American Journal of Agricultural and Biological Sciences

Original Research Paper

A Comparative Analysis on the Physiological Effects of the Physical and Chemical Properties of the Trihalomethanes on Nutrient Levels, Oxidative Stress and Sterol Compositions of Leaf Oils in *Solanum Lycopersicum* Cultivars

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Article history
Received: 07-11-2017
Revised: 08-03-2018
Accepted: 11-05-2018

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Abstract: Trihalomethane group of disinfection by-products was used to evaluate the magnitude of physiological changes induced on nutrient levels, oxidative stress and phytosterol content in two tomato cultivars. The tomato cultivars were grown as potted plants in a greenhouse and exposed to the varying concentrations of trihalomethanes and organochlorines via irrigation water for a period of 30 days. The concentration levels of nitrogen, potassium and boron in both cultivars significantly ($p<0.05$) decreased with increasing chlorination. A significant ($p<0.05$) increase in total polyphenol content, Ferric Reducing Antioxidant Power (FRAP) and guaiacol peroxidase activity was observed in both cultivars. Increase in chlorination could probably be partly responsible for the induction of a greater antioxidant response, as well as the increased free phytosterol content in the tomatoes leaf oils, while β-sitosterol content decreased. In general, the magnitude of the effect of the increasing number of chlorine atoms in the trihalomethane structure were greater than the effects of increasing concentration on nutrient levels. Although, trihalomethanes induced significant physiological changes in some of the measured parameters, no plant mortality was recorded even at the highest concentrations.

Keywords: Antioxidants, Halogenation, Nutrient Concentration, Oxidative Stress, ROS, Tomato

Introduction

The advent of industrialisation gave rise to the increased use of chlorinated and brominated refractory organics worldwide. Traces of these halogenated refractory organics have been found in the terrestrial, hydrological and atmospheric environments (Habartová *et al*., 2013; Li *et al*., 2015; Montelius *et al*., 2015). As such, concerns have been raised on the carcinogenic and mutagenic potential of these halogenated refractory organics (Woo *et al*., 2013; Pi *et al*., 2016).

The terrestrial environment receives a heavy load of these organohalogens; hence, plant exposure to these compounds was imminent. This generated the interest of the earlier scientific community aiming to understand the mechanisms of plant detoxification of organohalogens. Findings from a number of research studies suggested four possible mechanisms for the removal of organohalogens in plants. These include: the rapid sequestration and partitioning of organohalogens to lipophyllic plant cuticles (Moeckel *et al*., 2007); phyto-reduction of organohalogens to less halogenated metabolites (Nzengung and Jeffers, 2001); phyto-oxidation of organohalogens to halo-ethanols

or haloacetic acids (Reichenauer and Germida, 2008) and the assimilation of organohalogens as non-phytotoxic metabolites into plant tissues (Susarla *et al*., 2002). The role of plant oxygenase and dehalogenase enzymes on the isolation, structure and detoxification mechanisms of organohalogens was also reported (Wolfe and Hoehamer, 2003; Van Aken, 2011; San Miguel *et al*., 2013).

The initial contact physiological and morphological response mechanisms of plants to chlorinated organics have been reported (Faure *et al*., 2012; San Miguel *et al*., 2012; Ahammed *et al*., 2013; Guittonny-Philippe *et al*., 2015). Imfeld *et al*. (2009); Doucette *et al*. (2013); and Chen *et al*. (2014), also noted that some plants have the ability to stimulate the removal of chlorinated organic pollutants through plant uptake, phytovolatilization and/or phytodegradation. San Miguel *et al*. (2012) were the first to associate increasing halogenation with increasing oxidative stress, in their study on the physiological response of *Zea Mays* to monochloro-, 1,4-dichloro- and 1,2,4-trichloro-benzenes. However, there are several physical and chemical properties of organohalogens that influence their phytotoxic responses in different plant types.

Trihalomethanes and Plant Response

The trihalomethanes which include Bromodichloromethane ($CHBrCl_2$), bromoform ($CHBr_3$), chloroform ($CHCl_3$) and dibromochloromethane ($CHBr_2Cl$) are the organohalogens classified as persistent bioaccumulative toxins (PBT) (Wong *et al*., 2012). Despite this, they are widespread and detected in most surface water bodies. An evaluation of the molecular structures of the listed trihalomethanes revealed an increasing order of both bromination ($CHCl_3$<$CHBrCl_2$<$CHBr_2Cl$<$CHBr_3$) and chlorination ($CHBr_3$<$CHBr_2Cl$<$CHBrCl_2$<$CHCl_3$). Therefore, the molecular structure of trihalomethanes allows for the evaluation of the physiological response of plants (such as the tomato cultivars) with respect to the halogen reactivity series.

The non-structural content of plants such as phenolic compounds are known to perform a wide variety of functions which include acting as antioxidants (Ainsworth and Gillespie, 2007). Plants contain a wide range of antioxidant molecules and enzymes which are able to maintain intracellular Reactive Oxygen Species (ROS) levels. When exposed to chemical stressors, they undergo oxidative stress which results from the imbalance between the production of ROS and their removal by antioxidants (Phung and Jung, 2015; Talbi *et al*., 2015). Most antioxidant defences which are stimulated by abiotic factors such as light stress and drought in plants, are also stimulated by organic pollutants (Wang *et al*., 2008; Yang *et al*., 2008; Faure *et al*., 2012). They may as well exhibit non-enzymatic antioxidant responses which are measured using indices such as total Phenolic Content (TPC), Ferric Reducing Antioxidant Power (FRAP) and the Oxygen Radical Absorbance Capacity (ORAC). The FRAP assay can measure the antioxidant reducing potential of most biological fluids (Benzie and Strain, 1996), while ORAC assay measures the inhibition of free radical damages to a fluorescent probe by antioxidants. The delay in the degradation of the fluorescent probe may be suggestive of the ability of the pre-existing antioxidants to scavenge the free radicals (Prior *et al*., 2003).

Lipid oxidation for instance, is particularly dangerous because it propagates the production of free radicals through so-called ‘chain reactions. Severe lipid peroxidation leads to the breakdown of membrane function, followed by the disintegration of organelles, oxidation and dysfunction of proteins, DNA and RNA (Gutteridge and Halliwell, 2000; Farmer and Mueller, 2013). The end products of lipid peroxidation may include aldehydic secondary products (malondialdehyde, 4-hydroxy-2-nonenal, 4-hydroxy-2-hexenal and acrolein), which are markers of oxidative stress (Lykkesfeldt, 2007; Farmer and Mueller, 2013). They are easy to measure by using the thiobarbituric acid assay (TBARS) for malondialdehyde (Hodges *et al*., 1999). Common free phytosterols found in plant oils are stigmasterol, β-sitosterols and campesterol. Sterols have been known to exhibit antioxidant properties attributed to their ability to form allylic free radicals that isomerizes to other stable free radicals (Ramadan and Moersel, 2006).

Typical enzymatic responses for the scavenging of ROS in plants include the measurement of the activity of antioxidant enzymes such as Ascorbate Peroxidase (APX), Guaiacol Peroxidase (GPX) and Superoxide Dismutase (SOD) (Jebara *et al*., 2005). Superoxide Dismutase (SOD; EC 1.15.1.1) is an enzyme that catalyses the dismutation of the toxic superoxide (O_2^-) radicals into molecular Oxygen (O_2) and hydrogen peroxide (H_2O_2) (Barondeau *et al*., 2004). Ascorbate peroxidase (APX; EC 1.11.1.11) is the enzyme that detoxifies peroxide (H_2O_2) using ascorbate as a substrate, to transfer electrons to peroxide, producing dehydroascorbate and water as products (Pang and Wang, 2010). Guaiacol peroxidase (GPX; EC 1.11.1.7) is an enzyme in the peroxidase group that detoxifies peroxide (H_2O_2) using guaiacol (2-methoxyphenol) as its reducing substrate (Mika and Lüthje, 2003).

In this study, the effects of the increasing halogenation of trihalomethanes on the phytosterol content of leaf oils was investigated. The tomato cultivars popularly known as “moneymaker” and “star” were selected based on their popularity, high yields and stability in the Southern climate regions. More also, tomato plants was recently reported to demonstrate capacity for the uptake of organochlorine compounds (Zhang *et al*., 2015). The non-enzymatic and enzymatic responses to oxidative stress with the peroxidation of lipids membrane, induced by increasing concentration and halogenation of trihalomethanes in the tomato cultivars were evaluated. The physiological changes in the

tomato cultivars in response to increasing halogenation (a key chemical property) and concentration of trihalomethanes (a key physical property), may be evaluated in terms of the magnitude of the differences in the levels of selected key macro and micro nutrients, different levels in oxidative stress and sterol content in leaf oils. In addition, the effects of increasing halogenation on sterol compositions in plant leaf oils was also investigated in tomato plants.

Materials and Methods

Chemicals

The chemicals used in this study include bromodichloromethane ($CHBrCl_2$), bromoform ($CHBr_3$), chloroform ($CHCl_3$) and dibromochloromethane ($CHBr_2Cl$) which were purchased at Sigma-Aldrich (South Africa) as pure standards (99% of purity). They were all provided directly solubilized in methanol.

Plant Materials, Exposure Time and Treatments

The 2×4×5 factorial experiments were laid out in a completely randomized block design with four replications. The experiment commenced with the design of a 160 pot irrigation system fitted with timers and connected to 20 separate 68 L rough tote plastic reservoirs. Twenty 6 m long square plastic gutters containing 8 pots each were arranged on an elevated table in the greenhouse. The 12.5 cm plastic square pots were filled with Starke-Ayres organic potting soils with a Nitrogen, Phosphorus and Potassium (NPK) ratio of 3:1:5. Each pot was connected to 4 L per hour button dripper extended from 20 mm Low-Density Polyethylene (LDPE) irrigation pipe. Each 68 L plastic reservoir was fitted with 1,400 liters per hour submersible pump. Holes with diameters of 21 mm were made on each lid allowing the LDPE pipes connected to the submersible pumps to pass through. The reservoir lids remained closed to prevent loss of trihalomethanes to evaporation due to its classification as a semi-volatile compound. The irrigation timers were set to water the plants twice a day from 8.00 am-8.30 am and 8.00 pm-8.30 pm approximately giving each pot 4 L of water per day.

To eliminate nutrient stress, de-ionized water pre-treated with granular activated carbon removing other potential organic water pollutants was mixed with Starke-Ayres nutrified K2025 water-soluble fertilizer in the reservoirs for the entire duration of the experiment. Seeds of the *S. lycopersicum* cultivars tomato Money Maker (TMM) and the tomato Star (TS) were purchased from the Starke-Ayres Garden Centre and soaked in nanopure water for one hour to remove all preservatives. The first four pots in each plastic gutter contained seeds of the variety TMM and the last four pots contained seeds of the variety TS. All seeds emerged within 7 days after planting and the weaker seedling was uprooted leaving one seedling per pot 14 days after planting. The treatments began 16 days after emergence when all plants were within the range of 30-35 cm in height. The 160 pot plants were divided into four groups each containing 40 pot plants. The 1st group was exposed to varying Concentrations of Bromodichloromethane ($CHBrCl_2$), the 2nd group to bromoform ($CHBr_3$), the 3rd group to Chloroform ($CHCl_3$) and the last group to dibromochloromethane ($CHBr_2Cl$). In each group, plants in the first gutter continued to receive de-ionized water and the nutrient solution only (control). Plants in gutters 2-5 were treated differentially by varying the concentration of trihalomethanes in their reservoirs. Plants in gutters 2-5 were exposed to concentrations of 2.5 $mg.L^{-1}$, 5.0 $mg.L^{-1}$, 7.5 $mg.L^{-1}$ and 10 $mg.L^{-1}$ of the designated trihalomethane diluted with de-ionized water and nutrient solution. The treatment was terminated after a 30-day exposure of the plants to the trihalomethanes. The leaves of each plant were cut from the stem and immediately dipped in liquid nitrogen and stored in the Nuaire glacier ultra-low minus 86°C freezer (Nuaire, Plymouth, USA) for oxidative stress and lipid profile determination. The rest of the plants were washed with de-ionized water to remove all soil particles, put in paper bags and dried at 65°C for 72 h. to stop enzymatic activity for nutrient level analysis (Havlin *et al*., 2012).

Determination of Plant Mineral Nutrient Concentration

Changes in the primary macronutrients, Nitrogen (N), Phosphorus (P), potassium (K), the three secondary macronutrients, Calcium (Ca), Sulfur (S), magnesium (Mg) and the micronutrients Boron (B), Manganese (Mn), iron (Fe), zinc (Zn), copper (Cu) and sodium (Na) content of plant extracts were investigated. Homogenization of the dried plant material was achieved using the micro plant grinder model FZ102 (Tianjin taisite instruments, Tianjin, China). The homogenized plant powders were weighed and stored in 10 cm long airtight cylindrical glass vials. The air blower was used to clean the grinder before the next sample was homogenized so as to prevent sample mixing. The samples were sent off to Bemlab analytical laboratory where they were ashed at 480°C, shaken and digested with a 50:50 32% HCL/water solution for extraction through filter paper (Campbell and Plank, 1998). The primary and secondary macronutrients with the micronutrients content of the extract were measured with a Varian ICP-OES optical emission spectrometer (Varian Vista-Pro, Springvale, Australia) with the exception of nitrogen. Total nitrogen content of the homogenized plant leaves were determined by total combustion in a Leco N-analyzer (Leco Corp, Henderson, USA) (Sweeney and Rexroad, 1986). Nutrient concentrations in sample extracts were expressed in mg kg^{-1}.

Non-Enzymatic Antioxidant Extraction

The fresh leaf samples were taken from the freezer and lyophilized for 16 h. at minus 86°C using the Vir-tis genesis freeze dryer (SP Industries, Gardiner, NY,

USA). The lyophilized leaf samples were then homogenized into a fine powder using the micro plant grinder and stored in 2 mL vials at minus 40°C. Then 20-25 mg of lyophilized plant samples were weighed into a 15 mL screw-cape tube and 5 mL of 70% methanol in nanopure water was used to extract plant samples as described in (Lapornik *et al*., 2005). The samples were then loaded on the Intelli-mixer RM2 rotator/mixer for 24 h (ELMI Ltd, Latvia) and centrifuged at 4000 rpm for 5 min using the Eppendorf centrifuge 5810 R (Eppendorf, AG, Hamburg, Germany). The supernatant was then used directly for analysis after suitable dilution method was developed.

Determination of Total Polyphenol Content

Total polyphenols in plant extracts were determined by the Folin-Ciocalteu procedure as described by (Meyer *et al*., 1997). Total polyphenols were expressed in $mg.L^{-1}$ Gallic Acid Equivalent (GAE).

Determination of the Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing ability of the sample extracts were determined as described by (Benzie and Strain, 1996). The FRAP reagent was prepared by mixing 30 mL acetate buffer pH 3.6 (300 mM), 3 mL of 2, 4, 6, tripyridyl-s-triazine (TPTZ) (10 mM) prepared in 40 mM hydrochloric acid, 3 mL $FeCL_3$ (20 mM) and 6.6 mL distilled water (dH_20). 10 µl of sample was mixed with 300 µl of FRAP reagent in a 96-well plate and incubated at 37°C for 30 min. The change in absorbance due to the redox reaction occurring was monitored in a Thermo Scientific multiscan spectrum spectrophotometer (Thermo Fisher Scientific, Waltham, USA) at 593nm. FRAP were expressed in $mg.L^{-1}$ Ascorbic Acid Equivalent (AAE).

Determination of the Oxygen Radical Absorbance Capacity (ORAC)

The oxygen radical absorbance capacities of plant extracts were determined as described by (Prior *et al*., 2003). The reaction is initiated by the thermal decomposition at 37°C of the azo-compound 2, 2'-azo-bis (2-amidino-propane) dihydrochloride (AAPH) which serves as the source of peroxyl radicals. Control mixtures are then prepared using Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) solution. All reagents and standards were prepared in phosphate buffer (75 mM, pH 7.4). 138 µL of fluorescence and 12 µL of the sample were mixed in a black Nunclon 96-well plate. 50 µL of AAPH was then added last to the plate and fluorescence readings were taken using a Thermo Scientific fluroskan ascent 2.5 plate reader (Thermo Fisher Scientific, Waltham, USA) at 485 nm and 583 nm respectively. Antioxidant activity was expressed in Trolox equivalents.

Enzyme Extraction and Total Soluble Protein Determinations

Enzyme extraction of plant samples were performed as described by (Zhou *et al*., 2004) with some modifications. The extraction buffer contained 25 mM of 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethane sulfonic acid (HEPES) at pH 7.8, 0.2 mM of Ethylenediaminetetraacetic Acid (EDTA), 2% (w/v) polyvinylpyrrolidone (PVP), 1 mL of triton X-100 and 200 mM of potassium chloride (KCl) in 200 mL of nanopure water. Lyophilized samples weighing 30-35 mg were put into 15 ml screw cap tubes and 2 mL of extraction buffer were added to each tube. The tubes were loaded on the Intelli-mixer RM2 rotator/mixer for a period of 17hrs allowing thorough homogenization. The homogenates were centrifuged for 20 min at 12 000 rpm and the supernatants obtained were used for enzyme analysis. All operations were performed between 0 - - 4°C. An aliquot of each extract was used to determine total protein content using the Pierce Bicinchoninic Acid (BCA) protein assay kit according to the manufacturer's protocol (Scientific, 2011). The BCA protein assay is based on colorimetric detection and quantification of total protein. The working reagent was prepared by combining 50 mL of reagent A with 1 mL of BCA reagent B (50:1, Reagent A: B). 25 µL of the homogenized extract were transferred in triplicates into microplate wells. Then 200 µl of the working reagent was added to each well and plate was mixed thoroughly on a plate shaker for 30 seconds. The plate was covered and incubated at 37°C for 30 min. The plate was then cooled to room temperature and the absorbance was measured 562 nm on a Thermo Scientific multiscan spectrum plate reader. 1 mL ampule of 2 mg/mL of Bovine Serum Albumin (BSA) was sufficient to prepare a set of known protein concentration standards which were assayed alongside the unknown homogenized extract.

Determination of Superoxide Dismutase Activity

Superoxide dismutase activity (SOD; EC 1.15.1.1) of plant extracts were determined as described in (Ellerby and Bredesen, 2000) with some modifications. 170 µL of Diethylenetriaminepentaacetic Acid (DETAPAC) solution were added in triplicates to a visible 96-well plate. Then 12 µL of sample extract was added to each well with 18 µL of SOD buffer (50 mM of $NaPO_4$-buffer at pH 7.4 without triton X-100). Finally, 15 µL of 6-Hydroxydopamine (6-HD) was added to each well and immediately the auto-oxidation was recorded at 490 nm for 4 min with 1 min intervals. The activity of SOD was calculated from a linear calibration curve and SOD concentration was expressed as Units mg^{-1} of protein ($U\ mg^{-1}$).

Determination of Ascorbate Peroxidase Activity

Ascorbate peroxidase (APX) (APX; EC 1.11.1.11) activity in plant extract was determined (Nakano and

Asada, 1981) with some modifications. 180 µL of 50 mM K-PO_4 buffer (pH 7.0), 30 µL of EDTA, 30 µL of 5 mM ascorbate and 30 µL of homogenized plant extract were added in triplicates to an ultraviolet 96-well plate. The reaction was initiated by finally adding 30 µL of 0.1 mM hydrogen peroxide (H_2O_2) to each well and the reduction in ascorbate concentration was read by measuring the absorbance at 290 nm continuously for 180 seconds. The ascorbate oxidized was evaluated based on the extinction coefficient of ascorbate (E) = 2.8 $mM^{-1}cm^{-1}$ and the results expressed as APX units mg^{-1} of protein (U= 1 mM of ascorbate oxidized per min at 25°C).

Determination of Guaiacol Peroxidase Activity

Guaiacol peroxidase (GPX) activity (GPX; EC 1.11.1.7) was determined as described in (Bergmeyer *et al*., 1974) with some modifications. 180 µL of 0.1 M potassium phosphate buffer KH_2PO_4 (pH 7.0), 30 µl of guaiacol and 60 µl of homogenized plant extract were added in triplicates to a visible 96-well plate. The reaction was initiated by adding 30 µl of 0.1 mL of 30% hydrogen peroxide (H_2O_2) in 120 mL of nanopure water to each well and the rate of increase in absorbance at 436 nm was recorded using a linear portion of the curve with GPX activity expressed in Units mg^{-1} of protein (U mg^{-1}).

Lipid Extraction by Methyl-Tert-Butyl-Ether (MTBE)

The accurate profiling of lipidomes were obtained by MTBE extraction which allows faster and cleaner lipid recovery as described by Matyash *et al*. (2008) with some modifications. 80-100 mg of lyophilized plant homogenates were weighed into 15 mL screw cap tubes and 0.77 mL of methanol was added to each tube. The tubes were vortexed for 20 sec and 2.56 mL of MTBE solvent was added to each tube. The tubes were loaded on the intelli-mixer RM2 rotator/mixer for 1 h at room temperature after which phase separation was induced by adding 0.64 mL of nanopure water. After 10 min of incubation at room temp, samples were centrifuged at 13,750 xg for 4 min and the supernatant was removed with 2 mL disposable syringes and filtered using a 0.45 µm syringe filter. The filtrates were collected in 2 mL vials where 1-2 gm of sodium sulfite was added to each vial to remove all traces of water and a known concentration of 10 µL of a mixture of PC 18:0/18:0, PE 17:0/17:0, Phosphatidylinositol (PI) 17:0/17:0, C24:1 β-d-galactosylceramide and diacylglycerol 16:0/18 were used as internal standard.

Determination of Lipid Peroxidation (TBARS)

Lipid peroxidation in the homogenized extract was determined in terms of Malondialdehyde (MDA) content by Thiobarbituric Acid Reaction (TBARS) as described by (Heath and Packer, 1968) with some modifications. 100 µl of homogenized plant extract, 12.5 µl of 4 mM cold Butylated Hydroxytoluene (BHT) in ethanol and 100 µL of 0.2 M of orthophosphoric acid were mixed and vortexed for 10 sec in 2 mL vials. Then 12.5 µL of TBA reagent (0.11 M in 0.1 M NaOH) was added to each vial and vortexed for another 10 sec. The vials were heated at 90°C for one 1 h and cooled on ice for two min with further cooling at room temp for 5 min. 1000 µL of n-butanol with 100 µL of saturated NaCl were added to the vials for better phase separation. The vials were then centrifuged at 12,000 rpm for 2 min at 4°C then 300 µL from each vial was put in triplicates into a visible 96-well plate. MDA equivalent was calculated from the difference in absorbance at 532 and 572 nm using extinction coefficient of 155 mM^{-1} cm^{-1}.

Gas Chromatographic Profiling of Lipidomes

The lipid profile of the plant sample extracts was determined using the Agilent 6890 gas chromatographic mass selective detector (Agilent Technologies, Little Falls, DE, USA). and the method used to profile the lipids was developed from EPA method 551 (Hodgeson and Cohen, 1990). The initial oven temperature at 0.00 min was 70°C to reach a maximum temperature of 320°C at 0.25 min. The total runtime was 43.25 min per sample and the injector front inlet mode was splitless with initial temperature at 280°C and pressure at 60.2 kPa. The purge flow was 30 mL/min with a purge time of 3 min and a total flow of 33.8 mL/min with an injection volume of 1 µL and helium as the carrier gas. The capillary column used was model ZB 274305 (Phenomenex, Torrance, CA, USA) mainly used for semi volatiles with a nominal length of 30.0 m ×250 µm (diameter) ×0.25 µm (film thickness). The GC transfer line was maintained at 280°C with the mass selective detector operated at EMV mode and the resulting EM voltage to be 1800 with a maximum source temperature of 250°C and a solvent delay of 6 min. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the WILEY229 library data of the GC/MS system and published data.

Statistical Analysis of Data

All values reported in this study are means of four replicates. The data were Analyzed by two-way Analysis of Variance (ANOVA) and compared at $P \leq 0.05$ significance level by the Scheffe test using Statistical Analysis System (SAS). Below are tables reporting the significance of the two main factors and their interactions for both experiments.

Results

Effect of Trihalomethane Halogenation and Concentration on Nutrient Levels

After 30 days of single exposure to each trihalomethane chemical and halogenation kept at a constant, six of the twelve nutrients decreased

significantly at P≤0.05 with increasing trihalomethane concentration in both tomato cultivars. These nutrients include Nitrogen (N), potassium (k), Manganese (Mn), Copper (Cu), Zinc (Zn) and Boron (B) (Table 1).

Table 1: Effects of trihalomethane concentration and trihalomethane chemical species on nutrient concentrations of the dry matter yields of tomato plants (30 days treatment data)

Concentration mg.L^{-1}							
Nutrients mg kg^{-1}	Chemical species	0 control	2.5 mg.L^{-1}	5.0 mg.L^{-1}	7.5 mg.L^{-1}	10 mg.L^{-1}	Mean chem. Species
N	$CHBr_2Cl$	3.140	2.993	2.788	2.508	2.023	2.690
	$CHBrCl_2$	1.935	1.925	1.770	1.640	1.433	1.741
	$CHBr_3$	1.985	1.530	1.275	1.143	1.088	1.404
	$CHCl_3$	1.278	1.278	1.085	1.015	0.900	1.111
	Mean Conc.	2.085	1.932	1.730	1.577	1.361	
	CV%			1.93%			
	P≤0.05			*			*
	Interaction			NS			
P	$CHBr_2Cl$	0.940	0.859	0.845	0.790	0.790	0.845
	$CHBrCl_2$	0.888	0.765	0.730	0.613	0.563	0.712
	$CHBr_3$	0.670	0.653	0.603	0.593	0.420	0.588
	$CHCl_3$	0.583	0.568	0.518	0.425	0.395	0.498
	Mean Conc.	0.770	0.711	0.674	0.605	0.542	
	CV%			1.88%			
	P≤0.05			NS			*
	Interaction			*			
K	$CHBR_2Cl$	10.335	9.945	8.045	7.918	7.383	8.725
	$CHBRCl_2$	9.778	7.090	6.603	4.458	4.453	6.476
	$CHBr_3$	5.385	4.285	4.243	3.663	2.340	3.983
	$CHCl_3$	3.745	3.700	2.913	2.650	2.488	3.099
	Mean Conc.	7.311	6.255	5.451	4.672	4.166	
	CV%			2.92%			
	P≤0.05			*			*
	Interaction			*			
Ca	$CHBr_2Cl$	1.553	1.405	1.410	1.508	1.465	1.468
	$CHBrCl_2$	1.355	1.253	1.740	1.760	1.355	1.493
	$CHBr_3$	1.383	1.205	1.795	1.680	1.193	1.451
	$CHCl_3$	1.735	1.220	1.145	1.160	1.320	1.316
	Mean Conc.	1.506	1.271	1.523	1.527	1.333	
	CV%			2.10%			
	P≤0.05			NS			NS
	Interaction			*			
Mg	$CHBr_2Cl$	0.340	0.300	0.285	0.288	0.283	0.299
	$CHBrCl_2$	0.343	0.290	0.333	0.350	0.295	0.322
	$CHBr_3$	0.335	0.270	0.315	0.293	0.223	0.287
	$CHCl_3$	0.323	0.253	0.223	0.260	0.228	0.257
	Mean Conc.	0.335	0.278	0.289	0.298	0.257	
	CV%			1.68%			
	P≤0.05			NS			*
	Interaction			NS			
S	$CHBr_2Cl$	0.698	0.650	0.615	0.550	0.543	0.611
	$CHBrCl_2$	0.570	0.465	0.418	0.400	0.370	0.445
	$CHBr_3$	0.445	0.335	0.303	0.283	0.280	0.329
	$CHCl_3$	0.395	0.326	0.280	0.253	0.243	0.299
	Mean Conc.	0.527	0.444	0.404	0.372	0.359	
	CV%			2.71%			
	P≤0.05			NS			*
	Interaction			NS			
Na	$CHBr_2Cl$	857.50	804.75	1023.75	1016.50	812.25	902.95
	$CHBrCl_2$	1101.0	900.25	1152.75	1253.50	1019.50	1085.40
	$CHBr_3$	1161.2	841.25	1120.00	886.00	780.00	957.70
	$CHCl_3$	911.75	617.75	1032.00	892.25	758.75	842.50
	Mean Conc.	1007.88	791.00	1082.13	1012.06	842.63	

Table 1: Continue

	CV%			2.31%			
	P≤0.05			NS			*
	Interaction			NS			
Mn	$CHBr_2Cl$	61.50	58.50	50.50	42.50	26.25	47.85
	$CHBrCl_2$	44.25	43.00	38.00	31.75	28.75	37.15
	$CHBr_3$	52.50	38.75	36.00	34.75	27.00	37.80
	$CHCl_3$	50.25	42.75	41.00	35.50	33.25	40.55
	Mean Conc.	52.125	45.750	41.375	36.125	28.813	
	CV%			2.36%			
	P≤0.05			*			*
	Interaction			*			
Fe	$CHBr_2Cl$	1224.75	789.50	1251.25	1105.50	1105.50	1095.30
	$CHBrCl_2$	1603.50	847.50	507.25	966.25	971.50	979.20
	$CHBr_3$	3040.25	931.00	2352.75	1429.00	1492.50	1849.10
	$CHCl_3$	988.75	1465.25	3072.75	2634.00	2387.50	2109.65
	Mean Conc.	1714.31	1008.31	1796.00	1533.69	1489.25	
	CV%			10.79%			
	P≤0.05			NS			NS
	Interaction			NS			
Cu	$CHBr_2Cl$	9.25	8.75	8.25	8.00	7.50	8.35
	$CHBrCl_2$	7.75	6.75	6.50	6.25	6.00	6.65
	$CHBr_3$	8.50	7.75	5.75	5.25	4.75	6.40
	$CHCl_3$	7.00	6.75	6.50	5.25	4.00	5.90
	Mean Conc.	8.125	7.500	6.750	6.188	5.563	
	CV%			2.35%			
	P≤0.05			*			*
	Interaction			NS			
Zn	$CHBr_2Cl$	217.25	176.00	166.75	155.00	145.25	172.05
	$CHBrCl_2$	188.25	182.00	132.25	126.75	111.00	148.05
	$CHBr_3$	197.25	183.25	174.00	145.25	108.75	161.70
	$CHCl_3$	154.25	146.50	140.75	134.25	89.50	133.05
	Mean Conc.	189.25	171.875	153.438	140.313	113.625	
	CV%			2.85%			
	P≤0.05			*			*
	Interaction			NS			
B	$CHBr_2Cl$	40.00	36.00	33.50	32.50	31.75	34.75
	$CHBrCl_2$	39.75	35.75	31.25	27.25	27.25	32.25
	$CHBr_3$	31.25	25.50	25.00	22.75	20.75	25.05
	$CHCl_3$	25.50	23.00	23.00	20.00	19.25	22.15
	Mean Conc.	34.125	30.063	28.188	25.625	24.75	
	CV%			1.68%			
	P≤0.05			*			*
	Interaction			NS			

Values presented are the mean values calculated from the sum of both TMM = tomato moneymaker and TS = tomato star means divided by n = 2 each with n = 4 replicates, * = significance at P≤0.05 respectively; NS = not significant, $CHBrCl_2$ = Bromodichloromethane, $CHBr_3$ = Bromoform, $CHCl_3$ = Chloroform, $CHBr_2Cl$ = Dibromochloromethane

The average percentage decrease in nutrient levels from control plants to those exposed to 10 $mg.L^{-1}$ of trihalomethanes were N (34.72%), K (43.02%), Mn (44.72%), Cu (31.53%), Zn (39.96%) and B (27.47%). The nutrient levels of N, P, K, S, Cu, Zn and B in plant dry matter decreased significantly at P≤0.05 with increasing chlorine of the trihalomethane molecules (except for $CHBr_3$) at a constant concentration of 10 $mg.L^{-1}$ in the order of $CHBr_2Cl$>$CHBrCl_2$>$CHBr_3$>$CHCl_3$ in the tomato cultivars (Table 2).

It should be noted that the nutrients Cu and Zn displayed these trends only in the TMM cultivar. The average percentage decrease in nutrient levels from the effects of mono-chlorinated to the tri-chlorinated trihalomethanes was N (58.70%), P (41.07%), K (64.48%), S (51.06%), Cu (29.34%), Zn (22.67%) and B (36.26%).

The nutrients levels affected by both the effects of increasing halogenation and concentration of trihalomethanes in both tomato cultivars tested can be seen in Fig. 1.

A Comparison between the effects of increasing trihalomethane concentration and the effects of increasing chlorination on the percentage decrease in nutrient levels can be seen in Fig. 2. The effects of increasing halogenation induced a greater decrease on nutrient levels with the exception of copper and zinc where the effects of increasing trihalomethane dose appear to be greater.

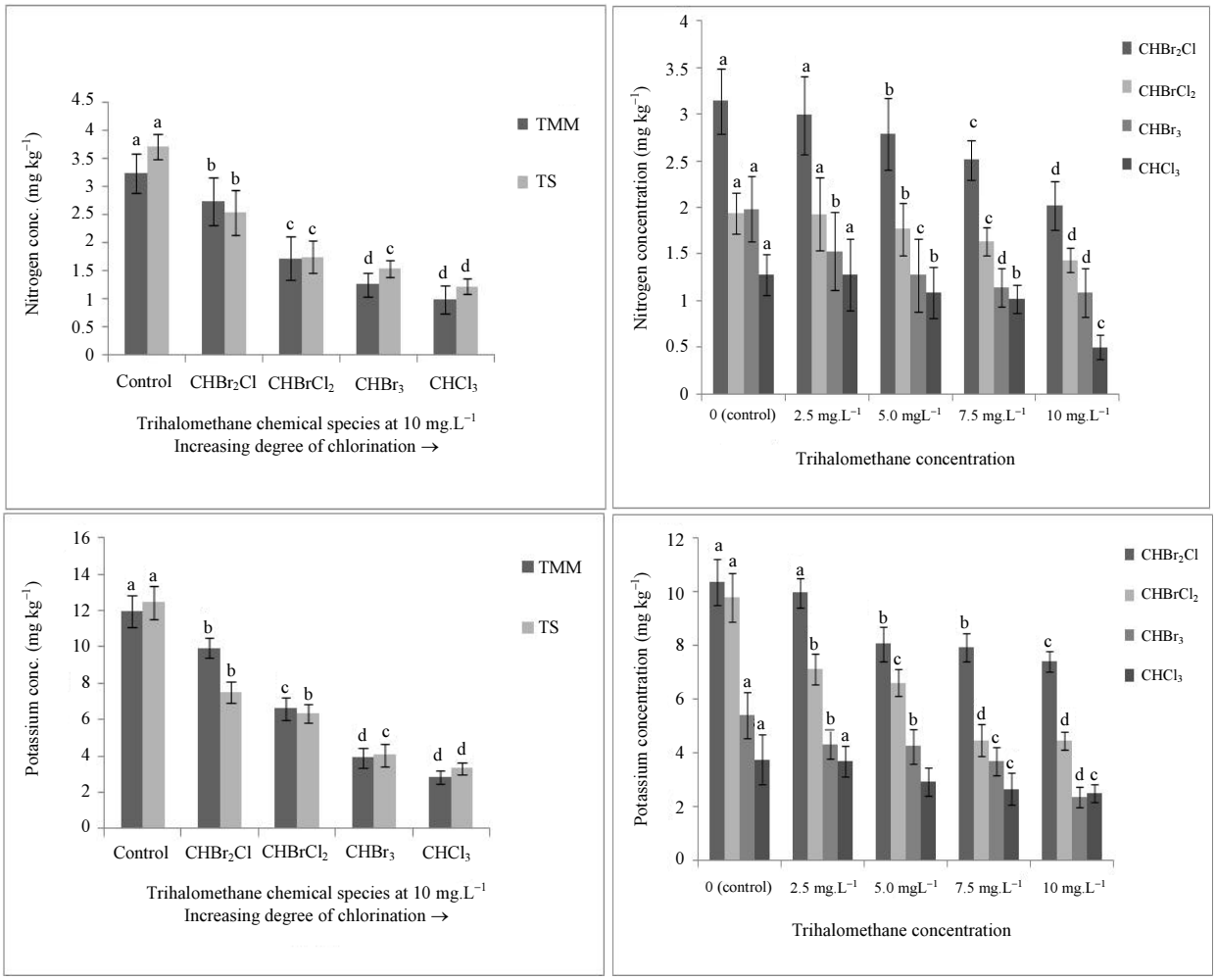
Table 2: Effects of trihalomethane chemical species at a concentration of 10 mgL^{-1} on plants nutrient concentration of the dry matter yields of the tomato cultivars (30 days treatment data)

Chemical species Nutrients mg kg^{-1}	Plant Varieties	$CHBr_2Cl$	$CHBrCl_2$	$CHBr_3$	$CHCl_3$	Mean p varieties
N	TMM	2.840	1.727	1.263	0.993	1.706
	TS	2.540	1.754	1.545	1.229	1.767
	Mean C varieties	2.690	1.741	1.404	1.111	
	CV%			1.93%		
	P≤0.05			*		NS
	Interaction			*		
P	TMM	0.836	0.710	0.543	0.461	0.638
	TS	0.853	0.713	0.632	0.534	0.683
	Mean C varieties	0.845	0.712	0.588	0.498	
	CV%			1.88%		
	P≤0.05			*		NS
	Interaction			NS		
K	TMM	9.951	6.617	3.906	2.855	5.832
	TS	7.499	6.335	4.060	3.343	5.309
	Mean C varieties	8.725	6.476	3.983	3.099	
	CV%			2.92%		
	P≤0.05			*		NS
	Interaction			*		
Ca	TMM	1.525	1.546	1.493	1.312	1.469
	TS	1.411	1.439	1.409	1.320	1.395
	Mean C varieties	1.468	1.493	1.451	1.316	
	CV%			2.10%		
	P≤0.05			NS		NS
	Interaction			NS		
Mg	TMM	0.285	0.323	0.287	0.248	0.286
	TS	0.313	0.321	0.287	0.266	0.297
	Mean C varieties	0.299	0.322	0.287	0.257	
	CV%			1.68%		
	P≤0.05			NS		NS
	Interaction			NS		
S	TMM	0.656	0.477	0.317	0.282	0.433
	TS	0.566	0.412	0.341	0.315	0.409
	Mean C varieties	0.611	0.445	0.329	0.299	
	CV%			2.71%		
	P≤0.05			*		NS
	Interaction			NS		
Na	TMM	946.2	1248.9	932.6	802.0	982.425
	TS	859.7	921.9	982.8	883.0	911.850
	Mean C varieties	902.95	1085.40	957.70	842.50	
	CV%			2.31%		
	P≤0.05			NS		NS
	Interaction			*		
Mn	TMM	56.8	40.8	35.5	38.7	42.950
	TS	38.9	33.5	40.1	42.4	38.725
	Mean C varieties	47.85	37.15	37.80	40.55	
	CV%			2.36%		
	P≤0.05			NS		NS
	Interaction			*		
Fe	TMM	1081.4	1192.0	1148.8	1779.3	1300.38
	TS	1109.2	766.4	2549.4	2440.0	1716.25
	Mean C varieties	1095.30	979.20	1849.10	2109.65	
	CV%			10.79%		
	P≤0.05			NS		NS
	Interaction			NS		
Cu	TMM	8.9	7.2	5.8	4.8	6.675

Table 2: Continue

	TS	7.8	6.1	7.0	7.0	6.975
	Mean C varieties	8.35	6.65	6.40	5.90	
	CV%			2.35%		
	P≤0.05			*		NS
	Interaction			*		
Zn	TMM	193.8	160.5	147.3	126.0	156.900
	TS	150.3	135.6	176.1	140.1	150.525
	Mean C varieties	172.05	148.05	161.70	133.05	
	CV%			2.85%		
	P≤0.05			*		NS
	Interaction			*		
B	TMM	37.6	33.6	26.1	22.2	29.875
	TS	31.9	30.9	24.0	22.1	27.225
	Mean C varieties	34.75	32.25	25.05	22.15	
	CV%			1.68%		
	P≤0.05			*		*
	Interaction			NS		

Values presented are means of n = 4 replicates, * = effect of trihalomethanes significant at P≤0.05 respectively; NS = not significant, TMM = tomato moneymaker, TS = tomato star, $CHBrCl_2$ = Bromodichloromethane, $CHBr_3$ = Bromoform, $CHCl_3$ = Chloroform, $CHBr_2Cl$ = Dibromochloromethane



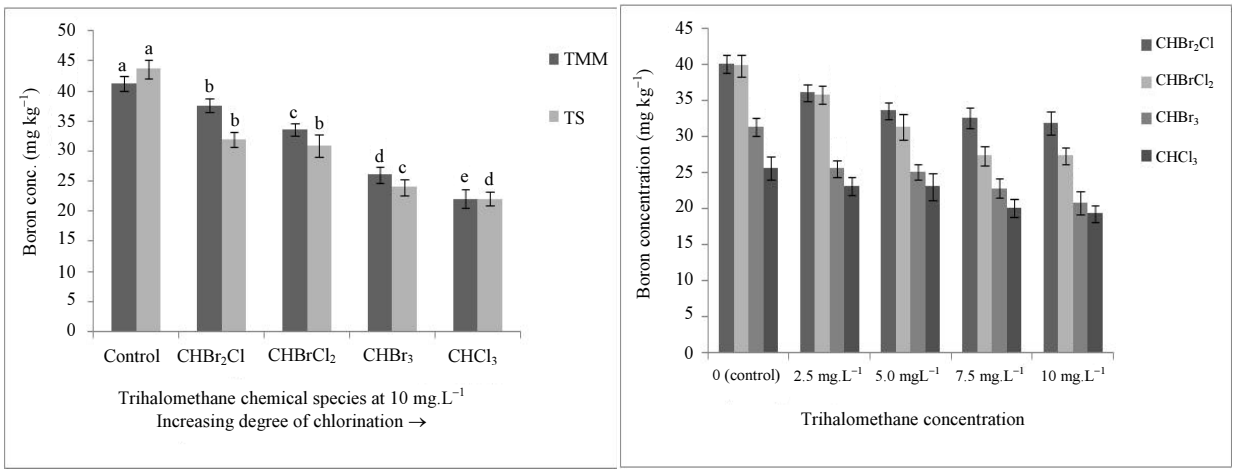


Fig. 1: Effects of increasing trihalomethane halogenation and concentration on the nutrient levels of the dry matter yields of both tomato cultivars. Control plants were exposed to deionized water containing plant nutrients only. Means followed by similar letter in a column are not significantly different from each other at P≤0.05 according to FSD. Vertical bars represent standard error of means (n = 4). Mean values from the same cultivar and the same chemical treatment were compared with each other only; Values presented in the concentration graphs are the mean values calculated from the sum of both TMM = tomato moneymaker and TS = tomato star means divided by n = 2 each with n = 4 replicates

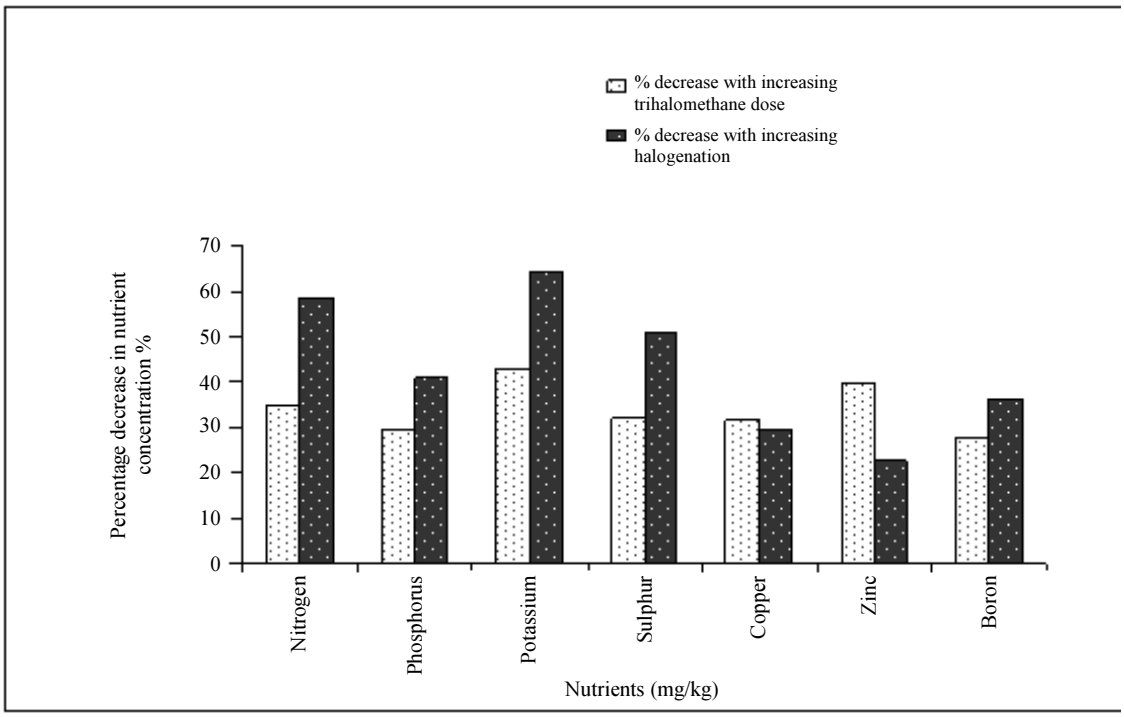


Fig. 2: Comparison between the effects of increasing trihalomethane dose and increasing halogenation on nutrient concentration of the dry matter of tomato plants after a 30 day exposure

Effects of Trihalomethane Halogenation And Concentration on Non-Enzymatic and Enzymatic Biomarkers of Oxidative Stress

Antioxidant parameters that increased significantly at P≤0.05 with increasing trihalomethane concentration and halogenation kept at a constant include total polyphenols, Ferric Reducing Antioxidant Power (FRAP), Oxygen Radical Absorbance Capacity (ORAC), peroxidase enzyme activities (APX and GPX) and thiobarbituric acid reacting substances (TBARS) in both tomato cultivars (Table 3).

Table 3: Effects of trihalomethane concentration and trihalomethane chemical species on non-enzymatic and enzymatic biomarkers of oxidative stress in lyophilized leaf tissues of tomato plants (30 days treatment data)

	Trihalomethane Concentration $mg.L^{-1}$						
	Chemical species	0 control	2.5 $mg.L^{-1}$	5.0 $mg.L^{-1}$	7.5 $mg.L^{-1}$	10 $mg.L^{-1}$	Mean chem. species
Polyphenols	$CHBr_2Cl$	2.361	3.665	3.907	3.958	4.285	3.635
$mg.L^{-1}$(GAE)	$CHBrCl_2$	5.284	6.805	6.875	7.491	9.103	7.112
	$CHBr_3$	9.590	11.625	11.824	12.216	15.200	12.091
	$CHCl_3$	15.096	15.324	16.363	17.008	18.224	16.403
	Mean Conc.	8.083	9.355	9.742	10.168	11.703	
	CV%		3.05%				
	P≤0.05		*				*
	Interaction		NS				
FRAP	$CHBr_2Cl$	14.707	21.743	21.971	22.086	23.348	20.771
$mg.L^{-1}$(AAE)	$CHBrCl_2$	20.777	26.567	33.505	34.737	37.131	30.543
	$CHBr_3$	31.537	32.181	34.616	42.827	48.107	37.853
	$CHCl_3$	36.824	39.927	40.502	44.988	52.903	43.029
	Mean Conc.	25.961	30.105	32.649	36.160	40.372	
	CV%		2.93%				
	P≤0.05		*				*
	Interaction		*				
ORAC	$CHBr_2Cl$	3.718	6.853	7.189	7.645	8.002	6.681
mM trolox	$CHBrCl_2$	7.565	10.912	11.100	11.275	12.404	10.651
	$CHBr_3$	7.117	7.133	7.932	9.279	11.238	8.540
	$CHCl_3$	18.852	21.680	21.971	28.717	32.102	24.665
	Mean Conc.	9.313	9.931	12.048	14.229	15.937	
	CV%		3.28%				
	P≤0.05		*				*
	Interaction		*				
APX activity	$CHBr_2Cl$	0.001	0.004	0.005	0.006	0.007	0.004
U mg^{-1}	$CHBrCl_2$	0.007	0.008	0.011	0.020	0.026	0.014
	$CHBr_3$	0.002	0.002	0.002	0.003	0.004	0.002
	$CHCl_3$	0.012	0.022	0.023	0.031	0.076	0.033
	Mean Conc.	0.006	0.009	0.010	0.011	0.028	
	CV%		24.54%				
	P≤0.05		*				*
	Interaction		NS				
GPX activity	$CHBr_2Cl$	0.005	0.009	0.011	0.012	0.014	0.010
U mg^{-1}	$CHBrCl_2$	0.006	0.010	0.013	0.019	0.035	0.017
	$CHBr_3$	0.004	0.004	0.005	0.006	0.007	0.005
	$CHCl_3$	0.009	0.019	0.019	0.037	0.039	0.025
	Mean Conc.	0.006	0.011	0.012	0.019	0.024	
	CV%		16.76%				
	P≤0.05		*				*
	Interaction		NS				
SOD activity	$CHBr_2Cl$	0.037	0.040	0.026	0.019	0.025	0.029
U mg^{-1}	$CHBrCl_2$	0.024	0.007	0.002	0.010	0.013	0.011
	$CHBr_3$	0.018	0.016	0.010	0.027	0.011	0.016
	$CHCl_3$	0.056	0.036	0.012	0.051	0.079	0.047
	Mean Conc.	0.034	0.025	0.012	0.027	0.032	
	CV%		7.15%				
	P≤0.05		NS				*
	Interaction		*				
BCA	$CHBr_2Cl$	101.67	87.771	72.176	67.668	52.380	76.320
mg/mL	$CHBrCl_2$	126.629	115.589	114.210	80.524	51.752	97.741
	$CHBr_3$	184.608	209.798	243.993	302.665	311.946	250.602
	$CHCl_3$	102.078	86.251	85.448	79.020	38.248	78.209
	Mean Conc.	128.746	124.852	128.957	132.469	113.582	
	CV%		4.18%				
	P≤0.05		NS				*
	Interaction		*				
TBARS	$CHBr_2Cl$	0.838	0.988	1.547	2.925	3.049	1.869
$mM^{-1}cm^{-1}$	$CHBrCl_2$	2.191	2.416	3.403	3.947	3.983	3.188
	$CHBr_3$	1.761	2.100	3.058	3.442	4.034	2.879
	$CHCl_3$	1.393	1.819	2.516	3.132	3.280	2.428

Table 3: Continue

	Mean Conc.	1.546	1.831	2.631	3.362	3.587	
	CV%		3.55%				
	P≤0.05		*				*
	Interaction		*				

Values presented are the mean values calculated from the sum of both TMM = tomato moneymaker and TS = tomato star means divided by n = 2 each with n = 4 replicates, * = significance at P≤0.05 respectively; NS = not significant, $CHBrCl_2$ = Bromodichloromethane, $CHBr_3$ = Bromoform, $CHCl_3$ = Chloroform, $CHBr_2Cl$ = Dibromochloromethane FRAP = Ferric reducing ability of plasma, ORAC = Oxygen radical absorbance capacity, APx = Ascorbate peroxidase, GPx = Guaiacol peroxidase, SOD = Superoxide dismutase, BCA = pierce bicinchoninic acid protein assay, TBARS = Thiobarbituric acid reaction for general lipid peroxidation

Table 4: Effects of trihalomethane chemical species at 10 mgL^{-1} on non-enzymatic and enzymatic biomarkers of oxidative stress in lyophilized leaf tissues of the tomato cultivars (30 days treatment data)

Chemical species						
	Plant Varieties	$CHBr_2Cl$	$CHBrCl_2$	$CHBr_3$	$CHCl_3$	Mean p variety
Polyphenols	TMM	3.832	6.965	12.435	16.755	9.997
$mg.L^{-1}$ (GAE)	TS	3.439	7.258	11.747	16.052	9.624
	Mean C varieties	3.635	7.112	12.091	16.40	
	CV%		3.05%			
	P≤0.05		*			NS
	Interaction		NS			
FRAP	TMM	22.172	31.169	40.241	43.801	34.346
$mg.L^{-1}$ (AAE)	TS	19.370	29.918	35.465	42.257	31.753
	Mean C varieties	20.771	30.543	37.853	43.029	
	CV%		2.93%			
	P≤0.05		*			NS
	Interaction		NS			
ORAC	TMM	7.175	10.726	7.755	26.020	12.919
mM trolox	TS	6.188	10.576	9.325	23.309	12.350
	Mean C varieties	6.681	10.651	8.540	24.665	
	CV%		3.28%			
	P≤0.05		*			NS
	Interaction		NS			
APX activity	TMM	0.005	0.016	0.002	0.024	0.012
U mg^{-1}	TS	0.004	0.012	0.002	0.042	0.015
	Mean C varieties	0.004	0.014	0.002	0.033	
	CV%		24.54%			
	P≤0.05		*			NS
	Interaction		NS			
GPX activity	TMM	0.010	0.021	0.005	0.026	0.016
U mg^{-1}	TS	0.011	0.013	0.005	0.023	0.013
	Mean C varieties	0.010	0.017	0.005	0.025	
	CV%		16.76%			
	P≤0.05		*			NS
	Interaction		NS			
SOD activity	TMM	0.031	0.012	0.020	0.044	0.027
U mg^{-1}	TS	0.028	0.010	0.013	0.050	0.025
	Mean C varieties	0.029	0.011	0.016	0.047	
	CV%		7.15%			
	P≤0.05		NS			NS
	Interaction		NS			
BCA	TMM	76.823	113.899	283.550	70.215	136.122
mg/mL	TS	75.817	81.583	217.654	86.203	115.314
	Mean C varieties	76.320	97.741	250.602	78.209	
	CV%		4.18%			
	P≤0.05		NS			*
	Interaction		*			
TBARS	TMM	1.872	3.220	3.005	2.329	2.607
$mM^{-1}cm^{-1}$	TS	1.867	3.156	2.753	2.526	2.576
	Mean C varieties	1.869	3.188	2.879	2.428	
	CV%		3.55%			
	P≤0.05		NS			NS
	Interaction		NS			

Values presented are means of n = 4 replicates, * = significance at P≤0.05 respectively; NS = not significant, TMM = tomato moneymaker, TS = tomato star. $CHBrCl_2$ = Bromodichloromethane, $CHBr_3$ = Bromoform, $CHCl_3$ = Chloroform, $CHBr_2Cl$ = Dibromochloromethane FRAP = Ferric reducing ability of plasma, ORAC = Oxygen radical absorbance capacity, APx = Ascorbate peroxidase, GPx = Guaiacol peroxidase, SOD = Superoxide dismutase, BCA = pierce bicinchoninic acid protein assay, TBARS = Thiobarbituric acid reaction for general lipid peroxidation

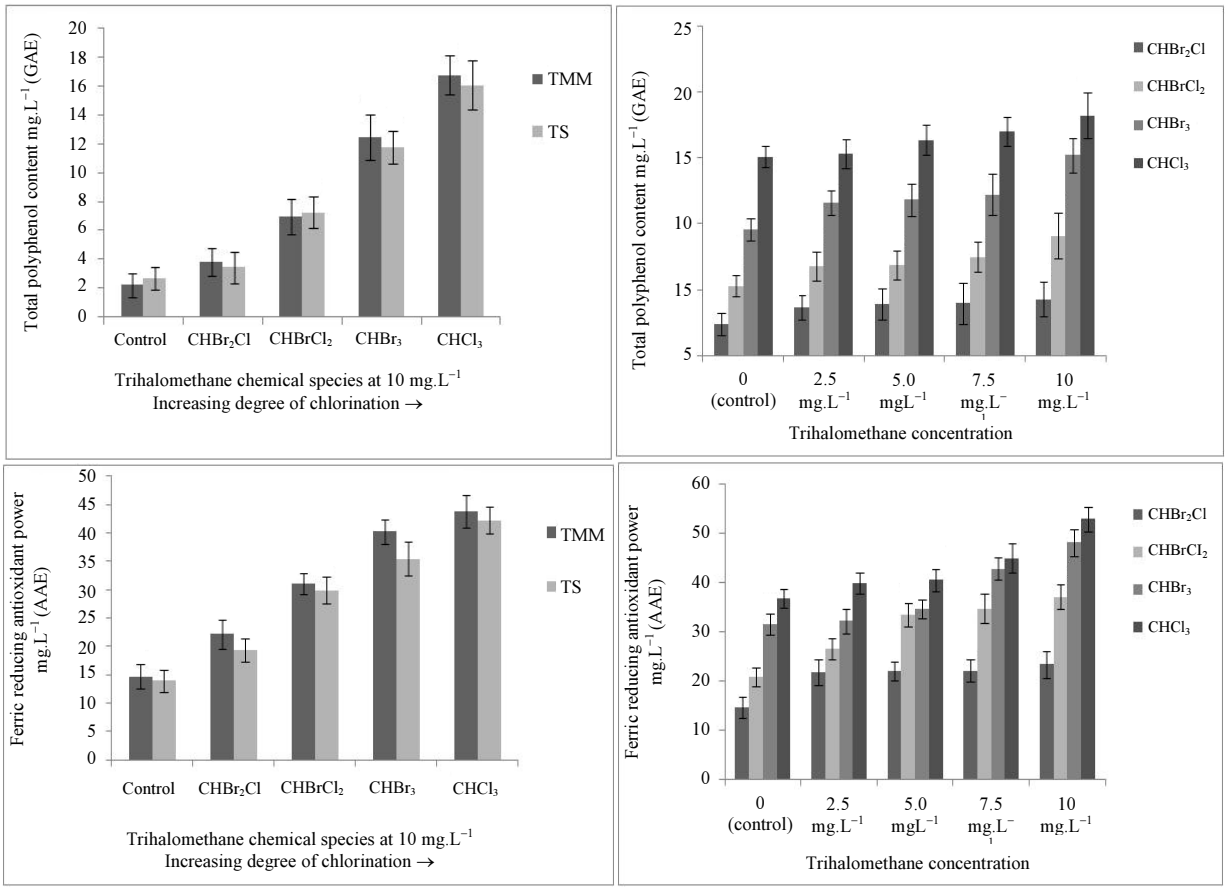
The average percentage increase in antioxidant parameters measured from control plants to those exposed to 10 $mg.L^{-1}$ of trihalomethanes were total polyphenols (44.79%), FRAP (55.51%), ORAC (71.13%), ascorbate peroxidase (APX) activity (366.67%), guaiacol peroxidase (GPX) activity (300%) and TBARS (132.02%). The antioxidant parameters that increased significantly at $P \leq 0.05$ as a response to an increasing chlorination order (except for $CHBr_3$) of $CHBr_2Cl < CHBrCl_2 < CHBr_3 < CHCl_3$ kept at a constant concentration of 10 $mg.L^{-1}$ in both tomato cultivars include total polyphenols and the Ferric Reducing Antioxidant Power (FRAP). The Oxygen Radical Absorbance Capacity (ORAC) increased significantly at $P \leq 0.05$ with a slightly different chlorination order of $CHBr_2Cl < CHBr_3 < CHBrCl_2 < CHCl_3$ in both tomato cultivars, while the activities of both Ascorbate Peroxidase (APX) and Guaiacol Peroxidase (GPX) increased significantly at $P \leq 0.05$ with the chlorination order of $CHBr_3 < CHBr_2Cl < CHBrCl_2 < CHCl_3$ in both tomato cultivars (Table 4).

The percentage increase in antioxidant parameters from the effects of mono-chlorinated to the tri-chlorinated trihalomethanes were in total phenolic content (351.25%), FRAP (107.16%), ORAC (269.18%), APX activity (725%) and GPX activity (150%). There was no response to increasing trihalomethane concentration or increasing halogenation observed in the activity of Superoxide Dismutase (SOD). The antioxidant parameters that were significantly affected at $P \leq 0.05$ by both increasing chlorination and concentration of trihalomethanes in both tomato cultivars tested can be seen in Fig. 3.

A comparison between the effects of increasing trihalomethane concentration and the effects of increasing chlorination on the percentage increase in antioxidant parameters can be seen in Fig. 4. The effects of increasing halogenation induced a greater response in all antioxidant parameters with the exception of the activities of guaiacol peroxidase that appear to be more sensitive to the effects of increasing trihalomethane concentration.

Effects of Trihalomethane Halogenation on the Sterol Content of the Tomato Leaf Oils

In general, the sterol content of the tomato leaf oils on exposure to the trihalomethanes increased with an increasing degree of chlorination with the exception of β-sitosterol which decreased with increasing chlorination. There was no significant difference at $P \leq 0.05$ in stigmasterol levels in leaf oils of control plants and the plants exposed to the mono-chlorinated trihalomethanes in both tomato cultivars as seen in Fig. 5.



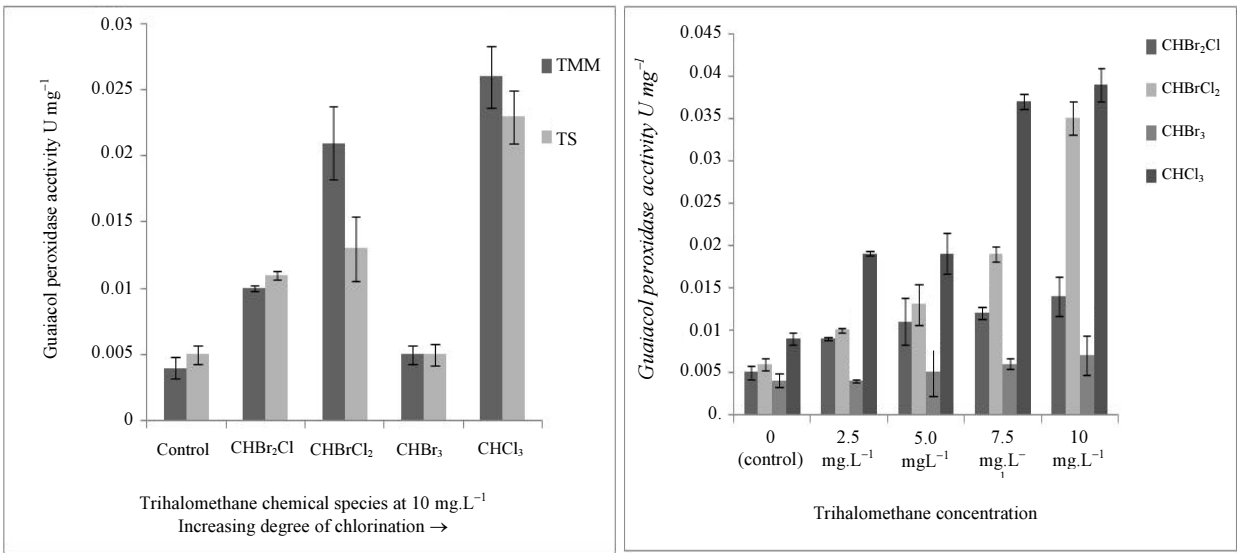


Fig. 3: Effects of the increasing order of trihalomethane chlorination and concentration on the antioxidant response of the both tomato cultivars. Control plants were exposed to deionized water containing plant nutrients only. Means followed by similar letter in a column are not significantly different from each other at P≤0.05 according to FSD. Vertical bars represent standard error of means (n = 4). Mean values from the same cultivar and the same chemical treatment were compared with each other only; Values presented in the concentration graphs are the mean values calculated from the sum of both TMM = tomato moneymaker and TS = tomato star means divided by n = 2 each with n = 4 replicates

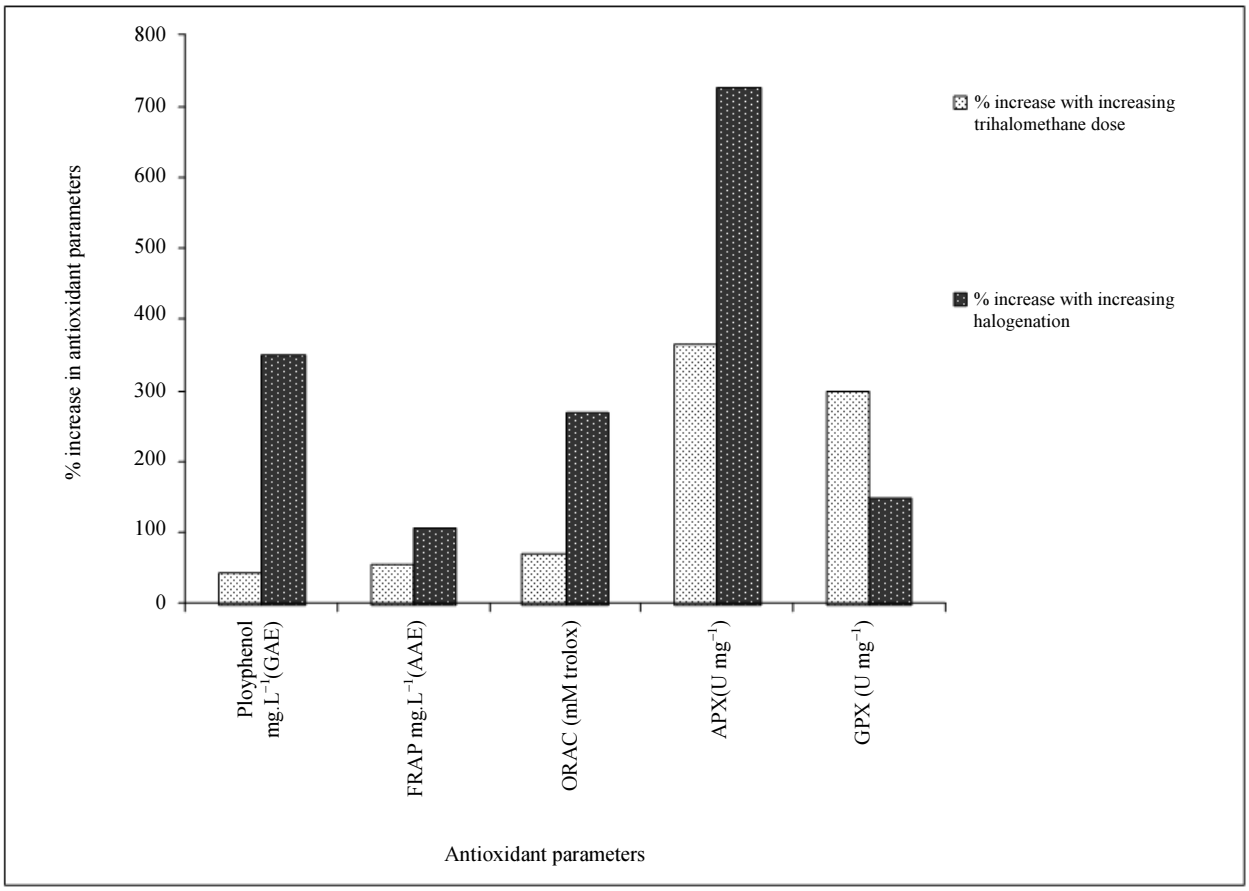


Fig. 4: Comparison between the effects of increasing trihalomethane dose and increasing halogenation on the antioxidant response of tomato plants after a 30 day exposure

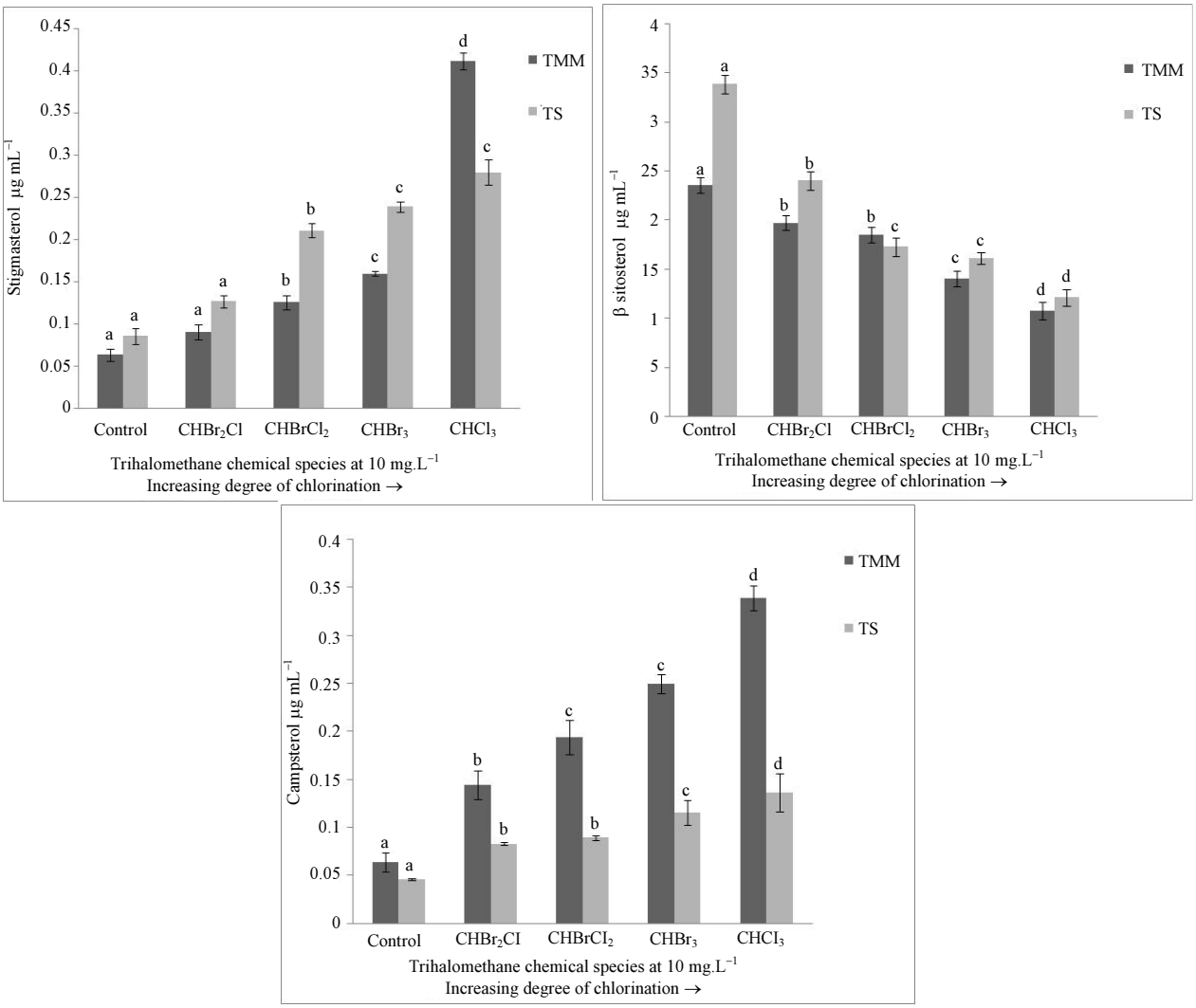


Fig. 5: Effects of the increasing order of trihalomethane chlorination on the sterol content of leaf oils in both tomato cultivars. Control plants were exposed to deionized water containing plant nutrients only. Means followed by a similar letter in a column are not significantly different from each other at P≤0.05 according to FSD. Vertical bars represent standard error of means (n = 4). Mean values from the same cultivar were compared with each other only

Discussion

The degree of the phytotoxic effect of a refractory organic on vascular plants in principle depends on the physical and chemical properties of the compound. Hence, subtle changes affecting crop yield as a result of chemical exposure can be monitored through biochemical parameters in plant physiology. The physical and chemical properties of halogenated organics in nature induce phytotoxic effects simultaneously on plants. Hence, it is often difficult to elucidate which physical or chemical property of a compound is responsible for the greater phytotoxic effect. To our knowledge, few studies have been carried out in this field mainly because it is not feasible for chemical manufacturers to test plant sensitivity of all products on every species and cultivar. However, trihalomethanes are commonly detected in all surface waters as a result of chlorine disinfection during water treatment (Al-Otoum *et al*., 2016).

In this study, a significant decrease at P≤0.05 in the nutrient levels of nitrogen, potassium and boron was induced by both the physical property of increasing concentration and the chemical property of the increasing number of chlorine atoms in trihalomethanes on both tomato cultivars (Fig. 1). Nitrogen deficiency in plants as been associated with loss of plant biomass, leaf area and leaf chlorophyll content (Zhao *et al*., 2005). Potassium plays a significant role in alleviating the detrimental effects of oxidative stress in plants but its

deficiency causes increased loss in photosynthetic CO_2 fixation and impaired partitioning and utilization of photosynthates (Cakmak, 2005). Boron deficiency in plants has been known to affect water uptake by inhibiting root and shoot growth, affect the photosynthetic mechanism and induce adverse metabolic pathways in leaves (Wimmer and Eichert, 2013). These symptoms of boron deficiency were considered as secondary effects of boron induced changes in membrane permeability (Pilbeam and Kirkby, 1983).

In general, the chemical property of the increasing number of chlorine atoms in trihalomethanes induced a greater decrease in nutrient levels when compared with the effect of increasing concentration (Fig. 2). A possible theory suggests that during the detoxification of chlorinated compounds by peroxidases in plants (Park *et al*., 2000; Talano *et al*., 2012), mono-oxygenase enzymes actively participate in the dehalogenation process leading to the formation of free Cl^- radicals that may damage nutrient transport proteins in the tomato cultivars. However, the effect of concentration was greater on the decrease in nutrient levels of the divalent micronutrients Cu^{2+} and Zn^{2+} when compared with the effect of increasing halogenation in both tomato cultivars (Fig. 2). In the past, research has revealed that plant genomes contain several gene families specifically involved in the transport of divalent micro-nutrients (Mäser *et al*., 2001). This suggests that the mechanism of uptake and translocation of divalent cations in plants may differ from other nutrient categories and hence the effects of chemical exposure may also differ.

The antioxidant parameters that increased significantly in response to the effect of increasing concentration and the effect of the increasing halogenation of the trihalomethanes in both tomato cultivars include the total polyphenol content, the Ferric Reducing Antioxidant Power (FRAP) and the activity of Guaiacol Peroxidase (GPX) (Fig. 3). Plants are known to increase their phenolic content as a non-enzymatic response to free radical production from oxidative stress (Nogués *et al*., 2014). The exposure of plants to chlorinated organic compounds has been reported to induce the production of the peroxide H_2O_2 free radical (Menone *et al*., 2008; Michalowicz and Duda, 2009; San Miguel *et al*., 2012). The FRAP assay measures the total antioxidant power of biological fluids and as such most non-enzymatic activity (scavenging of free radicals) is mediated by redox reactions. The antioxidant activity of plant phenols mainly due to their redox potential has been extensively documented (Agati *et al*., 2012; Dangles, 2012; Martín *et al*., 2015). Hence, strong correlations have been reported between the increase in plant phenols and the FRAP values (Reyes-Carmona *et al*., 2005; Bunea *et al*., 2011; Ma *et al*., 2011). The increase in the activity of the enzyme guaiacol peroxidase by both the effect of the increasing trihalomethane concentration and halogenation in both tomato cultivars further confirms the production of the peroxide H_2O_2 radical due to chemical exposure. Guaiacol peroxidase, located in the vacuole, cytosol, cell wall and apoplast forms part of the Peroxidase (POX) complex of enzymes that detoxifies peroxide (H_2O_2) using guaiacol (2-methoxyphenol) as its reducing substrate (Mika and Lüthje, 2003).

In general, the effect of the increasing degree of chlorination in trihalomethanes induced a greater measure of oxidative stress in the tomato cultivars when compared to the effect of increasing concentration (Fig. 4). However, the peroxidase enzymes responded differently with the activity of Guaiacol Peroxidase (GPX) being more sensitive to increasing trihalomethane concentration as opposed to halogenation. The activity of the Ascorbate Peroxidase (APX) enzymes, on the other hand, proved more sensitive to increasing trihalomethane halogenation. Ascorbate Peroxidase (APX) enzyme is the most important in H_2O_2 detoxification to water using ascorbate as substrate and has been reported to be the first line of defense and major H2O2 scavenger in plants (Jebara *et al*., 2005). Many studies report the increase in the activity of the Peroxidase (POX) enzymes to environmental stressors (Sulmon *et al*., 2015; Czégény *et al*., 2016) but our findings suggest that their sensitivities may vary based on the properties of a chemical stressor.

The effects of the increasing chlorination of trihalomethanes resulted in an increase in the levels of stigmasterol and campesterol in leaf oils with the exception of β-sitosterol where a decrease was observed Fig. 5). The decrease in β-sitosterol has been reported to increase the permeability of plasma membrane for ions and SH-containing molecules (Valitova *et al*., 2011). This alteration in membrane function as a result of β-sitosterol depletion may be a mechanism in the tomato cultivars to combat decreasing nutrient levels by increasing membrane permeability for nutrients. However, many studies report the increase in phytosterol levels in plants under environmental and chemical stressors (Briceño *et al*., 2012; Ruggiero *et al*., 2013; Kumar *et al*., 2015).

In this study, the magnitude of the physiological response of tomato cultivars to the increasing degree of chlorination in trihalomethanes was greater in most parameters observed. It must be stated that at the concentration levels and chemical properties tested, no plant death was recorded in these experiments. While the limits for most chlorinated organics in international guidelines are concentration based, attention should be given to some chemical properties of the chlorinated organics in assessing potential phytotoxicity.

Conclusion

Data from the current study reports for the first time the probability of a halogenated compound such as the

trihalomethanes displaying a disruptive effect on nutrient levels, inducing oxidative stress and altering phytosterol levels in tomato cultivars. These physiological alterations in the tomato cultivars due to chemical exposure appear to be more a function of the degree of chlorination rather than their concentration levels. The theory proposed in this study suggests that the increase in the amount of free radical $Cl^{\cdot}$ ions released during the dehalogenation processes in the tomato cultivars may cause more physiological harm than an increase in concentration. The physiological responses to the increasing trihalomethane concentration and halogenation were non-fatal since no plant death was recorded even at the highest concentrations tested.

Author's Contributions

Babatunde C. Akande: Researcher.

Olalekan S. Fatoki: Research supervisor and analytical/environmental chemistry.

James P. Odendaal: Research co-supervisor and ecotoxicology.

Jeanine L. Marnewick: Biochemical oxidative stress.

Patrick Ndakidemi: Plant diversity and taxonomy.

Olatunde S. Olatunji: Analytical/environmental chemistry.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and there are no ethical issues involved.

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