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Influence of Combined Biotic and Abiotic Stress on Nutritional Quality Parameters in Tomato (*Solanum lycopersicum*)

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Supporting Information

ABSTRACT: Induction of abiotic stress in tomato plants has been proposed as a mechanism for improving the nutritional quality of fruits. However, the occurrence of biotic stress can interfere with normal abiotic stress responses. In this study, the combined effect of water stress and infection with plant-parasitic nematodes on the nutritional quality of tomato was investigated. Plants were exposed to one or both stresses, and the levels of phenolic compounds, carotenoids, and sugars in fruits were analyzed as well as physiological responses. Levels of carotenoids lycopene and β-carotene were lower in water-stressed tomatoes but exhibited a different response pattern under combined stress. Nematode stress was associated with increased flavonoid levels, albeit with reduced yields, while chlorogenic acid was increased by nematodes, water stress, and the combined stress. Sugar levels were higher only in tomatoes exposed to both stresses. These results emphasize the importance of studying plant stress factors in combination.

KEYWORDS: Water stress, drought, plant-parasitic nematodes, *Meloidogyne incognita*, tomato, *Solanum lycopersicum*, stress interaction, nutritional quality, flavonoids, carotenoids, sugars

INTRODUCTION

Tomatoes (*Solanum lycopersicum*) contain various compounds that are potentially beneficial to human health. As the world's third most important vegetable after potato and cassava (http://faostat.fao.org), tomato plays a significant role in diet and nutrition globally. With increasing interest in so-called functional foods, tomato has become the focus of many studies investigating the factors that influence nutritional quality. The levels of beneficial compounds in tomatoes are known to vary depending on the cultivar, ripening stage, and growth conditions, as well as their level of exposure to environmental stress.[1−3]

Agriculturally, the most damaging abiotic stress is water-deficit leading to drought.[4] Exposure to water or osmotic stress triggers the production of active oxygen species that can be extremely harmful to plant cells, causing oxidative damage and inactivation of enzymes. To minimize damage, cells produce antioxidants that scavenge active oxygen species.[5] Several of these compounds including carotenoids, flavonoids, and other phenolic compounds are of interest nutritionally, as when present in the diet they confer health benefits related to their antioxidant activity.[6−11] Abundant in tomato, carotenoids are potent antioxidants within the plant and are also crucial at times of water deficit for dissipating excess heat in chloroplasts.[1,5,12] Their bright color is an important attractant in pollination and seed dispersal.[13] Lycopene accounts for 80−90% of total carotenoids in tomato and when consumed is associated with a reduction in the risk of prostate and other cancers, as well as protection against cardiovascular disease.[6] Health benefits are also conferred by the vitamin A precursor β-carotene.[7] Flavonoids are a group of bioactive phenolic compounds important in plant stress responses both as antioxidants and as signaling molecules.[14] They are also associated with protection against cardiovascular disease, cancer, and age-related diseases in humans.[8,9] The most abundant flavonoids in tomato are chalconaringenin, rutin, and naringenin.[15] Chlorogenic acid is one of the principle nonflavonoid phenolic compounds in tomatoes. As well as being a potent and widespread antioxidant, it has anticarcinogenic and antidiabetic properties.[10,11] Plants produce phenolic compounds as a defensive mechanism in response to attack by pests or pathogens such as insects, fungi, or nematodes[16,17] as part of the lignification process,[18] and in response to abiotic stresses.[17] In particular, chlorogenic acid is thought to be important in the response of resistant *Solanaceous* plants to infection with root-knot nematodes of *Meloidogyne* spp.[19]

Plant-parasitic nematodes are agriculturally important pathogens that infect almost every species of crop plant, causing extensive damage to yields worldwide and a global loss of over $100 billion per year.[20] Root-knot nematodes (*Meloidogyne* spp.) are the most damaging of all plant-parasitic nematodes in terms of yield loss due to their broad range of host plants, which includes most *Solanaceae*.[21] This pathogen is well studied in the laboratory and provides an excellent model for biotic stress in plants. Root-knot nematodes are sedentary endoparasites that invade and migrate through the root before initiating specialized feeding cells and causing characteristic root galls. As a result, the uptake of water and nutrients by the roots can become severely disrupted, leading to reduced shoot growth and biomass accumulation, reduced photosynthesis, and wilting. The disruption of plant water relations due to infection by plant-parasitic nematodes can thus have severe consequences for plants suffering from drought stress, often exacerbating yield losses.[22]

Because of the connection between plant antioxidants and human health benefits, it has been proposed that a cultivation

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system exposing tomato plants to controlled levels of stress could be of use in improving the nutritional quality of fruits.[23,24] The concentration of sugars in tomato fruits is often used as an assessment of nutritional quality, because of contribution to flavor parameters and also because vitamin C is synthesized from sugars supplied through photosynthesis.[1] Varied levels of success in increasing carotenoid and sugar concentrations have been reported as a result of water deficit or salinity stress.[25−27] However, even low levels of stress can have a negative impact on the yield and fruit ripening time, often counteracting the benefit of such measures.[1,23] Little work has been done to examine the effect of a combination of stresses on the nutritional qualities of tomato. Transcriptome studies have revealed that plants respond very differently to combined stress than to each individual stress, to the extent of activating an entirely new program of gene expression.[28] It is also known that the signaling pathways for abiotic and biotic stress responses may interact and inhibit each other, allowing the plant to adapt most efficiently to the environmental situation.[29] Therefore, it cannot be assumed that the concentrations of nutritional compounds that accumulate due to water stress or pathogen attack would be additive if the two stresses occurred together.

The purpose of this study was to determine the effect of water stress and root-knot nematode-induced biotic stress on the levels of carotenoids, flavonoids, chlorogenic acid, and sugars in tomato fruits. Furthermore, by imposing these two stresses in combination, the possible synergistic or antagonistic effects of the biotic and abiotic stresses could be investigated. The effect on yield and ripening time was also examined to evaluate the impact of the stresses on physiological parameters.

MATERIALS AND METHODS

Nematodes. Root-knot nematodes of species *Meloidogyne incognita* (originally donated by Imperial College, London) were maintained on the roots of tomato plants, cv. Ailsa Craig (Tozer Seeds, Surrey, United Kingdom), grown at 25 °C in a glasshouse. When nematodes reached maturity and egg masses were visible on the surface of the roots, the tomato root balls were removed from the soil and washed with tap water. The root systems were finely chopped and an egg count carried out on a 1 g sample. This sample was shaken in 0.5% sodium hypochlorite for 5 min to release the eggs from the roots, and then, the eggs were counted under a stereobinocular microscope (Wild Heerbrugg, Leica Geosystems Ltd., Milton Keynes, United Kingdom). The number of eggs/g of infected root tissue was determined, and nematode infection of tomato plants was carried out by mixing infected root tissue with compost (Sinclair Potting & Growing Medium, East Riding Horticulture, York, United Kingdom) to achieve a final infection rate of 10 eggs/g of soil.

Plants and Growth Conditions. Seeds of tomato (*S. lycopersicum*) cv. Shirley F1 (Tozer Seeds) were sown in trays of compost in a glasshouse with a constant temperature of 25 °C. Sodium Grolux lamps provided light for 16 h a day. Plants were irrigated with tap water (pH 7.4). After 2 weeks, plants were transferred to 9 cm pots containing standard 3−4 month slow-release fertilizer (Scotts Osmocote Exact Standard, Everris Ltd., Ipswich, Suffolk) at 25 g/L soil. Following a further 3 weeks of growth, plants were divided into four groups for treatment: unstressed, water stress, nematode infection, and joint stress (comprising combined water stress and nematode infection). Plants were then transferred to 18 cm pots of either normal compost or compost containing chopped roots infected with *M. incognita*. The tomato plants were irrigated to field capacity for the following 12 days, to allow time for the juvenile nematodes to hatch and invade the tomato root system. Water stress was then initiated in the water stress and joint stress treatment groups. The plants were subjected to a daily watering regime whereby the well-watered plants received an equal amount of water to that evapotranspired the previous day, as measured by weighing the entire pot after watering and again 24 h later. Plants undergoing water stress treatment received only 80% of the water evapotranspired the previous day. This treatment was continued for 3 weeks, after which all plants were watered to field capacity for the remainder of the experiment. Stomatal conductance was measured before and after the period of water stress (SC-1 Leaf Porometer, Decagon, United States). Flowers were tagged on the day of anthesis to determine ripening time, and fruits were harvested at red ripe stage, the ripening stage at which the fruit is usually consumed. Fruits were considered red ripe on the first day that red color covered more than 90% of the epidermis.[30] For the analysis of nutritional compounds, three plants were sampled per treatment group. Six tomatoes were harvested per plant: three from truss 2 and three from truss 5. Thus, 18 tomatoes were individually sampled for each stress treatment. Truss 2 was a lower region of the plant that produced fruits at an early time point (ripening approximately 108 days after sowing). Truss 5 was at the top of the plant, and the fruits developed later (ripening approximately 126 days after sowing). For the study of physiological characteristics, data from 8 to 9 plants were collected, and the data were combined from five trusses per plant.

Chemicals. Chalconaringenin and morin were obtained from Apin Chemicals (Oxon, United Kingdom). Naringenin, chlorogenic acid, and rutin were from Extrasynthese (Geney, France). Lycopene, β-carotene, fucose, glucose, sodium hypochlorite, high-performance liquid chromatography (HPLC)-grade ethanol, and formic acid were purchased from Sigma (Gillingham, United Kingdom). Fructose was obtained from BDH Chemicals. Sucrose and HPLC-grade acetonitrile were from Fisher Scientific (Leicestershire, United Kingdom). HPLC-grade methyl-*tert*-butyl ether (MTBE), ethyl acetate, and methanol were from VWR International (Leicestershire, United Kingdom).

Extraction and Quantification of Phenolic Compounds. Extraction of phenolic compounds was performed according to Giuntini et al.[31] with some modifications. The pulp and seeds were removed from tomato fruits, and sections consisting of peel and pericarp were freeze-dried and ground to a powder. Each fruit was sampled separately, and extraction was performed once on each. A 25 mg portion of tomato powder was added to 2 mL of 40% aqueous ethanol containing 12.5 μg/mL of an internal standard (morin) and homogenized using an Ultra Turrax T-10 (IKA, Staufen, Germany) for 5 min at approximately 20000 rpm. No additional antioxidant was included, as preliminary tests showed no benefit in terms of yield of phenolics when samples were incubated with either ascorbic acid or sodium metabisulfite (data not shown). The addition of antioxidants caused a decrease in the yield of chlorogenic acid and rutin over the time scale of preparation and analysis, despite the wide use of antioxidants in previous studies on phenolics.[31,32] After centrifugation at 12200*g* for 10 min, the supernatant was filtered using a 0.2 μm polytetrafluoroethylene (PTFE) filter (VWR International) and used for liquid chromatography−mass spectrometry (LC-MS) analysis.

Quantification of tomato polyphenols was conducted using an Agilent LC-MS-MS system (Agilent Technologies UK Ltd., Berkshire, United Kingdom). The rapid resolution front end comprised a 1200 series microdegasser, Binary SL pump, SL autosampler with a chiller module (set to 4 °C), column oven (set to 35 °C), and SL diode array detector. Tomato extract (5 μL) was injected onto a 150 mm × 2 mm 3 μm Luna PFP column (Phenomenex, Cheshire, United Kingdom), and separation was achieved using a binary HPLC gradient of 0.2% aqueous formic acid (solvent A) versus 0.2% formic acid in LC-MS grade acetonitrile (solvent B). The flow rate was 0.3 mL/min, the gradient starting at 15% solvent B, rising to 40% over 13 min, holding at 40% for another 2.2 min. To wash the column, the gradient then moved to 95% solvent B over 3.6 min, held for a further 3.6 min, then returned to 15% over 3.6 min. The column was re-equilibrated for a further 8.5 min before the next injection.

The eluent was directed into an Agilent 6410 triple quadrupole mass spectrometer. The electrospray source was operated in negative mode, with a capillary voltage of 4000 V, a drying gas temperature of 350 °C flowing at 11 L/min, and a nebulizer pressure set to 30 psi. Tomato polyphenols of interest were quantified via multiple reaction monitoring (MRM). Commercial standards were used to determine optimal fragmentor and collision energy values, as well as the most favorable transitions to observe. Briefly, these were 353.1 ≫ 190.9 for chlorogenic acid, 609.1 ≫ 299.9 (quant)/300.9 for rutin, 271 ≫ 150.9 (quant)/118.9 for both naringenin and chalconaringenin, and 301 ≫ 150.9 (quant)/124.9 for morin internal standard, which was used to normalize the response from other analytes. Compound identification was achieved by comparing their retention times with those of commercially available standards and by analysis of their unique fragmentation patterns into known daughter ions. The concentration of target polyphenols was determined using external standard curves. In a subset of tomato segments, the peel and pericarp were separated, weighed, and analyzed individually for phenolic compounds.

Extraction and Quantification of Carotenoids. Freeze-dried sections consisting of peel and pericarp were ground, and the carotenoids were extracted according to Lacker et al. with some modifications.[33] Forty milligrams of tomato powder was mixed with 1 mL of water and 1 mL of MTBE in a two-phase separation. The organic phase was centrifuged for 5 min at 11500*g*, filtered using a 0.2 μm PTFE filter, and injected directly in HLPC. Quantitative determination of compounds was achieved using a reverse-phase HPLC system consisting of a LC-20AD liquid chromatograph, autosampler, and SPD20A UV/vis spectrophotometric detector (Shimadzu UK Ltd.). Separation was accomplished using a C_{30} carotenoid column (4.6 mm × 250 mm, 5 μm particle diameter, YMC). Chromatography was carried out according to Ishida et al.[34] using an isocratic method and a mobile phase of MTBE/methanol/ethyl acetate (45:40:15) and a flow rate of 1 mL/min for 27 min. Lycopene and β-carotene were identified by their retention times compared to commercially available standards and by their classic absorption spectra.[35] Quantification was achieved by comparing against a standard curve. Lycopene and β-carotene were quantified at 450 nm. Chromatograms were analyzed using LCsolution software.

Extraction and Quantification of Sugars. Sugars were extracted from fresh tomato halves by homogenizing the fruit and adding 1 mL of homogenate to 4 mL of 100% ethanol and then vortexing. Fucose was added as an internal standard to a concentration of 125 μg/mL. The samples were centrifuged at 4000*g* for 5 min, and then, 120 μL of the supernatant was evaporated and resuspended in 600 μL of water before filtering and analysis by anion-exchange chromatography. Samples were analyzed using a Dionex system with a pulsed amperometric electrochemical detector (ED50) (Dionex Ltd., Camberley, United Kingdom). The anion exchange column used was a CarboPac PA20 (3 mm × 150 mm, Dionex), suitable for the analysis of mono- and disaccharides. Separation was carried out at a flow rate of 0.4 mL/min, using a gradient of 60 mM NaOH for 10 min, during which time the sugars eluted, followed by column regeneration with 100 mM NaOH for 5 min, and re-equilibration with 60 mM NaOH for the remaining 12 min. Detection was achieved using a gold working electrode and a three-step waveform. Chromatography was conducted at 30 °C. Chromatogram acquisition was performed using Chromeleon 6.5 software.

Statistical Analysis. Results with a normal distribution were analyzed by one-way or two-way analysis of variance (ANOVA) using SPSS statistical software (version 16.0), and mean differences were compared between each stress treatment and the unstressed plants by the Student−Newman−Keuls (SNK) test. Data with a right-skewed distribution were normalized by taking the square root of the values before analyzing with ANOVA, while data with an extremely right-skewed distribution were normalized by transformation into log values. Nonparametric data were analyzed using the Kruskal−Wallis H test and differences between treatments determined by Mann−Whitney U test with a Bonferroni correction. A p value of <0.05 was considered statistically significant.

RESULTS

Physiological Response to Stress. Tomato plants were exposed to either individual biotic or abiotic stress or a combination of both stresses (joint stress). Biotic stress consisted of infection with the plant-parasitic nematode *M. incognita* 5 weeks after sowing. This stress continued for the duration of the experiment, as the nematodes would have completed their life cycle of approximately 6 weeks and their juvenile offspring reinfected the plant roots.[20] The stress was more severe toward the end of the experiment as the nematodes increased in numbers. Abiotic stress consisted of a moderate water stress lasting 3 weeks during the time of flowering. The effects of the water stress were assessed by measurements of gas exchange and growth. At the end of the period of water stress, the stomatal conductance of the treated plants was only 30.5 ± 3.8% of that of the well-watered plants. This indicates a lower level of gas exchange due to the reduced aperture of the stomata and, thus, a reduction of photosynthesis in a manner typical of plants undergoing drought.[4,24] The stomatal conductance of nematode-infected plants was no different from the control. Plants from all three stress treatments also showed a significant height reduction when measured after the period of water stress treatment, as compared to the unstressed plants. Nematode-stressed plants were on average 8.0 ± 2.0% shorter than unstressed plants, while water-stressed and joint-stressed plants were 22.7 ± 2.6 and 20.8 ± 2.0% shorter, respectively ($p < 0.001$).

The time taken for the plants to flower and fruit after planting was observed. Plants that had undergone water stress or joint stress flowered significantly later than those that were well-watered, resulting in a delay of approximately 2 days ($p < 0.001$) (Figure 1A). The fruit ripening period, as defined by the number of days from anthesis to red ripe stage, was also severely affected by the stress treatments (Figure 1B). Water stress alone significantly increased the ripening time from 59.6 ± 0.7 to 62.6 ± 0.8 days, whereas fruit from nematode-infected and joint-stressed plants ripened significantly faster (54.5 ± 0.4 and 53.4 ± 0.5 days, respectively) ($p < 0.001$). In addition, stress treatments affected the yield of tomatoes on an individual fruit weight basis. Water stress alone did not influence the weight, but fruits from plants infected with nematodes and those undergoing joint stress were significantly lighter than those from their nonparasitized counterparts, with average weight decreasing from 50.2 ± 1.3 g in the control group to 39.0 ± 1.2 g in the nematode treatment and 38.3 ± 1.3 g in the joint treatment ($p < 0.001$) (Figure 1C). A comparison of in flowering time, fruit ripening time, and fruit yield for each truss position can be found in Figure 1 in the Supporting Information. At the end of the experiment, the tomato plants infected with nematodes were wilted and had diminished foliage as compared with plants that had not been infected. At this point, the stomatal conductance of the water-stressed, nematode-treated, and joint-stressed plants was 76, 41, and 21%, respectively, of the unstressed plants, implying a maintained level of stress throughout the experimental period, even after rewatering of plants.

The percentage dry matter in collected fruit segments was calculated by comparing fresh and freeze-dried weights. Early- (truss 2) and late-harvested (truss 5) fruit from water-stressed plants had a significantly lower percentage dry matter than control

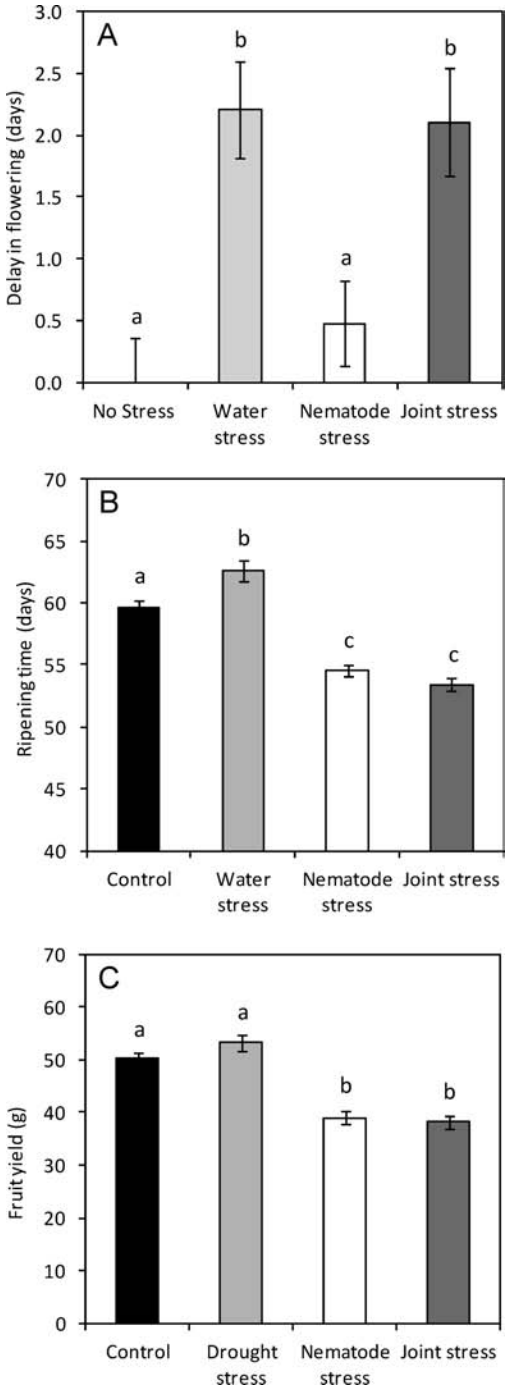

Figure 1. (A) Delay in flowering, (B) fruit ripening time from anthesis, and (C) average fruit weight of plants under water-deficit, nematode stress, joint water and nematode stress, or no stress (control). Delay in flowering time is the number of days delay to first flowering as compared to the control plants. Fruits were harvested and weighed at red ripe stage. Data shown are the mean values of fruits from all five trusses of 8−10 plants per treatment (n = 150−200). Bars represent the standard error of the mean. Means with different letters are significantly different at the 5% level according to the Mann−Whitney U test.

fruits (p < 0.001) (Figure 2). This is in contrast with previous studies that have found a higher proportion of dry matter in water-stressed fruits.[24,36] Both nematode and combined stress caused a differential effect on the dry matter accumulation in the tomato

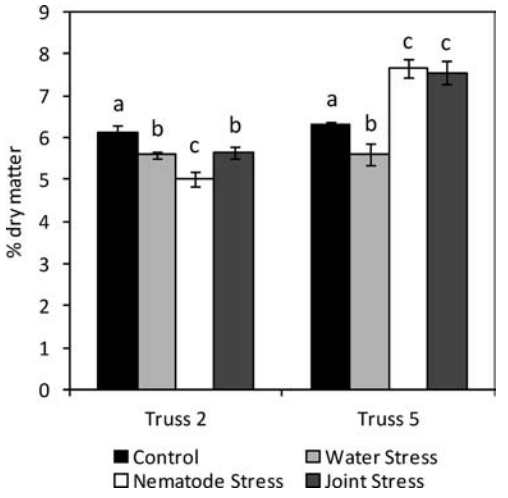

Figure 2. Percentage dry matter of tomatoes from plants under water deficit, nematode stress, joint water and nematode stress, or no stress (control). After they were harvested at red ripe stage, fruit segments were weighed before and after freeze-drying, and the percentage dry weight was calculated. Data shown are mean values from several plants (n = 10−20). Bars represent the standard error of the mean. Means with different letters are significantly different at the 5% level according to the SNK test for truss 2, and the Mann−Whitney U test for truss 5.

fruits. In fruits harvested early, there was a significantly lower percentage dry matter as a result of these stress treatments (p < 0.001), while in later harvested fruits, there was a significantly higher proportion of dry matter than in control fruits (p < 0.001), indicating that the more severe nematode stress at the later time point caused the plants to produce drier fruit.

Phenolic Compounds. The effect of plant stress treatments on the concentration of phenolic compounds in tomato fruits was investigated by analyzing the levels of flavonoids and chlorogenic acid in peel and pericarp sections using LC-MS. The most abundant compound detected was chalconaringenin, followed by rutin, chlorogenic acid, and then naringenin in trace amounts. This supports the results of previous studies that have found chalconaringenin and rutin to be the most abundant flavonoids in fresh tomatoes and chlorogenic acid to be the next most abundant phenolic antioxidant.[15] Flavonoids are reported to be most highly concentrated in the peel of tomatoes,[31] a finding corroborated in the current study. Despite only accounting for 9% of the sample weight, the peel contained 61% of the rutin, 55% of the naringenin, and 99% of the chalconaringenin. This concentration in the epidermis of the fruit may allow the flavonoids to protect the tissues below from the damaging effects of UV-B.[16] In contrast, 9% of the chlorogenic acid was present in the peel, indicating an equal concentration in the peel and pericarp. The concentration of phenolic compounds is given as a proportion of fresh weight, as this is the more commonly used measurement.[3]

Stress treatments affected the levels of phenolic compounds in truss 5 tomatoes, which were harvested at a late point in the experiment (Figure 3). Significantly higher levels of rutin were observed in truss 5 tomatoes from plants exposed to either nematode stress (3.6 ± 0.3 mg/100 g) or joint stress (3.3 ± 0.4 mg/100 g), as compared to the controls (2.3 ± 0.3 mg/100 g), resulting in an increase of 56 and 40%, respectively (p < 0.001) (Figure 3A). Naringenin concentrations were also heightened by nematode stress in truss 5, showing an increase of 62% (1.0 ± 0.1 μg/100 g as compared to a control value of 0.6 ± 0.1 μg/100 g, p < 0.01)

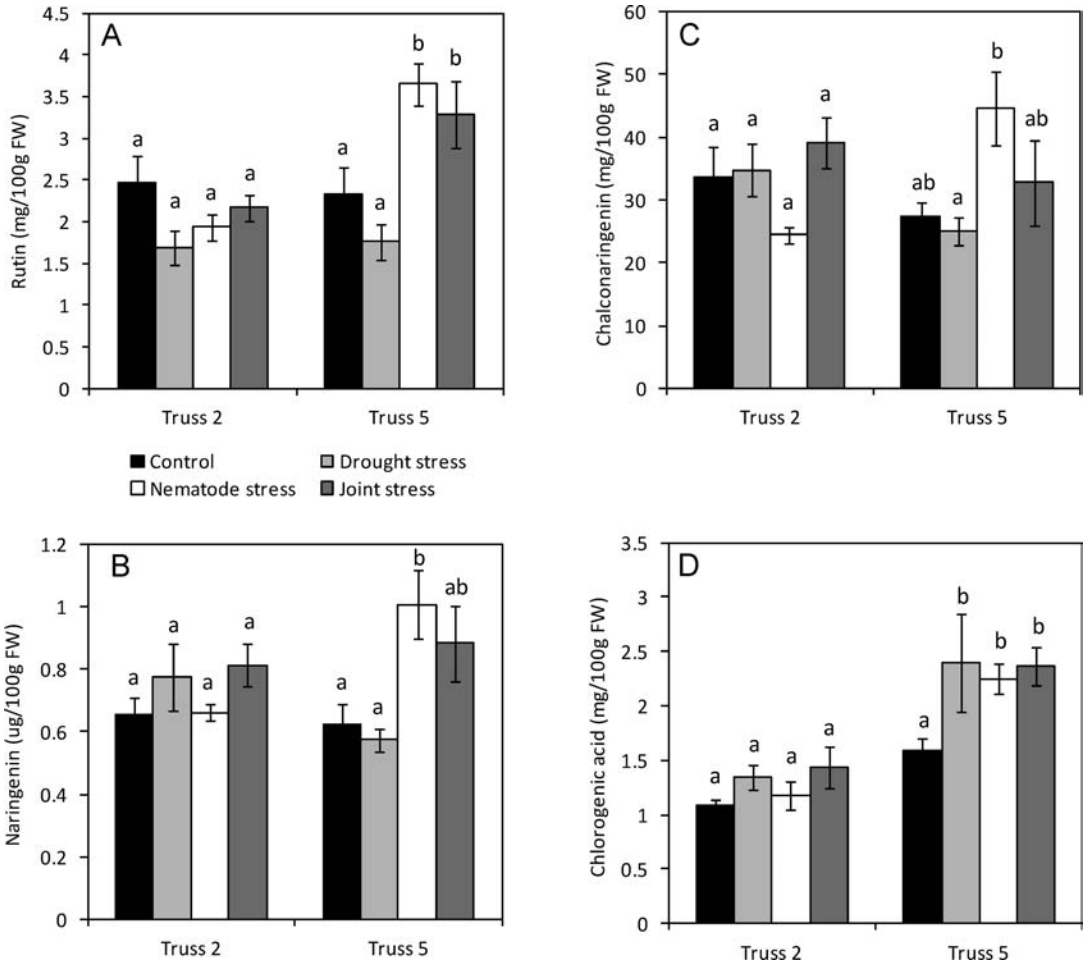

Figure 3. Concentrations of the phenolic compounds (A) rutin, (B) naringenin, (C) chalconaringenin, and (D) chlorogenic acid in tomato fruits from plants under one of four conditions (water stress, nematode stress, joint stress, or no stress). Fruits were harvested either early (truss 2) or late (truss 5) in the experiment. Concentrations are expressed per 100 g of FW. Bars represent the standard error of the mean ($n = 9$). Means with different letters are significantly different at the 5% level according to the SNK test. Bars displaying two letters show no difference from either group.

(Figure 3B). The chalconaringenin concentration in fruits from nematode-stressed plants was not significantly different from the controls; however, a significant difference was observed between the water-stressed and the nematode-stressed fruits, resulting in an increase of 78% (44.6 ± 5.9 μg/100 g as compared to 25.1 ± 2.1 μg/100 g, $p < 0.05$) (Figure 3C). Water stress alone did not affect the levels of chalconaringenin and naringenin. Furthermore, when the two stresses were applied together, the heightened concentrations seen under nematode stress were reduced and thus not significantly different from the control or water-stressed plants. No difference in rutin, naringenin, or chalconaringenin concentrations was observed in fruits harvested at an early stage (truss 2). The concentration of chlorogenic acid was significantly affected by all three stress treatments in truss 5 fruits (Figure 3D). Water stress and nematode stress increased chlorogenic acid levels by 49 and 46%, respectively, as compared to the control, while the two stresses in combination gave an increase of 51% [control, 1.6 ± 0.1 mg/100 g fresh weight (FW); water stress, 2.4 ± 0.4 mg/100 g; nematode stress, 2.3 ± 0.1 mg/100 g; and joint stress, 2.4 ± 0.2 mg/100 g, $p < 0.05$]. Chlorogenic acid levels in fruits harvested early (truss 2) were not affected significantly by any stress. When tomatoes from truss 2 and truss 5 were analyzed together, an interaction was observed between stress treatment and truss position for the flavonoids rutin ($p < 0.01$), naringenin ($p < 0.05$), and chalconaringenin ($p < 0.01$), although not for chlorogenic acid (Table 1). Truss position significantly affected rutin ($p < 0.001$) and chlorogenic acid ($p < 0.001$) concentration but not naringenin or chalconaringenin.

Carotenoids. Carotenoids were analyzed in peel and pericarp sections of tomatoes that had been exposed to single or combined stress. As expected, the most abundant carotenoids were identified as lycopene and β-carotene. The concentration of lycopene in the tomato samples ranged between 3.6 and 14.7 mg/100 g FW. β-Carotene was present at approximately 1/10 of the abundance of lycopene, varying from 0.3 to 1.2 mg/100 g FW. Similar concentrations for each compound have been reported by other authors in various studies of fresh tomatoes, as summarized by Dumas et al.[2]

The relative levels of carotenoids were influenced significantly by different stress treatments. The lycopene concentration was significantly lower in truss 2 fruits from plants that were exposed to water deficit or joint stress, resulting in a decrease in concentration of 34 and 30%, respectively (from 11.3 ± 0.9 mg/100 g FW in the unstressed controls to 7.5 ± 0.6 mg/100 g in the water stressed and 7.9 ± 0.7 mg/100 g in the joint treatment, $p < 0.01$) (Figure 4A). The concentration was not affected by nematode stress in these fruits. In truss 5, when the

Table 1. Concentration of Nutritional Compounds in Fruits from Tomato Plants Subjected to Individual or Combined Water and Nematode Stress[†]

		mg/100 g FW				mg/100 g FW		mg/g FW	
truss position	treatment	chlorogenic acid	rutin	chalconaringenin	naringenin	lycopene	β-carotene	glucose	fructose
truss 2	control	1.08 ± 0.2	2.47 ± 1.0	33.68 ± 14.3	0.65 ± 0.2	11.29 ± 2.6 a	0.87 ± 0.2 a	13.70 ± 1.3	15.07 ± 1.2
truss 2	water stress	1.35 ± 0.3	1.69 ± 0.6	34.74 ± 12.6	0.78 ± 0.3	7.50 ± 1.7 b	0.62 ± 0.1 b	13.47 ± 1.1	14.58 ± 1.3
truss 2	nematode	1.17 ± 0.4	1.93 ± 0.5	24.40 ± 3.7	0.66 ± 0.1	9.27 ± 3.0 ab	0.84 ± 0.2 a	13.12 ± 1.0	14.68 ± 1.1
truss 2	joint stress	1.44 ± 0.6	2.17 ± 0.5	39.05 ± 12.3	0.81 ± 0.2	7.90 ± 2.0 b	0.72 ± 0.2 ab	14.22 ± 1.0	15.83 ± 1.3
ANOVA		NS	NS	NS	NS	**	*	NS	NS
truss 5	control	1.59 ± 0.3 a	2.34 ± 0.9 a	27.40 ± 6.5 ab	0.63 ± 0.2 a	7.58 ± 2.2 a	0.57 ± 0.1 ab	15.92 ± 2.4 a	17.87 ± 2.7 a
truss 5	water stress	2.40 ± 1.3 b	1.76 ± 0.6 a	25.05 ± 6.1 a	0.57 ± 0.1 a	5.16 ± 0.9 b	0.53 ± 0.1 a	15.48 ± 1.7 a	17.61 ± 1.9 a
truss 5	nematode	2.25 ± 0.4 b	3.65 ± 0.7 b	44.64 ± 17.5 b	1.01 ± 0.3 b	9.04 ± 2.4 a	0.75 ± 0.1 b	17.31 ± 2.5 a	18.77 ± 2.5 a
truss 5	joint stress	2.37 ± 0.5 b	3.28 ± 1.2 b	32.74 ± 20.4 ab	0.88 ± 0.4 ab	7.31 ± 2.0 a	0.61 ± 0.1 ab	19.56 ± 2.1 b	21.81 ± 2.3 b
ANOVA		*	***	*	**	**	*	**	**
truss 2 + 5	control	1.34 ± 0.4 a	2.40 ± 0.9 a	30.54 ± 11.3	0.64 ± 0.2 a	9.44 ± 3.0 a	0.72 ± 0.2 a	14.81 ± 2.2 a	16.47 ± 2.5
truss 2 + 5	water stress	1.84 ± 1.0 b	1.72 ± 0.6 b	30.18 ± 11.0	0.68 ± 0.3 ab	6.33 ± 1.8 c	0.58 ± 0.1 b	14.48 ± 1.7 a	16.10 ± 2.2
truss 2 + 5	nematode	1.71 ± 0.7 b	2.79 ± 1.1 a	34.52 ± 16.1	0.84 ± 0.3 b	9.16 ± 2.7 ab	0.79 ± 0.2 a	15.21 ± 2.8 a	16.72 ± 2.8
truss 2 + 5	joint stress	1.90 ± 0.7 b	2.72 ± 1.1 a	35.90 ± 16.7	0.85 ± 0.3 b	7.59 ± 2.0 bc	0.65 ± 0.2 ab	16.89 ± 3.2 b	18.82 ± 3.6
ANOVA	treatment	**	***	NS	**	***	**	***	NS
	truss position	***	***	NS	NS	***	***	***	***
	treatment × truss position	NS	**	**	*	NS	NS	*	NS

[†] Concentrations of phenolic compounds and carotenoids are given as mg/100 g FW ± SD. Sugar concentrations are given as mg/g FW ± SD. Means for each compound were compared between treatment groups. "Truss 2 + 5" indicates the results of the two trusses together as analyzed by two-way ANOVA. The significance of differences between factors is given as follows: NS, not significant; $*p < 0.05$; $**p < 0.01$; and $***p < 0.001$. Means with different letters are significantly different at the 5% level according to the SNK test.

nematode stress was more severe, water stress alone resulted in a 32% decrease in lycopene concentration (7.6 ± 0.7 mg/100 g FW in the control as compared to 5.2 ± 0.3 mg/100 g in the water deficit group, $p < 0.01$), while joint water and nematode stress had no effect. β-Carotene levels followed a similar pattern in truss 2, where a 28% lower concentration was observed in the water-stressed plants as compared to the control (0.9 ± 0.1 mg/100 g FW in control as compared to 0.6 ± 0.04 mg/100 g in water-stressed, $p < 0.05$). The β-carotene concentration in joint-stressed plants was also lower than unstressed controls but not to a significant level ($p = 0.085$) (Figure 4B). In truss 5, a different pattern of results was observed. Although none of the stress treatments were significantly different from the control, in each case, the β-carotene concentration with respect to the control was higher than in truss 2. The truss position significantly affected both lycopene ($p < 0.001$) and β-carotene ($p < 0.001$) concentrations, giving lower concentrations in the later harvested fruits (Table 1), a finding previously documented by Dumas et al. (after Cabibel and Ferry).[2] When results from the two trusses were analyzed together, the effect of the stress treatments became more significant for both lycopene ($p < 0.001$) and β-carotene ($p < 0.01$), although no interaction effect was observed between the stress treatments and the truss position (Table 1).

Sugars. The hexose sugars glucose and fructose were detected in the truss 2 tomato fruits at concentrations of 13.6 and 15.0 mg/g FW, respectively. Truss 5 concentrations were significantly higher than those of truss 2 ($p < 0.001$) (Table 1), a difference of approximately 20%. These concentrations are similar to those previously described.[26,27,37] Sucrose was not detected in the tomatoes. This absence is consistent with previous studies that have failed to detect sucrose in ripe tomatoes or have found it present only in trace amounts and indicates that by this stage of fruit development all of the sucrose had been converted to starch.[26,37] Water stress and nematode stress on their own had no effect on glucose or fructose concentration in tomato fruits. However, when the two stresses were applied in combination, a significantly higher concentration of both sugars was observed in truss 5 fruits, resulting in a 23% increase in glucose (from 15.9 ± 0.80 mg/g FW to 19.6 ± 0.69 mg/g, $p < 0.01$) and a 22% increase in fructose (from 17.9 ± 0.80 mg/g FW to 21.8 ± 0.78 mg/g, $p < 0.01$) as compared to the controls (Figure 5). An interaction was observed between the effects of stress treatment and truss position for glucose ($p < 0.05$) but not for fructose (Table 1).

DISCUSSION

Physiological Response to Stress. The exposure of tomato plants to individual or combined abiotic and biotic stress affected flowering time, ripening time, fruit yield, and influenced the accumulation of phenolic compounds, carotenes, and hexose sugars in the fruits. The results demonstrate a different pattern of response depending on the stress encountered and the time of harvesting, as well as a complex interaction between the effects of the stresses in combination. As plants are likely to experience more than one stress at any time, this differential response may be necessary to conserve resources and focus on the most damaging stress. Water deficit caused the tomato plants to flower later than the unstressed controls (Figure 1A). The inhibition of growth caused during the water deficit period may have led to a delay in the establishment of normal developmental and reproductive patterns.[4] In a continuation of this trend, fruit from water-stressed plants also ripened more slowly (Figure 1B). However, in contrast to previous studies, there was no effect of water deficit on yield (Figure 1C). Severe water stress is known

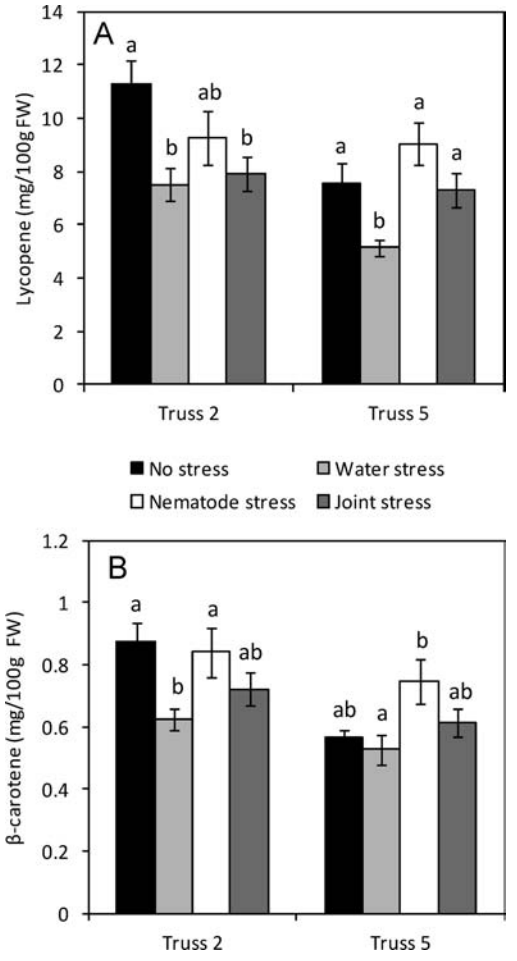

Figure 4. (A) Lycopene and (B) β-carotene concentrations of tomato fruits from plants under water deficit, nematode stress, joint water and nematode stress, or no stress (control). Fruits were harvested either early (truss 2) or late (truss 5) in the experiment. Concentrations are expressed per 100 g of FW. Bars represent the standard error of the mean ($n = 9$). Means with different letters are significantly different at the 5% level according to the SNK test.

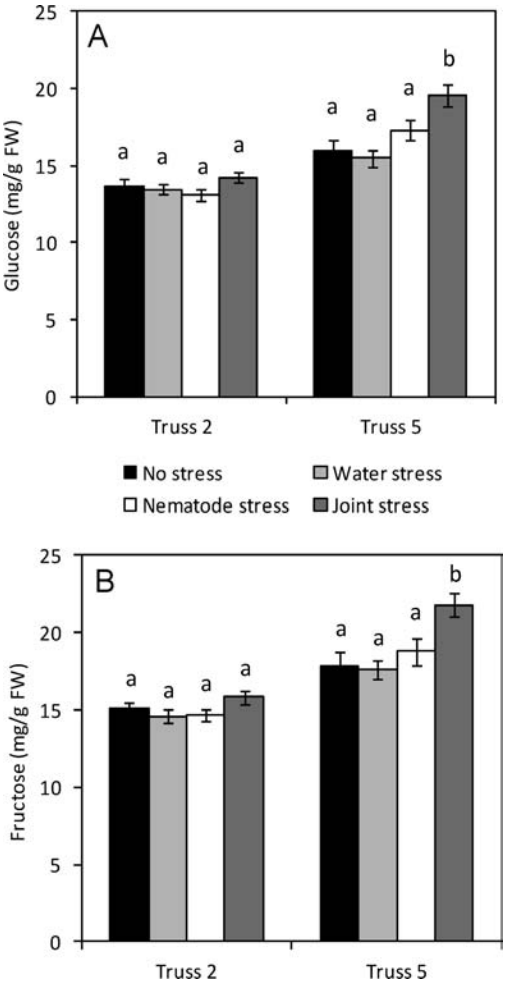

Figure 5. (A) Glucose and (B) fructose concentrations of tomato fruits from plants under water deficit, nematode stress, joint water and nematode stress, or no stress (control). Fruits were harvested either early (truss 2) or late (truss 5) in the experiment. Concentrations are expressed per gram of FW. Bars represent the standard error of the mean ($n = 9$). Means with different letters are significantly different at the 5% level according to the SNK test.

to negatively influence yield in terms of kilograms per plant or per hectare,[23,24] although the weight of individual fruits often remains the same[26] or can actually increase,[24] perhaps explaining why no reduction in fruit size was seen in this study. Infection with nematodes caused a severe yield impediment, producing fruits that were 20% lighter and that ripened much faster than the controls (Figure 1C). The reduction of yield in tomato plants infected with *Meloidogyne* spp. is well characterized, occurring due to the disruption of water and nutrient transport from the roots.[38] The late-harvested fruits from infected plants also had a significantly lower water content than the unstressed fruits, suggesting that water relations in the plant were disrupted to a greater extent by the severe nematode stress than by water stress itself (Figure 2). Water deficit actually increased the water content of tomato fruits, a surprising finding in light of previous studies that found the contrary to be true.[23,36] The earlier timing of the water stress in the current study may account for differences between results. Interestingly, when water deficit and nematode infection occurred in combination, the plant's physiological response was most similar to that of water stress alone in the early harvested tomatoes but to nematode stress alone in the late-harvested tomatoes. These results support the hypothesis that plant stress responses are specifically tailored to the exact combination of environmental stresses encountered, to the extent that the plant responds to whichever stress is most severe, over-riding the pathway for the lesser stress.[29]

Phenolic Compounds. Flavonoids are a diverse group of phenolic secondary metabolites known to have several functions in plants. They act in the protection of plant tissues during oxidative stress from UV-B damage, as insect antifeedants induced during defense responses such as lignification, as signaling molecules in establishing symbiotic relationships with rhizobia, and as regulators of auxin transport.[16,31] The current study found that as a result of severe nematode stress the levels of the flavonoids rutin, chalconaringenin, and its isomer naringenin all increased significantly in tomato fruits (Figure 3). An interaction was observed whereby the effect of nematode stress was greater in truss 5 fruits. The activation of the flavonoid synthesis pathway has previously been described in response to infection with both cyst nematodes and root-knot nematodes, but this was localized in the roots during the establishment of the nematode feeding site. It has been proposed that

flavonoids may be necessary to influence local auxin transport pathways and thus allow the establishment of feeding cells.[39] However, little is known about the influence of nematode infection on the nutritional status of the fruit, as reported here. Plants may thus respond to root-knot nematodes by activating a systemic defense system whereby flavonoid antifeedants accumulate throughout plant tissues. Under severe biotic stress, there may be a shift in carbon allocation toward the production of chemical defense compounds rather than growth. Water stress has previously been reported to influence flavonoid levels in plants. Pernice et al. reported that although the accumulation of total flavonoids was heightened in fruits from plants under moderate water stress, the concentration of naringenin was actually lower under extreme water deficit.[25] Rutin and chlorogenic acid have been shown to accumulate in the foliage of tomato plants as a result of drought stress.[17] However, the current study found no such effect in the tomato fruits, as little or no change in flavonoid concentration was observed as a result of water stress. This suggests that the water status of the plant does not always affect the process of stress-responsive flavonoid accumulation in the fruit. Interestingly, when both stresses were applied to the plant in combination, the increase in flavonoid content was lower than under nematode stress alone. Therefore, the water stress, although not significant in itself, may act to temper the biotic stress response induced by the nematodes and thus maintain the flavonoid content at more normal levels. Abscisic acid is known to accumulate in response to abiotic stress and in turn inhibits the transcription of defense and pathogen-response genes.[29,40] This phenomenon may thus explain the observed interaction of the two stresses, leading to the inhibition of the nematode-induced flavonoid accumulation. Chalconaringenin was detected at a somewhat higher concentration than has been found in whole red tomatoes in previous studies as summarized by Slimestad and Verheul[3] where, depending on cultivar, the values ranged between 0.9 and 18.6 mg/100 g. This difference can be attributed to the localization of chalconaringenin in the peel, giving a higher concentration in peel/pericarp sections than in the whole fruit. Accordingly, studies examining peel in isolation have reported much higher concentrations.[41] There is some debate as to whether naringenin is naturally present in ripe tomatoes or whether its detection is an artifact resulting from the spontaneous isomerization of chalconaringenin during extraction, a process that can occur at low pH conditions.[15,42] Many studies have previously treated chalconaringenin and naringenin as the same compound, reporting a single combined figure for both. However, this is now considered erroneous due to their very different spectral absorbencies[3] and the fact that they can be separated via HPLC. In this study, naringenin itself was detected at extremely low concentrations. This may be an indication of a more stable extraction procedure than previously documented, causing less isomerization of chalconaringenin during sample preparation. Rutin and chlorogenic acid were detected at levels similar to those reported previously.[3,15] Chlorogenic acid has previously been shown to accumulate in tomato leaves in response to drought stress[17] and in tomato and pepper roots in response to root-knot nematodes, where it is thought to act as a crucial component of nematode resistance, particularly in resistant cultivars.[19] Chlorogenic acid, although not itself a toxic compound, may be produced as part of a pool of available phenylpropanoids that are broken down into activated defense components such as caffeic acid.[18,19] In the current study, the compound accumulated to a higher level under all three of the stress treatments in late-harvested fruits, indicating that chlorogenic acid is part of a generalized systemic stress system and not just a local response to pathogens. Its role as a potent antioxidant during other abiotic stresses such as UV-B exposure may explain its induction in fruit from plants under water stress.[43]

Carotenoids. Studies on the effect of water stress on carotenoid levels in tomato fruits have previously revealed inconsistent results.[2] Water deficit has been associated in some cases with a reduction in the levels of carotenoids such as lycopene,[24,36] while other studies demonstrated a higher level of lycopene and total carotenoids.[25,27] β-Carotene levels have been reported to increase or remain unchanged in response to water stress[27,36] or in one study to decrease with moderate water stress but increase with severe water stress.[25] The analysis of carotenoids in the current study revealed a negative effect of water stress on lycopene in both trusses and β-carotene accumulation in truss 2 (Figure 4). Carotenoids are important in the plant stress response as they act as scavengers for damaging oxygen radicals and also protect plant tissues by absorbing excess light.[1,5] Therefore, it could be expected that carotenoid levels would increase under osmotic stress conditions, as opposed to the observed decrease. However, it has been proposed that this inhibition in carotenoid accumulation may be related to the antagonism between abscisic acid and ethylene.[1] Ethylene is crucial in regulating carotenoid accumulation in response to UV-B stress, and lycopene and β-carotene in particular correlate positively with ethylene concentration in tomato fruits.[44] However, abscisic acid is produced rapidly in response to drought and osmotic stress in plants and is central in orchestrating stress response pathways.[40] The signaling pathways of ethylene and abscisic acid are known to inhibit one another,[29] and so the large-scale induction of abscisic acid in response to water stress in the tomato plants may be the cause of the reduced carotenoid levels observed. A lack of ethylene may also explain the prolonged ripening time in the fruits that had undergone water stress. There was no significant effect of nematode infection on carotenoid levels in the tomato fruits.

Sugars. The concentration of hexose sugars in the tomato fruits was significantly increased as a result of combined water deficit and nematode infection, even though each individual stressed showed no effect (Figure 5). Higher hexose concentrations have frequently been reported in fruits under water deficit or salinity stress, thus contributing to a higher fruit quality due to increased fruit sweetness and flavor.[26,37] However, in several cases, the difference is not maintained when taken as a proportion of dry weight,[23,27] and thus, the effect seen could be attributed to the lower water content of the fruit. In the current study ,when the sugar concentration was calculated as a proportion of dry weight, a significant increase was still observed between the nematode-stressed fruits and those under joint stress (although neither were different to the control), suggesting that the results were not merely due to fruit water content. Under conditions of water or osmotic stress, the sink strength of tomato fruit may be increased to achieve a maintained level of assimilate translocation and accumulation of dry matter.[26,37] To increase the sink strength in fruits of stressed plants, sucrose is hydrolyzed more rapidly by the enzymes sucrose synthase and invertase and converted into starch, thus maintaining a sucrose gradient between the leaves and the fruit. During ripening, the starch is converted back into the sugars glucose and fructose.[45] Thus, although nematode stress or water deficit alone did not affect the process of sucrose translocation into the fruit, in the jointly stressed plants, the reduced plant growth rate due to nematode infection combined with higher sink activity in the fruit due to osmotic stress may have caused a switch of carbohydrate allocation away from vegetative growth, thus channeling a higher level of sucrose into the fruits.[26]

Concluding Remarks. There is much interest in the possibility of improving the nutritional quality of tomato fruit by adjusting agronomic conditions to incur plant stress.[1] The results of this

study highlight the influence of environmental stresses on nutrition and yield parameters of tomatoes and indicate a complex interaction between the environment and the water status, growth, and reproduction within the plants. Inflicting water stress has previously produced some success in improving levels of carotenoids and sugars.[23−26] However, this has usually incurred a yield penalty. The current study has found that water deficit can furthermore delay flowering and ripening and may actually diminish the levels of antioxidants such as carotenoids and some flavonoids, while having little effect on other nutritional compounds. An interesting comparison can be made with the effect that a biotic stress has on tomatoes: Infection with root-knot nematodes actually had a positive effect on the nutritional qualities of tomato fruits, albeit with greatly reduced yield.

There has been little research into the confounding effect of multiple stresses on nutritional quality in tomatoes or their impact on a system designed to induce controlled water stress. This study has shown that the simultaneous imposition of biotic and abiotic stress results in a new profile of the levels of nutritional compounds that does not bear close resemblance to that of either stress individually. Certainly, the effect of the combined stresses on antioxidant and sugar concentrations was not additive and would have been difficult to predict. In normal growing conditions, plants are frequently exposed to more than one stress at any one time; therefore, care should be taken when proposing a set of environmental conditions to try and maximize quality parameters.

■ ASSOCIATED CONTENT

Supporting Information. Figure showing days from sowing to flowering, fruit ripening time from anthesis, and average fruit weight of plants under water-deficit, nematode stress, joint water and nematode stress, or no stress (control). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

MTBE, methyl-*tert*-butyl ether; LC-MS, liquid chromatography−mass spectrometry; PTFE, polytetrafluoroethylene; MRM, multiple reaction monitoring; FW, fresh weight; HPLC, high-performance liquid chromatography; SNK, Student−Newman−Keuls test

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