

Isolate and Cultivate Three Species of Blue-Green Algae from Soil Southern of Iraq and Study the Effect of Purified Microcystins from Alga *Oscillatoria Pseudogeminata* on Seed Germination of Tomato Plant *Lycopersicon Esculentum*

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Abstract

The present study included the diagnosis, isolation, purification and cultivation of three blue-green algal species belong to three genus represented by *Aphanothece clathrata*, *Microcystis flos-aque* and *Oscillatoria pseudogeminata* from the moist soil in the Al-Dafas orchards area, which is adjacent to the Tigris River in the district of Amara / Maysan province and Basra southern Iraq. The algae were cultivated in the BG-11 medium .The growth constant (k) and generation time (G) was measured for 60 days. Microcystins were purified from algae and detected qualitatively and quantitatively by using ELISA technique reach to 0.34 and 0.734 $\mu\text{g/L}/50$ mg of dry weight for the first and second algae respectively, While the alga *O. pseudogeminata* demonstrated its ability to produce microcystins at high concentration of 1.47 $\mu\text{g} / \text{L} / 50$ mg of dry weight of alga. The seeds of tomato plant *Lycopersicon esculentum* were treated with purified microcystins (MCs) from alga *O. pseudogeminata* for 7 days with three concentrations of 0.5, 3 and 6 $\mu\text{g} / \text{L}$, in addition to the control group which was treated with distilled water. The results showed a significant decrease $P \leq 0.05$ in the germination percentage in the first and second day of the experiment under concentrations 3 and 6 $\mu\text{g} / \text{L}$ reached to 0% in both compared with control group and concentration of 0.5 $\mu\text{g} / \text{L}$ which their reach 26.6%. On the third to seven days of exposure, the germination percentage was range between 3.3- 86.6% when exposed to 6 $\mu\text{g/L}$ compared with control group (100%). Also results showed a significant decrease $p \leq 0.05$ in the rate of germination speed, mean length of the radicle and plumule, fresh and dry weight with increasing of period exposure and purified microcystins concentration exactly at high concentration 6 $\mu\text{g} / \text{L}$.

Keywords: Purified microcystins, *A. clathrata*, *M. flos-aque* *O.pseudogeminata*, Seed germination, *L. esculentum*

1. Introduction

Microcystins are considered secondary metabolites which produce by some blue-green algae and causes toxicity in higher organisms such as human health, plants and food supplements (Drobac *et al.*, 2013, Schmidt *et al.*, 2014). But the effect of blue green algae and their toxins in agriculture is not yet understand especially microcystins-LR (Praena *et al.*, 2014). At present many species of cyanobacteria (Blue-green algae) reach approximately more than 80 species can produce hepatotoxin (Microcystins only) (EPA, 2014). Most of the blue-green algae blooms occur in open water systems such as oceans, rivers, lakes and ponds, but they can also appear in water for plant irrigation, including crop plants. For this reason, it has been assumed that microcystins can accumulate in the tissues of food they then pose a potential risk to food safety and human health through the food chain (Xiao *et al.*, 2009). In this regard, microcystins were found in irrigated fields of chestnut plant. Their concentrations in plants reached 7 $\mu\text{g} / \text{kg} / \text{dry weight}$, as found in tomato plant fields, and its concentrations of microcystins accumulated in fruits reached 1.16 $\mu\text{g} / \text{kg}$. (Romero *et al.*, 2014). The accumulation was found in many other plants crops irrigated with contaminated groundwater, with a concentration of 70-1200 $\mu\text{g} / \text{kg w.w.}$ (Mohamed and Al Shehri 2009). The study of Crush *et al.*, (2008) showed the clover, kelp and lettuce plants accumulated toxins of 0.2-1.45 $\text{mg} / \text{kg} / \text{D.W.}$ when exposed to a concentration of 2.1 mg / L of those toxins for 15 days.

The negative effects of blue -green- algae toxins in crop plants can cause reducing growth, productivity and crop quality. The study of Kós *et al.* (1995) showed that exposure to different concentrations of MCs inhibited the growth of *Nustapis alba* mustard , However, some studies have shown that these toxins have a negative effect on seed germination and root development (Máthé *et al.*, 2009, Al-Khalloufi *et al.*, 2012). It has been shown recently that it inhibits the complexity and growth of Rhizobia with a potential reduction in nitrogen uptake in leguminous crops (El Khalloufi *et al.*, 2012; Lahrouni *et al.*, 2012). The present study was aimed to isolate and purify and cultivate of the blue – green algae which inhabit the soil environment diagnosis the susceptibility to the production of hepatotoxins (microcystins) and extracting and purifying toxins from them and testing their toxic effect on one of the seeds of one of the local field crops (tomato plants *Lycopersicon esculentum*).

2. Material and Methods

2.1. Sample collection and isolation of soil blue-green alga *Oscillatoria pseudogeminata*

The algal samples were collected from wet soil areas of the Al-Dafas orchards / Maysan and Basra governorate/southern of Iraq at a depth of 3 cm from the soil surface by using clean sealed plastic containers. Algal samples were brought directly to the laboratory to isolate and cultivate the algal species. The isolation was made according to the method of Stien (1975). One gram of soil sample were mixed with distilled water (10 ml). The mixture was centrifuged at 3000 rpm for 10 min. The surface of the precipitate was scrapped and diluted by 10 mL of distilled water. Glass slides were made from this solution to diagnose algae by using the Olympus optical microscope type CX21. Five milliliter of algal solution was washed by using centrifuge type TLE-Danger several times with distilled water at 3000 rpm for 10 minutes. Then 1 ml of washed samples were put in a test tubes and the size completed to 5 ml from the sterile liquid media (BG-11). The culture was incubated for 7-10 days in growth chamber with light and dark illumination 12:12 under the light intensity (130-150 $\mu\text{E}^2\text{Sec}^{-1}$). After to then the unialgal cultures was done according to the method of (Stein, 1975).

2.2. Purification of unialgal cultures

The method of Weidman *et al.*, (1984) was used to obtain the axenic culture of blue-green algae according to the following: The unialgal cultures of alga were washed with sterile distilled water by using centrifuged at 3000 rpm for 5 minutes. The precipitate washed with sterilize distilled water again and repeat this process 10 times. The methods of (Stein, 1975) were used to ensure that the bacteria and fungi did not grow.

2.3. Classification of alga: The alga was classified according to Desikachary (1959) and Prescott (1975).

2.4. Extraction and purification of Microcystins

The method of Luukkainen *et al.*, (1993) was used to extract microcystins from the three soil algal isolates. Fifteen milligram of lyophilized biomass from alga was taken and then mixed with mixture solution (MBW): Water: Methanol: n-Butanol in portion 15: 1: 4 ml respectively in 100 mL conical flasks with a magnetic stirrer for one hour. The mixture was centrifuged at 3000 rpm for 10 minutes. This process was repeated three times; finally the total supernatant was collected and concentrated to 5 mL by using hot dry air. Microcystins purification was made according to the method of Namikoshi *et al.*, (1993) which depending on column chromatography with some modification, a glass column of 15 x 2 cm was used. The column filled with silica gel (mesh size 200-100 μ). The concentrated extract in the above paragraph was loaded in the column and was washed with three solvents: 20 ml of ion-free distilled water, 20% and 80% methanol respectively at flow rate 3 ml/ min. The last eluent was concentrated and stored at (-8 C⁰) in refrigerator until analyzed. by ELISA technique From Abraxis Company (United States) kit was used according to Fischer *et al.*, (2001).

2.5. Effects of purified microcystins on seed germinations

Lycopersicon esculentum seeds class Hoda was prepared (Dutch origin) from Dabana Agricultural Company/ Baghdad governorate/Iraq, which is resistant to harsh conditions, seeds sterilized with 5% Sodium hypochlorite solution for 5 minutes and then washed with distilled water, then planted on filter paper in Petri dishes (12 dish) clean and sterile (9 cm in diameter) and 10 seeds per dish. A dish was divided into four groups. Three groups were treated with different concentrations of purified microcystins from the *O. pseudogeminata* because it has a highly concentrations of purified microcystins. This represented by concentrations of 0.5, 3, and 6 $\mu\text{g} / \text{L}$ in addition to control group irrigation with distal water. The concentration 0.5 $\mu\text{g} / \text{L}$ are considered less than the universally allowed concentrations according to the classification of world health organization WHO (1999). Each group was added 5 ml to each of the concentrations above. The dishes were covered with their own cover. The germination rate (%) was calculated daily for seven days from the start of the experiment.

2.6. Seeds germination percentage: The rate of seeds germination was measured daily for seven days from the start of the experiment depending on the method of Lee and Woolhouse, (1969) as follows:

Germination percentage % = Number of seeds grown / total number of seeds x 100

2.7. Germination speed: The germination speed was measured daily depending on the method of Camargo and Vanghan (1973) and according to the following equation:

Germination speed (Seed / days) = number of seeds grown / number of days since the beginning of planting.

2.8. Length of the root and the feather (centimeter): The length of the root and the quill was measured using a standard measuring ruler for tenths of centimeters and a length of 20 cm at the end of the experiment (seventh day) and expressed in length by centimeter units.

2.9. Wet weight of the root and the feather (g): The wet weight of the root and the feather was measured using the sensitive balance at the end of the experiment (day 7) and express the weight in gram units.

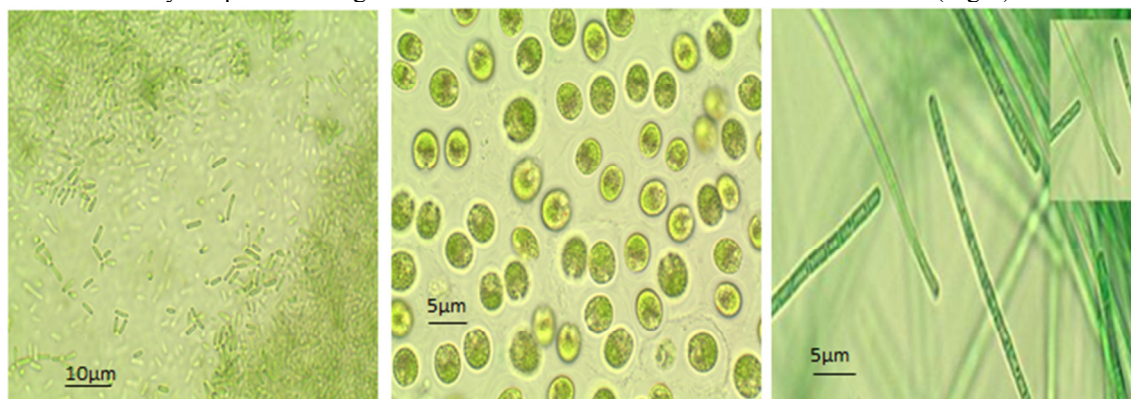
2.10. Dry weight of the root and the feather (gm.): The root and feather was dried in the oven at 65 °C for 72 hours. The weights were then measured by the sensitive balance and the filter paper was neglected.

2.11. Statistical analysis: Statistical Analysis for Social Sciences program SPSS (Version-22) was used to analyze data using one-way anova analysis and below the probability level $p \leq 0.05$. The value of revised least significant differences (R.L.S.D) was made to compare the mains according to Al-Rawy and Khalaf-Allha (1980).

3. Results

3.1: Description and classification of soil blue-green algae

In the current study, three species of blue- green algae were identified, isolated and purified from soil in Maysan and Basra governorates in Southern Iraq represented by species *Aphanothece clathrata*, *Microcystis flos-aque* and *Oscillatoria pseudogeminata* (Fig-1). The alga *A. clathrata* is characterized by a bluish green alga that appears pale under the light microscope in the form of single or a double cell in the form of short bars range in length of the cell between 1-8 μm and width between 0.7-2 μm microns and it grows adherently on the walls of the conical flasks. As for alga *M. flos-aque* it tends to form irregular colonies (palmelloid colonies), the cells of this species are spherical. Their diameter range between 4.2 6.6 μm . The presence of gaseous vesicles that appear in the cytoplasm of the cell is clearly marked as black dots and characterized by a clear mucosa surrounding the cells. The third species *O. pseudogeminata* is characterized by the fact that it is composed of long, irregular, tangled, non-moving filaments, and the cells in the line are clear. The alga shows a green color under the light microscope. The length of each cell is between 2.3-3 μm and 1.1-1.4 μm in width. The specie is characterized by the presence of gaseous vesicles and the threads end with a round cell (Fig-1).



A. clathrata

M. flos-aque

O. pseudogeminata

Figure-1: The blue green algal species isolated from soil

3-2: Growth curve

The growth curves of the selected species were measured in terms of dry weight. The alga *A. clathrata* shows the highest growth constant (k) and the least generation time reach 0.211 and 1.424 respectively it is on the order compared to the other two species Table-1 and Fig 2-4.

Table (1): Growth curve periods for blue –green algae isolation from soil.

Algal species	Lag phase	Exponential phase	Stationary phase	Harvested days	Growth constant	Generation time
<i>A. clathrata</i>	5	30	14	39-43	0.211	1.425
<i>M. flos-aque</i>	2	24	16	32-36	0.17	1.718
<i>O.pseudogeminata</i>	3	27	17	33-40	0.144	2.09

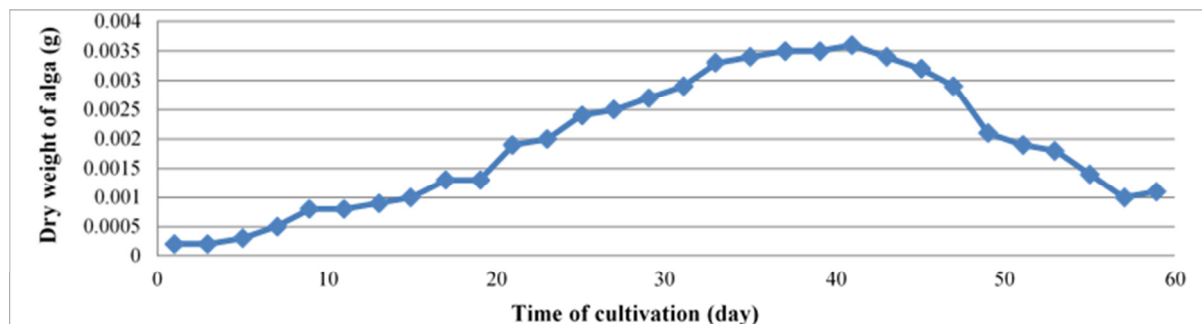


Figure-2: Growth curve of soil blue green alga *A. clathrata*

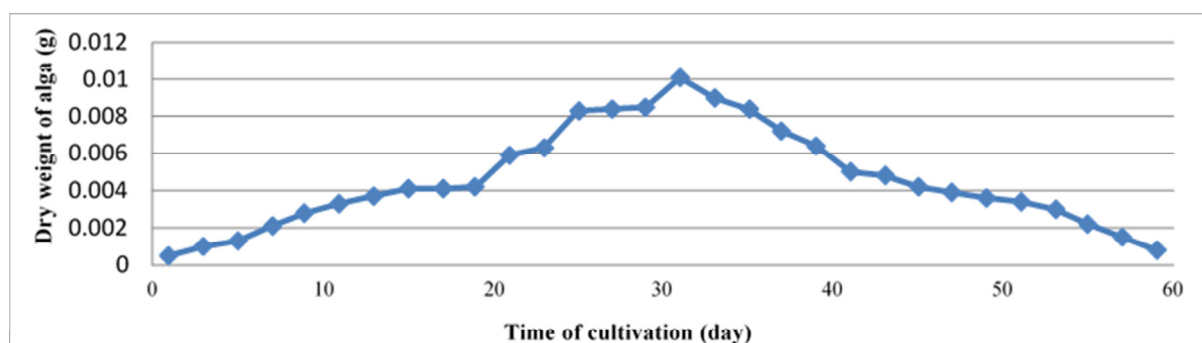


Figure-3: Growth curve of soil blue green alga *M. flos-aque*

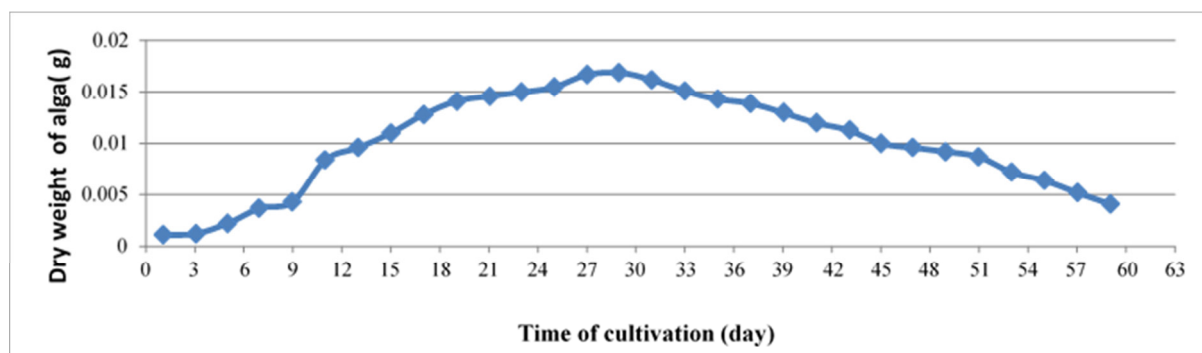


Figure-4: Growth curve of soil blue green alga *O. pseudogeminata*

3.3. Purified microcystins concentration in algal species

The study showed that the blue-green alga *O. pseudogeminata* have the highest susceptibility to the production of microcystins reach to 1.47 $\mu\text{g}/50\text{mg}$ of algal dry weight followed by alga *M. flos-aque* 0.734 $\mu\text{g}/50\text{mg}$ and the lowest toxicity is found in alga *A. clathrata* reach to 0.324 $\mu\text{g}/50\text{mg}$ Fig-5.

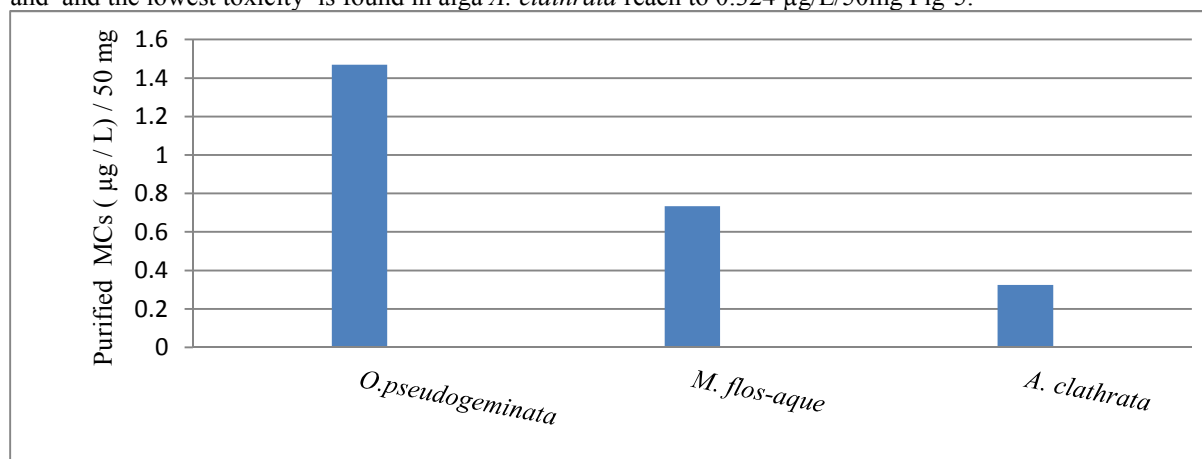


Figure-5: The concentrations of purified microcystins from soil algal isolates

3.4. Effects of purified microcystins from alga *O.pseudogeminata* on seed germination of *L. esculentum*

3.4.1. Seeds germination percentage (%)

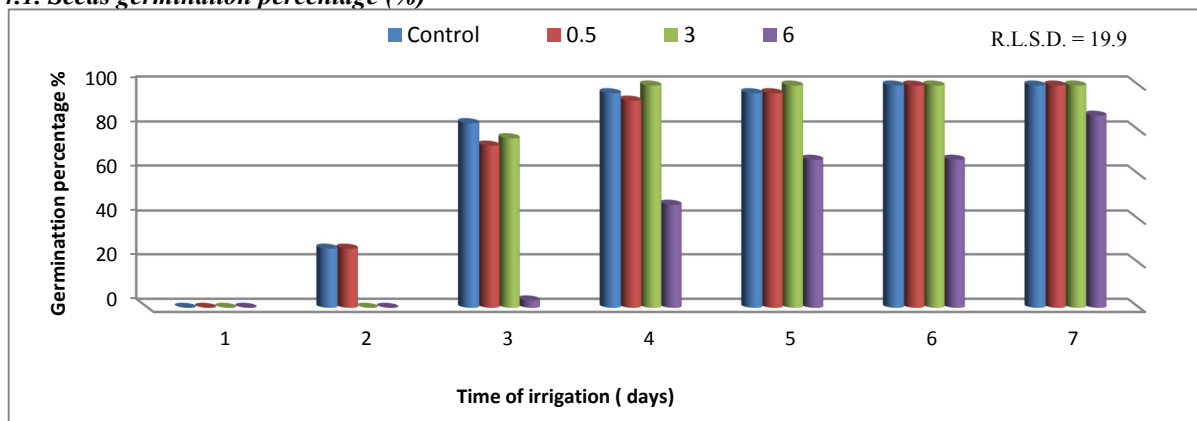


Figure-6: Effects of purified microcystins from blue green alga *O. pseudogeminata* on seed germination of *L.*

The results showed that exposure tomato seeds to purified microcystins caused inhibition and delayed germination, especially when treated with concentrations 3 and 6 µg / L on the first and second day of the experiment compared with control group and concentration 0.5 µg /L as the proportion of germination in them 26.6 %. The effect of inhibition was continued in all day of the experiment especially at concentration 6 µg/L resulted in a significant decrease in germination rate 66.6% on day six and seven compared to control group Fig.6.

3.4.2. Effects of purified microcystins on germination speed

The treatment of tomato seeds with purified microcystins resulted in a significant decrease $p \leq 0.05$ in the rate of germination speed, especially when treated with concentrations 3 and 6 µg/L reach to (0 seed/day) compared to concentration 0.5 µg/L and control group which reach to (1.3 seed/day). The negative effect of purified microcystins on germination speed increased to the sixth day of experiment at the highest concentration 6 µg/L reach to (1.23 seed/day) compared with the other concentrations and control group which reach to (1.42 seed /day), whereas on the seven day there was no significant decrease in the rate of germination speed for all treatments Table-2.

Table-2: Effect of purified microcystins from *O. pseudogeminata* on mean of germination speed of *L. esculentum*

Purified MCs(µg/L)	Time of exposure (days)						
	1st	2nd	3rd	4rd	5th	6th	7th
Control group	0	1.3	2.7	2.4	1.9	1.66	1.42
0.5	0	1.3	2.4	2.3	1.9	1.66	1.42
3	0	0	2.5	2.5	2	1.66	1.42
6	0	0	0.11	1.16	1.33	1.11	1.23

3.4.3. Toxic effects of purified microcystins on length of radicle and plumule of tomato plant *L. esculentum*

Finding showed the irrigation of tomato seeds with different concentrations of purified MCs for 7 days was led to significantly reduced the length of the radicle under level $P \leq 0.05$ especially in the treated group at the highest concentration 6 µg / L reach to 1.3 cm compared with the control group 5.2 cm. the irrigation of seed with 0.5 µg / Land 3 µg/L showed significant increasing in length of radicle reach 6.1 and 7 cm respectively compared with control group. (Fig.7). Results also showed significant decreasing $P \leq 0.05$ in the length of the plumule when treated with highest concentration of 6 µg / L of purified MCs reach to 0.7 cm compared to the control group 1.8 cm ,while treated with 0.5 and 3µg / L was led to significant increasing in length reach to 2.06 and 2.5 cm respectively (Fig-8).

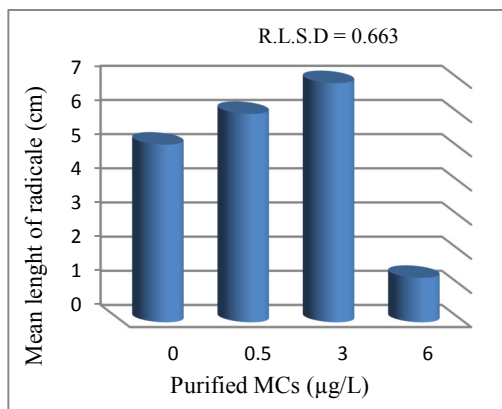


Figure-7: Mean radicle length of tomato *L. esculentum* plant under different concentrations of purified MCs from alga *O. pseudogeminata*

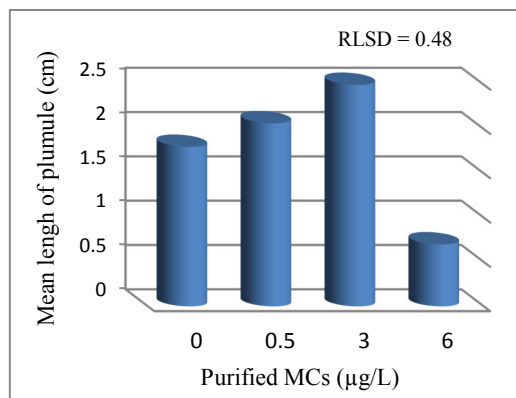


Figure-8: Mean plumule length of tomato *L. esculentum* plant under different concentrations of purified MCs from alga *O. pseudogeminata*

3.4.5. Toxic effects of purified microcystins on fresh and dry weight of radicle of tomato seeds *L. esculentum*.

The results in Fig. 9 was showed a significantly decrease in the fresh weight of the tomato seeds radicle when treated with highest concentration 6 reach to 2.466 mg compared to the control group 5.333 mg ,while other concentrations of 3and 0.5 µg / L, which ranged between 5.8 - 6.633 mg respectively with non-significant differences with control group. In addition (Fig.10) showed a significant decreasing $P \leq 0.05$ in dry weight of radicle of tomato seeds after treated with 6 µg / L of purified microcystins only reach to 0.22 mg compared with the control group and other treatments which do not Showed significant differences between them .

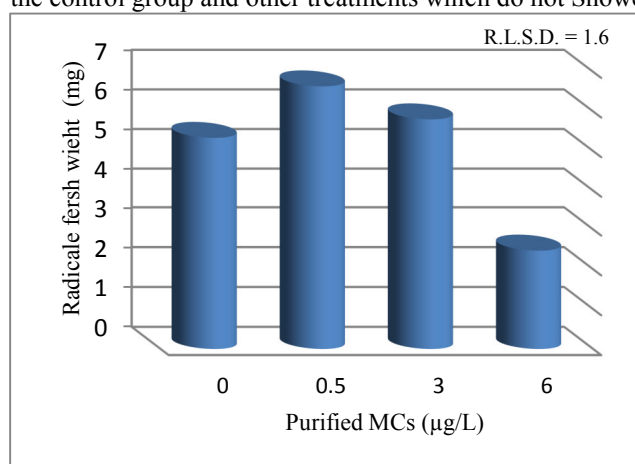


Figure-9: Mean of radicle fresh weight of tomato seeds *L. esculentum* plant under different concentrations of purified MCs from alga *O. pseudogeminata*.

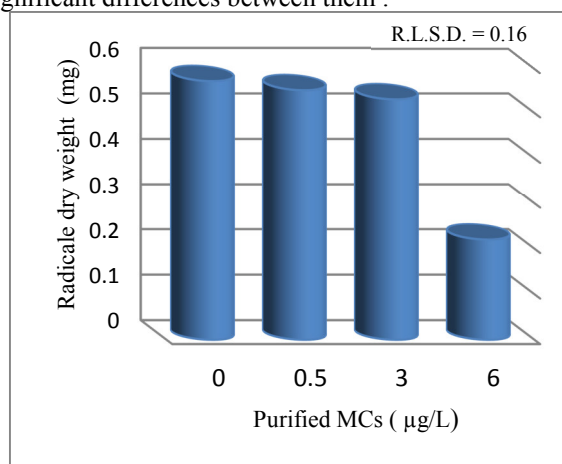


Figure-10: Mean of radicle dry weight of tomato seeds *L. esculentum* plant under different concentrations of purified MCs from alga *O. pseudogeminata*.

3.4.6. Toxic effects of purified microcystins on fresh and dry weight of plumule of tomato seeds *L. esculentum*.

Finding showed that the treatment of tomato seeds with different concentrations of purified microcystins for seven days especially at the highest concentration 6 µg / L resulted in a decrease significantly $p \leq 0.05$ in the fresh weight of plumule reach to 5.066 mg compared to control group of 9.9 mg, while treated with 0.5 and 3 µg / L was led to increasing significantly in fresh weight ranged between 14.3-16.166 mg respectively compared with control group (Fig.11). As for the dry weight of plumule showed significant decreasing in dry weight when treated with 6 µg / L only reach to 0.7 mg compared to the control and other group which showed non-significant differences between them Fig-12.

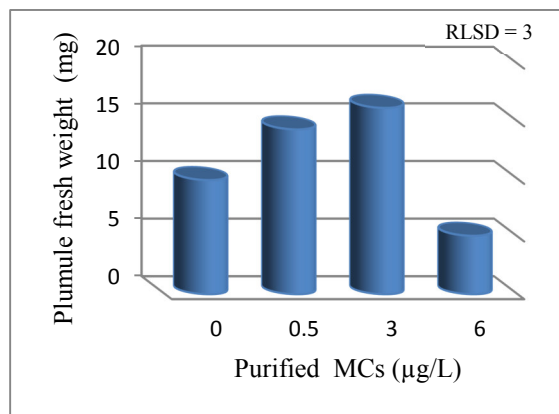


Figure-11: Mean of plumule fresh weight of tomato seeds *L. esculentum* plant under different concentrations of purified MCs from alga. *pseudogeminata*

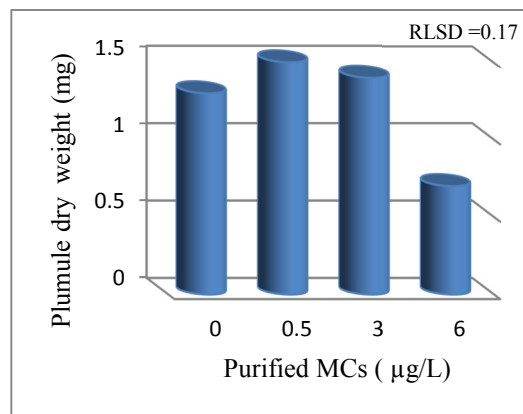


Figure-12: Mean of plumule Dry weight of tomato seeds *L. esculentum* plant under different concentrations of purified MCs from alga. *pseudogeminata*

4. Discussion

The current study is considered the first of its kind in the country to isolate, purify and cultivate of three species of blue green algae from soil and proved its ability to produce highly concentrations of microcystins in terms of purify microcystins and study their toxic effects on seeds germination of tomato plant. because the previous studies were interested isolation and purification of blue-green algae and their toxins from the fresh aquatic environments only (AL-Aarajy and AL-sultan 2008; Sultan, 2011; Talib 2013 ; Sultan and Obaid 2017), Therefore, the findings showed that the soil is a suitable environment for the growth of different types of blue-green algae and demonstrated its ability to produce microcystins and the possibility of laboratory cultivation those algae and purification of these toxins from them. Results appeared the growth curve of isolated species revealed that there is a clear difference in the growth constant (k) and the generation time (G) values, some of which were similar and some differed from those in previous studies. (Al-Sultan 2007, Al-sultan and Aubaed, 2017, Talib 2013) because those algae were isolated from fresh water environments and may be cause by to using different media or different conditions.

Findings shows that there was a significant variation in the susceptibility of algae to the production of microcystins after they were cultivated in laboratory and as a pure cultures after the stabilization of all the environmental factors in the laboratory, the culture medium and the dry weight extracted. This difference may be due to genetic and phylogenetic differences between species. The species *O. pseudogeminata* showed was the most productive of microcystins from other species, but the concentration of toxin produced was lower than that found in the study of AL-Sultan and Audaed, (2017) on the same species but isolated from the freshwater environment and because their using the Chu-10 medium while in the present study we using BG-11 liquid medium . The concentrations of microcystins in the algae *Lyngbya rubida* , *Microcystis flos-aque* and *Stigonema informe* were close to the MCs concentration in the alga *O. pseudogeminata* in the present study which reached 1.753, 0.734 and 1.309 µg/L /50 mg D.W respectively . This indicates that the one species and the different environment isolated from may it has the same ability to produce toxins and concentrations.

The exposures of tomato seeds *L. esculentum* to purified microcystins from blue-green alga *O. pseudogeminata* led to significantly reduction the germination rate, speed of germination, the growth of radicle and plumule and reduce their fresh and dry weight especially at concentration 6µg/L of purified microcystins. These results are consistent with study of El Khalloufi *et al.*, (2012) when treating the seeds of tomato plant and seedlings with the blue-green algae extracts containing microcystins (MC). Seed germination and root growth in *Medicago sativa* were inhibited at 5 µg / L of microcystins (Pflugmacher *et al.*, 2006). Other studies have also shown that seeds germination of several species of plants such as *Lens esculenta* , *Brassica napus*, *Oryza nap*, *Triticum durum*, *Zea mays*, *Pisum sativum* and *Lactuca sativa* have been negatively affected by algal extracts containing microcystins (Corbel *et al.*, 2014; Bittencourt-Oliveira *et al.*, 2015). The finding is in line with previous studies, which indicated that blue-green algae toxins, especially MCs, can accumulate in various crops which irrigated with water contaminated with blue-green algae or toxins (Hereman, 2012; Gutierrez-Praena *et al.*, 2014 ; Bittencourt-Oliveira, 2016 ; Cordeiro-Araújo *et al.*, 2016 ; Machado *et al.*, 2017). 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 (Mackintosh *et al.*, 1998; Dawson, 1998) .The results confirm that purified microcystins from alga *O. pseudogeminata* toxicity negatively effect on seed germination rate and that the effect is inversely proportional and concentrated. This result is also consistent with the study of Sengar *et al.*, (2010) also that the seeds of the *Vigna radiata* bean in the extract blue-green alga *Microcystis*

aeruginosa caused a reduction in root growth in a relative manner with increasing concentration of the extract as it increased concentration decreased growth.

Findings of this study also agreed with the study of Pflugmacher, (2007a). It was reported that the exposure of *Zea mays* seeds to 5 µg / L of microcystins had an effect on germination rate, root length, stem and total chlorophyll content (a + b). Pflugmacher *et al.*, (2006) also reported that the exposure of clover-type *Medicago sativa* to 5 µg / L of algal toxin type MC-LR had a significant effect on germination rate and root length. This is due to the fact that MCs reduce plant uptake of water, leading to reduced growth (El Khalloufi *et al.*, 2012; Wang *et al.*, 2011) and water is one of the main requirements for seed germination. If the seed fails to obtain the appropriate amount of water, it will negatively affect its germination (Creelman *et al.*, 1990). In addition to the effects MCs in plants such as inhibition of seed germination and negative effects on plant growth, some studies have reported oxidative stress and lipid peroxidation (Saqrane *et al.*, 2008; Prieto *et al.*, 2011).). Oxidative stress occurs through the production of ROSs, which may be an important in biochemical mechanism which caused by MC-LR toxicity in both mammals and plant cells (Pichardo and Pflugmacher, 2011; Zegura *et al.*, 2011; Zhou *et al.*, 2015). Some studies have revealed that plants stimulate defensive reactions against oxidative stress by activating a secondary anti-oxidation enzyme that limits the free radicals that emerge after exposure to blue-green algal toxins. Pflugmacher *et al.*, (2007b) reported that treating *Spinachia oleracea* with 0.5 µg / L of blue-green algal toxins for 6 weeks resulted in an increase in antioxidant activity in both catalase (CAT) and enzyme peroxidase (POD) and enzyme (SOD) superoxide dismutase compared to control group plants. El Khalloufi *et al.*, (2012) found that there was an increase in phenolic compounds and the activity of peroxidase in tomato plant exposed to blue-green algal toxins, as this increase may be related to the detoxification process. Bittencourt-Oliveira *et al.*, (2016) showed that the lettuce plants exposed to low concentrations of pure microcystins MCs from blue-green algae were able to control the oxidative stress oxidative stress by the use of glutathione-S-transferase (GST) and also noted a significant increase in the activity of the enzyme in the roots, Hence , all these studies confirm that the seeds of plants and seedlings that are exposed to the toxins of green-blue algae MCs stimulates oxidative stress, which requires cooperation between different forms of antioxidant enzymes to remove stress, which is a specific path of detoxification as well, which may be attributed to the significant increase in some indicators vegetative growth of plants.

5. Conclusions

The study showed that the soil environment is considered a rich source of toxic blue-green algae it has the ability to produce microcystins and the alga *O. pseudogeminata* has the ability to produce at high concentration of microcystins reach to 1.47 µg/L /50 mg of dry weight compare with other algae. The purified microcystins from this species , especially the concentration 6 µg/L has a negative impact on the seeds germination of the tomato plant *L. esculentum* as well as the length of the radicle and plumule and the their dry and fresh weight, but the low concentration of purified microcystins 0.5 µg/L don't showed any significant differences compared with control group .Also we concluded the purified microcystins between 3-6 µg/L and more it is harmful to economic field crops because of its great effect on seed germination especially on tomato plant seeds and may be accumulate in their tissues and fruits.

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