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Effects of Increased Atmospheric Carbon Dioxide Levels on Tobacco Mosaic Virus and Root-Knot Nematodes in Genetically Resistant and Susceptible Tomato Plants

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By

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Effects of Increased Atmospheric Carbon Dioxide Levels on Tobacco Mosaic Virus and Root-Knot Nematodes in Genetically Resistant and Susceptible Tomato Plants

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ABSTRACT

Rising atmospheric carbon dioxide (CO_2) may affect plant/pathogen interactions. This study focused on the effects of elevated CO_2 on Root-Knot Nematode (*Meloidogyne arenaria*) and Tobacco Mosaic Virus (TMV) infection in genetically resistant versus susceptible tomatoes (*Solanum lycopersicum*). Both resistant and susceptible tomatoes were grown in chambers with either ambient CO_2 or CO_2 elevated to 750 ppm and infected with *M. arenaria* or TMV. Measurements were taken at regular intervals to determine the effects of the pathogens on the plants. Resistant plants infected *with M. arenaria* maintained resistance while susceptible plants remained susceptible at both CO_2 levels. Resistant plants inoculated with TMV maintained their resistance in both CO_2 levels. Susceptible plants inoculated with TMV took longer to demonstrate infection in elevated CO_2.

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INTRODUCTION AND LITERATURE REVIEW

According to the United States National Oceanic and Atmospheric Administration, the global average of measured carbon dioxide (CO_2) levels are rising. In 2014 the global mean was 396ppm, and by the end of 2019 that average had risen in steady growth to 411.76ppm (NOAA, 2020). These levels are expected to exceed 450 ppm by 2030 and are predicted to continue to increase to levels between 750 ppm and over 1300 ppm by 2100 (IPCC, 2014). With this increase of CO_2 levels across the globe, it is important to understand how these changes will affect food supply by studying the effects of elevated CO_2 on plants and their pathogens.

Studies have been done to measure the effects of elevated CO_2 levels on plants, and changes to multiple aspects of plant functions have been discovered. While there are many factors, both environmental and within the plant, that influence stomatal aperture, there is evidence that elevated levels of CO_2 consistently reduce stomatal aperture (Ainsworth & Rogers, 2007). Photosynthesis changes under high levels of CO_2 are also well documented. Plants that undergo C_3 photosynthesis show increased photosynthetic activity with increased atmospheric CO_2 concentrations, but how much it increases is dependent on the growth form of the plant and the environment in which it is

grown (Ainsworth & Rogers, 2007). C_4 photosynthesis levels also increase at elevated CO_2 levels, but evidence is increasing to support the hypothesis that this “is an indirect effect resulting from the interaction of water stress with reduced stomatal aperture at elevated CO_2” (Ainsworth & Rogers, 2007, p.263). In addition to increased photosynthesis, both C_3 and C_4 plants undergo changes to their leaf protein content, thickness, and carbohydrate content. This reduces their nutritional quality for the herbivores and omnivores that depend on them for food. Ehleringer, Cerling, & Dearing (2002) found that non-structural carbohydrate content increased, and nitrogen content decreased in leaves grown at elevated CO_2 levels. Mammals and insects that feed on these plants have demonstrated slower growth rates when consuming them (Ehleringer et al., 2002).

Studies on the effects of elevated CO_2 on plants have found that it has limitations on how much it affects photosynthesis and, if levels rise too high, can cause detrimental effects on the plants. Granados & Korner (2002) found that in tropical climbing plants the elevated CO_2 growth effects level off at 700ppm. The availability of nutrients such as nitrogen can down-regulate a plant’s photosynthesis rate regardless of the concentration of CO_2 that the plant is exposed to (Zheng et al., 2018). Leaf biochemical composition can also affect photosynthesis in plants in elevated CO_2, associated with reductions in activity or amounts of Rubisco (Zheng et al., 2018). Adverse effects on some plants were

found when CO_2 levels reached 1,000ppm or higher. A study on tomato plants found that at 1,000ppm CO_2, the chloroplasts and leaves became deformed and eventually withered (Kramer, 1981). Different species of plants have different optimal CO_2 concentration levels and different levels at which it becomes dangerous to the plant (Zheng et al., 2018).

Studies conducted on plant-pathogen systems in differing levels of CO_2 have produced independent results. In some studies, elevated CO_2 levels have reduced the ability of the pathogen to affect the plant, in others, parasitic activity increased, and in yet other studies of plant-pathogen systems, no effect was seen with increased CO_2 (Zhang et al., 2015). Trebicki et al. (2015) found that the virus titre of BYDV-PAV increased by 36.8% in wheat exposed to elevated CO_2 environmental levels when compared to wheat grown in ambient CO_2. Chitarra, Siciliano, Ferrocino, Gullino, & Garibaldi (2015) found in their study that both increased CO_2 and higher temperatures caused an increase in fungal infection of rocket plants. The lower nitrogen content in the leaves of plants grown in elevated CO_2 environments causes insect herbivores, such as *Spodoptera litura*, a tobacco cutworm, to eat more leaves to compensate. Kumari and Verma (2017) demonstrated higher levels of leaf damage on bell pepper plants grown in elevated CO_2 levels compared to those grown in ambient CO_2 levels. In another study Kumari and Verma (2017) suggested that aphids, herbivores that feeds on the phloem of plants, benefit from plants being grown in elevated CO_2 and

temperature conditions, and the numbers of them found on bell pepper plants is significantly higher in elevated CO_2 conditions compared to ambient conditions. However, Dáder, Fereres, Moreno, & Trebicki (2016) found that elevated CO_2 negatively impacts aphid life history and reduces the damage to the plants.

This study examined elevated CO_2 levels on the two plant-pathogen systems of tomatoes and root-knot nematodes and tomatoes and Tobacco Mosaic Virus. By growing genetically resistant and genetically susceptible tomato plants under ambient and artificially elevated CO_2 concentrations and infecting them with the pathogens, we attempted to ascertain if the elevated CO_2 causes the plant to lose resistance to these pathogens or if resistance is enhanced or not affected.

Root-knot nematodes (*Meloidogyne spp*.) are parasitic worms that can cause extensive changes to a plant's roots (Taylor and Sasser, 1978). During the second stage of life, the nematode penetrates a root just above the root cap and works its way up the root to the zone of cell elongation. It pierces cell walls nearby and injects secretions that induce the infected cells to hypertrophy into giant cells as well as causing nearby cells to undergo hyperplasia and form galls (Taylor & Sasser, 1978). A set of 3,373 genes have been identified that show significant changes in expression during the formation of giant cells and galls, demonstrating the complexity of the influence of the nematode on the plant (Jammes et al., 2005). The cytoskeletons of the giant cells are rearranged, and

metabolism increases so the nematodes can feed from them (Jammes et al., 2005). The giant cells are multinucleated from undergoing repeated nuclear divisions without cytokinesis. Many of the nuclei are aneuploid with highly variable chromosome counts (Wiggers, Starr, & Price, 1990). These changes in the roots affect the ability of the plant to take up water and partition nutrients (Milligan et al., 1998).

Tobacco Mosaic Virus (TMV) is a single-strand RNA virus that is part of a larger genus of viruses called tobamoviruses (Knapp & Lewandowski, 2001). TMV infects solanaceous plants that include tobacco, tomato, and green peppers, all of which are economically important crops. The virus particle makes its way into the plant through lesions and begins to reproduce and infect one cell at a time moving through plasmodesmata, then more widely infects the plant through the phloem. TMV causes mottling and curling of the leaves in addition to necrotic lesions. Flowers and fruits can also become mottled and distorted, and growth of the entire plant may be stunted (Agrios, 2005). The distinctive yellow and green mottling of the leaves is caused by chlorosis induced by TMV, possibly by the reduction of Ferrodoxin I in the chlorotic portions of the leaves (Banerjee, Wang, & Zaitlin, 1995; Ma et al., 2008). In addition to the chlorosis, reduction in photosynthetic activity is seen in infected plants due to reduced number and size of chloroplasts and lower CO_2 fixation efficiency (Wilhelmová, Procházková, Sindelarova, & Sindelar, 2005).

TMV is extremely stable and remains potent after many years in cigars and cigarettes made with contaminated tobacco plants. It is recommended that once a field has been infected, plants that are susceptible to TMV should not be planted there for at least 2 years. Transmission in commercial supplies of tomatoes is often caused by workers handling contaminated plants with their hands or tools, then touching noncontaminated plants. Washing hands and tools with soap is recommended to help control the virus, and soaking hands and tools in milk has been found to inhibit infection (Agrios, 2005).

In both soil and air, a higher level of CO_2 affects root-knot nematode interactions with plants. It changes the interactions of the parasites with tomato plants of different genotypes (Sun, Cao, Yin, Kang, & Ge, 2010). Tomato plants with genotypes that are defense-dominant reduced their defenses when exposed to higher levels of CO_2. Tomato plants without defense-dominant alleles had no change in their defenses. All tomato plant types, however, had increased biomass and size under elevated CO_2 conditions, which allowed the plants to better withstand the infection of species of *Meloidogyne*. The plants showed very little damage from the nematodes despite high numbers of galls in the roots (Sun et al., 2010).

In a long-term study on the nematode populations in soil treated with elevated CO_2, Yeates and Newton found that overall numbers of parasitic nematodes were significantly higher in the soil treated with elevated CO_2 than in

soil at ambient CO_2 levels (2009). For numbers of the *Meloidogyne* species specifically, there were significantly more after 4 years in elevated CO_2 soil than in ambient soil (Yeates & Newton, 2009).

King (2003) found that elevated levels of CO_2 affected *Meloidogyne arenaria* grown on the host plant *Vicia faba*. Nematode-induced giant cells accumulated DNA faster than in the plants grown at ambient CO_2. Additionally, the number of eggs produced by the nematodes at higher CO_2 levels was significantly higher than the number of eggs from those at ambient CO_2 (King, 2003).

Several studies have been conducted to determine the effects of elevated CO_2 on the tobamovirus family. Zhang et al. (2015) found that at CO_2 levels of 800ppm, the effect of TMV on tomato plants decreased compared to an ambient CO_2 level of 380ppm. They discovered an increase in a plants' use of the salicylic acid signaling pathway that is an integral part of plant stress response to TMV (Zhang et al., 2015). Another study done on this family of viruses found that aphids feeding on plants grown under elevated CO_2 levels had decreased transmission rates of Cucumber Mosaic Virus (CMV). More research needs to be done to determine if this result was caused by the negative effects of elevated CO_2 on the aphids or on the virus itself (Dáder et al, 2016). Del Toro et al. (2015) studied CMV in *Nicotiana benthamiana*, a close relative of tobacco, at elevated CO_2 levels and found infection symptoms to be comparable to ambient CO_2

levels, however, the ratio of virus to plant protein was increased because as the plants grew larger under elevated CO_2 their protein content per leaf unit decreased.

Defense-dominant resistance responses in plants, also called R-protein-mediated responses, are coded by a class of genes known as R-genes (Knepper & Day, 2010). The largest class of R-genes contains a nucleotide-binding site (NB) at the amino-terminus and a leucine rich region (LRR) at the C-terminus (Knepper & Day, 2010). The NB is necessary for ATP or GTP binding, and the LRR is for protein-protein interactions and peptide/ligand binding (Knepper & Day, 2010). In addition to the specific structures necessary for R-genes to function, many have also been found to require additional protein interactions (Knepper & Day, 2010). Milligan, et al. (1998) found that R-genes are only effective if the parasite they are resisting has an avirulence gene because R-genes function in gene to gene interactions. There is no clear evidence, however, that R-genes recognize the avirulence factor of the pathogen. Indirect evidence points to the R-gene being expressed in plant immune response when the changes caused by the pathogen begin (Nimchuk, Eulgem, Holt, & Dangl, 2003). When expressed, R-genes cause a hypersensitive response (HR) in a plant that is a form of programmed cell death which is triggered, along with other defenses, by production of reactive oxygen species, nitric oxide, and salicylic acid messengers (Nimchuk et al, 2003). The resistance genes that have been

discovered that prevent tomato plants from becoming host to either root-knot nematodes or TMV are R-genes in the NB-LRR class.

Resistance to *Meloidogyne spp* is conferred by the gene *Mi-1*/*Meu-1* which was previously thought to be two separate genes. However, Vos et al. (1998) found that these two genes are the same. This gene has been introgressed from *Lycopersicon peruvianum*, also called *Solanum peruvianum* and commonly named the Peruvian tomato, into modern cultivars of tomatoes (Vos et al., 1998). *Mi-1* is in a subclass of NB-LRR R-genes that has a leucine zipper motif (Milligan et al., 1998). In addition to resistance to root-knot nematodes, *Mi-1* also confers resistance to whiteflies, aphids, psyllids and other sap-sucking insects (Guo et al., 2016). When a genetically resistant tomato plant is infected with nematodes, it undergoes a HR instead of creating the giant cells and galls. This *Mi-1* associated response occurs approximately 12 hours after inoculation of the plant with juvenile nematodes, when the nematode would be injecting secretions into the cell (Milligan et al., 1998). The nematodes still move through the root to the feeding site, but the feeding site doesn't develop. The nematode will then either die or leave the root (Milligan et al., 1998). Similarly to other R-genes, *Mi-1* requires other protein interactions to function. Without the heat shock protein *Hsp90*, nematodes can successfully parasitize tomato roots even if the *Mi-1* gene is intact and functioning (Bhattarai et al., 2007).

Because *Mi-1* confers resistance and not immunity, studies have been done to determine possible variables that could affect the effectiveness of the *Mi-1* gene. Many studies have focused on the effects of temperature changes. In temperatures consistently held higher than 30° C for multiple days, resistance to root knot nematodes from the *Mi-1* gene is less effective (Cooper, Gia, & Goggin, 2005; Haroon, Baki, & Huettel, 1993). de Carvalho et al. (2015) found that a 3-hour single heat spike of 35° C, which is comparable to rising temperatures that occur during the hottest part of the day, reduced the effectiveness of *Mi-1*, as evidenced by the increased number of galls formed in tomato plant roots. However, continuing to expose the plants to these temperature spikes each day caused the plant to adjust, and resistance increased, demonstrated by there being fewer galls that formed on these plants compared to the tomatoes treated with only one day of increased temperature (de Carvalho et al., 2015). There are also virulent species of *Meloidogyne* upon which *Mi-1* has no effect (Cooper et al., 2005). In 1995 farmers in California found that tomato fields with the *Mi-1* gene were heavily infested with galls and giant cells indicative of a species of root-knot nematode. Studies of the parasites determined these galls were formed by *M. incognita*. The response there has been to rotate crops between tomato plants and other plants that are not hosts for *M. incognita* (Kaloshian, Williamson, Miyao, Lawn, & Westerdahl, 1996).

Guo et al. (2016) studied the effects of elevated CO_2 levels on the effectiveness of *Mi-1* resistance on Tomato Yellow Leaf Curl Virus which is transmitted by the sap-sucking whitefly *Bemisia tabaci*, another parasite *Mi-1* codes for resistance against. They found that disease incidence in plants without the *Mi-1* resistance gene decreased when grown in higher levels of CO_2. In plants with the *Mi-1* resistance gene, the increased levels of CO_2 actually increased the disease incidence regardless of whether the plants were inoculated with the virus by *B. tabaci* or were agroinoculated (Guo et al., 2016).

Four different genes have been identified that can confer resistance to TMV and other viruses in the tobamovirus family. Cultivated tomato plants have *Tm-1*, *Tm-2*, and *Tm-2²* genes that control viruses in this family (El-Aziz, Guirgis, Roshdy, & Kheder, 2016). These genes are all R-genes that induce localized cell death as part of the resistance response. *Tm-2²* has been found to mediate the most effective resistance to TMV, and, like the *Mi-1* gene, is a NB-LRR gene and requires *Hsp90* to function (Qian et al., 2018).

The fourth resistance gene is the N gene from *Nicotiana glutinosa*, a species of tobacco (Knapp & Lewandowski, 2001). The N gene induces a more effective HR against more strains of tobamoviruses than any other resistance genes (Whitham, McCormick, & Baker, 1996). The N gene has a NB-LRR with a Toll/Il-1 receptor domain. The N gene induces a HR within 48 hours to restrict the spread of the virus, and after an initial exposure, the plant becomes more

resistant to subsequent exposure through systemic acquired resistance (Marathe, Anandalakshmi, Liu, & Kumar, 2002). As with the other resistance genes already discussed, the N gene requires other proteins to function properly (Marathe et al., 2002). Because this gene is from a tobacco plant, tomato plants must be transformed to benefit from this gene. When a tomato was transformed with N from tobacco, the tomato plant produced the same level of HR when exposed to TMV as the tobacco plant (Whitham et al., 1996).

The *Tm* genes can lose their effectiveness at providing TMV resistance to tomato plants. *Tm-1* and *Tm-2* can be overcome by naturally occurring strains of TMV (Weber, Schultze, & Pfitzner, 1993). Two base substitutions in TMV can overcome resistance in tomatoes with *Tm-1*, a C → G substitution causing a Gln-979 → Glu amino acid change, and a C→ U that causes a His-984→ Tyr amino acid change. These substitutions happen in the same protein of TMV. (Meshi et al., 1988). Two different substitutions in TMV remove resistance in plants with *Tm-2*. These cause the changes Cys-68→Phe and Glu-133→Lys in the TMV 30-kD movement protein (Meshi et al., 1989). *Tm-2*^{2} resistance has been overcome by amino acid substitutions Ser-238→Arg and Lys-244→Glu, also in the 30-kD movement protein (Weber et al., 1993).

Inactivation of the N gene is less common than inactivation of the *Tm* genes. It can be inactivated by high temperatures (Marathe et al., 2002) of 28°C and above, but HR is restored when the temperature is brought back below 28°C

(Whitham et al., 1996). The Ob strain of tobamovirus has also been found to overcome N gene-mediated resistance (Padgett & Beachy, 1993). Additionally, N gene function requires the gene *Rar1*, which interacts with SGT1 and then associates with COP9 signalsome. Suppressing COP9 and SGT1 compromises resistance conferred by N (Marathe et al., 2002).

Both TMV and *Meloidogyne spp.* can have detrimental effects on economically important crops grown for human consumption. Plants that are genetically resistant to parasites are critical in the efforts of the agricultural industry to ensure a sufficient food supply for people and livestock. It is important that we understand how the changes in the earth's atmosphere will affect plant-pathogen systems so that food production can continue to meet global demands. To address this concern, this study was designed to investigate how elevated CO_2 affects genetically resistant tomatoes infected with either TMV or *M. arenaria*. The alternative hypothesis for this experiment is elevated CO_2 will affect a tomato plant's resistance or susceptibility to *M. arenaria* or TMV. If the plant's susceptibility or resistance is not affected, however, the null hypothesis that elevated CO_2 will not affect the plant's resistance or susceptibility to these diseases will be accepted.

Two growth chambers were used, one with ambient CO_2 concentrations and one with elevated CO_2 concentrations. Genetically resistant and genetically susceptible ragdolls infected with the nematodes were placed in each chamber,

and samples were taken every 9 days to count the number of feeding sites per plant and the number of nuclei from the large cells in these feeding sites. For the TMV experiment, pots of genetically susceptible tomatoes and pots of genetically resistant tomatoes were grown in each chamber and then infected with the virus. They were sampled every 10 days beginning with the day of inoculation. Measurements taken included plant height, visual scale of plant healthiness, TMV presence in the leaves, and chlorophyll content of the leaves. Comparisons were made between the susceptible and resistant plants infected with *M. arenaria* grown at both ambient and elevated CO_2 levels, and between the susceptible and resistant plants infected with TMV grown at both CO_2 levels.

MATERIALS AND METHODS

Growth Chambers

Two 705-liter growth chambers located in a greenhouse were used. Each chamber had a vent and a fan to allow air exchange throughout the chamber twice per minute (King, 2003). One chamber circulated outside air, allowing for ambient CO_2 levels of approximately 410ppm. To elevate the CO_2 level of the other chamber to 750ppm (±25ppm), a compressed CO_2 gas cylinder with a flow meter was attached. Other than the level of CO_2, conditions in the chambers were almost identical (King, 2003). Temperatures in the chambers ranged from approximately 16° C at night up to 35°C during the day. The temperature rose above 35° C on four nonconsecutive days of the study, however, this temperature did not last more than a few hours at a time.

Tomato Plants

Two varieties of tomatoes were used for this study. Druzba is an heirloom variety that has no known genetic resistance to pathogens, and Bush Early Girl Hybrid is genetically resistant to both TMV and *Meloidogyne spp*. For each of the pathogens studied, a set of Druzba and a set of Bush Early Girl Hybrid tomato plants were grown from seed in both the ambient CO_2 and elevated CO_2 growth chambers. They were infected with pathogens after the second set of leaves

grew in for those in pots, approximately four weeks after being planted, and after roots had grown to 10mm long in the ragdolls, approximately two weeks after sprouting.

Pathogens

Roots infected with *M. arenaria* were provided by Terry Wheeler, Texas A&M University. To maintain a continuous supply of nematodes, Druzba variety tomatoes were grown from seed on the greenhouse bench and were periodically inoculated with nematodes to allow them to reproduce. Eggs of *M. arenaria* were taken from the roots of these supply plants and used for inoculating test plants. Roots were agitated in 20% bleach solution for 5 minutes then filtered to remove debris and hatchlings and isolate the eggs.

To obtain TMV sap, Druzba variety plants were grown on the greenhouse bench and inoculated with a small amount of TMV obtained from a sample in storage at Stephen F. Austin State University. These plants were allowed to grow so the virus could replicate and spread within them. TMV was extracted from these infected plants by grinding the plants and mixing the resulting sap in a phosphate buffer. This created a liquid sap of TMV that could be applied to leaves of plants. One leaf of each plant to be inoculated was heavily abraded with diatomaceous earth, and the TMV extract was applied to the abrasion with a small brush.

Ragdolls and Nematode Feeding Sites

Seeds of Druzba and Bush Early Girl Hybrid tomatoes were placed in four separate ragdolls (Carter, Nieto, & Veech, 1977). One ragdoll of each tomato variety was grown in each CO_2 growth chamber until roots were approximately 10mm long. Nematode eggs were isolated from roots of infected tomato plants and placed in water. 3mL of this egg solution was then pipetted onto the miracloth of the ragdolls where the eggs would hatch and infect the roots of the seedlings growing in the ragdolls.

Samples of seedlings from each of the four ragdolls were taken every nine days after inoculation. These samples were placed in a 3:1 fixative of 95% ethanol to acetic acid and stored at 4°C for 1-3 days. The 3:1 fixative was then poured off the samples and replaced with a 70% ethanol solution for storage at 4°C (Wiggers, Starr, & Price, 1990). These root samples were then Feulgen stained (Wiggers, Starr, & Price, 1990) and the average number of feeding sites per plant was calculated. Feeding sites were dissected out of the roots and the number of nuclei per feeding site was counted.

Tobacco Mosaic Virus

36 tomato plants were grown to test the tomato-TMV plant-parasite system. In each growth chamber, nine resistant and nine susceptible plants were grown in pots and staked to cause them to grow up straight and prevent them contacting other plants nearby as much as possible within the confined space of

the chamber. One susceptible plant and one resistant plant in each chamber were left uninoculated to serve as controls. The remaining eight plants of each variety in each chamber were inoculated with TMV. On the day of inoculation and every 10 days after, samples were taken from each of these plants to measure height, TMV presence, chlorophyll content, and visually score the plants for signs of TMV infection. The final samples were taken 50 days after inoculation, for a total of 6 samples for each plant.

Height was measured in millimeters by following the stem growth from the top of the soil to the youngest branch bud nearest the end of the tallest piece of the stem. TMV presence was detected by testing a single leaf randomly taken from each plant, freezing it for storage, then using the Agdia ImmunoStrip® for TMV that gives a clear response if TMV is present. Chlorophyll amounts were measured to determine the extent of the chlorosis caused by TMV because chlorosis “is always accompanied by a reduction in chlorophyll content” (Goodman, Király, & Wood, 1986, p. 53). This was accomplished by placing a small, standardized piece of leaf material in a jar with a N,N-Dimethylformamide (DMF) extraction buffer. These jars were placed in the dark at 4°C for 1-3 weeks while the chlorophyll dissolved out from the leaf piece (Inskeep & Bloom, 1985). The chlorophyll absorbency of the DMF in the sample jars was then measured using a spectrophotometer at 657nm, 665nm, and 750nm to measure

chlorophylls a and b and the total chlorophyll amount of each sample, which was reported as chlorophyll per unit leaf area (mg/cm^2).

The visual scale used to determine the extent of visible signs of TMV was modified from a scale developed to score potato plants infected with various viruses (Islam et al., 2015). Each plant was given a score of 0-5 each time samples were taken.

Table 1: Visual Disease Rating for Plants Infected with TMV

Score	Visual Symptoms
0	No visible sign of TMV
1	Some leaves are lightly mottled yellow
2	All leaves are lightly mottled
3	All leaves affected with some leaves curling or completely yellow
4	All leaves completely yellow and curling
5	All leaves completely yellow and curling and plant in state of distress, dying

Statistical Analysis

To analyze the numbers of feeding sites per plant and the number of nuclei per feeding site for each tomato variety and CO_2 treatment in ragdolls infected with root-knot nematodes, one-way ANOVAs were used.

When analyzing the results of the TMV experiment, one-way ANOVAs and Tukey HSD tests were used to interpret height, chlorophyll content, and visual scale data to determine what differences existed between the susceptible and resistant plants at each CO_2 level. TMV presence was analyzed with Chi-squared tests. Infected plants were compared to uninfected control plants of the same tomato variety and in the same CO_2 treatment for samples taken every 10 days. Infected plant groups given different treatments were also compared to each other for samples taken every 10 days.

RESULTS

Root-Knot Nematodes

The number of feeding sites per plant was significantly lower for both CO_2 treatments of resistant Bush Early Girl Hybrid plants than both CO_2 treatments of the susceptible Druzba tomatoes ($F=3.9497$, $p<0.0358$). There was no significant difference in the number of feeding sites per plant between the two groups of resistant plants ($F=0.0765$, $p<0.7913$) or between the two groups of susceptible plants ($F=0.4342$, $p<0.5344$). (Figure 1).

There was also a significant difference in the number of nuclei per feeding site between the resistant groups and the susceptible groups. The susceptible plants from each treatment had many more nuclei than the resistant from each treatment ($F=4.4488$, $p<0.0254$). There was no difference seen in the number of nuclei per feeding site between the two groups of resistant plants ($F=0.1520$ $p<0.7101$) or between the two groups of susceptible tomatoes ($F=0.0344$, $p<0.8589$). (Figure 2).

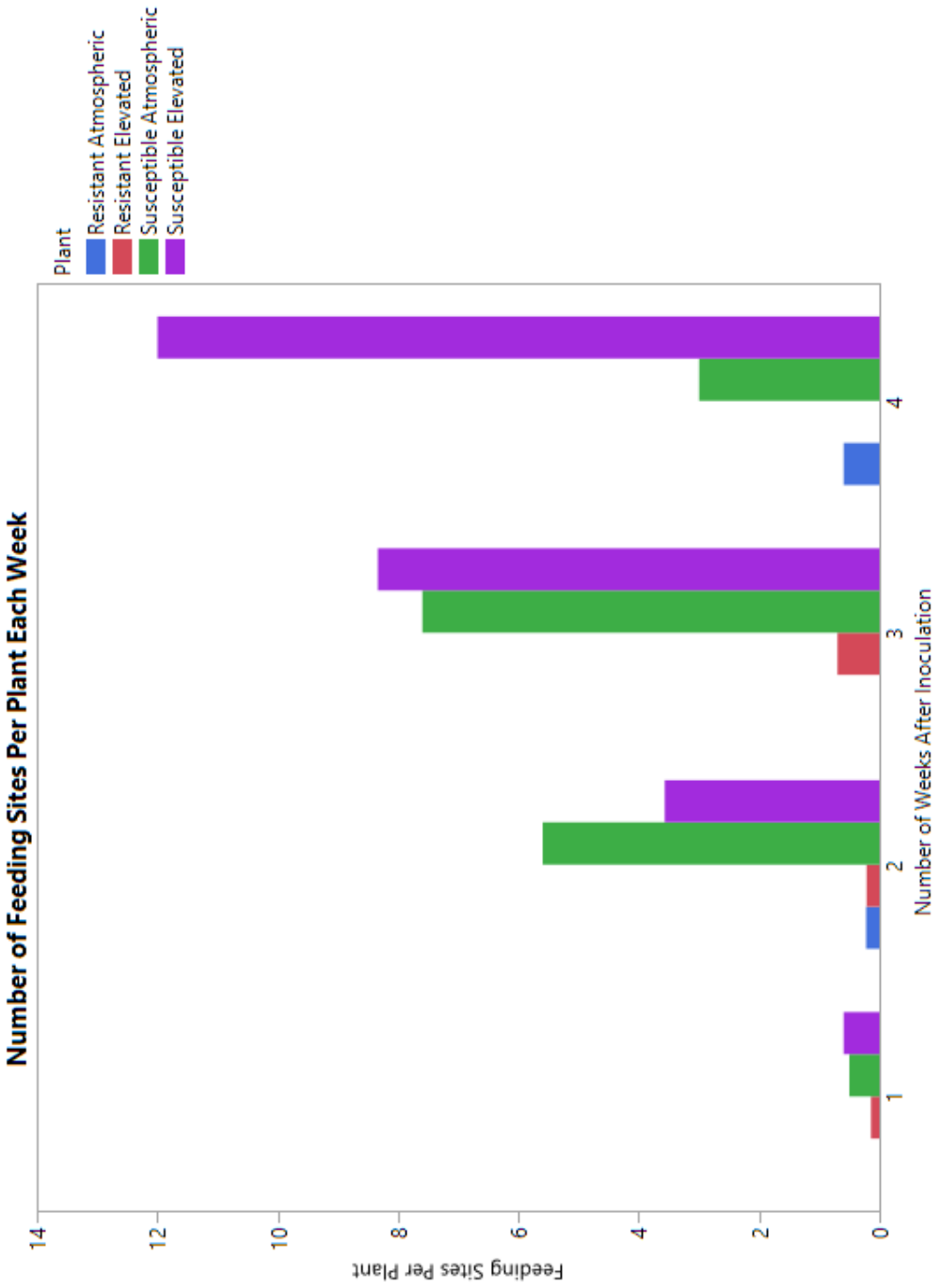

Figure 1: Number of Feeding Sites per Plant by Treatment Each Week

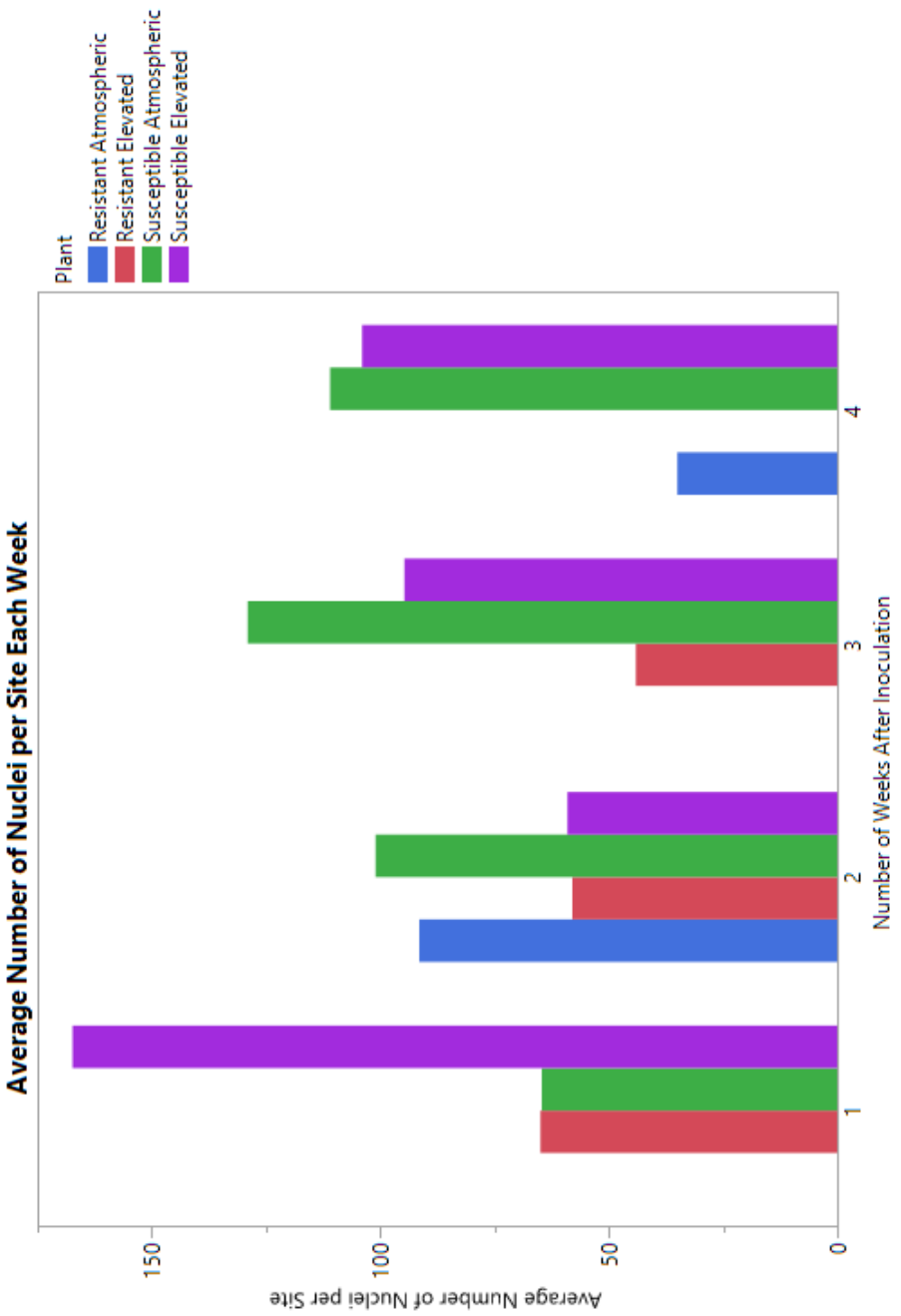

Figure 2: Average Number of Nuclei per Plant by Treatment Each Week

Tobacco Mosaic Virus

Resistant tomatoes grown in the chamber kept at ambient CO_2 concentration and infected with TMV did not show any significant difference in height or TMV presence in their leaves during the 50 days of testing compared to the noninfected plant with the same treatment. When the chlorophyll content of the infected tomatoes was compared with that of the noninfected control plant, the data was inconclusive. 30 days after inoculation, the infected plants had significantly more chlorophyll than the control plant ($F=6.4309$, $p<0.0389$), and 10 days later, the control had higher levels of chlorophyll than the infected plants, but not enough to be significant. There was also no significant difference in their chlorophyll levels by day 50 ($F=1.5809$, $p<0.2490$). It took until 50 days after inoculation for the infected plants to show significantly more visual signs of TMV than the uninfected plants ($F=1.87x10^{16}$, $p<0.0001$).

Resistant tomatoes grown in elevated CO_2 showed no differences between the control and the infected plants until 50 days after inoculation. At 50 days the only difference was that infected plants showed more visual signs of TMV infection ($F=7.000$, $p<0.0331$).

Susceptible tomatoes grown at ambient CO_2 began showing significant differences between the control and the infected plants by 10 days after inoculation. TMV presence was detected in 7 of the 8 inoculated plants demonstrating a difference between infected and controls ($X^2=3.938$, $p<0.0472$).

By 20 days after inoculation and throughout the remainder of the sampling time period, all inoculated plants in this group tested positive for TMV giving a clear difference between the control and the infected plants (X^2=9.000, p<0.0027). Visually the infected plants also showed significantly more symptoms than the non-infected control by 20 days after inoculation (F=32.11, p<0.0008) and throughout the rest of the experiment. No difference was measured between height and chlorophyll content for the infected susceptible tomatoes grown at atmospheric CO_2 and the control.

Susceptible tomatoes grown in elevated CO_2 only showed visual symptom differences between infected and the non-infected control. At 20, 30, 40, and 50 days after inoculation, the infected plants showed more visual symptoms of TMV than the control (F=1.87x10^{16}, p<0.0001, F=9.000, p<0.0199, F=12.7034, p<0.0092, F=18.7185, p<0.0035).

On the day of inoculation, four weeks after planting the seeds and placing them in the growth chambers, both the resistant and susceptible plants growing in elevated CO_2 were significantly taller than all the plants growing at ambient CO_2 (F=9.5921, p<0.0002). This trend continued throughout the sampling period. (Figure 3).

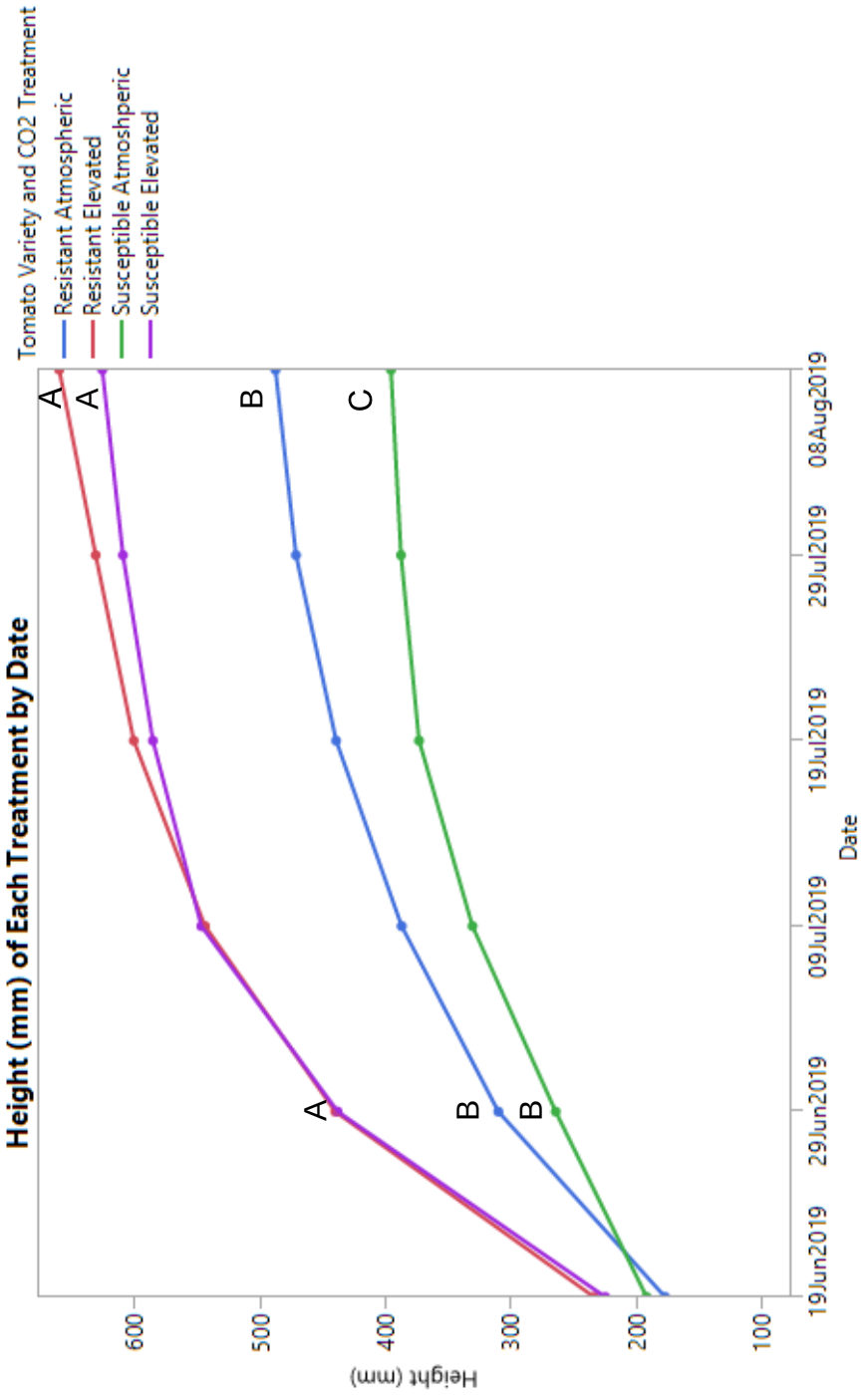

Figure 3: Height of Each Treatment by Date

Significant differences in visual symptoms between the four inoculated groups of tomatoes were seen from 10 days post inoculation through 50 days post inoculation. (10 days $F=3.7207$, $p<0.0228$; 20 & 30 days $F=35.6667$, $p<0.0001$; 40 days $F=8.400$, $p<0.0004$; 50 days $F= 18.1754$, $p<0.0001$) The susceptible plants grown in both the ambient and elevated CO_2 consistently showed more visual signs of TMV than the resistant plants grown in each treatment. (Figure 4).

Seven of the susceptible tomato plants grown in the ambient CO2 chamber began testing positive for TMV 10 days after inoculation, which was different than all other test groups in which no plants tested positive for TMV ($X^2=26.880$, $p<0.0001$). At 20 and 30 days post inoculation, all susceptible tomatoes in ambient CO_2 tested positive, and one resistant in the atmospheric chamber did. 40 days after inoculation 25% of the susceptible plants in the elevated CO_2 chamber tested positive for TMV and by day 50, five of the eight tested positive for TMV infection, which, when analyzed with the susceptible in the ambient growth chamber, was significantly different than the resistant plants in both chambers ($X^2=20.825$, $p<0.0001$).

Significant differences were seen in chlorophyll amounts between the infected test groups each time samples were taken. On the day of inoculation, both plant groups in the elevated CO_2 had significantly more chlorophyll than both groups in the ambient CO_2 ($F=28.7605$. $p<0.0001$).

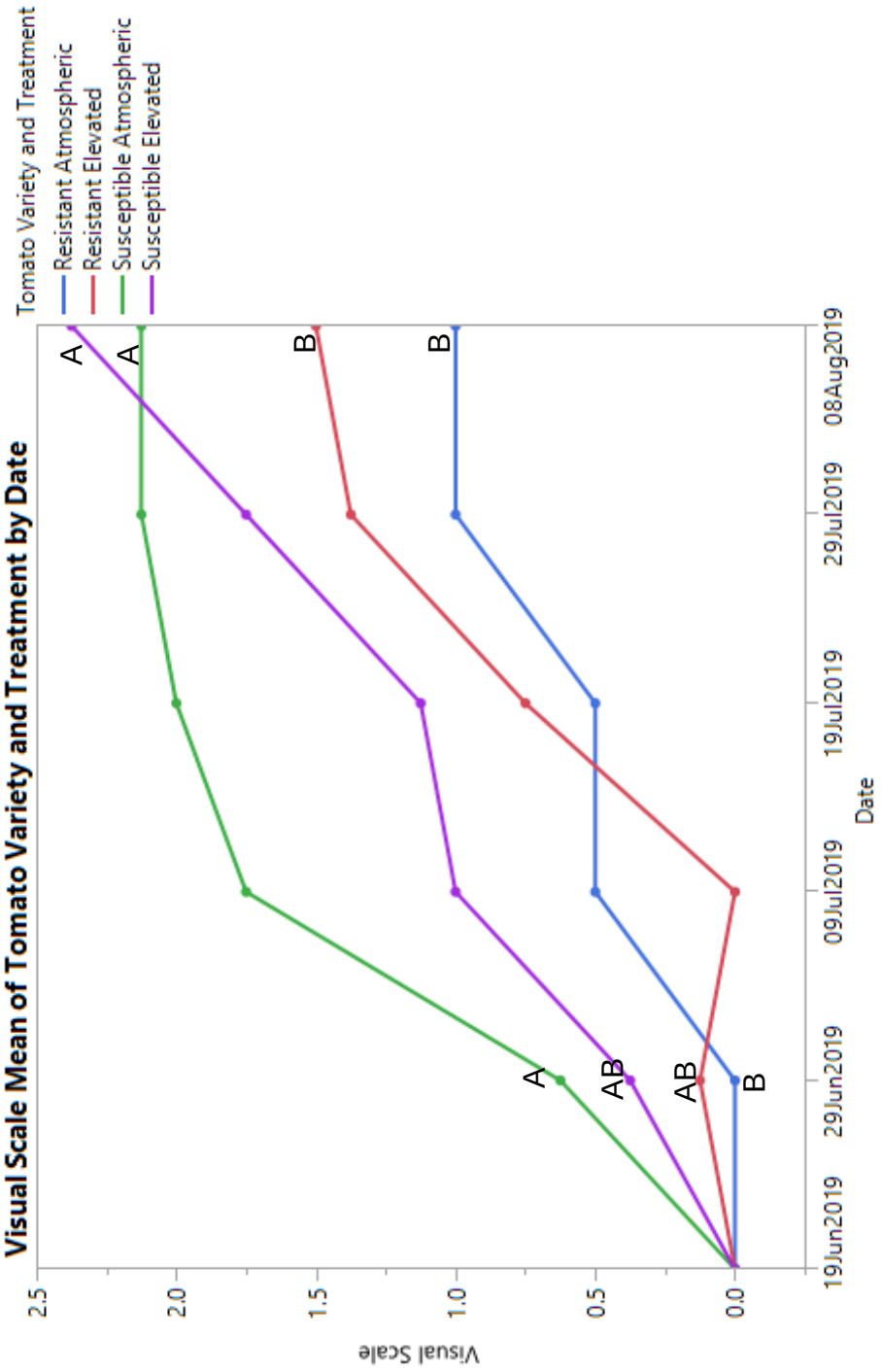

Figure 4: Visual Scale of Each Treatment by Date

10 days after inoculation the resistant plants in ambient CO_2 had more chlorophyll than both susceptible groups, and the resistant plants in elevated CO_2 had chlorophyll concentrations between these groups ($F=9.0959$, $p<0.0002$). At 20 and 30 days, both elevated CO_2 test groups once again had more chlorophyll than both groups in the ambient CO_2 chamber ($F=11.6163$, $p<0.0001$). By the end of the experiment at day 50, the resistant plants grown in ambient CO_2 had significantly more chlorophyll than the susceptible grown in elevated CO_2, and the other test groups' chlorophyll concentrations were between these two ($F=4.9808$, $p<0.0068$).

Table 2: Chlorophyll Concentrations Day 50

Tomato Variety	CO_2 Treatment	Chlorophyll a & b (mg/cm^2)
Bush Early Girl Hybrid	Elevated	5.30
Bush Early Girl Hybrid	Ambient	6.23
Druzba	Elevated	3.00
Druzba	Ambient	4.75

DISCUSSION

Root-Knot Nematodes

When the resistant plants grown at elevated CO_2 and inoculated with *M. arenaria* were compared to the resistant plants inoculated with *M. arenaria* and grown at ambient CO_2, no significant difference was seen in the level of infection. Elevated CO_2 did not change the resistance of the tomatoes to this parasite in a manner detectable by this experiment. It was also found that elevated CO_2 did not affect the susceptibility of the tomatoes to *M. arenaria*. The resistant tomatoes remained resistant to root-knot nematodes and the susceptible tomatoes remained susceptible to these infections in both levels of CO_2. For this pathogen, the null hypothesis that elevated CO_2 would not affect genetic resistance or susceptibility to *M. arenaria* is supported.

Tobacco Mosaic Virus

The Druzba tomatoes grown in ambient CO_2 showed signs of TMV infection earlier and more consistently throughout the sample period. By 10 days post inoculation, 7 of the 8 infected plants tested positive for TMV, and 10 days later all 8 plants in this group tested positive for TMV and continued to test positive through the remainder of the experiment. These plants were also shorter than all other plant groups by the 50th day after inoculation. The chlorophyll

concentrations in the leaves of the Druzba at ambient CO_2 were also lower than chlorophyll concentrations in the leaves of the other groups. These results were expected because this variety does not have any known genetic resistance to TMV. The data from this study supports the understanding that when exposed to TMV under current ambient CO_2 conditions, the Druzba tomatoes will continue to become infected and show symptoms typical of TMV.

The Druzba tomatoes grown in elevated CO_2 had different results than the Druzba in ambient CO_2. These plants were also expected to test positive for TMV early in the experiment and develop the characteristic symptoms of TMV. However, this is not what occurred. Because of the elevated CO_2 exposure, these plants grew taller than both varieties of tomatoes grown in ambient CO_2 and held off the symptoms of TMV longer than anticipated. It took 40 days after inoculation for any of these plants to test positive for TMV, and even then, only 2 tested positive. 10 days later 5 total plants in this group had tested positive for TMV. Rating these plants visually overall, they showed fewer symptoms of TMV than the Druzba grown at ambient CO_2. The chlorophyll concentration in the leaves of these plants also remained higher than the concentration in the Druzba variety at ambient CO_2. While the elevated CO_2 did not confer resistance to these plants, it did give them an advantage compared to the same variety grown at the ambient CO_2 level. They were able to delay expression of TMV symptoms and slow down the spread of the virus through the plant for over 5 weeks. Testing at

the molecular level would explain if this is because the plant's immune system was strengthened by the extra CO_2, or if these results are due to increased growth from the additional CO_2.

The resistant variety of tomatoes, the Bush Early Girl Hybrid, maintained its resistance to TMV in both CO_2 treatments. The resistant tomatoes were less visibly stressed by TMV exposure throughout the growth period. 20 days after inoculation only one resistant tomato plant at ambient CO_2 tested positive for TMV, and by 30 days it was no longer testing positive for TMV. Another plant tested positive at 30 and 40 days but did not test positive for TMV by day 50. A third resistant plant tested positive for TMV only at the data collection on day 50. Because it was not the same plant each time that tested positive and because 2 of the 3 that did give a positive TMV test did not test positive at a later date, it indicates that these plants were able to continue resisting the disease even after the virus had spread within the plant.

By the end of the data collection period, the resistant tomatoes under ambient CO_2 also demonstrated their ability to resist the chlorosis typically caused by TMV, and their visual symptoms were less than both groups of susceptible tomatoes. At day 50 of data collection, these plants had the highest level of chlorophyll in their leaves compared to all three other inoculated groups and had the fewest visual symptoms, which was significantly less than the Druzba groups, but comparable to the resistant tomato plants in elevated CO_2.

These tomatoes grew taller than the Druzba in the same level of CO_2 but did not grow as tall as either variety in the elevated CO_2. This result was expected because it is well documented that elevated CO_2 causes an increase of mass, including increased height, in plants grown in CO_2 levels of 560ppm to at least 970ppm (Del Toro et al., 2015; Granados & Korner, 2002; Trebicki et al., 2015).

The resistant variety of tomatoes grown in elevated CO_2 were the least affected by TMV inoculation. These tomatoes were the tallest on the day of inoculation and at days 10, 40, and 50 post-inoculation. On days 20 and 30, they were only shorter than the susceptible tomatoes in elevated CO_2, but even this was not a significant difference. Measuring these plants with a visual scale, they had few to no symptoms of TMV comparable to the resistant plants at ambient CO_2. Only one resistant plant at elevated CO_2 tested positive for TMV throughout the study. It occurred at the 40-day data collection and no longer tested positive by day 50. These plants had either the highest levels of chlorophyll at each measurement from 20 days onward or were not significantly different from whichever other group had a higher chlorophyll content. At 10 days their chlorophyll level was not significantly different from the resistant tomatoes at ambient CO_2 which was the only group with a higher chlorophyll level.

For the plant-pathogen system of TMV and tomatoes, the data shows that genetically resistant plants will remain resistant as the CO_2 level in the atmosphere increases, supporting the null hypothesis that resistance would not

be affected by elevated CO_2. Higher atmospheric CO_2 is a benefit to genetically susceptible plants because the increased CO_2 gives them an advantage over the plants grown in current atmospheric CO_2 levels. Corroborating a previous study (Zhang et al., 2015), susceptible tomato plants infected with TMV showed a decrease in infection under elevated CO_2 levels compared to current levels of CO_2. For this cultivar of tomatoes, the data supports the alternative hypothesis because its susceptibility to TMV was decreased under elevated CO_2.

The results of these experiments add to the growing evidence that the rising levels of CO_2 in our atmosphere are going to affect plants that are necessary for human consumption. The results also continue to demonstrate that each plant and each pathogen within plant-pathogen systems may respond differently to these changes. While both varieties of plants infected with nematodes showed no difference in withstanding or succumbing to these parasites in elevated CO_2, the susceptible tomatoes inoculated with TMV were able to better withstand the disease caused by that virus. The agricultural industry can continue to grow tomatoes in the predicted elevated CO_2 atmospheric conditions as long as genetically resistant plants are cultivated or susceptible plants are grown in a way that keeps them away from root-knot nematodes and TMV.

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