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INFLUENCE OF SYMBIOSIS BETWEEN FUNGUS, VIRUS, AND TOMATO PLANT IN COMBATING HEAT STRESS

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ABSTRACT

The influence of the three-way interaction between fungus (*Curvularia protuberata*), virus (CThTV), and tomato (*Solanum lycopersicum*) in combating heat stress was evaluated in this study. The plants were grown under greenhouse conditions of 400 ± 150 µmol $m^{-2}s^{-1}$ photon flux density, 45 to 50% relative humidity (RH), and 30 ± 2°C. Tomato seeds were germinated and inoculated with the combination of the fungus and virus at the seedling stage. Plants were allowed to grow for two weeks before treatment application. Plants were placed in geothermal soil simulators containing autoclaved, organic potting soil. Randomly selected plants were equally distributed between two treatments. The treatments included non-symbiotic (NS) and symbiotic (An) plants. Four separate soil simulators were utilized in this experiment. Three were set at day/night temperatures of 31.3/26.4°C, and the other three were at 41.8/38.8°C. Initially the plants were allowed to adjust to the soil simulators for one week before heat was applied. Plants were grown under heat stress for two weeks before physiological measurements were determined. The three-way symbiotic interaction appeared to have no significant influence on tomato photosynthetic rate at both examined soil temperatures. However, under the symbiotic interaction, the photosynthetic rate was significantly reduced at 41.8/38.8°C in comparison to that obtained at the lower temperature (31.3/26.4°C). Tomato stomatal conductance, internal CO_2 concentration, water potential, and soluble sugar content were not significantly affected by the treatments. Visual examination of the non-symbiotic plants exposed to high temperature (41.8/38.8°C) showed symptoms of wilting and chlorosis, whereas symbiotic-growing plants appeared much less stressed under the same conditions.

INTRODUCTION

The daily variation between day and night temperature and the overall variation in temperature during plant growth subject plant roots and shoots to different temperatures, due to the difference in the thermal conductivity of the soil. As a result, plants are continually exposed to heat stress after air temperature cools down, especially during the summer months (Fredlund, 1992). Variation in the root zone temperature was reported to influence plant growth and

development by modifying some major physiological responses such as hormonal balance, shoot meristematic activity (McMaster *et al*., 2003), plant growth, carbon partitioning, and photosynthetic rate (Monje *et al.*, 2007). However, plant response to root zone temperature was highly dependent on the plant species and temperature range.

Tomato (*Solanum lycopersicum* L.) growth was found to be reduced by increasing the root zone temperature from 25 to 36°C after 19 days of growth (Klock *et al.*, 1997). Optimum tomato growth and photosynthetic occured at a root zone temperature around 30°C (Hurewitz and Janes, 1983). Gradual detrimental decline in plant growth followed as the temperature elevated. Similarly, wheat (*Triticum aestivum* L.) growth and photosynthetic rate were severely negatively affected at a root zone temperature of 35°C (Monje *et al.*, 2007). The growth rate of peanut (*Arachis hypogaea* L.) was the highest at soil temperatures of 25, 32, and 40°C (Awal *et al.*, 2003). The same study also reported that chlorophyll *a*, chlorophyll *b*, photosynthetic rate, and water use efficiency were the highest at 32°C.

Plant adaptation to heat stress was reported to be improved through symbiotic relationships between plants and selected fungi (Rodriguez *et al.*, 2004b). Fungal association with plants was predicted to be one of the major factors in facilitating the transition of plants to the terrestrial habitat (Pirozynski and Malloch, 1975). There are three classes of fungi symbiotically associated with plants and widely studied in the literature: mycorrhizae and two classes of endophytes (Rodriguez *et al.*, 2005). Endophytes were reported to play important roles in inducing heat tolerance in tomato (Morsy *et al.*, 2010). Successful induction of heat tolerance in tomato appeared to be reliant on the presence of a virus (*Curvularia* thermal tolerance virus, CThTV) (Márquez *et al.*, 2007). This three-way symbiotic relationship induced tolerance to soil temperatures reaching 65°C. However, individually, the plant, fungus, and virus failed to survive at such high temperatures. Therefore, it was concluded that the presence of all three symbionts was required to induce plant stress tolerance.

Osmotic adjustment and scavenging of reactive oxygen species (ROS) was reported to be one of the major stress factors responsible for inducing heat tolerance (Rodriguez *et al.*, 2008b). Soluble sugars such as trehalose were considered the key contributors to maintaining plant osmoprotection during high soil temperature (Morsy *et al.*, 2010). Most of these studies were carried out at very high temperature ranges that exceed what is expected under natural habitat conditions. Additionally, the specific physiological responses of tomato to elevated soil temperatures are lacking. Therefore, the main objective of this study was to evaluate the impact of soil presence of *C. protuberata* and CThTV on selected physiological responses of tomato to elevated soil temperatures. Two selected day/night temperatures were imposed (31.3/26.4°C and 41.8/38.8°C). Plant gas exchange measurements and osmoprotection evaluations were carried out at the conclusion of the experiment to evaluate heat tolerance of tomato at various treatments.

MATERIALS AND METHODS

Plant material

Tomato plants (cultivar 'Rutgers') were established from seeds. The seeds were acquired from Tomato Grower's Supply (catalog number #4050) and germinated in small plastic trays containing commercial potting soil. The seedlings were allowed to grow under greenhouse conditions to approximately 10 cm. To inoculate the individual seedlings with *C. protuberata* and CThTV, the procedure outlined by Morsy *et al.* (2010) was followed. Individual seedlings were removed from the soil and washed thoroughly with autoclaved water to remove debris. They were then placed in a 50 mL beaker filled with autoclaved 0.035% agarose solution containing fungal spores contaminated with CThTV. The seedlings were allowed to remain in the contaminated beakers for two days under light conditions. This procedure was repeated with spores uncontaminated with the virus to ensure virus-free fungus. Next, the seedlings were transplanted into small plastic trays containing autoclaved potting soil and allowed to grow for an additional 10 days. Individual plants were then placed in geothermal soil simulators containing autoclaved, organic potting soil (Redman *et al.*, 2002). Soil simulators were set at two different temperatures (Table 1). The soil pH was 5.6, and concentrations of nitrogen, phosphorus, and potassium were 179, 21, and 200 ppm, respectively. Plants without fungus and virus are referred to as the non-symbiotic (NS) plants, and symbiotic plants inoculated with both the fungus and the virus are labeled as the anastomosis (An) plants, which refers to the hyphal fusion of the fungus. Four replicates (samples) were prepared for each treatment. The plants were allowed to grow in the soil simulators for approximately one week under greenhouse conditions prior to high-temperature treatment imposition. Physiological measurements were conducted from all treatments when the plants showed visible wilting.

Table 1. The average day and night temperature for both treatments

Temperature	Day	Night
T_1	31.3	26.4
T_2	41.8	38.8

*The data was collected using digital temperature loggers taking continuous measurement throughout the experiment.

Gas exchange measurements

Simultaneous measurements of CO_2 assimilation, stomata conductance, and internal CO_2 concentrations were taken from each of the four samples for each treatment using a Li-Cor 6200 portable photosynthesis system (Lincoln, NE, USA). Measurements were taken 6 h after the onset of the light period utilizing the second most fully expanded leaf near the apical meristem. The leaf was enclosed in a flow-through Plexiglas assimilation chamber (4.5 x 11.8 x 7.3 cm) as described by McDermitt *et al.* (1989). The measurement conditions were 400 µmol $m^{-2}s^{-1}$ photon flux density, 45 to 50% relative humidity (RH), and 27°C.

Photosynthetic pigment determination

Chlorophyll *a*, chlorophyll *b*, and carotenoids were measured from the same leaf used for gas exchange measurements. Four leaf diskettes with a total area of 0.785 cm^2 were taken from each of the six samples for each treatment. Leaf diskettes were placed in vials and incubated in 5 ml of N,N-Dimethylformamide (DMF) solution. The individual vials were wrapped with aluminum foil and placed in the refrigerator at 4˚C for 48 h. Chlorophyll *a* and *b* were determined spectrophotometrically at wavelengths of 647 and 664.5 nm as described by Inskeep and Bloom (1985). DMF extracts were further utilized to spectrophotometrically determine the carotenoid concentration at a wavelength of 470 nm, and the concentration was calculated following the formula reported by Doong *et al.* (1993).

Water potential measurement

Leaf water potential was determined from each of the samples of each treatment following the procedure outlined by Al-Hamdani *et al.* (1990). Randomly selected leaves from individual plants were homogenized using a mortar and pestle. A 100 µL aliquot of cell sap was loaded on a paper disc and placed in a vapor pressure osmometer (model 5520, Wescor, Logan, UT).

Soluble sugar analysis

Root total soluble sugar content was analyzed following a modified procedure of Chatterton *et al*. (1987). Freeze-dried root samples were ground through a 1 mm screen and 33 mg was weighed from each of the four samples of each treatment. Each aliquot was placed in a test tube with 5 ml of 95% (v/v) ethanol, and the tube was wrapped with aluminum foil. Test tubes were placed in a dry bath incubator at 80°C for 20 min. The solution was allowed to settle before removal of the supernatant and was then placed in a separately labeled test tube. An additional 5 ml of 95% (v/v) ethanol was added to the precipitate, and a glass stirring rod was used to re-suspend the contents. The test tubes were placed in the dry bath incubator for an additional 20 min. Again, the supernatant was extracted and combined with the first supernatant extraction to be used for the total sugars assay.

The colorimetric method for sugar determination reported by Dubois *et al.* (1956) was followed. Standards of 0, 130, 150, 200, 250, 300, and 350 mg/ml were prepared first. A 0.15 ml aliquot from each of the combined ethanol extraction samples was mixed with 0.5 ml of 5% phenol in a 10 ml test tube. The sample was vortexed for approximately 10 seconds before the addition of 2.5 ml of H_2SO_4. The sample was vortexed an additional 10 seconds and left to cool down before being read on a spectrophotometer at a wavelength of 490 nm. Using the equation generated from the standard curve, the total soluble sugar content was calculated for each sample.

Statistical analysis

This experiment was carried out as a completely randomized design (CRD). The experiment was repeated twice, and the combined data from both experiments were analyzed using ANOVA as a complete randomized block design (CRBD). This was done to reduce

experimental error resulting from the different times of carrying out the experiment. Mean separations for the values that showed significant F values (P = 0.05) of the ANOVA analyses were based on the least significant difference (LSD) test.

Results and Discussion

The three-way symbiotic interaction between tomato, *C. protuberata*, and CThTV appeared to have no significant influence on tomato photosynthetic rate at both examined soil temperatures (Table 2). However, under the symbiotic interaction, photosynthetic rate was significantly reduced at 41.8/38.8°C in comparison to that obtained at the lower temperature (31.3/26.4°C). Tomato stomatal conductance and internal CO_2 concentrations showed similar responses to all treatments throughout the experiment (Table 2). Visual examination of the non-symbiotic tomato plants exposed to high temperature (41.8/38.8°C) showed symptoms of wilting and chlorosis. In contrast, symbiotic-growing plants appeared much less stressed under the same conditions. It was predicted that photosynthetic rate for the symbiotic plants would perform at a significantly higher rate, and the data was numerically in agreement with this hypothesis. However, the results were not statistically significant, perhaps due to the interference of elevated experimental error.

Table 2. Gas exchange determination of tomato plant as influenced by the symbiotic interaction with *C. protuberata* and CThTV at two selected soil temperatures

	Soil Temperature (°C)					
	CO_2 Assimilation (µmol $m^{-2}s^{-1}$)		ta Conductance (mol $m^{-2}s^{-1}$)		Internal CO_2 (µL L^{-1})	
ction						
	ʌa**	ʌa	ʌa	ʌa	33Aa	33Aa
	ʌa	ʌb	ʌa	ʌa	57Aa	57Aa

*NS = non-symbiotic; An = anastomosis

**Means within columns followed by the same uppercase letter are not significantly different based on the least significant difference (LSD) test (*P* = 0.05).

Means within rows followed by the same lowercase letter of the same corresponding measurements are not significantly different based on the LSD test (*P* = 0.05).

***T_1 and T_2 represent mean day/night temperatures at which the experiments were carried out, 31.3/26.4 and 41.8/38.8, respectively.

Temperature stress effects on photosynthetic rate are well documented in the literature, which was reported to negatively impact photosystem II (PSII) (Strasser, 1997; Murata *et al.*, 2007), as well as the dark reactions by reducing Rubisco activity (Law and Crafts-Brandner, 1999; Ashraf and Harris, 2013; Bowes, 1991). Reductions in CO_2 assimilation affected by temperature stress were reported to be associated with damage in the chloroplast ultrastructure and increased oxidative damage resulting from reactive oxygen species

(ROS) (Chen *et al.*, 2012). In the present study, leaf temperature was the same between the treatments (30 ± 2°C) and appeared independent of the root temperature and very close to the air temperature. This could be one of the reasons that the gas exchange measurements were relatively unaffected by the elevated root zone temperature (Table 2). Leaf temperature in this study was very close to the optimum temperature for photosynthesis, which was reported to be between 25-30°C (Khavari-Nejad, 1980). Similarly, root zone temperature around 30°C was reported to increase tomato photosynthetic rate in comparison to lower temperatures (Hurewitz and Janes, 1983). They attribute the increase in photosynthetic rate to enhanced uptake of nutrients such as phosphorus. Klock *et al.* (1997) reported that elevation of root zone temperature to 36°C reduced phosphatase activity and P uptake in tomato, which was reflected by a reduction in plant growth. Reduction in photosynthetic rate has also been found in peanut as the root zone temperature increased from 32-40°C, and they attribute the reduction to non-stomatal factors (Awal *et al.*, 2003). These findings appear to be consistent with the results of the current study, specifically for the symbiotic plants, which showed a reduction in photosynthetic rate as root zone temperature elevated to 41.8/38.8°C (Table 2). This reduction was independent of the influence of internal CO_2 concentrations, which were the same at all treatments. Moreover, photosynthetic pigments were the same in response to the elevated temperature and symbiotic interaction (Table 3). The exception was chlorophyll *a* content, which was significantly reduced in non-symbiotic plants as root zone temperature increased. It appeared that the symbiotic interaction conferred partial protection from heat stress by maintaining concentrations of chlorophyll *a.* Singh *et al*. (2011) reported that another fungal endophyte (*Piriformospora indica* Sav. Verma, A. J. Varma, Rexer, G. Kost & P. Franken) conferred protection from drought stress by preventing the degradation of chlorophylls and thylakoid proteins.

Table 3. Effect of high temperature on photosynthetic pigment content in tomato plants with selected fungus and virus interaction

	Soil Temperature (°C)					
	T_1***	T_2	T_1	T_2	T_1	T_2
Interaction	Chl *a* (mg g^{-1} FW)		Chl *b* (mg g^{-1} FW)		Caro (µg g^{-1} FW)	
NS*	13.554^{Aa**}	8.706^{Ab}	8.450^{Aa}	8.579^{Aa}	4.825^{Aa}	3.094^{Aa}
An	13.736^{Aa}	11.176^{Aa}	8.590^{Aa}	7.236^{Aa}	4.662^{Aa}	3.402^{Aa}

*NS = non-symbiotic; An = anastomosis

**Means within columns followed by the same uppercase letter are not significantly different based on the least significant difference (LSD) test ($P = 0.05$).

Means within rows followed by the same lowercase letter of the same corresponding measurements are not significantly different based on the LSD test ($P = 0.05$).

***T_1 and T_2 represent mean day/night temperatures at which the experiments were carried out, 31.3/26.4 and 41.8/38.8, respectively.

Additionally, protective mechanisms involving osmolyte regulation influence the survival of plants under heat stress (Bray, 1997; Wang et al., 2003). Osmotic potential is the major

contributor to the overall value of plant water potential, which is highly influenced by the concentration of soluble sugars (Li *et al.*, 1993). In this study, soluble sugar concentration and water potential were insignificantly affected by the various treatments (Figures 1 and 2). Rodriguez *et al*. (2008) concluded that osmolyte concentration was not a factor in combating heat stress as influenced by the plant-fungal interaction.

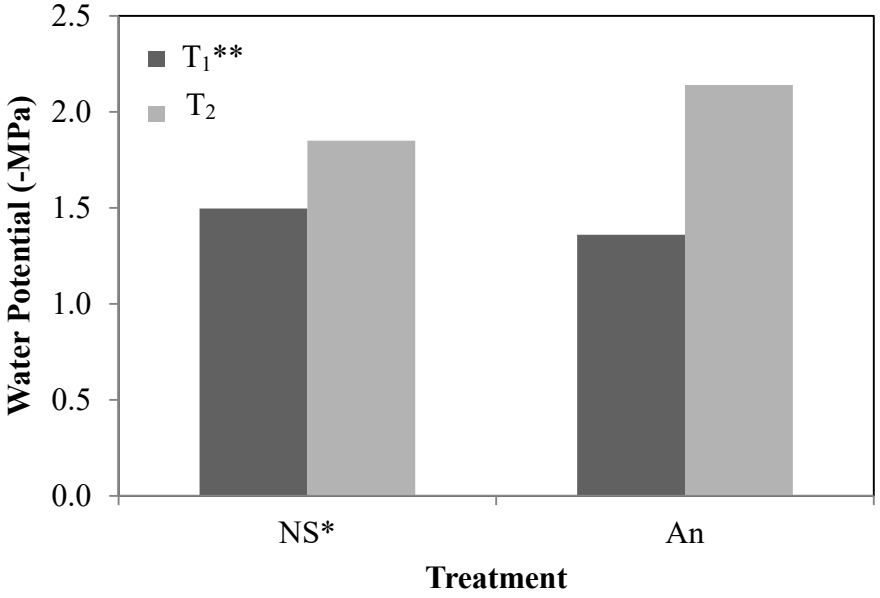

Figure 1. Effect of high temperature on leaf water potential in tomato plants with selected fungus and virus interaction
***NS = non-symbiotic; An = anastomosis**
****T_1 and T_2 represent mean day/night temperatures at which the experiments were carried out: 31.3/26.4 and 41.8/38.8, respectively.**

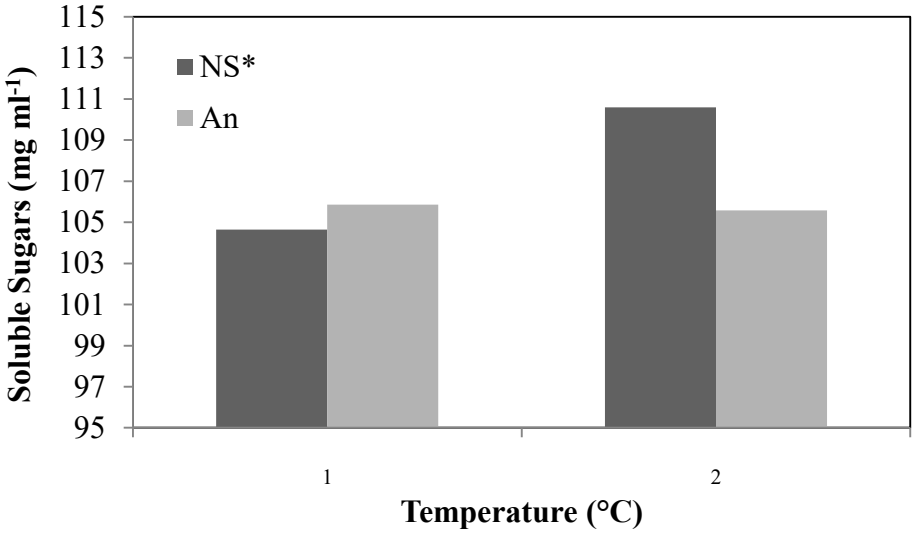

Figure 2. Effect of high temperature on soluble sugar content in tomato plants with selected fungus and virus interaction There was no significant difference based on the LSD test (P = 0.05).
***NS = non-symbiosis; An = anastomosis**
****T_1 and T_2 represent mean day/night temperatures at which the experiments were carried out, 31.3/26.4 and 41.8/38.8, respectively.**

Fungal endophytes have been shown to interact with a wide range of plant species in various capacities to enhance growth and development, and to confer tolerance to high temperature stress (Singh *et al*., 2011). However, there is very limited information available dealing with the impact of this three-way symbiosis on the physiological responses of tomato plants. The few studies available dealing with this specific interaction reported the molecular responses or determined the rate of plant survival to elevated root zone temperature (Redman *et al*., 2002; Márquez *et al*, 2007; Morsy *et al*, 2010). The combinatory influence of the fungus and virus in this study had limited impact on the physiological responses of tomato to elevated root zone temperature. Overall, the three-way symbiotic interaction might confer heat tolerance by inducing different mechanisms than those examined in this study. It appears that this relationship is very complex and includes a wide range of physiological, biochemical, and other responses, which should be considered.

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