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Toxic Effects of Purified Microcystins from Soil Blue-Green Alga *Oscillatoria pseudogeminata on* Tomato Plant *Lycopersicon esculentum*

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Abstract:

The current study included the isolation, purification and cultivation of blue-green alga *Oscillatoria pseudogeminata* G.Schmidle from soil using the BG-11liquid culture medium for 60 days of cultivation. The growth constant (k) and generation time (G) were measured which (K=0.144) and (G=2.09 days).

Microcystins were purified and determined qualitatively and quantitatively from this alga by using the technique of enzyme linked immunosorbent assay (Elisa Kits). The alga showed the ability to produce microcystins in concentration reached 1.47 µg/L for each 50 mg DW. Tomato plants (*Lycopersicon esculentum*) aged two months were irrigated with three concentrations of purified microcystins 0.5 , 3.0 and 6.0 µg/L for 24 days. The results showed that the highest bioaccumulation of these toxins was found in the plant group treated with concentration 6.0 µg/L, with an average of 12.278 µg/L in the entire plant body, with a daily accumulation rate of 0.511 µg/day compared with control group and other treatments ($p \leq 0.05$).

The highly accumulation of toxin was found in roots followed in descending order by stems and leaves respectively and were directly proportional to the increased concentration of purified microcystins. The study indicated that there were significant changes ($p \leq 0.05$) in the plant height, length of roots and surface area of the leaves and the leaves appeared pale green in color, which increased with increasing the concentration of purified toxin. The obtained results also showed a significant decrease in the concentration of carbohydrates and chlorophylls by increasing concentrations of purified toxins.

Key words: *Lycopersicon esculentum*, Microcystins toxins, *Oscillatoria pseudogeminata*, Tomato plant.

Introduction:

Cyanobacteria (blue-green algae) found in different habitats including fresh, brackish, marine water and soil (1). Several genera belonging to cyanobacteria as *Microcystis, Anabaena, Oscillatoria, Haapalosiphon, Radiocystis, Stigonema, Lyngbya, Pseudoanabaena and Phormidium* are known to produce microcystins (Hepatotoxins) and neurotoxins or both. (2, 3, 4). Many species of blue green algae reach approximately to 80 species can produce hepatotoxin (Microcystins only) (5). The safe levels of microcystins are approximately 0.3 µg/L in drinking water for children, while for other ages reach to 1.6 µg/L (6), but the concentration of these toxins may reach to 100 µg/L in some water bodies (7). The proliferation of toxic and non-toxic blue-green algae in aquatic habitats is well known as a famous phenomenon. However, the expansions of toxic species to a wide range may cause harmful effects on ecosystems (8).

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Water blooms of blue-green algae have caused many damages to environment conditions and human health in recent decades and impediment to biological waste water treatment (9, 10). Microcystins are secondary metabolites produced by blue-green algae and causes toxicity to higher organisms including humans, plants as well as food supplements (11, 12), the effect of blue green algae and their toxins in agriculture is not yet understood especially microcystins-LR (13). On the other hand, the water used for irrigation often comes from natural water systems or ponds constructed for agricultural purposes which are not subject to the examination and treatment or monitoring of microcystins. In addition, the cyclic composition of these toxins provides a high chemical stability with its ability to stay in the soil for a long time. Recently, the half-life of these toxins in agricultural soil is estimated as 56 days (14). Consequently, the presence of these toxins in both irrigation and soil water can have a negative impact on soil organisms as well as on the growth and development of crop plants (7). The cyanotoxins in the soil may be transported to water bodies again by runoff

,leaching process or accumulation in soil ,consequently , this cause contamination of plants especially leaves and fruits (15). The current study is devoted to determining the toxic effects of hepatotoxins (Microcystins) which was isolated and purified from soil blue-green alga *Oscillatoria pseudogeminata* with very low concentrations less than the permitted concentrations worldwide, as well as higher concentrations on one of the economically important tomato plants (*Lycopersicon esculentum*).

Materials and Methods:

Sample collection of soil blue-green alga

Wet soil samples containing developing algae were collected in the area of the Dafas orchards located adjacent to the Tigris River in the district of Maysan governorate. Clean sealed plastic containers were used to collect the soil samples. Samples were collected at a depth of 3 cm from the soil surface, and then were brought directly to the laboratory to isolate and cultivate the algal species.

Isolation of blue-green algae from soil samples

1 g of wet soil sample was taken and mixed with 10 ml distill water and put in a test tube and then inserted into the Centrifuge type (TLE-Danger) quickly 3000 rpm for 10 min. After that the algae were scrapped from the surface of the precipitate and increased by 10 mL of distilled water. Several drops were taken for making glass slides from each sample to identify their algal content by using the Olympus CX21 optical microscope. (Under the magnification X4, X10, X40 and X100). Then 5 ml of algal solution was taken and washed several times with distill water using centrifugation at 3000 rpm and 10 min that in order to get rid of soil, plankton and impurities, then 1 ml of washed samples were put in a test tube and completed to 5 ml by sterile liquid media and left in the incubator for 7-10 days. After the algae growth on the walls of test tubes was obtained, the dilution method was made to get unialgal cultures (16).

Purification of unialgal cultures of blue-green alga

After obtaining algal culture of alga *O. pseudogeminata*, Weidman *et al*., (17) method was used to obtain the axenic culture according to the following. Unialgal cultures were washed with sterile distilled water and then centrifuged at 3000 rpm for 5 minutes, leave the leachate and recombine the precipitate with distill water again and repeat this process 10 times. To ensure the purity of the isolates, the method described by stein (16), which included the cultivation of algal isolate in nutrient agar medium and incubated at 37 ° C for 24 hours to ensure that the absence of bacterial growth and for a week to ensure the absence the fungi growth.

Classification of blue-green alga *O. pseudogeminata*

The blue green alga was classified based on morphological identification (18, 19).

Extraction of Microcystins

Luukkainen *et al*., (20) method was used to extract hepatotoxins (MCs), taking 50mg from the algal biomass and then mixed with the water solution (MBW): Water: Methanol: n-Butanol in proportion 15 : 1: 4 ml respectively in 100 mL conical flasks with tin and cotton foil blocked to prevent evaporation. The samples were then mixed thoroughly using a magnetic stirrer for one hour. The supernatant was collected by centrifugation at 3000 rpm for 10 min. The process was repeated three times; the total supernatant was collected and concentrated to 5 mL using hot dry air.

Purification of Microcystins

The method of Namikoshi *et al*, (21) was used to purify toxins using column chromatography. After some modification, a glass column of 15 x 2 cm was used to purify MCs filled with silica gel with mesh size 200-100 µ. The extracted and concentrated sample in the previous paragraph was loaded in the separation column and was washed with three solvents: 20 ml of ion-free distilled water, 20% methanol, and 80% methanol respectively at flow rate 3 ml/ min. The last eluent was concentrated and preserved at a temperature of (-8 C^0) in refrigerator until analyzed using Enzyme linked Immuno-Sorbent Assay (ELISA) technique. Abraxis Company (United States) kit was used and method was adopted (22).

Effect of purified microcystins from alga *O. pseudogeminata* on total root and shoot (vegetative) of tomato plant.

The experiment of cultivating tomato plant and irrigated with algal toxins was done in laboratory / Faculty of Education-University of Basra/Iraq for the during spring season 2017 in controlled laboratory conditions. The seeds of the tomato *Lycopersicon esculentum* CV (Cultivar) were prepared by Hoda Dutch origin from Dubban Agricultural Company in Baghdad, Iraq, which is resistant to harsh conditions. Three concentrations of purified microcystins from alga *O. pseudogeminata* represented by 0.5, 3.0 and 6.0 µg/L were used to irrigate the tomato plant periodically and for irrigation every three days depending on the field capacity and for 24 days

after reaching the plant two months of age over the control group irrigated with distal water.

The seeds were sown in cork plates containing 99 hole with diameter 4 cm and height of 6 cm filled with peat moss and planted one seed in each hole on 10/3/2017 and placed on a stand inside the laboratory. The seedling was sown with 12.8 cm diameter and 11 cm high. They contained sandy clay soil with 1: 1 peat moss (500 g of mix). The seedlings were sprayed as foliage application by urea at a concentration of 0.5 g / L after 16 and 23 days from planting and to protect it from a disease of Damping off were sprayed with Ridomil Gold at a concentration of 0.5 ml /L at 10 days from planting and was protected from the worms by spraying with a 0.4 mL/L at 19 days of planting. The experiment included four groups, each group containing three replicates (each containing three plants). Three groups were treated with three purified microcystins concentrations mentioned above in addition to the control group (which is not treated with purified microcystins).

Statistical analysis

Statistical package for social sciences (Version-22) was used to analyze data using One-Way analysis of variance (ANOVA) at the probability level ($p \leq 0.05$). The value of revised least significant differences (R.L.S.D.) was applied to compare the differences means according to AL-Rawy and Khalaf-Allah (23).

Results:

Description and classification of the blue-green alga *O. pseudogeminata*

This blue-green alga inhabits wet soils, it was long threads (tricomes) wrapped irregularly, tangled and immobile, thread cells have clear boundaries and the threads appear green under light microscope. The algal cells contain visible gas vacuoles, which are regularly spread in the cytoplasm of the cell, which appear as black dots on the boundary between the cells forming the dermal thread and the thread ends with an around shape. The length of cells range between 2.3-3 µm and the width 1.1-1.4 µm as shown in Fig.1.

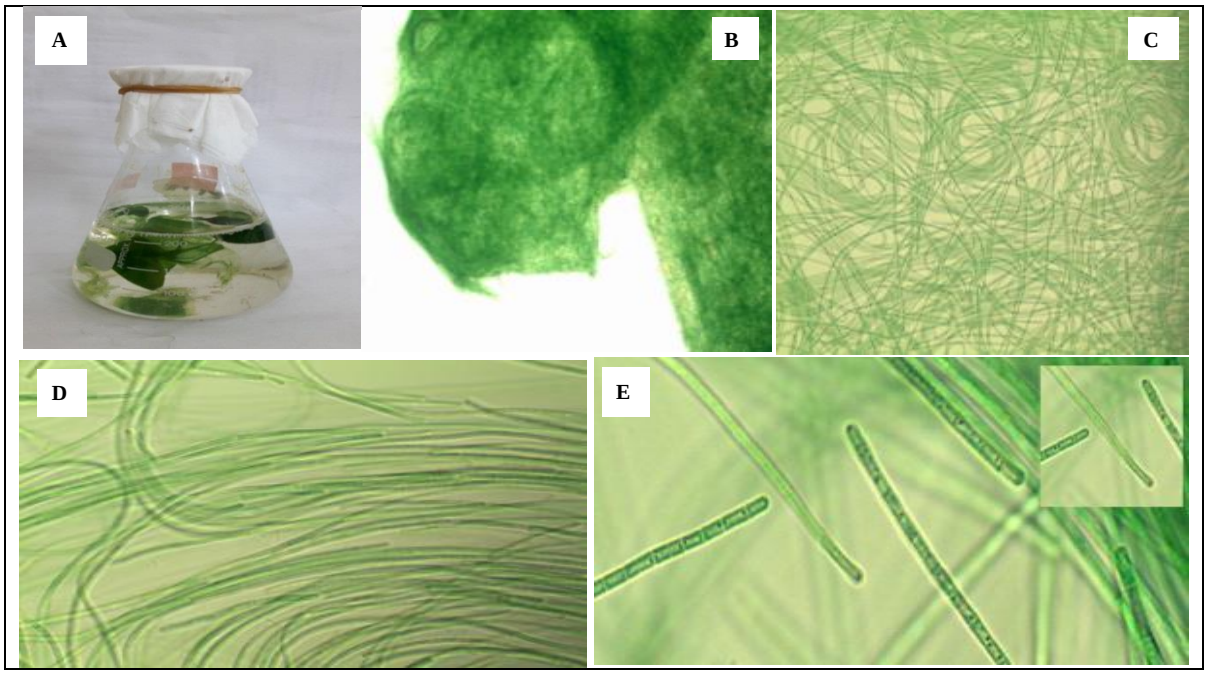

Figure 1. (A): The pure liquid culture of alga *O. pseudogeminata* after a period of 25 days of cultivation. (B): 4X, (C):10X, (D):40X and (E): 100X

Growth curve

The initial phase of the alga *O. pseudogeminata* took three days, followed by the 27-day exponential phase, with a steady speed in growth, which continued until the 30th day. Stationary phase, which continued until the 47th day, Decline phase on the forty-eighth day to the sixtieth day and therefore the alga was harvested in the middle of the stability phase between the thirty-third day and the 40th day. Estimation of growth rate (K = 0.144) and the generation time of (G = 2.09) of days of the investigated alga as recorded in Table-1.

Table 1. Growth curve periods (days) of blue-green alga *O. pseudogeminata* for 60 days of cultivation in BG-11 liquid medium.

Algal species	Lag phase	Exponential phase	Stationary phase	Harvested days	Growth constant	Generation time
O.pseudogeminata	**3**	**27**	**17**	**33-40**	**0.144**	**2.09**

Qualitative and quantitative assessment of Microcystins in alga

The qualitative and quantitative assessment of hepatotoxicity of microcystins (MCs) after purification was determined using enzyme-linked immunosorbent assay (ELISA) technique. The results showed that *O. pseudogeminata* was able to produce MCs with a concentration of 1.47 µg / L for 50 mg algal dry weight.

The ability of tomato plant (*L. esculentum*) to bio-accumulate purified microcystins

The results shown in Fig.3 showed significant differences at (P ≤0.05) between tomato plant groups treated with different concentrations of MCs purified from *O. pseudogeminata* for 24 days of exposure to these toxins (irrigation periods). The highest concentration was found in the plants of the treated group with 6.0 µg / L with a concentration rate of 12.278 µg / L in the whole plant body with a daily accumulation rate of 0.511 µg / L and the highest concentration in the plants group treated with 6.0 µg / L was found in the roots at an average of 4.818 µg / L followed in descending order by stems 4.091 µg/L and leaves 3.369 µg/L. While, the mean concentration of toxins in the entire plant body in plants group treated with 3.0 µg/L was 3.856 µg / L with a daily accumulation rate of 0.16 µg / L per day with concentrations of 1.717 µg / L in the root and 1.499 µg / L in the stem and 0.640µg/L in leaves . The lowest concentration of accumulated microcystins was found in the plant group treated with 0.5 µg / L and with a daily bioaccumulation rate of 0.069 µg / L per day. The highest concentration of toxins was 0.687 µg / L in the roots followed by the concentration of 0.588 µg / L in stems then in the leaves at a concentration of 0.425 µg / L (thus the total concentration of 1.670 µg / L in the whole plant body) as shown in Fig.4, 5.

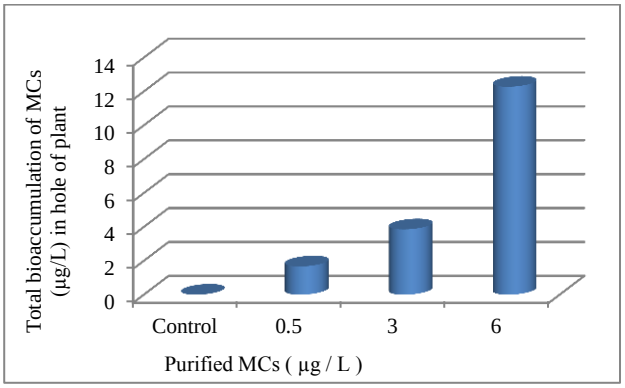

Figure 3. Purified MCs bioaccumulation in plant body (µg / L) for each 250 g D.W).

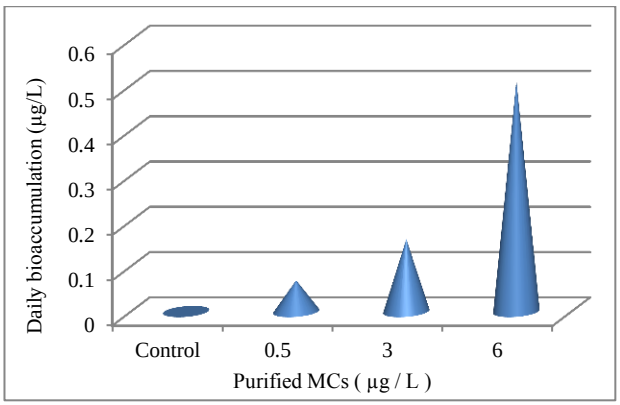

Figure 4. Daily bioaccumulation of purified toxins (MCs) in whole plant body (µg /L).

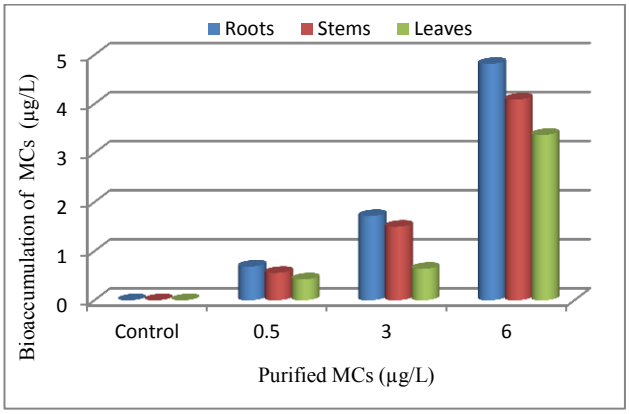

Figure 5. Purified MCs bioaccumulation in different plant body stem roots and leaves (µg / L) for each 250 g DW

Effect of purified MCs on mean length of plant stems, roots and surface area of leaves.

The obtained results showed a significant decrease (p≤0.05) in stems length of plant, especially when irrigated by the concentration of 6 µg / L which reached to 20.2 µg/L compared with control group 28.3 µg/L, while there were no significant differences between the rests of transactions Fig.6 and7. Also the root length decreased significantly (p≤0.05) especially under dose 3 and 6 µg /L which reached to 5.733 and 6.133 µg /L respectively, but the lowest concentration 0.5µg/L did not show a significant difference with the control group (Fig.8).The surface area to tomato leaves decreased only under concentration 6µg/L reaching to 23.3 decimeter^2 compared to the other concentrations which showed non-significant differences with control plants Fig.9.

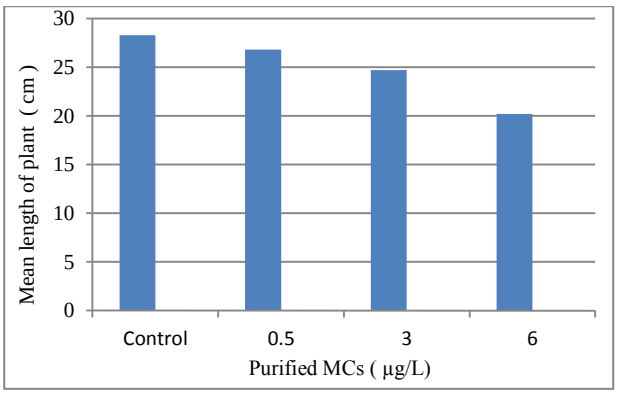

Figure 6. Plant stems length under different conc. of purified MCs.

Figure 7. Toxic effects of Purified MCs on length of tomato plant under different conc. of toxins.

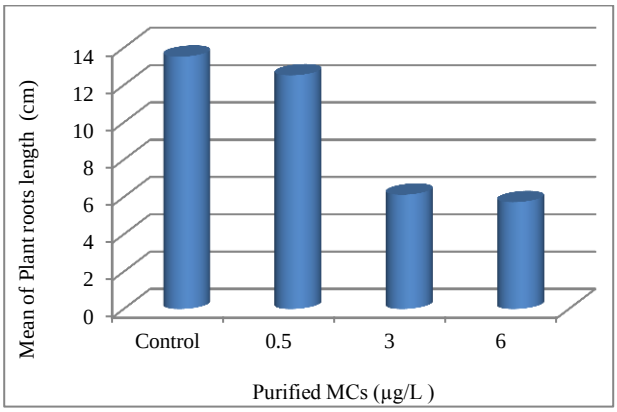

Figure 8. Plant root length under different conc. of purified MCs.

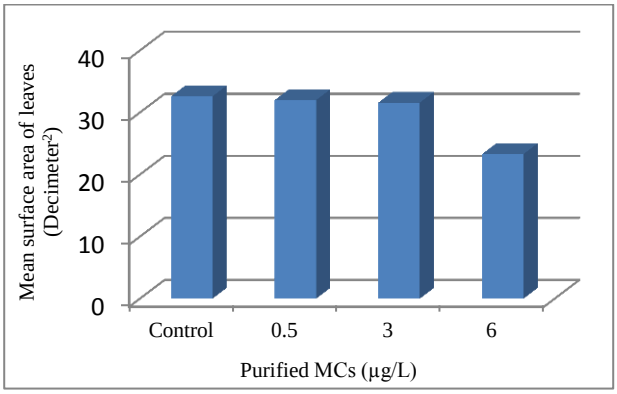

Figure 9. Surface area of plant leaves under different conc. of purified MCs.

Toxic effects of purified MCS on total chlorophyll and carbohydrate concentrations in plant leaves

Results showed significant differences ($p \leq 0.05$) in total chlorophyll concentration with increasing purified toxin concentration especially under 6.0 µg/L concentrations which reached 0.138 mg/100g ww. followed by other concentrations and compared with control group which reached to 0.283 mg/100g ww and the leaves appeared pale green in color with increasing toxin concentrations. On the other hand, the increase in purified toxins led to a significant decrease in carbohydrate concentration especially under 6.0 and 3.0 µg/L purified toxins which reached to 33.64 mg/g dw and 38.73 mg/g dw respectively compared with that produce under toxin concentration 0.5 µg/L and control group which reached to 45.94 and 55.95 mg/g dw respectively is illustrated in Fig.10,11and 12.

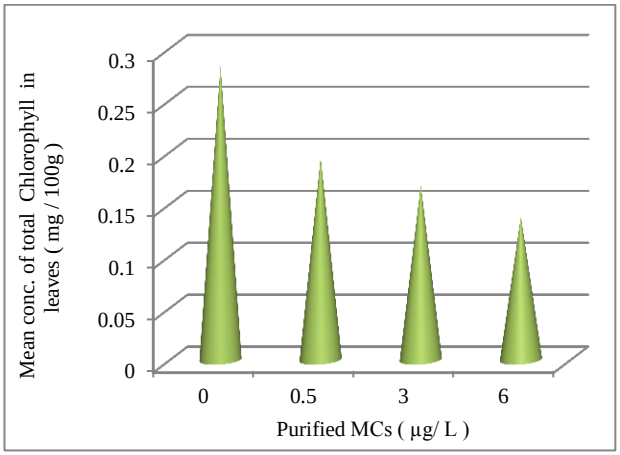

Figure 10. Total chlorophyll conc. in leave of plant under different conc. of purified MCs.

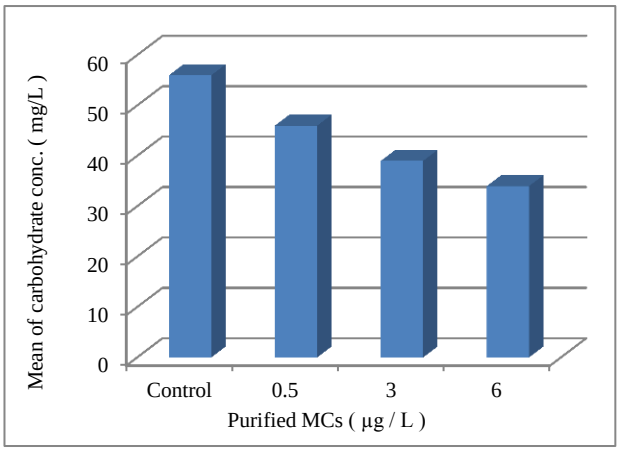

Figure 11. Carbohydrate conc. in leave of plant under different conc. of purified MCs.

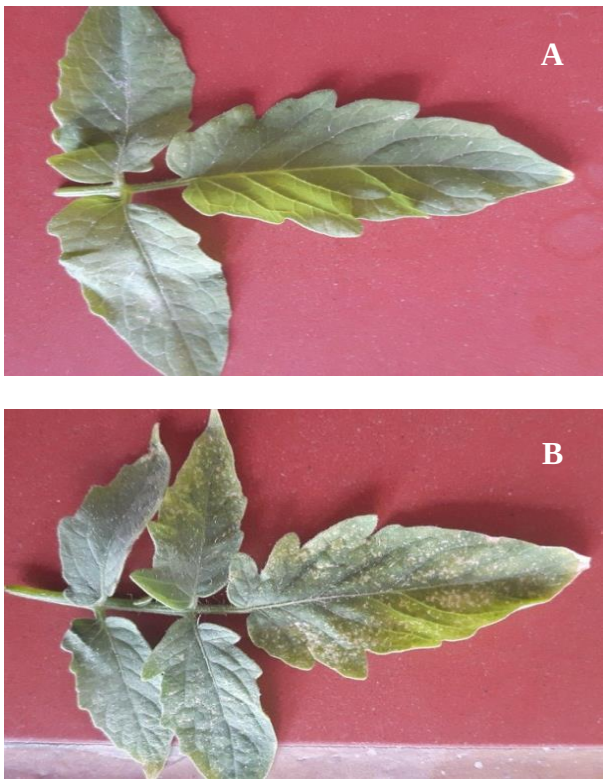

Figure 12. Toxic effects of purified MCs on color of leaves. (A): Control group, (B): Treatment group with 6 µg/L of purified toxins.

Discussion:

Several previous studies have been concerned with blue green algae and their toxins production in the aquatic environments and purified toxins (MCS) on mice, fish and snails (24, 25, 26, 27, 28, and 29). However, the current study is concerned with the toxicity of algae inhabiting the wet land (Soil algae) and its ability to produce hepatotoxins, especially the microcystins and their effects on one of the economically important plants. Results showed the ability of soil alga *O. pseudogeminata* to produced high concentration of microcystins reached to 1.47 µg / L for 50 mg of algal dry weight, equal to 870 µg / L for 1g (1000 mg) of algal dry weight, so this result is considered the first attempt to the isolation, purification of blue green algae from Iraq soil and study their ability to produce microcystins, This algae showed producing high amount to microcystins compared with other species isolated from sewage and fresh water . The growth curve of this alga in BG-11 liquid medium showed less growth constant from the same species ,but isolated from water environment (fresh water) (Shutt Al-Arab river southern of Iraq) and cultivation in Chu-10 liquid medium as reported by Aubaed (30).

In this study, the bioaccumulation of purified MCs from soil blue-green alga *O. pseudogeminata* was studied to identify its impact on different parts of the tomato plant (*L. esculentum*) represent in roots, leaves and stems after irrigation for 24 days with water containing different concentrations of these purified toxins. This study is different from most of the other studies where it neglect using high toxin concentrations to using concentrations that near to the natural environment concentration in water bodies represented by concentration 0.5 µg / L, which is considered to be less than the acceptable concentrations (1 µg/L) according to the WHO classification (31).In addition to apply high concentration 3.0 and 6.0 µg /L.

Concerning the previous studies, which indicated that blue-green algae toxins, especially MCs, can be bio-accumulated in various crops when irrigated with water contaminated with blue-green algae or toxins (32, 33, 34, 35 and 36). The main mechanism of hepatotoxicity in both animals and plants is inhibition of serine / threonine proteins phosphatases 1 and 2A (PP; PP1 and PP2A) by associating with covalent bonds (37, 38). Although the target molecules look the same enzymes in both animals and plants, the plant's absorption mechanism for MCs is not yet explored and specific vectors have not yet been identified in the plants. However, these carriers may be similar to peptides and amino acids in plants (39). Since microcystins are peptide compounds, it is probable to suggest that peptides may be involved in the transfer of microcystins in plants (40). Finding appeared that the plants treated with high dose of purified toxin showed highly accumulation of toxins in different plant tissues when compared with the control group. These results are consistent with the study of Hereman and Bittencourt-Oliveira (41) they found that the amount of concentrations measured in plants depends on the amount of concentrations in the water used for irrigation.

The significant differences in the accumulation of MCs in different parts of the tomato plant with increasing purified toxins in irrigation, specifically in the root were recorded in this study. Microcystins was accumulated in the body of the plant with increased concentrations treated and the highest concentration of toxins was found in the roots of plants treated with all concentrations compared to the stems and leaves. In spite of the fact that the root of the tomato plant is not the part used for nutrition by the human, but in many plants where the root is the edible part increase the risk of toxin accumulated in the plant roots exposed to irrigation with container water on blue-green algae or their toxins, especially the daily exposure allowed for microcystins developed by WHO (31) Is 0.04 µg / kg B.W / day. These results were in agreement with the study of Gutiérrez-Praena *et. al*., (33). Their found that the highest concentration of microcystin in root after irrigating

the tomato plant every three days with 500 ml of water containing 100 µg / L of crude microcystins for two weeks. Studies on Broccoli and Mustard *Sinapis alba* showed that microcystins were found only in the roots of these plants with concentrations ranging from 0.9-2.4 µg / kg for broccoli and 2.5-6.6 µg / kg of mustard weight after exposure to microcystins concentration between 1 and 10 µg / L for 20 and 19 days respectively (42). The study of Machado *et al*., (36) also reported that the accumulation of hepatotoxin type MC-LR in carrot type *Daucus carota* was 5 µg / kg wet weight when exposed to hepatotoxin type MC-LR at 10 and 50 µg / L for 32 days after reaching the plant to one month of age. Since the root is the edible part of the carrot plant, there are fears that toxins can spread through the food chain and threaten human health.

Several significant changes in the vegetative growth indicators of tomato plant when exposed to the purified toxins (MCs), denoted by decreasing the length of the stems, roots and surface area of the leaves. The results are consistent with the study of Dao *et al*., (43), despite the use of high concentrations of microcystins reach to 20 and 200 µg / L affected significantly the plant seedlings of the type *Brassica rapa* and turnip type *B. narinosa* and watercress plant type *Nasturtium officinale* which led to a decrease in the length of the stem and root .The study of Pereira *et al*., (44) also showed inhibited root growth in lettuce *Lactuca sativa* when exposed to the extract of hepatotoxins (MCs) with concentrations of 5.9 to 56.4 µg / L. These results were also parallel with the study of Pflugma Cher et al (45) who recorded very clear decrease in the surface of the leaves of spinach (*Spinacia oleracea*) leaf area type when exposed to microcystins (45). In addition to the morphological changes, the pale green color (Chlorosis) of leaves of the tomato plant with increasing in MCs , caused decrease in chlorophyll concentration and carbohydrates reserves ,this results may be due to or associated with disorders in the metabolism of plants exposed to such toxins, Such alteration of metabolism may be as inhibition of photosynthesis, reduction of total chlorophyll content and the difference of chlorophyll concentrations in the tissue of tomato leaves was reversely proportional with the concentration of exposed microcystins (46,47).

Conclusion:

We concluded from the present findings that blue-green algae specifically the alga *O. pseudogeminata* may affect directly the growth of plants. The irrigation of agricultural crops by water contaminated with blue-green algae or their toxins does not only raise economic and environmental problems, but also health problems for consumers, especially field crops, due to the accumulation of these toxins in plant tissues and their transmission through the food chain, Even though the concentrations of toxins are very low.

Conflicts of Interest: None.

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التاثيرات السامة للمايكروسستينات المنقاة من طحلب التربة الاخضر المزرق *Oscillatoria pseudogeminata* على نبات الطماطم *Lycopersicon esculentum*

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الخلاصة:

اشتملت الدراسة الحالية على عزل وتنقية وزراعة الطحلب الاخضر المزرق *Oscillatoria pseudogeminata* G.Schmidle من التربة باستخدام الوسط الزرعي الغذائي السائلBG-11 ولمدة 60 يومًا. تم قياس ثابت النمو (k) وزمن تكاثر الجيل (G) والذي بلغ (K = 0.144) و (G = 2.09 يوم) .تم تنقية السموم الكبدية Microcystins وتشخيصها نوعا وكما من الطحلب باستخدام تقنية الامتزاز المناعي المرتبط بالإنزيم Enzyme linked immunosorbent assay. أظهرت النتائج قابلية الطحلب على إنتاج السموم الكبدية وبتركيز بلغ 1.47 ميكروغرام / لتر لكل 50 ملغرام من الوزن الجاف للطحلب. عومل نبات الطماطم بعمر شهرين بالسم المنقى وبثلاث تراكيز 0.5 و 3 و 6 ميكروغرام / لتر لمدة 24 يوما. أظهرت النتائج أن أعلى تراكم حيوي لتلك السموم وجد في مجموعة النباتات المعاملة بالتركيز 6 ميكروغرام / لتر بمعدل بلغ 12.278 ميكروغرام / لتر في جسم النبات الكامل مع معدل تراكم يومي قدره 0.511 مايكروغرام/ يوم مقارنةً بمجموعة الكونترول والمعاملات الاخرى ($p\leq0.05$). كما بينت النتائج اعلى مراكمة للسموم الكبدية المنقاة وجدت في الجذور والسيقان والأوراق على الترتيب تنازليا وازدادت بزيادة تركيز السم المنقى. أشارت الدراسة إلى وجود انخفاض معنوي ($p\leq0.05$) في ارتفاع النبات وطول الجذور ومساحة سطح الأوراق وقد بدت الأوراق باللون الأخضر الباهت والتي ازدادت بزيادة تركيز السم المنقى. وأظهرت النتائج أيضا انخفاض معنوي في تركيز الكربوهيدرات والكلوروفيل بزيادة تركيزالسموم الكبدية المنقاة.

الكلمات المفتاحية: الطحلب الخضر المزرق *Oscillatoria pseudogeminata* ، المايكروسستينات (السموم الكبدية)، نبات الطماطم *Lycopersicon esculentum*