.4	18.5	3.8	23.1
2012	TIFGA2	$\text{BLS} = 5.66 \text{ Fe:Zn} - 0.12 \text{ Mn} - 0.0007 \text{ Cu} + 3.69 \text{ K} + 18.3$	0.04	0.43	1.2	12.8	2.5	35.3
2013	TIFGA3	$\text{BLS} = 8.3 \text{ Fe:Zn} + 166.7 \text{ Cu:Fe} - 9.5$	0.009	0.79	1.1	3.4	0.34	59.2
2013	TIFGA4	$\text{BLS} = 4.82 \text{ Fe:Zn} - 0.89 \text{ Mn} + 4.18 \text{ Cu} - 0.0004 \text{ K} + 30.8$	0.004	0.63	1.7	5.1	0.85	43.8
2014	TIFGA5	$\text{BLS} = 17.04 \text{ Fe:Zn} - 0.5 \text{ B} - 0.22 \text{ S} + 0.002 \text{ K} - 0.0005 \text{ N} + 50.47$	0.03	0.52	2.5	8.5	1.72	32.5
2014	ECGA1 ^f	$\text{BLS} = 14.1 \text{ Fe:Zn} - 2.33 \text{ Mn} + 0.23 \text{ Cu} - 0.006 \text{ K} + 208.4$	0.005	0.99	1.5	2.2	0.15	75.8

^a Name based on sample site and order of model development at that site.

^b Adjusted (Adj.) R^2 .

^c Maximum variance inflation factor (VIF) for the independent variables of the model on that line is listed.

^d Root mean square error (RMSE).

^e Akaike's information criterion correction (AICc) used for model selection.

^f Model selection for further study and validation (ECGA1) was based on consistent representation of the same independent variables, statistical level of significance (P value), the adj. R^2 value, the maximum VIF value ≤ 5.0 , Mallow's Cp, RMSE, and AICc.

(Bio-Rad Laboratories Inc., Hercules, CA) and qPCR was conducted on a Smart Cycler System (Cepheid, Sunnyvale, CA) using the iQ SYBR Green Supermix (Bio-Rad Laboratories Inc.) and specific primer pairs for the marker genes (Table 2). For real-time PCR, 5 µl of cDNA was amplified in 25 µl of PCR master mix containing 12.5 µl of SsoFast EvaGreen Supermix (Bio-Rad Laboratories Inc.) and 1 µl each of 25 µM primers per sample. Real-time PCR was conducted in a Cepheid Smart Cycler, with specific thermal conditions depending on the set of primers used (Table 2). The *CaActin* gene was used as a control, and was assessed using the following primers: 5'-CACTGAAGCACCTTGAACCC-3' and 5'-GAGACAACACCGCTGAATAGC-3'. The primer sequence and PCR conditions for *PR1* gene were from Choi et al. (2007). The cycle threshold (Ct) values thus obtained were converted into relative fold differences of marker genes in treated samples compared with the untreated samples and relative to the endogenous control gene using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001; Schmittgen and Livak 2008). This was done after verifying the stability of the endogenous control genes and that the primer pairs had high, comparable PCR efficiencies (Schmittgen and Livak 2008). Relative fold changes of target genes were calculated (Schmittgen and Livak 2008) and compared for each treatment by Fisher's LSD at the $P < 0.05$ level or by Student's t test.

RESULTS

Model development. Significant regression models were developed with BLS severity as the dependent variable and composition of nutrients in pepper fruit tissues as independent variables. Interestingly, 100% of the models consistently contained Fe:Zn ratios as one of the independent variables evaluated (Table 1). In addition, in 50% of the models, Cu, Mn, and K with the Fe:Zn ratio occurred as independent variables (Table 1). The adjusted (adj.) R^2 values for all models ranged from 0.39 to 0.99 and the maximum VIF values were all 2.5 or less, indicating that a problem with collinearity was unlikely. The Mallow's Cp and root mean square errors for the model ranged from 2.2 to 18.5 and 0.15 to 3.8, respectively. The AICc for the models ranged from 23.1 to 75.8. Among these regression models, ECGA1 had the highest adj. R^2 (0.99) and AICc (75.8) values and the lowest Mallow's Cp (2.2) and root mean square error (0.15) values. In addition, the ECGA1 model was significant, with a P value of 0.005, and did not have an issue with collinearity (VIF = 1.5).

The relative mean expression of the *NPR1* gene from pepper fruit sampled from plots with 10% BLS severity (10.2) was significantly higher than the samples collected from plots that displayed 70% BLS severity (2.1) ($P = 0.002$) (Fig. 1A). Relative expression of *PR1* was also significantly higher in pepper fruit sampled from plots with 10% BLS severity (12.5) compared with fruit from the plots with

70% (1.8) ($P = 0.014$) (Fig. 1B). The SA concentrations were significantly higher for pepper fruit collected from low-BLS plots (3.6 ppm) compared with high BLS-plots (0.75 ppm) ($P = 0.041$) (Fig. 1C). Based on the P values, adj. R^2 , VIF, Mallow's Cp, AICc, and root mean square error values plus the relative expression of *NPR1*, *PR1*, and SA concentrations, model ECGA1 ($\text{BLS} = 14.1 \text{ Fe:Zn} - 2.33 \text{ Mn} + 0.23 \text{ Cu} - 0.06 \text{ K} + 208.4$) was selected for further evaluation and validation.

Model validation. BLS was observed in all three trials for two consecutive years. The mean final BLS severity ratings for high- and low-risk plots in Worth County in 2015 were 51.6 and 25.4%, respectively, and they were significantly different ($P < 0.05$). In Colquitt County, the final BLS rating for the low-risk plot (31%) was significantly lower than the high risk (64%) plots ($P < 0.05$) in 2015. In Tift County (2016), low-risk plots had significantly lower final BLS severity (23.5%) compared with higher BLS severity (43.2%) ($P < 0.05$). Natural infection of *X. euvesicatoria* was observed and confirmed, because 100% of the suspected colonies isolated from infected tissue displayed positive amplification using species-specific PCR. When AUDPC values for two field sites (Colquitt and Worth Counties) in 2015 were pulled together and plotted against ECGA1 predicted values for both sites, the resulting relationship was highly significant ($P = 0.0001$), with an R^2 value of 0.92 (Fig. 2A). A significant relationship between observed AUDPC versus predicted values was also observed in Tift County in 2016 ($P < 0.001$; adj. $R^2 = 0.98$) (Fig. 2B).

Gene expression of *NPR1*, *PR1*, and SOD (*CuZnSOD*, *FeSOD*, and *MnSOD*) genes. Relative gene expression of *NPR1* as measured by the fold change was greater and highly significant for pepper sampled from predicted low-BLS plots compared with those from high-BLS plots in both Colquitt and Worth Counties (Fig. 3A and B). In Colquitt County (2015), the mean expression level of *NPR1* gene was significantly higher for fruit collected from low-risk (6.8) than for high-risk (0.87) plots (Fig. 3A). Similar trends of significantly higher levels of *NPR1* gene expression were also observed for low-risk plots compared with high-risk plots at Worth County (high risk = 0.38 and low risk = 3.2) (Fig. 3B). In Tift County (2016), expression of *NPR1* was also significantly higher in predicted low-risk (5.2) compared with high-risk (1.8) plots (Fig. 3C).

Similar results were observed for the relative expression of *PR1* because the fold change was highly significant for pepper sampled from predicted low-risk plots compared with those from high-risk plots for all locations in both years (Fig. 3D to F). In 2015, significantly higher levels of *PR1* expression were observed for fruit collected from low-risk than high-risk plots at Colquitt (high risk = 0.45 and low risk = 3.1) and Worth (high risk = 0.18 and low risk = 3.3) Counties (Fig. 3D and E). Mean *PR1* gene expression was also significantly higher for samples from low-risk plots (8.2) compared with samples from high-risk plots (2.1) in Tift County in 2016 (Fig. 3F).

TABLE 2. List of genes, primer sequence, and quantitative polymerase chain reaction (PCR) protocols used in this study

Gene	Primer sequence ^a	Comments	PCR conditions
<i>Cu-Zn SOD</i>	5'-GAAGCCATGAAGTCCAGGTT-3' (F)	This study	95°C for 3 min; 35 cycles of 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s; and final extension of 72°C for 10 min
	5'-TGTGTTAGTGGCACCATCCT-3' (R)	...	
<i>FeSOD</i>	5'-AGTTCACCAGATGGCTCTCC-3' (F)	This study	95°C for 3 min; 35 cycles of 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s; and final extension of 72°C for 10 min
	5'-TCTCCTTCCACCATTCAACA-3' (R)	...	
<i>MnSOD</i>	5'-AAGCTGCATGATTTACCAC-3' (F)	This study	95°C for 3 min; 35 cycles of 95°C for 10 s, 56°C for 30 s, and 72°C for 30 s; and final extension of 72°C for 10 min
	5'-TTCTAACATTCCGGCAACAA-3' (R)	...	
<i>NPR1</i>	5'-TTCAGTTGACGCTCTTCTGG-3' (F)	This study	95°C for 3 min; 35 cycles of 95°C for 10 s, 58°C for 30 s, and 72°C for 30 s; and final extension of 72°C for 10 min
	5'-GCTTCTTTCCACGTTGTTCA-3' (R)	...	
<i>PR1</i>	5'-CACTGAAGCACCTTGAACCC-3' (F)	...	Primers from Choi et al. (2007)
	5'-GAGACAACACCGCTGAATAGC-3' (R)	...	

^a F = forward and R = reverse.

DISCUSSION

In the current study, significant relationships between plant nutrition and BLS severity were observed in pepper from 2012 to 2014. Soils were sampled at different locations and analyzed for nutrient content. Multiple regression models were developed using

stepwise regression. BLS rating was the dependent variable and independent variables evaluated included nutrient concentrations, ratios of different nutrient concentrations, and soil pH values. Independent variables such as soil pH which did not significantly improve or contribute to the model were removed until the models with the best statistical parameters remained. In particular, the concentrations of Cu, K, and Mn as well as the ratio of Fe:Zn in pepper fruit were significantly correlated with BLS severity in all years. Among the independent variables in the models from 2012 to 2014, Fe:Zn appeared in 100% of the models. Also, Mn and K each appeared in 66.6% of the models. Based on the model selection criteria, model ECGA1 was chosen for further validation in commercial pepper fields in 2015 (two field trials) and 2016 (one field trial). It was also observed that relative expression of *NPR1* and *PR1* genes and concentration of SA (in parts per million) in pepper fruit collected were significantly higher for plots displaying 10% versus 70% BLS severity in a field where the ECGA1 model was

Fig. 1. Mean relative expression of nonexpressor pathogenesis-related protein 1 (*NPR1*) and pathogenesis-related protein 1 (*PR1*) genes and salicylic acid (SA) concentrations in pepper (*Capsicum annuum*) fruit from Echols County, GA (ECGA1 model, 2014). Healthy-appearing fruit (5 fruit/plot/severity ratings) from plots with lowest (10%) and highest (70%) bacterial leaf spot (BLS) severity at harvest were used for gene expression study and SA estimation. An asterisk (*) denotes P value ≤ 0.05 .

Fig. 2. Regression of predicted bacterial leaf spot (BLS) values versus observed area under the disease progress curve (AUDPC) for three commercial pepper fields. **A**, Pooled predicted versus observed values from Colquitt and Worth Counties, GA in 2015 and **B**, predicted values versus observed AUDPC from Tift County in 2016.

developed. These results indicate that SAR responses may be directly affected by nutrient concentrations or that cross-talk may occur between pathways governed by SAR genes responding to BLS infections and other pathways affected by intracellular nutrient concentrations, including mineral homeostasis.

The ECGA1 model was used for prediction of BLS risk in three commercial pepper fields (2015 and 2016). For two consecutive years of model validation, ECGA1 was able to accurately predict AUDPC. The AUDPC values for two field sites (Colquitt and Worth Counties) in 2015 were plotted against ECGA1-predicted values for both sites, which resulted in a highly significant relationship ($P = 0.0001$), with an R^2 value of 0.92. The significant relationship was also observed between observed AUDPC versus predicted values in Tift County in 2016 ($P < 0.001$; adj. $R^2 = 0.98$). These results also indicate that nutrient concentrations in the soil can be an important predictor for BLS risk.

In our models, Cu, Fe, Mn, and Zn consistently occurred as independent variables. The modes of action or the roles that these nutrients are playing are not clearly understood. However, we observed a significant connection between plant disease resistance genes *NPR1* and *PR1* and risk sites identified by the nutrient model.

Relative gene expression of *NPR1* was greater and highly significant for pepper sampled from predicted low-risk BLS plots compared with those from high-risk BLS plots in all predicted field sites for two consecutive years (Fig. 3A to C). Similar results were observed for the relative expression of *PR1* because the fold change was highly significant for pepper sampled from predicted low-risk plots compared with those from high-risk plots for all locations in both years (Fig. 3D to F). These results suggest a potential direct or indirect relationship among soil nutrient concentrations, induction of plant defense genes in pepper, and BLS risk.

In addition to the complexities of soil interactions affecting plant conditions, macro- and microenvironment conditions can also influence disease development in the context of the disease triangle (Roberts and Boothroyd 1973). This could affect predictions of disease severity from models based solely on nutrient levels (soil). Because of the potential variation in levels of final disease severity at different locations or in different years due to weather that was favorable or unfavorable for disease development, we chose to evaluate the models in terms of risk. Equations from the ECGA1 regression model predicting BLS risk were solved using soil nutrient concentrations collected from different research sites.

Fig. 3. Mean relative expression of nonexpressor pathogenesis-related protein 1 (*NPR1*) and pathogenesis-related protein 1 (*PR1*) genes in commercial pepper (*Capsicum annuum*) fruit sampled from healthy-looking plants from areas predicted to be low- or high-risk sites for bacterial leaf spot severity (BLS) in three different southern Georgia counties. Healthy-appearing fruit (5 fruit/predicted risk plots) at harvest were used for gene expression study. **A to C**, *NPR1* expression from Colquitt and Worth Counties in 2015 and Tift County in 2016; **D to F**, *PR1* expression from Colquitt and Worth Counties in 2015 and Tift County in 2016; ** indicates P value ≤ 0.01 and * indicates P value = 0.05.

Locations producing the higher predicted BLS values were deemed high-risk sites. Likewise, areas with lower predicted BLS values were designated low-risk sites. However, risk is a relative term and the final levels of BLS will also be dependent upon weather conditions for each locale or for each year.

The expressions of *MnSOD* and *FeSOD* were observed to be significantly higher in pepper fruit sampled from plots displaying 10 versus 70% BLS severity (Supplementary Fig. S1). During the model validation phase, *MnSOD* and *FeSOD* expressions were highly significant for pepper fruit sampled from predicted low-risk BLS plots compared with the expressions from high-risk BLS plots in all predicted field sites (Supplementary Fig. S2) for two consecutive years. However, *CuZnSOD* expression was not significantly different for fruit collected from two risk sites in both years (Supplementary Fig. S3). This gene was also not significant during the model development phase in the ECGA1 site. Together, the results suggest that SOD gene activity, especially *MnSOD* and *FeSOD* expression levels, may also play a role in SAR in pepper. However, the exact mechanism is not clearly understood. Hence, these findings warrant further research on the role of *MnSOD* and *FeSOD* in SAR and, in particular, a time-course study under controlled conditions seems appropriate.

The SOD are known to be involved in detoxifying reactive oxygen species (ROS) produced in routine metabolic pathways such as the Calvin Cycle and ATP production via the electron-transport system. During early stages of infection with certain plant pathogens, there is rapid generation of ROS and accumulation of H_2O_2 following recognition of signals produced by the pathogen. This sudden and large generation of ROS is known as the oxidative burst (Lamb and Dixon 1997). Among other mechanisms, the ROS formed during the oxidative burst may be detoxified by SOD. The intermediate product H_2O_2 from the detoxification process can be detoxified by enzymes such as catalase. However, it is believed that SA inhibits catalase and, thus, there is an accumulation of H_2O_2 . This accumulation leads to the production of plant disease resistance protein such as *PR1* (Chen et al. 1993). Increased levels SOD as a response to infection and as a precursor to SAR fits within our hypothesis of SAR activation. Increased SOD activity associated with SAR has been observed by others (Cao et al. 2005; Deepak et al. 2006). In those studies, plants were treated with acibenzolar-S-methyl, a known SAR-inducing chemical. The authors speculated that the resulting accumulation of H_2O_2 from SOD activity did not only regulate *PR1* proteins but also acted as a substrate for peroxidases. *PR1* is a marker for SAR induced via the SA pathway (Durrant and Dong 2004; Zhang et al. 2010). The authors proposed that pathogenic infections induce SA synthesis by upregulating isochorismate synthetase 1, a component of the shikimic acid pathway; and, as SA accumulates, it activates the downstream components, *NPRI*, and three transcription factors. Redox reactions cause a reduction in *NPRI*. The reduced forms accumulate in the nucleus where they activate defense genes. Finally, in our investigation, we observed that *FeSOD*, *MnSOD*, *NPRI*, and *PR1* may be involved in a cascade of events induced by the nutrient concentrations in the soil or tissue. Detailed study is required to tease out how nutrient concentrations in soil or pepper tissue affect SOD, *NPRI*, and *PR1* genes.

In conclusion, we developed a BLS risk model (ECGA1) using concentrations of the combination of Fe:Zn, Cu, Mn, and K as independent variables. ECGA1 was able to accurately predict risk under field conditions in three different commercial sites for two consecutive years. We acknowledge that other edaphic factors can potentially affect nutrient uptake or distribution of the nutrients within the plant, thereby affecting model accuracy. Soil types and varying topographies could also affect nutrient uptake and model accuracy. Also, environmental conditions such as temperature, free water affecting foliar disease levels, and evapotranspiration and soil moisture could also be affecting nutrient uptake and model accuracy. It is interesting that, in the pepper model and two other models for different pathosystems (TSW in tobacco and sour skin of

onion), a ratio of Fe with either Cu, Mn, or Zn is an important independent variable in the models.

Finally, ECGA1 shows promise as a means of predicting risk for choosing pepper planting locations. Because the data support the hypothesis that SAR activity is increased at the low-risk sites, the model would not likely be affected differentially by different races of the pathogen because SAR is a race-nonspecific type of resistance. Additional research is planned to evaluate how these nutrients affect SAR and BLS severity and whether the model continues to accurately produce predictions of risk with pepper produced in different soil types and under different environmental conditions. We also plan to study the interaction of SOD with these nutrients and how they may affect BLS severity and SAR activity.

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