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Effect of Irrigation with Lake Water Containing Cylindrospermopsin Toxin on Seed Germination and Seedlings Growth of Cucumis Sativus and Lycopersicon Esculatum

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Abstract

Cylindrospermopsin (CYN) phytotoxicity, was investigated on seeds and seedlings of tomato *(Lycopersicon esculatum L.)* and cucumber *(Cucumis sativus L.)*, after irrigation by water contaminated with this toxin. Our results showed that seed germination was reduced after exposure to higher concentrations of CYN. The cucumber seeds were more resistant than tomato seeds to this toxin. The CYN affected also the growth and productivity of seedlings. A significant reduction in the length of stems, roots (principle and lateral) and the number and size of leaves, appeared on the seedlings exposed to CYNs.

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These morphological changes were associated with alterations in primary xylem as a result of the inhibition of root growth and the reduction of water absorption. In addition, altered metabolism of tomato and cucumber seedling exposed to CYNs was manifested by chlorosis and leaf necrosis following a reduction in the chlorophyll content, photosynthesis inhibition and induction of oxidative stress.

Keywords: Cylindrospermopsin; irrigation; cucumber; tomato; water quality**.**

1. Introduction

Several lakes and reservoirs throughout the world suffer from eutrophication process that promotes the development cyanobacterial blooms. Some cyanobacteria species can produce cyanotoxins that threaten human health [1].

In Lebanon, farmers use the water reservoir of Lake Karaoun contaminated by cyanobacteria to irrigate crops and pose real economic problems [2]. Cyanotoxins produced by Cyanobacterial bloom, can be absorbed by plants, transported to the stem, and accumulated in the tissues. Many studies have reported the accumulation of cyanotoxins in plant tissues such as lettuce [3-5], Corn [6,7], peas wheat and lentils [7], ryegrass, clover [3], rape [3,8], rice [8], broccoli and mustard [9] and apple cultivation shoot [10]. The accumulation of these toxins in plant tissues and their passage to the man through the food chain can cause severe health risks. Cylindrospermopsin (CYN), a hepatotoxic alkaloid produced by *Chrysoproum ovalisporum* forming the bloom (may-June) in Lake Karaoun and this probably related to the temperature of the water (18- 22 ° C). The CYN inhibits the synthesis of enzyme proteins irreversibly and causes disturbances in cellular metabolic activity and thus leads to cell necrosis. Unlike other hepatotoxic, CYN is not an inhibitor of protein phosphatases 1 and 2A in the synthesis of amino acids serine and threonine [11].

Irrigation of seeds, fruits and vegetables with water containing cyanotoxins has the potential to carry these toxins over into the food chain, thereby representing a risk to human health. The tolerable daily intake established by the WHO is 0.04 µg/kg body weight/day. The effects of cyanotoxins on plants is dependent of the plant species, toxin concentration, plant stage of development, and the time of exposure. Cyanotoxins can reduce of pollen germination and inhibit root and shoot elongation. Pereira and his colleagues in 2009 reported that microcystin concentration higher than 5.9 µg L^{-1} inhibited the root growth in *Lactuca sativa* [12]. Exposure of the seeds to the crude extract (containing 22.24 mg MC/ mL) caused a reduction of germination up to 85%. High concentrations of microcystin toxin significantly inhibited the growth and photosynthesis of rice (*Oryza sativa*) seedlings [13]. Meanwhile some studies where performed on the effect of microcystin toxins on plant physiology, little or no studies where performed on other types of cyanotoxins.

The purpose of this research is to study the effect of cyanobacterial bloom producing CYNs on seed germination and plant growth of tomatoes and cucumber. The physiological and metabolic changes were also monitored after irrigation of seeds and seedlings with water containing CYNs.

2. Materials and methods

2.1. Water sampling

Water samples were taken in May and June from Lake Karaoun during cyanobacterial blooms. The ecological state of the lake was oligotrophic to eutrophic. The degradation of water quality was announced by the appearance of *Chrysosporum ovalisporum*, a cylindrospermopsin (CYN) producing cyanobacterium. This species had annual fluctuations (in the last 5 years) where it appeared in May-June and disappeared in July probably due to the variations in water temperature [14]. During 2015, the occurrence of *C. ovalisporum* took place in mid-March and form a thick bloom in May-June with total exclusivity.

The concentration of CYNs was 10 µg/l. Details about the ELISA method used to quantify the toxin concentration in this reservoir can be found in Fadel and his colleagues in 2014 [1]. Seed and tomato plants were exposed to concentrations of 2, 5 and 10 µg/l, respectively. The used concentrations aim to mimic the use of the lake eutrophic to oligotrophic enriched cyanobacteria in irrigation.

2.2. Seed exhibition to CYNs

The seeds were disinfected with a 5% sodium hypochlorite solution for 5 min, then rinsed with distilled water. They were then placed in Petri dishes and treated with various concentrations of CYNs (0, 2, 5 and 10 µg/l) and incubated in the dark in the presence of a tank containing water in the middle of incubation. The experiment was repeated 3 times with 15 seeds for each concentration. The control seeds were exposed to distilled water. The germination rate was determined after 8 days of incubation.

2.3. Exposure of seedlings to CYNs

Seeds were sown in soil pots and irrigated with distilled water. The pots were placed in a culture chamber (green house) at 25 °C and natural photoperiod. After seed germination and emergence of seedlings (elongation of the hypocotyl and first leaves appear), exposure to the different concentrations of CYNs was performed every 3 days for a duration of 28 days for cucumbers and this and 21 days for tomatoes.

After harvesting, the length of the stems and roots was measured. The plant biomass was weighed and expressed as fresh weight (g /m^2/ day). The number of leaf was also monitored for each concentration.

2.4. Plant Anatomy

Very thin cross sections were performed at the stems of the plants that have been exposed to CYNs. These sections were then stained by the double staining technique (carmine-green iodine) to identify the cellulose and lignin.

2.5. Measuring the chlorophyll

The chlorophyll content was measured on 3 leaves (from bottom to top of the stem) of each plant exposed to CYNs directly using a Chlorophyll meter. The unit of measurement is the "value SPAD" (index value correlates with the density and chlorophyll content).

2.6. Stress index

Leaves were grounded in the presence of 90% acetone. After complete homogenization of the solution, they were placed in a pure acetone tube. After incubation in dark for 24 hours at 5 °C, the tubes were centrifuged at 3000 rpm for 10 min at 20ºC. The extracts were then separated into pure solution, and 10 ml of HCl (37%) was added. The absorbance of the solutions was measured by a spectrophotometer at 665 nm to calculate the stress index by dividing the absorbance without HCl to the absorbance with HCl (37%).

2.7. Statistical analysis

To verify the results and to ensure that they are significant and are not obtained randomly, ANOVA (one -way analysis of variance) was performed with a probability level of $p \leq 0.05$ and the comparison of means using SPSS statistical 20. Thus, the correlation coefficient was calculated to evaluate the correlation between the CYNs concentration used and the studied parameters.

3. Results

3.1. CYNs effect on seed germination

Exposure of tomato seeds to CNYs induced an inhibitory effect on the germination process. The results show a significant inhibitory effect on the seed exposed to concentrations of 10 $\mu g.l^{-1}$ toxin (Fig. 2). The germination rate of tomato seeds was reduced after exposure to various concentrations 2; 5 and 10 μg CYN.l^{-1}, in the order of 30%, 38.8% and 50% respectively compared to control (Fig. 1 and Fig. 2)

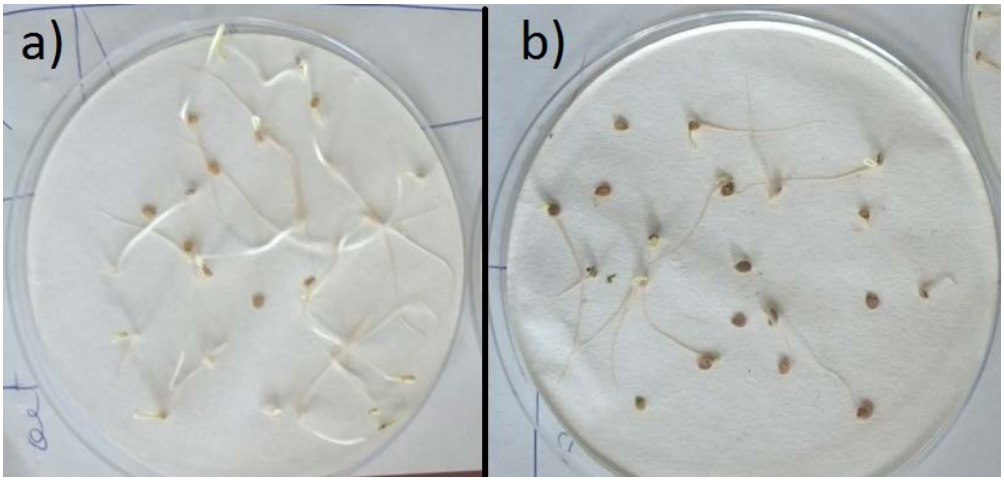

Figure 1: The germination of tomato seeds: a) control and b) with 10 $\mu g.l^{-1}$ of CYN

However, the seed of cucumbers appeared to be less affected by CYNs during germination. These results are observed after exposure of seed of cucumbers to the same concentrations (2; 5 and 10 $\mu g.l^{-1}$), where all the seeds have germinated. However, a difference was observed in the length of the radicles according to the various concentrations when compared with controls (Fig. 3 and Fig. 4)

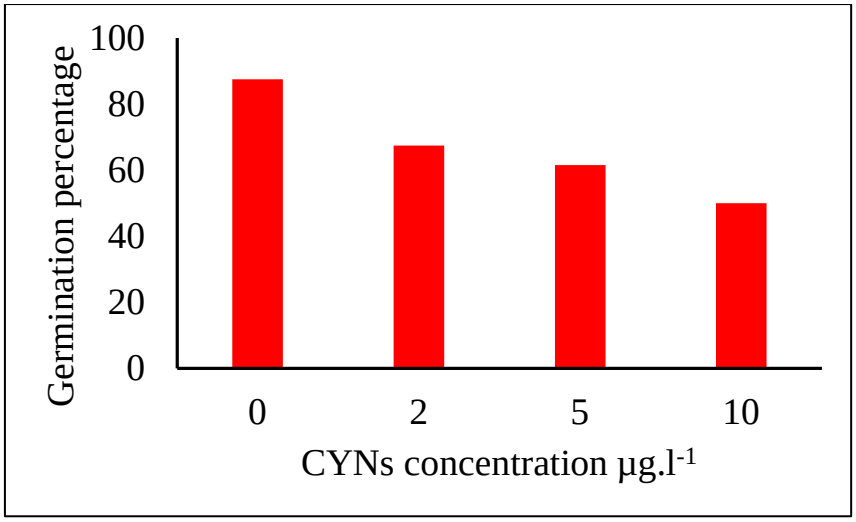

Figure 2: Variation of the growth rate of tomato seeds in function of CYN concentrations

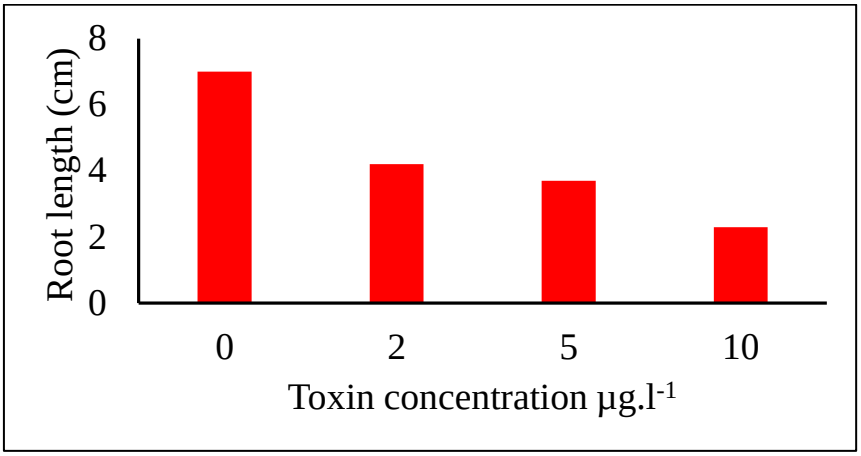

Figure 3: Variation of the roots length (cm) of cucumber seeds in function of CYN concentrations.

Figure 4: Difference in the length of radicles after exposure to increasing concentrations of CYNs (from left to right 0, 2, 5 and 10 µg.l^{-1})

3.2. Effect of CYNs on plant development

From morphological point of view, the control seedlings in both species that irrigated with distilled water, had a good growth. They had a long and rigid stems, persistent cotyledons and many green and large sized leaves. In addition, the root system was well developed with long tap roots and numerous lateral roots.

However, the growth of seedlings vulnerable to CYNs was affected in a manner dependent on toxin concentration (Fig. 5).

On the other hand, the tomato seedlings seemed more susceptible to CYNs than cucumbers. Hence the effects of the toxins started to appear in the second week in tomatoes and in the fourth week in cucumbers (Fig. 5).

After two weeks of exposure to CYNs; tomato seedlings exhibit low growth, reduced leaves size and number (2 µg.l-^{1} CYNs) and yellowing (5 µg.l^{-1} and 10 µg.l $^{-1}$ CYNs).

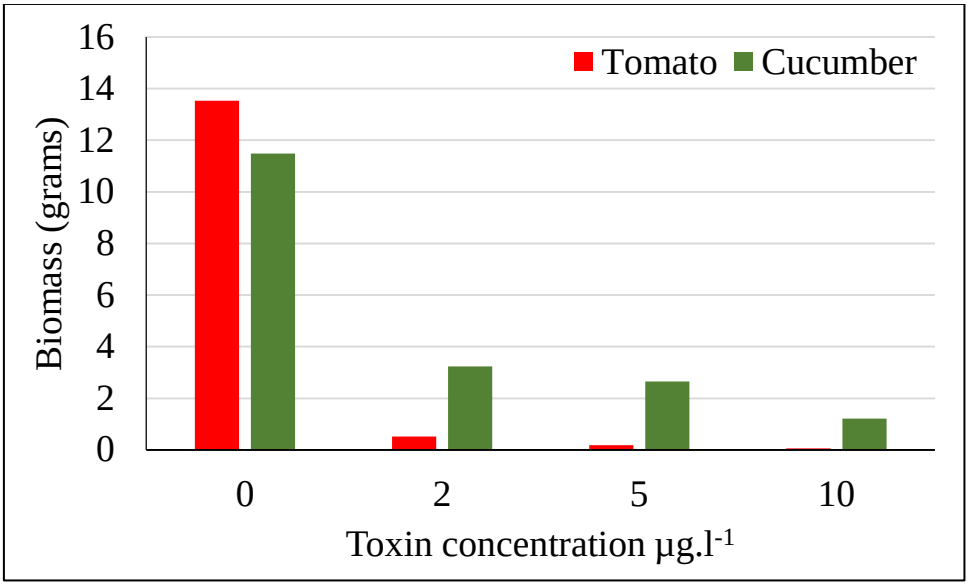

Figure 5: Changes in productivity of seedlings of tomatoes and cucumbers as concentrations CYNs

At the end of the third week of exposure to CYNs, plants with 2 µg.l-^{1} had soft stems and started to turn yellow with brown spots (chlorosis) on leaves. Also, most of the leaves with 5 µg.l^{-1} were yellow and brown, while at 10 µg.l^{-1} there were wilting and browning of leaves accompanied by necrosis and drying of the plant. Noting that the control tomato seedlings on their stems have white trichomes. However, the plantlets exposed to CYNs did not.

In seedlings of cucumbers, changes began to appear after 3 weeks of exposure to CYNs. The cotyledons became yellow and the leaves wilted in the 2 µg.l^{-1} CYNs. In addition the leaves at 5 µg.l^{-1}, was an abnormal compared with the control. At 10 µg.l^{-1}, brown spots with yellow margins were present on the leaves (Fig. 6).

Figure 6: Chlorosis and necrosis on cucumber leaves 10 µg.l^{-1} CYNs.

Towards the end of the fourth week; changes became increasingly severe, where at 2 µg.l^{-1} CYNs leaves and petioles were mole and dry, at 5 µg.l^{-1}, the leaves were rolled. In addition, at 10 µg.l^{-1} the rolled leaves were brown. Noting that the stem in various concentrations was soft, yellow and with small diameter. In addition, the control plants are characterized by twists which extend from the stem which were absent in seedlings exposed to toxins.

The lengths of the stem in both species were significantly reduced proportionally to the concentrations of CYNs compared to controls. In addition, the results confirm that CYNs act negatively on root growth. Thus, the development of the main root becomes weaker with progressive levels with the disappearance of lateral roots after exposure to 10 µg.l^{-1} CYNs (Table 1 and Table 2).

Table 1: CYNs effect on the length of the stems, roots and the number of leaves in the tomato seedlings. Each value is the average of three values of replicates. Each value is verified by ANOVA test with P 0.05. The correlation coefficient is significant at $P \leq 0.01$.

CYNs concentration	Stem lengh (cm)	Root lengh (cm)	Number of leaves
0	33	26	37
2 µg.l^{-1}	10.5	4.5	9
5 µg.l^{-1}	7.5	2	5
10 µg.l^{-1}	4.5	0.2	2
F value	61.533	69.997	170.459
P value	0.000	0.000	0.000
Correlation	- 0.883	- 0.847	- 0.872
P value	0.000	0.01	0.000

Table 2: CYNs effect on the length of the stems, roots and the number of leaves of the cucumber seedlings. Each value is the average of three values of replicates. Each value is verified by ANOVA test with P 0.05. The correlation coefficient is significant at P≤0.01

CYNs concentration	Stem lengh (cm)		Root lengh (cm)	Number of leaves
	Hypocotyl	epicotyl		
0	29	15	6.9	6
2 µg.l^{-1}	19.25	12	3.9	3
5 µg.l^{-1}	13.06	9.3	2.5	3
10 µg.l^{-1}	9	7.5	0.5	2
F value	48.063	84.063	487.660	35.000
P value	0.000	0.000	0.000	0.000
Correlation	- 0.960	- 0.977	- 0.985	- 0.947
P value	0.000	0.000	0.000	0.000

The estimation of plant productivity in both species showed a reduction in fresh biomass dependently on concentrations. Reducing the productivity of cucumber was estimated around 71.8%, 77%, 98% respectively. However, those of tomatoes were more affected than the cucumber, where they were reduced by about 96%, 98%, and 99% after exposure to 2; 5 and 10 µg.l^{-1} CYNs.

Histologically, there were clear differences between the anatomy of the stems of control seedlings and those exposed to CYNs. Cucumber control section rods showed that from outside inward, they were made of: a cortex comprising a cutinized epidermis with stomata, a collenchyma, cortical parenchyma chlorophyll and sclerenchyma; a primary conductive tissues (xylem and phloem), the primary xylem consists of protoxylem and a metaxylem; and a pith (Fig. 7).

After exposure to CYNs, the size of the vascular bundles were affected. Thus, metaxylem gradually regressed. At 10 µg.l^{-1} CYNs, the primary xylem was completely reduced, the vessels were closed and the phloem was no longer visible in the cups. This observation was also correlated with the chlorosis of the stem at this stage (Fig. 8). In addition, the outer surface of this stems irregularities were increasingly marked with significant concentrations, and cortical parenchyma cells died and their contents emptied. This can be attributed to the drying and softening the stems. Similar results were observed in the stems of tomatoes, but with a shorter

exposure time (3 weeks) than that observed in cucumbers (4 weeks).

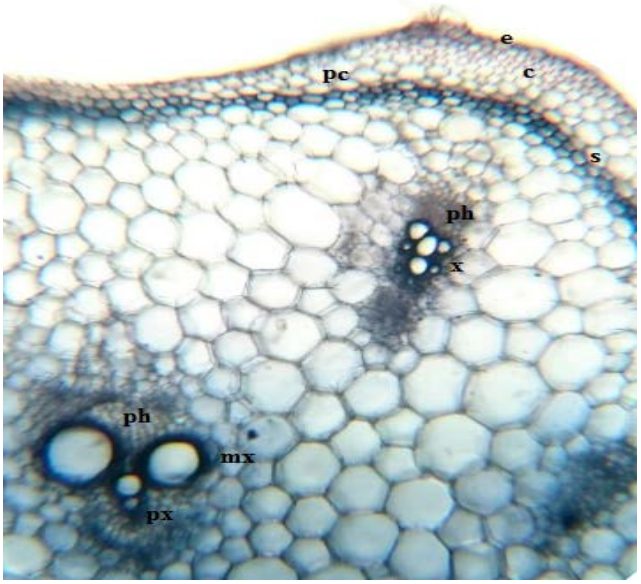

Figure 7: Cross section of control stem cucumber . e, epidermis ; c, collenchyme ; pc, parenchyme cortical ; s, sclerenchyma ; ph, primary phloem; x, primary xylem ; mx, metaxylem ; px, protoxylem

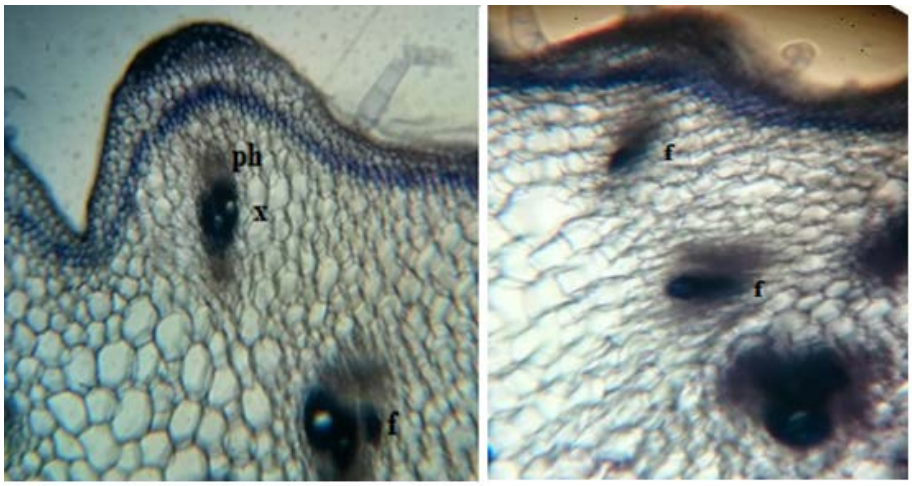

Figure 8: Cross-section of stem cucumber, after exposure to 5 µg.l-1CYNs (left) and 10 µg.l-1 (right) of CYNs. On the left, the outer surface of the stem is marked by irregularities, cells are necrotic cortex and the vascular bundle leads are reduced. B, the irregularities are accentuated and primary conductive tissue (f) have atrophied.

3.3. Effet of CYNs on photosynthetic activity

The results show a reduction in the chlorophyll content in relation to the concentration of CYNs. Disturbance of the photosynthetic process is significant especially in seedlings exposed to 10 µg.l-^{1} with a higher sensitivity in tomatoes than in cucumbers (Table 3 and Table 4).

Table 3: Variation of chlorophyll content in tomatoes at various concentrations of CYNs. Each value is the average of three values of replications. Each value is verified by ANOVA test with P 0.05. The correlation coefficient is significant at $P \leq 0.01$

CYNs concentration	1st leave	2nd leave	3rd leave
0	35.8	46.7	58.6
2 $\mu g.l^{-1}$	9.8	13.7	18.3
5 $\mu g.l^{-1}$	5.6	10.4	13
10 $\mu g.l^{-1}$	1.2	4.2	6
F value	413.818	235.389	511.522
P value	0.000	0.001	0.000
Correlation	- 0.889	0.855	- 0.887
P value	0.001	0.000	0.000

Table 4: Variation of chlorophyll content in cucumber at various concentrations of CYNs. Each value is the average of three values of replications. Each value is verified by ANOVA test with P 0.05. The correlation coefficient is significant at $P \leq 0.01$

CYNs concentration	1st leave	2nd leave	3rd leave
0	29.8	46.3	59
2 $\mu g.l^{-1}$	22.3	38.8	44.3
5 $\mu g.l^{-1}$	12.9	32.6	39.4
10 $\mu g.l^{-1}$	8.5	27.6	34.2
F value	166.845	53.608	475.692
P value	0.000	0.000	0.001
Correlation	- 0.981	-0.974	- 0.957
P value	0.002	0.002	0.001

3.4. Stress index

The results show a positive relation between the stress index and the concentration of CYNs, The stress became more significant at a concentration of 10 µg.l^{-1} (Fig. 9).

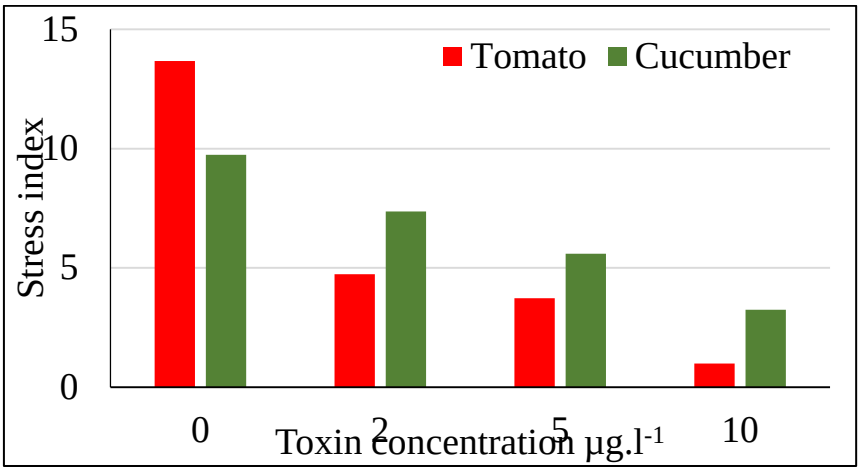

Figure 9: Variation of stress index in tomato and cucumber with respect to CYN concentrations

4. Discussion

The exposition of the seeds and seedlings of tomato (*Lycopersicon esculatum L.*) and cucumber (*Cucumis sativus L.*) to CYNs induced a reduction in seed germination rate and affected the growth, the productivity and the development of the seedlings. Comparable results were obtained by El Khallouffi and his colleagues in 2012 after exposure of tomato seeds and seedlings to another type cyanotoxins called microcystin, MCs [5]. Other studies also demonstrated that the germination of seeds of several types of plants (*Lens esculenta, Brassica napus, Oryza napa, Triticum durum, Zea mays* and *Pisum sativum*) were affected by the extracts of microcystin, MC-LR [8,15,16]. These results confirm that the cyanotoxins affect seed germination.

The effect of CYNs on the seed germination depends on the applied concentration and the species itself. Sengar and his colleagues in 2010 showed that soaking *Vigna radiata* seeds with a cyanobacterial extract of *Microcystis aeruginosa* caused a decrease in the growth of the rootlets in a relatively proportional way with the increasing concentration of cyanobacterial extract [17]. Seeds germination and roots growth were also inhibited at a MC concentrations of 5µg /l MCs in *Medicago sativa* [18] and 800 µg /ml in Zea mays [6].

However, the cucumber seeds are less sensitive to CYN than those of tomatoes and can germinate at various toxin concentrations but with variations in the length of radicles. This shows that certain species are more resistant to CYNs than others, for instance, Chen and his colleagues in 2004 found that rice seeds are more resistant than colza to MCs [8].

In addition to the inhibitory effect on seed germination, morphological changes such as reduction in the length of stems, roots (main and lateral) and the reduction in the number and size of leaves clearly appeared in tomato

and cucumber seedling exposed to CYNs. Dao and his colleagues in 2014 studied the effect of MCs at concentrations of 20 and 200 µg /l on seedling growth of *Brassica rapa-chinesis*, *B. narinosa* and *Nasturtium officinale*, measuring the fresh weight and stem length and roots [19]. He found that higher concentrations of cyanobacterial toxins had greater effects on the fresh mass of exposed seedlings. MC reduces the water absorption by the seedlings resulting in growth limitation and therefore reduction of fresh weight in seedlings [5], [20]. The CYNs acted like MCs, they affected the development of roots and stems, reducing their lengths. *Lactuca sativa* root growth was inhibited when exposed to extracts from 5.9 to 56.4 µg.l-1of microcystins [12]. It has been also shown that the number of fronds of aquatic plants *Spirodela oligorrhiza* [21] and *Lemna gibba* [22] as well as the number and size of the leaves of spinach, *Spinacia oleracea var. Ballat* and *var. Sharan* were significantly reduced after exposure to microcystins [23].

Histological observations indicated an alteration of the vascular bundles of the seedlings exposed to high concentrations of CYNs. The regression of primary xylem was correlated with the inhibition of root growth and consequently the water absorption reduction of seedlings. The problem of root uptake accompanied by mineral nutrients deficiency are all elements essential to plant growth and the chlorophyll synthesis.

In addition to morphological changes, chlorosis and necrosis appeared on the leaves of the tomato and cucumber seedlings that were exposed to increasing concentrations of CYNs. Studies confirm that the start of the necrosis is linked to a disturbance in the metabolism of the exposed plants, such as inhibition of photosynthesis and reduction of the chlorophyll content. The concentration of the latter in the leaf tissue of tomato and cucumber varied inversely with the concentration of CYNs. It has been shown by El Khalloufi and his colleagues in 2012, that the inoculation of the leaves of tomato *Lycopersicon esculentum* by MCs (2.22-22.24 $\mu g.ml^{-1}$) resulted in the appearance of necrosis of leaf tissue and a reduction in chlorophyll a and b contents and was followed by a reduction in the photosynthesis of the order of 50% [5]. Similar results were obtained on the *Nicotiana tabacum* leaves after their exposure to 60 and 120 $\mu g.ml^{-1}$ MC-RR, inducing apoptosis of cells exposed to a low concentration, and necrosis to those exposed to a higher concentrations [24].

The chlorosis of cotyledons of cucumber seedlings exposed to CYNs can be due to the reduction in chlorophyll content; and possibly disruption of enzymes present in the cotyledons, which are responsible for the degradation and transport of reserves in the growing seedling.

In addition, exposure to CYNs induced oxidative stress in seedlings of tomato and cucumber. The measurement of the stress index affirms that the stress becomes increasingly significant with increasing dose of toxin. Noting that the stress index is greater when its value is lower.

It has been shown that plants induce defense reactions against oxidative stress by activation of anti-oxidative secondary metabolites that neutralize free radicals of oxygen appearing upon exposure to cyanotoxins. According to Pflugmacher and his colleagues in 2007 , the exhibition of different varieties of spinach, *Spinacia oleracea*, 0.5 $\mu g.l^{-1}$ for 6 weeks, caused an increase in the activity of antioxidant enzymes (POD, CAT, SOD) compared to the control plants [23]. Furthermore; analyzing the enzyme activity of GST (cytosolic and microsomal), involved in the route of biotransformation of MCs, and revealed a clear increase of this enzyme in

all tested varieties. Chen and his colleagues in 2004 demonstrated an increase of two antioxidant enzymes (SOD and POD) in seedlings of *Brassica napus* and *Oryza sativa* submitted to microcystin stress manifested by oxidative stress [8]. Also, according Khalloufi EL and his colleagues in 2012, the increase of phenolic compounds and peroxidase activity in the tomato seedlings, *Lycopersicon esculatum* exposed could be connected to the detoxification process [5]. All these results confirm that seeds and seedlings exposure to MCs as well CYNs induce oxidative stress, which requires cooperation between the different antioxidant enzyme forms to remove stress as a specific detoxification pathway.

Simultaneously, tomatoes control stems are provided with trichomes, those cucumbers develop tendrils enabling him to cling to supports and push upwards. Seedlings exposed to CYNs are devoid of trichomes and tendrils. Probably due to the disruption of the metabolic activities of these seedlings. Tomatoes without trichomes, some glandular, can no longer secrete a fragrant essential oil. As for no frills cucumber seedlings become more fragile and unstable unable to settle to lift the leaves to light.

5. Conclusion

In this study, we showed that both hepatotoxins (CYN and MC) have phytotoxicity on seed germination and seedling development. The exposition of seeds and seedlings to water containing these cyanobacterial toxins can cause negative effects on the life cycle of the plant and cause damage on yield and crop quality. Therefore, irrigation of crops by contaminated water does not only raises economic problems, but also health problems for consumers of these crops. Indeed, the CYN can accumulate in plant tissues and transfer thereafter through the food chain. Phytotoxicity CYNs appeared clearly on the tested crops that showed significant reduction in the length of the shoots and roots, leaf number, biomass, and the content of chlorophyll. In addition, abnormal color and leaf shape. Phytotoxicity induced by exposure to CYNs confirms that the cyanobacterial bloom has severe negative impacts to ecological levels, economic and health; which requires strong needs for monitoring the quality of the water used for irrigation.

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