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The accumulation of the cyanobacterial toxin, microcystin, in cherry tomato (Solanum lycopersicum) and bush bean (Phaseolus vulgaris) plants

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Abstract

We aimed to develop a high-sensitivity method to detect microcystin toxins in fruit tissue and to determine if irrigation with water containing toxic cyanobacteria may result in accumulation of microcystin toxins in fruit tissue and affect fruit development. In a greenhouse experiment bush beans (*Phaseolus vulgaris*) and tomato plants (*Solanum lycopersicum)* were grown in the summer under natural light and temperature between the months of September and August. Mature plants received treatments of toxic, *Microcystis aeruginosa*, applied twice weekly to the soil. To simulate naturally contaminated irrigation water, the *M. aeruginosa* were applied to plants as a suspension of intact cells. After harvesting, fruiting bodies (beans and tomatoes) were homogenized and extracted with 80% methanol (MeOH) and analyzed by ELISA for microcystins. The first extraction method tested the extraction of 0.45 g fruit tissue in 1.5 mL MeOH, buffered with PBS after 24 hr and yielded MC concentrations just above detectable limits of the ELISA. The second extraction method concentrated samples using a SpeedVac and yielded MC concentrations in range of the ELISA. The third method filtered samples from Method 2 as a preliminary investigation into matrix effects and reduced MC concentration on an average of 84%. To determine if *Microcystis* affected the growth of the plant's fruit, all harvested beans and tomatoes were individually measured, weighed and photographed before processing their tissue for ELISA. The presence of cyanobacteria stimulated bean growth (t-Test $P<0.05$), although there was no effect on the size or growth of tomatoes. Treated plants produced more fruit than the controls, although the difference was not statistically significant. The high-sensitivity method of MC extraction allows for detection of the cyanotoxins and microcystins, in the fruiting bodies of plants and an assessment of the health risk to humans and livestock.

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Introduction

Cyanobacteria are photosynthetic bacteria that thrive in a wide range of climates and are therefore ubiquitous. The eutrophication of freshwater fosters the growth of this organism, resulting in surface blooms that can pose serious threats to water quality and human health. Many species of cyanobacteria produce toxins; one of the most common cyanotoxins is microcystin (MC), a hepatotoxin (liver toxin) produced by several cyanobacterial genera such as: *Microcystis, Anabaena,* and *Oscilllatoria* (Carmichael, 1992). Microcystins are cyclic hepatapeptides containing D amino acids, N-methyl-α,β-dehydroalianine (MDHA), 3-amino-9-methoxy-2,6, 8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA) that is unique to the toxin and various forms of L amino acids (Botes *et al*., 1982). MC-LR is one of the most toxic and commonly found variant of the toxin. It is a specific inhibitor of protein phosphate enzymes 1 and 2A that control many cellular functions in animals and higher plants (MacKintosh *et al*., 1990). Acute hepatotoxicosis, a liver disease, is the most frequent animal toxicosis involving cyanobacteria (Carmichael, 1992). The prevalence and toxicity of cyanobacteria has recently triggered investigations into its implications on irrigation of agricultural crops with contaminated water, especially through surface water irrigation.

Although the accumulation of cyanotoxins in agricultural crops and its potential threat to human

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health has been established, most reports solely discuss the accumulation of cyanotoxins in vegetative portions of plant tissue (Crush *et al.*, 2008; McElhiney *et al.* 2001; Codd *et al.*, 1999). Watering lettuce by spray irrigation with water containing *Microcystis*, can result in MC-containing *Microcystis* cells adhering to the leaf surface of lettuce (Codd *et al.*, 1999). When *Microcystis* contaminated water is applied only to the roots of plants, MC can be detected within vegetative tissues of the plant, such as the roots, shoots, and leaves, (Mohamed and Shehri, 2009; Crush *et al.* 2008; McElhiney *et al.*, 2001). Although accumulation of microcystins in vegetative plant tissue poses a threat to consumers of root and leaf crops, current research has yet to address the accumulation of toxins in the fruiting bodies of plants, which is often the portion that is most consumed by humans (Milligan, 2010).

Microcystins can also alter the growth of agricultural crops. MC can impair the vegetative growth of several plant species (Chen *et al.*, 2010; Crush *et al.*, 2008; Bibo *et al.*, 2008; McElhiney *et al.*, 2001) through oxidative stress and cell death (Peuthert *et al.*, 2007). In contrast, MC may also facilitate growth in rapeseed (*Brassica napus*) and cabbage (*Brassica chinensis*) (Bibo *et al.*, 2008; Crush *et al.* 2008). The varying inhibitory and stimulatory effects of MC on plant growth have not been well recognized.

In this study, bush bean and tomato plants were grown from seed in a greenhouse and irrigated with a monoculture of the toxic cyanobacteria, *Microcystis aeruginosa*. The accumulation of the cyanotoxin microcystin in the plant's fruiting bodies was the subject of this investigation. We also examined the effects of irrigation with toxic *Microcystis* on the yield of the plants and on the development of the fruit. Due to there being no conventional method for extraction of microcystins from fruit tissue, this study also tested techniques to increase the sensitivity of the methanol extraction method for ELISA analysis.

Methods

Experimental Methods – Cherry tomato (*Solanum lycopersicum*) and bush bean (*Phaseolus vulgaris*) plants were chosen for this experiment because they are consumed in large quantities and are simple to grow in controlled settings. Eight plants of each species were grown from seed to allow for four replicates of the treatment and control conditions. The plants were maintained in a greenhouse under natural light and temperature between the months of August and September. The seeds were germinated and grown in a combined peat/vermiculture/perlite potting soil medium. Initially they were maintained in sprouting cells (6 cm) and were transferred to larger pots (3-10 L) after seedlings produced a second leaf pair. The potted plants were side-dressed with fertilizer (nitrogen, phosphorous, potassium mix) until reproductive maturity.

The control and treatment plants were irrigated with well water every other day. Once plants approached reproductive maturity through axillary flower bud development prior to fertilization, the treatment of cyanobacteria was applied to the soil of treatment plants twice a week. To simulate a scenario of surface water irrigation, *Microcystis* was applied to the treatment as a suspension of intact cells, rather than applying a concentrated MC extract (Chen *et al.*, 2010; McEliniey *et al.*, 2010). The cyanobacteria, *Microcystis aeruginosa* (UTEX # 2385), used in this experiment was cultured in a laboratory cold room in a BG-11 medium kept at 25 °C. At the time of treatment, the culture had approximately 10^8 cells mL^{-1} and 2 μg MC mL^{-1}.

Once bean plants began to transition from floral primordia to form fruit, the beans were harvested (harvest 1). After three days, fruits reached full maturity and were harvested again (harvest 2). The last harvest (harvest 3) was collected twenty days after the first harvest. Tomato plant fruit were first harvested once fruit reached full maturity and once again five days later. Beans collected from individual plants at each harvest were combined (n=1) while tomatoes were kept separate (n=4).

Analytical Methods

Growth and development of fruiting bodies – All harvested beans and tomatoes were weighed, measured and photographed before processing their tissue for ELISA to determine whether control and treatment fruits differed in size. Beans were measured by length and weight. Tomato volume was determined by using an ellipsoid volume equation: tomatoes were measured by two dimensions to determine intermediate and minor axis. After tissue was processed, a subsample (~0.2 g) was measured in a pre-weighed aluminum foil boat and dried in an oven at 60 °C for 24 hr to determine the wet weight to dry weight conversion factor.

Processing Tissue for ELISA – In a whole fruit analysis for MC, unbiased random sub-samples were taken from tomato and bean harvests. Three tomatoes (or less if fewer than three were harvested) were taken from each replicate group of treatment and control plants from both harvests. The tomatoes were sliced in half from proximal to distal ends using a scalpel in a glass dish. One half from each tomato was frozen at -40 °C in a plastic bag for future use and the other half was processed for ELISA. Due to the beans from replicate plants being combined at harvest, nine pods were randomly selected and distributed into three replicate groups per harvest. These were used as treatment and control plants that were processed for whole fruit analysis. Thus, this subsampling provided a measure of variability between beans, but not between individual plants. When the three beans selected from harvest 1 did not provide enough tissue for analysis, a forth bean was selected and homogenized with the other beans. To increase the sensitivity of our MC extraction technique we tested two different sample processing methods (Figure 1).

Method 1: Standard Extraction – Bean pods were thinly sliced horizontally and macerated to a paste with a mortar and pestle. This was then tripled rinsed with distilled water followed by a 70% ethanol wash and final distilled water rinse for 1 min, then was extracted at room temperature for 24 hr wrapped in aluminum foil to block out light. After 24 hr, the samples were sonicated for 1 min and buffered with 13.5 mL of PBS (Phosphate Buffer Solution) to obtain a final MeOH concentration of 8%. The sample was then vortexed for 1 min. Supernatant (~1.0 mL) was then extracted with a syringe and filtered through a syringe filter (nylon Fox Scientific 25 mm diameter, 0.2 µm) into a new 1.5 mL centrifuge tube. Samples were frozen at -40 °C until ELISA analysis. Samples from this method were analyzed using the high-sensitivity 50 µL method recommended by the ELISA manufacturer Envirologix.

Method 2: SpeedVac Concentration – Sliced bean pods and tomato halves were macerated using an electric homogenizer, rather than a mortar and pestle, which was tripled rinsed with distilled water followed by a 70% ethanol wash and final distilled water rinse between each sample to remove residual toxin. Subsamples (~3 g) of homogenized fruit tissue were weighed into a 15 mL glass centrifuge tube and extracted with 10 mL of 80% MeOH in milli-Q water. In order to accommodate the low tissue weight of beans from the first harvest, 1 g samples were extracted in 3.3 mL MeOH, preserving the same ratio used for 3 g samples. All subsamples were then sonicated and vortexed for 1 min and extracted at room temperature for 24 hr wrapped in aluminum foil to block out light. After 24 hr, the samples were sonicated and vortexed for 1 min and centrifuged (5,000 rpm) for 10 min. Supernatants were then concentrated to approximately one-fifth the volume of 5 mL samples and one-half the volume of 2 mL samples using a SpeedVac. After concentration, the vials were swirled lightly in hand and weighed to determine exact volume, using a factor of 0.7751 to convert from weight to volume of 80% MeOH. The vials were then centrifuged for 10 min to separate residue from the supernatant. Lastly, supernatants (~1 mL) were transferred to vials using a Pasture's pipette and frozen at -40 °C until ELISA analysis. Samples from this method were analyzed using the standard 20 µL method. According to Envirologix Inc. Portland, Maine, USA, it is not necessary to dilute the methanol to <10% when using the 20 µL sample size as there is

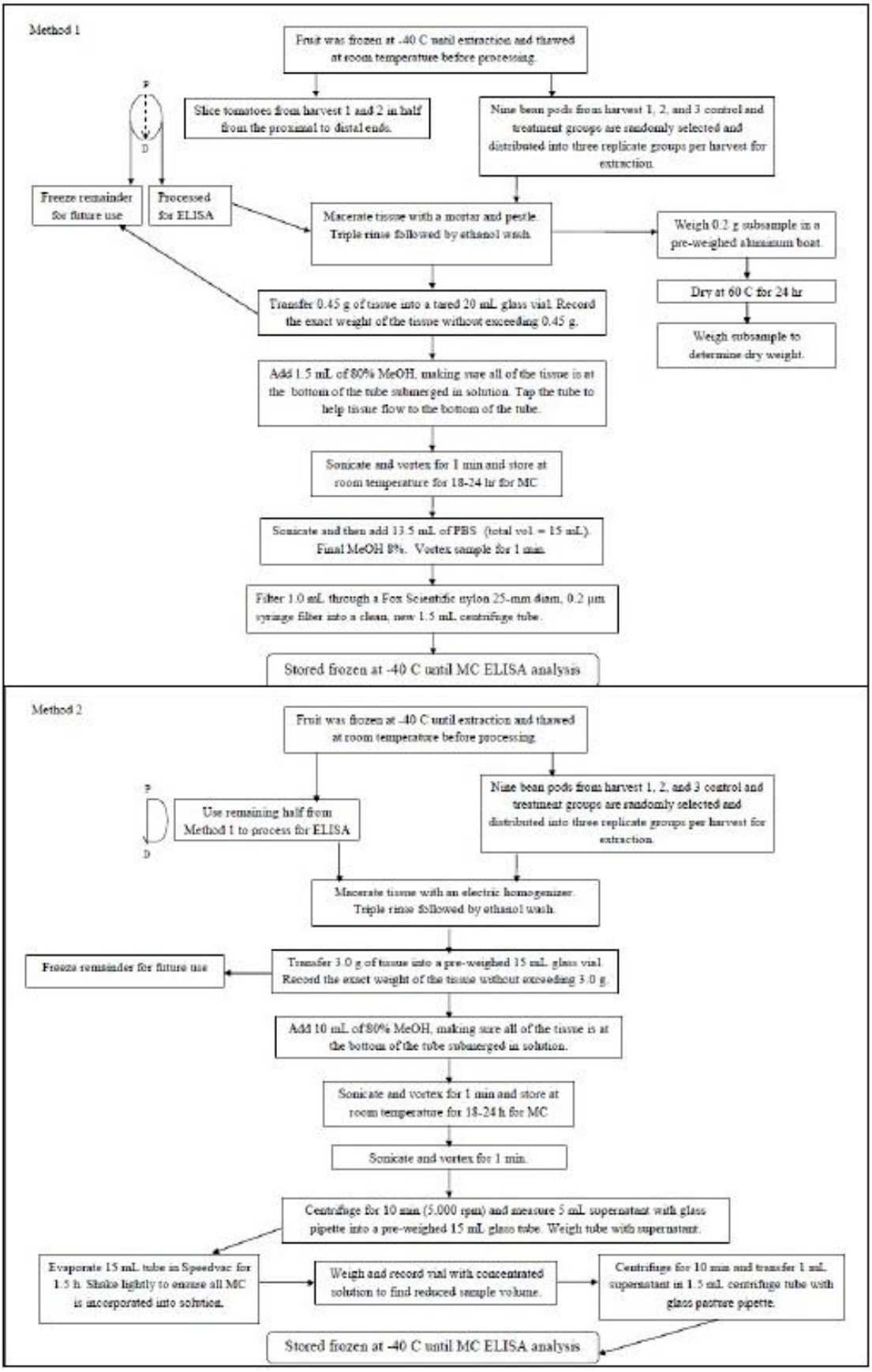

Figure 1. Comparison of Method 1; Standard Extraction and Method 2; SpeedVac Concentration.

Table 1. Total number of fruit at each harvest (H1, H2, H3) from replicate bean plants (n=4) and tomato plants (A, B, C, D) and total fruit produced from control and treatment plants from combined harvests.

	Bush Beans					**Cherry Tomatoes**								
	H1	H2	H3			H1				H2				
				Total		A	B	C	D	A	B	C	D	**Total**
Control	23	17	16	56		2	2	0	2	4	5	5	5	25
Treatment	21	49	51	121		2	2	3	3	21	16	5	7	59

no matrix effect when using this small quantity of MeOH. To ensure samples from Method 2 were not altered by MeOH, samples of 80% MeOH were also analyzed as controls for the ELISA.

Method 3: Matrix Filtration – Some of the bean samples in the second method had suspiciously high MC concentrations. Thus, samples with high MC concentrations from Method 2 were filtered, following MC extraction, through a 0.2 µm nylon syringe filter to remove any material that may have contributed to a matrix effect on the ELISA. This method was a preliminary investigation into possible matrix effects.

ELISA Analysis – The concentration of MC was determined by analyzing the supernatant extracts using an ELISA Quantiplate Kit for Microcystins

Table 2. Comparison of toxin extraction methods by MC concentration. Values are reported as the mean MC concentration in fruit from each harvest. Method 1 reports the mean value of duplicated samples analyzed by ELISA. Method 1 samples reported by the ELISA that were below the lowest standard but above the control were extrapolated to estimate MC content. Others are reported as below detectable limits (BDL).

	MC Concentration (ng kg^{-1})						
		Method 1		**Method 2**		**Method 3**	
		Treatment	**Control**	**Treatment**	**Control**	**Treatment**	**Control**
Tomato	**H1**	BDL	BDL	1363.9	1883.6ǂǂ	*	59.2ǂǂ
	H2	0.15ǂ	BDL	2091.0ǂǂ	1436.8	698.7ǂǂ	*
Bean	**H1**	BDL	0.13	2917.2	2836.3	*	*
	H2	0.14	0.11ǂ	1156.9	13826.0ǂǂ	*	16499.7ǂǂ
	H3	BDL	BDL	2401.2	1292.8	260.3ǂǂ	*

ǂ Raw value of a single sample read by ELISA.

ǂǂ Average of duplicated sample read by ELISA.

* Sample not tested.

and Nodularins (Envirologix Inc. Portland, ME). A standard curve was constructed using three calibrators supplied by the kit (0.16, 0.6, and 2.5 ppb MC-LR) as well as three dilutions of the calibrators (0.025, 0.060, 0.250 ppb) in order to extend the curve of detection.

Statistical Methods

The data were analyzed using Sigma Plot (Eleventh Edition, version 11.0.0.75). Differences between treatment and control bean and tomato plants were investigated using t-Test and linear regression analysis. Data were transformed to a log scale in order to best fit a linear regression. Beans yielded from replicate plants were combined at each harvest therefore, measurements of individual beans were used for statistical analysis. Tomatoes yielded from replicate plants at each harvest were preserved separately therefore mean values from each replicate plant yield were used for t-tests while individual measurements were used for linear regression analysis. At the first tomato harvest, replicate control plant C did not yield any fruits, therefore only three mean values were considered by statistical tests for that plant (Table 1).

Results

Crop Analysis – The presence of *M. aeruginosa* increased crop yield of bean plants by 116% and tomato plants by 140% (Table 1).

Bean Growth Analysis – The addition of *Microcystis* suspensions to the potting soil had a stimulatory effect on bean development. Bean weight was not significantly affected by the application MC (T-test $P<0.05$) (Figure 2). However, beans collected from treated plants at the third harvest were, on average, greater in length by 16% than untreated beans ($P=0.003$) (Figure 3). Fruit development of all bean plants followed a linear regression (Figure 4).

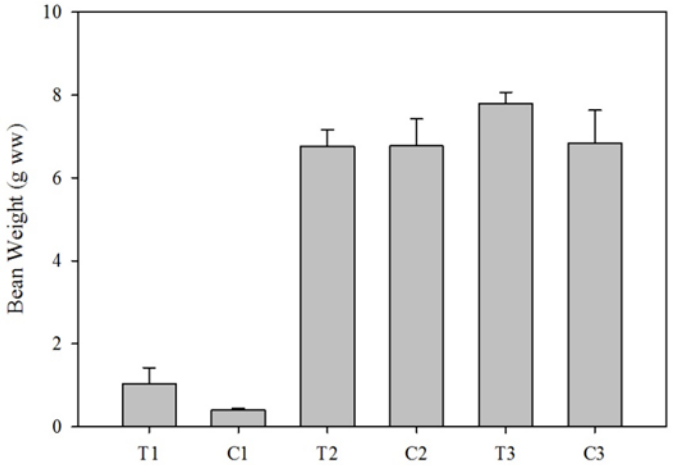

Figure 2. Comparison of individual bean wet weight (ww) between treatment and control plants (n=4) (T, C) at each harvest (1, 2, 3). The mean weight of treatment and control beans did not differ (t-Test P<0.05) at harvest 1 (P=0.099), harvest 2 (P=0.987), or harvest 3 (P=0.148). Bar indicates mean, error bar indicates standard error.

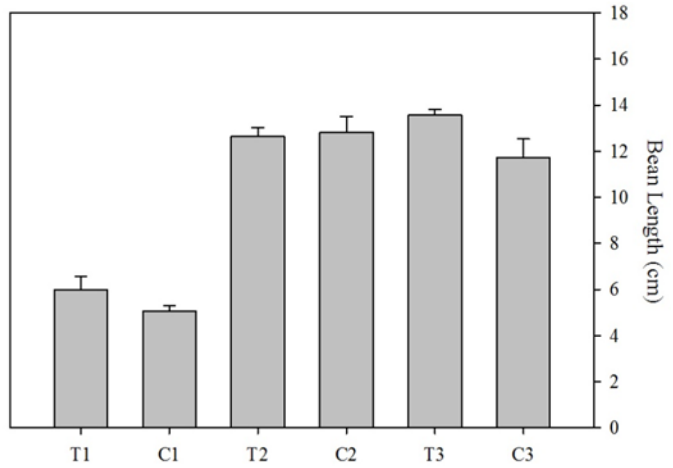

Figure 3. Comparison of individual bean length between treatment and control plants (n=4) at each harvest (1, 2, 3). The mean weight of treatment and control beans did not differ (t-Test P<0.05) at harvest 1 (P=0.131) or harvest 2 (P=0.806) but did differ at harvest 3 (P=0.003). Bar indicates mean, error bar indicates standard error.

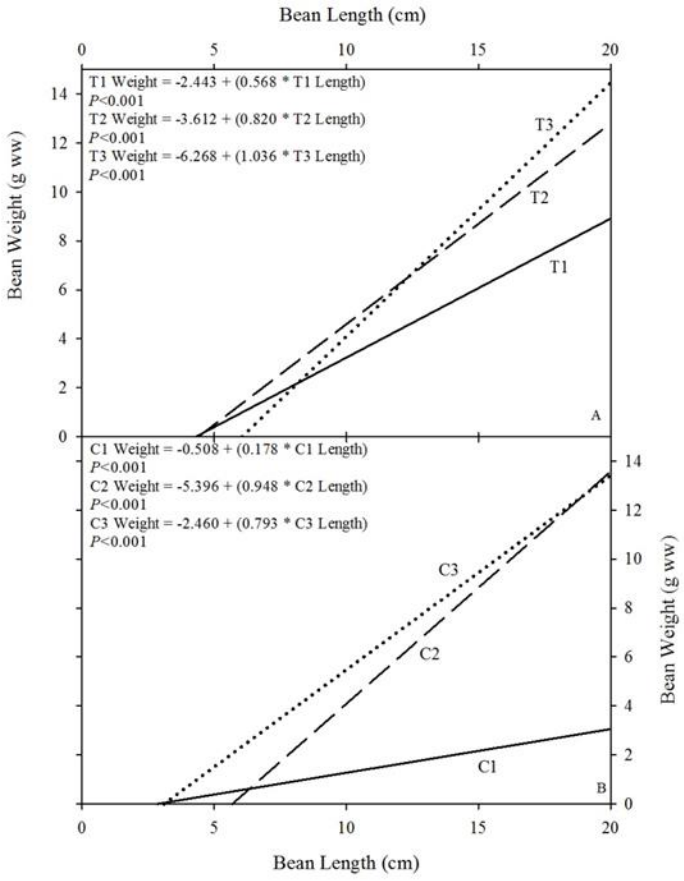

Figure 4. Comparison of length and wet weight (ww) regression relationships of treatment and control (T, C) beans from three harvests (1, 2, 3). Harvest 1 treatment (Adj R^2=0.697) and control (Adj R^2= 0.807), harvest 2 treatment (Adj R^2=0.633) and control (Adj R^2=0.958), and harvest 3 treatment (Adj R^2=0.692) and control (Adj R^2=0.649) fruit development fit a linear regression.

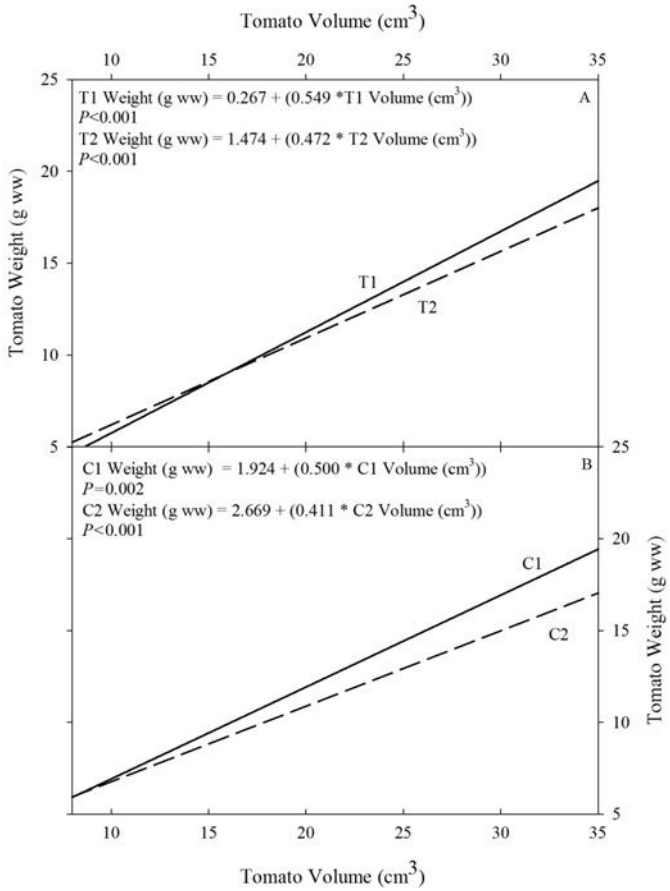

Figure 5. Comparison of volume and wet weight (ww) regression relationships of treatment and control (T, C) tomatoes from two harvests (1, 2). Harvest 1 treatment (Adj R^2=0.877) and control (Adj R^2=0.899) and harvest 2 treatment (Adj R^2=0.884) and control (Adj R^2=0.737) fruit development fit a linear regression.

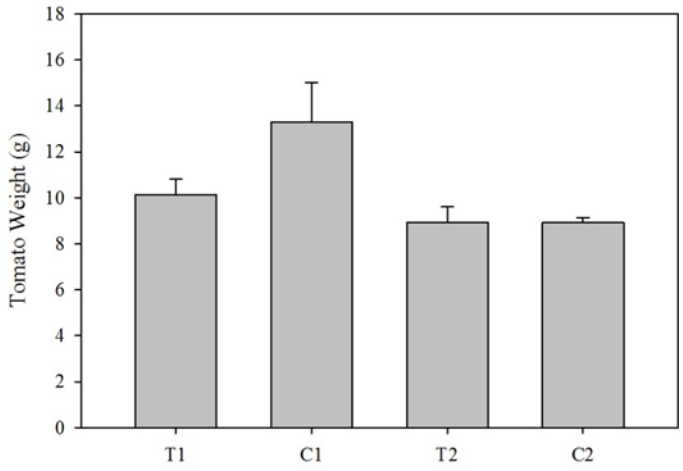

Figure 6. Comparison of mean tomato wet weight (ww) from treatment and control (T, C) plants (n=4) at the first harvest (1) and second harvest (2). The mean weight of treatment and control tomatoes did not differ (t-Test P<0.05) at harvest 1 (P=0.112) or harvest 2 (P=0.989). Error bars indicate 1 standard error of the mean.

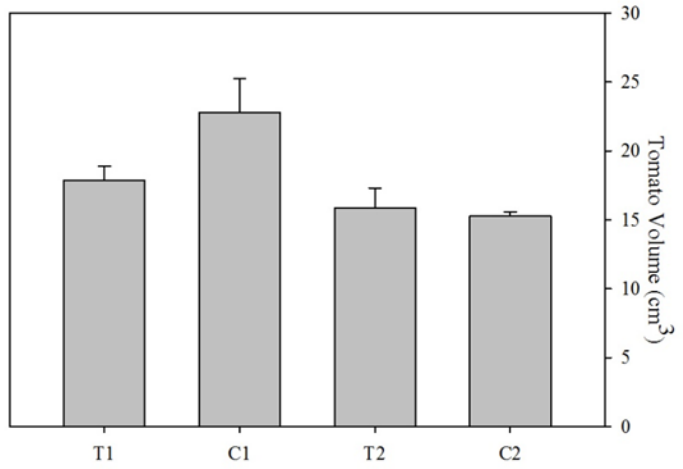

Figure 7. Comparison of mean tomato volume between four treatment and control (T, C) plants at the first harvest (1) and second harvest (2). The mean volume of treatment and control tomatoes did not differ (t-Test P<0.05) at harvest 1 (P=0.097) or harvest 2 (P=0.702). Error bars indicate 1 standard error of the mean.

Tomato Growth Analysis – The application of MC to potting soil did not significantly affect the growth of tomatoes. The fruit development of all tomato plants followed a linear regression (Figure 5). Tomato weight and volume were not altered in the presence of MC (t-Test P<0.05) (Figures 6-7).

Chemical Analysis

Methanol – All methanol samples were below detectable limits of the ELISA indicating the 80% MeOH used for MC extraction did not cause false positives in the MC tests.

Vegetable Tissue – ELISA analysis on some samples extracted using Method 1 had MC concentrations lower than the lowest standard but higher than the negative control. To estimate the MC concentration of these samples, their values were extrapolated using an inverse third order curve that best fit our standard values. All fruit tissue samples extracted using Method 2 had detectable microcystins. Total MC concentrations in tomatoes and beans from treated plants varied between harvests and averaged 1621.2 ng kg^{-1} and 2158.4 ng kg^{-1} DW for tomato and bean tissue, respectively. Total MC concentrations in tomatoes from the second harvest was greater than the first harvest (t-Test P=0.03). Interestingly, MC concentration in beans from the first harvest was greater than the second harvest (P=0.008). MC concentration of filtered samples was reduced by an average of 84%, with the exception of one particularly high sample, a control bean sample from the second harvest that increased in MC concentration from 13826 ng kg^{-1} to 16499.7 ng kg^{-1}.

Soil – Soil from the UNH greenhouse was investigated to explore possible causes for the MC concentrations found in control samples. The soil was collected in the summer of 2014 and was the same permaculture soil that would have been used in this experiment. The soil was tested using the same freeze/thaw method described above and yielded detectable levels of MC, 380 pg g^{-1} dry weight soil. The soil was also tested for the presence of cyanobacteria cells. The cells were washed from the soil and analyzed on a filter by epifluorescence microscopy under 572 nm excitation to fluoresce cells that had phycocyanin. The analysis found a cell count of 23,000 cells per gram of dry weight soil.

Discussion

Fruit Development – The number of fruit produced as well as the growth of treatment beans were stimulated by the addition of *Microcystis aeruginosa* to the developing plants, in contrast to many studies that have shown microcystin (MC) can inhibit plant growth (Bibo *et al.,* 2008; Chen *et al.,* 2010; Crush *et al.,* 2008; McElhiney *et al.,* 2001). The number of fruit produced by plants grown with contaminated water was greater by 116% and 140%, for bean and tomato plants respectively. Beans grown in the presence of *Microcystis aeruginosa* were, on average, 16% longer than beans grown with well water. Bibo *et al.* (2008), found low concentrations of MC-RR (<10 μg L^{-1}) could accelerate growth in agricultural crops. While the MC released from *Microcystis* in the soil could have assisted plant growth, it is also possible that the nutrients in the BG-11 culture medium, and the nutrients from the degrading cells, may have stimulated fruit production and development. Many plants may benefit from nitrogen fixation by cyanobacteria, which could have also played a role in facilitating fruit production and bean growth (Quesada and Valiente, 1996; Meeks, 1998, Valiente *et. al.,* 2000). Further investigations of MC accumulation and its effects on plant development should consider "controls" with an equivalent amount of BG-11 to untreated plants.

Method 1: Standard Extraction – The fruit tissue samples had MC concentrations lower than our range of standards using Method 1; however the ELISA reported several samples to be above the control value. This indicated the possibility that trace amounts of MC may have been present. The PBS added to the extracted tissue in methanol diluted the samples 15 times. Additionally, a small amount of tissue (0.45 g) was extracted in this method. These two factors may have attributed to the trace MC concentrations. According to the ELISA manufacturer, it is no longer necessary to dilute samples with PBS when extracting with methanol if 20 μL samples are used. Fruit tissue was therefore tested with an undiluted and more concentrated method.

Method 2: SpeedVac Concentration – Method 2 revealed detectable concentrations of MC in fruit tissue. This appears to be the first report of MC accumulation in the fruiting bodies of agricultural crops. The average total MC concentration measured in tomato tissue was 1621.2 ng kg^{-1} and bean tissue was 2158.4 ng kg^{-1} in tomato tissue. This study indicates yet another path for consumption of MC. The World Health Organization (WHO) established the daily consumption limit of MC as 40 ng kg^{-1} for a 60 kg person. Based on the results from this method, a serving of ten contaminated beans (26 ng) and five tomatoes (6 ng) would provide approximately 80% of the maximum allowable MC. While these concentrations were obtained using a methanol extraction, Metcalf and Codd (2000) revealed boiling and microwaving to extract similar concentrations of MC as methanol extraction. This indicates the possibility for consumers to unintentionally release MC from fruit tissue making it available for consumption. Interestingly, Milligan (2010) reported consumption limits of agricultural crops based on MC in shoots, which over estimated MC content of fruit in comparison to our results. Our study investigated preliminary methods to detect microcystin in fruit tissue, it is necessary to further develop and examine routes of microcystin extraction. In order to track where MC is retained, further studies should consider examining the relative concentrations of MC throughout the study plants' roots, stems and leaves and in the soil to which the *Microcystis* was applied.

Method 3: Matrix Filtration – Although supernatants from Method 2 were centrifuged before extracting samples to be analyzed by ELISA, it is possible that substances released during extraction may have caused a matrix effect on the ELISA plate. Passing samples through a 0.2 μm filter reduced MC concentrations by an average of 84%. Since only four samples were filtered and one sample increased in MC concentration while the others decreased, it is still unclear as to what caused this reduction. It is possible some loss of MC may have been caused by MC adhering to the plastic filter or syringe (Bell and Codd, 1996).

MC Accumulation in Control Fruit – Surprisingly, all ELISA tests revealed MC accumulation in control fruit (Table 1). The presence of MC may have resulted from variables in the experimental design, or even perhaps the natural biology of the plants. It is also possible that some of the MC detected may have been from background contamination with cyanobacteria from the soil or well water used to grow the plants in this study (Quesada and Valiente, 1996; Valiente, 2000). The post-experiment soil results suggest MCs could have been present in the control plants' potting soil at the time of the experiment. While we did not attempt to measure MCs in the well water, however if the irrigation water had been contaminated, dissolved MC would be more biologically available than it was in the treatments of whole *Microcystis* cells. Water and soil should be monitored for MC in future experiments.

Although we have no evidence, it is also conceivable that MC in control fruit may have come from a natural symbiotic relationship between the plants and cyanobacteria. Many cyanobacteria species have the ability to respond to signals from plants and have formed symbiotic relationships with plants from all four phylogenetic divisions of terrestrial plants, providing plants with nitrogen (Meeks and Elhai, 2002; Meeks 1998). Plants that are apt for cyanobacterial symbiosis have 'symbiotic cavities' that foster and attract the cyanobacteria and in some cases support other species of bacteria. Interestingly, these structures exist in the absence of cyanobacteria (Meeks and Elhai, 2002). These investigations make a case especially for the MC found in bean plants, known for their symbiotic relationship with n-fixing bacteria.

While ELISA has been adapted to test plant tissue in this and previous studies, the manufacturer intends the system to identify microcystins in surface water samples (Envirologix, 2010). Plants naturally produce a vast array of natural toxins, known as phytotoxins (Hoerger *et. al.*, 2009). While it is unknown how these phytotoxins react with ELISA antibody sites, it is possible some could have similar binding effects to the ELISA plate as microcystin.

Conclusion

This study describes a method for extracting low concentrations of microcystins from fruit tissue. In future studies, the investigation of toxin accumulation should cover the entire plant to identify where the cyanotoxins are most concentrated, if cyanobacteria are fixing nitrogen for the plant, and if the bacteria are naturally occurring in control plants. Water and soil samples should also be confirmed as nontoxic to avoid exposure to control plants. Our preliminary results also suggest that substances released during MC extraction from fruit tissues may create false positive results with the ELISA plate. It is recommended that tissue samples also be reexamined using liquid chromatography-mass spectrometry, which would confirm if toxins are present and identify cyanotoxin variants.

References

BELL, S.G. AND G.A. CODD, 1996. Detection, analysis and risk assessment of cyanobacterial toxins. Agricultural Chemicals and the Environment, Issues in Environmental Science and Technology **5**:109-122.

BIBO, L., Y. GONG, X. BANGDING, L. JIANTONG AND L. YONGDING. 2008. A laboratory study on risk assessment of microcystin-RR in cropland. Environmental Management **86**: 566-574.

BOTES, D.P., H. KRUGER, AND C.C. VILJOEN, 1982. Isolation and characterization of four toxins from the blue-green alga, *Microcystis aeruginosa*. Toxicon **20**: 945-954.

CARMICHAEL, W.W. 1992. Cyanobacterial secondary metabolites—the cyanotoxins. Journal of Applied Bacteriology **72**: 445-459.

CHEN, J., J. DAI, H. ZHANG, C. WANG, G. ZHOU, Z. HAN, AND Z. LIU. 2010. Bioaccumulation of microcystin and its oxidative stress in the apple (*Malus pumila*). Ecotoxicology **19**: 796-803.

CODD, G.A, J.S. METCALF, AND K.A. BEATTIE. 1999. Retention of *Microcystis aeruginosa* and microcystin by salad lettuce (*Lactuca sativa*) afterspray irrigation with water containing cyanobacteria. Toxicon **37**: 1181–1185.

CRUSH, J.R., L.R. BRIGGS, J.M. SPROSEN, AND S.N. NICHOLS. 2008. Effect of irrigation with lake water containing Microcystins on microcystin content and growth of ryegrass, clover, rape, and lettuce. Environmental Toxicology **23**: 246-252.

ENVIROLOGIX. 2010. Product Insert: QuantiPlate Kit for Microcystins - EP 022. Portland, ME, USA.

HOERGER, C.C., J. SCHENZEL, B.W. STROBEL, T.D. BUCHELI. 2009. Analysis of selected phytotoxins and mycotoxins in environmental samples. Anal Bioanal Chem **22**: 436-442.

HYENSTRAND, P., J.S. METCALF, K.A. BEATTIE, AND G.A. CODD. 2001. Losses of the cyanobacterial toxin Microcystin-LR from aqueous solution by adsorption during laboratory manipulations. Toxicon **39**: 589-594.

MACKINTOSH, C., K.A. BEATTIE, S. KLUMPP, P. COHEN, AND G.A. CODD. 1990. Cyanobacterial MC-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. February Letters **264**: 187-192.

MCELHINEY, J., L.A. LAWTON, C. LEIFERT. 2001. Investigations into the inhibitory effects of Microcystins on plant growth, and the toxicity of plant tissues following exposure. Toxicon **39**: 1441-1420.

MEEKS, J.C. 1998. Symbiosis between nitrogen-fixing cyanobacteria and plants. University of California. THESIS.

MEEKS, J.C. AND ELHAI, J. 2002. Regulation of Cellular Differentiation in Filamentous Cyanobacteria Free-Living and Plant-Associated Symbiotic Growth States. Microbiology and Molecular Biology Reviews **66**: 94-121.

METCALF, J.S. AND G.A. CODD. 2000. Microwave oven and boiling waterbath extraction of hepatotoxins from cyanobacterial cells. FEMS Microbiology Letters **184**: 241-246.

MILLIGAN, A. J. 2010. Cyanobacterial toxins impact crops and accumulate to levels considered unsafe for consumption. Oregon State University. THESIS.

MOHAMED, Z.A. AND A.M. AL SHEHRI. 2009. Microcystins in groundwater wells and their accumulation in vegetable plants irrigated with contaminated waters in Saudi Arabia. Journal of Hazardous Materials **172**: 310-315.

PEUTHERT, A., S. CHAKRABARTI, AND S. PFLUGMACHER. 2007. Uptake of Microcystin-LR and –LF (Cyanobacterial toxins) in seedlings of several important agricultural plant species and the correlation with cellular damage (Lipid peroxidation). Environmental Toxicology **22**: 436-442.

QUESADA, A. AND E. FERNADEX-VALIENTE. 1996. Relationship between abundance of N_2-fixing cyanobacteria and environmental features of Spanish rice fields. Microb Ecology **32**: 59-71.

VALIENTE, E.F., A. UCHA, A. QUESADA, F. LEGANES AND R. CARRERES. 2000. Contribution of N_2 fixing cyanobacteria to rice production: availability of nitrogen from ^{15}N-labelled cyanobacteria and ammonium sulphate to rice. Plant and Soil **221**: 107-112.