ISSR-PCR-Based Genetic Diversity Analysis on Copper-Tolerant Versus Wild Type Strains of the Unicellular Alga *Chlorella vulgaris*

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Abstract

The unicellular alga *Chlorella vulgaris* was isolated from Al-Asfar Lake, Al-Ahsa province of Saudi Arabia. Two different isolates were subcultured under laboratory conditions. The wild type was grown under a regular concentration of copper, whereas the other isolate was grown under a progressively increasing copper concentration. An Inter Simple Sequence Repeats (ISSR) analysis was performed using DNA isolated from the wild type and tolerant strains. The sum of the scored bands of the wild type was 155, with 100 (64.5%) considered to be polymorphic bands, whereas the resistant strain displayed 147 bands, with 92 (62.6%) considered to be polymorphic bands. The sum of the scored bands of a mixed sample was 117 bands, of which only 4 (3.4%) were considered to be polymorphic. The average Nei's genetic diversity (*h*) and Shannon-Weiner diversity indices (*I*) were 0.3891 and 0.5394, respectively. These results clearly indicate that the adaptation to a high level of copper in *Chlorella vulgaris* is not merely physiological but rather driven by modifications at the genomic level.

Key Words: *Chlorella vulgaris*, copper tolerance, genetic diversity, Green Algae, ISSR-PCR.

Introduction

Microalgae organisms could be both prokaryotic, such as cyanobacteria, and eukaryotic microorganisms, such as green algae. They are autotrophs, producing proteins, lipids, and carbohydrates. There is a large variety of these organisms estimated at 50,000 species, of which only approximately 30,000 species have been identified (Richmond, 2004). These organisms perform various biological roles such as the bioremediation of wastewater (Oswald, 1992), serving as a source rich in organic molecules for aquaculture (De Pauw and Persoone, 1992), serving as human and animal food (Becker, 1992), producing pigments (Johnson and An, 1991), and acting as heavy metal scavengers (Wilde and Benemann, 1993). Chlorella vulgaris is a microalgae that has been widely used as a food source and is currently used as a dietary supplement (Halperin *et al.*, 2003). *C. vulgaris* has also been utilized for metal recovery from solutions, and its use has

been advantageous compared to other types of similar biomasses (Fraile *et al.*, 2005).

The occurrence of heavy metals in the environment is increasingly posing a worldwide problem due to their harmful effects on human health, flora, fauna, and the entire ecological systems (Meena *et al.*, 2008). Therefore, it is very important to locate efficient and economically sound methods for the removal of heavy metals from the environment, especially wastewaters. The most commonly used conventional approaches for removing heavy metals from wastewater include chemical precipitation (Grau and Bisang, 1995), ion exchange (Denizli *et al.*, 1999), activated carbon adsorption (Hu *et al.*, 2003). and reverse osmosis, evaporation, membrane filtration (Molinari *et al.*, 2004), The type of treatment technique required for a particular industry depends on the nature, composition, and flow rate of the effluent together with the quality control desired to be achieved. However, most of these methods are expensive and, therefore, not feasible for small-scale industries (Kobya *et al.*, 2005). Furthermore, these methods have inadequate efficiencies at low concentrations (Wilde and Benemann, 1993; Kapoor and Viraraghavan, 1995). Therefore, a more economical method for the removal of metals, biosorption, has been employed as an innovative technique with the aim of utilizing dead and living biomasses to sequester heavy metals from polluted water sources. This technique is non-polluting, highly selective and easy to operate (Shim *et al.*, 2001; Monser and Adhoum, 2002). Algae are potentially qualified as efficient biosorbent materials owing to their abundance and high absorption uptake rate (Klimmek *et al.*, 2001). The surfaces of algal cell walls contain some macromolecules, such as carbohydrates, proteins, and lipids, with functional groups that can bind to heavy metals such as cadmium, lead, phosphorus, zinc, and copper (Holan and Volesky 1994; Yu *et al.*, 1999).

The metal resistances of chlorophyta from environments polluted by heavy metals have been studied extensively (Foster, 1982; Bates *et al.*, 1985; Kuwabara, 1985; Pettersson *et al.*, 1988; Fathi and Falkner, 1997; Fathi and El-Shahed, 2000; Yoshida *et al.*, 2006). Streams and ponds with high levels of heavy metals often show differences in their compositions of algae species compared to environments lacking such enrichments. Metal uptake in tolerant organisms is equal to or greater than the uptake in non-tolerant organisms (Foster, 1977; Fathi and Falkner, 1997). Several examples have been reported for marked differences in metal resistance within a single species, which almost certainly reflect genetic and biochemical variations.

Rai *et al*., (1991) studied the copper tolerance in Cyanophyta (Anabaena doliolum) by comparing the physiological properties of its wild type and Copper-tolerant strains. They reported a concentration dependent reduction of growth, pigments, protein, sugar, lipid and ATP content. Furthermore, photosynthetic electron transport chain, O_2 evolution, carbon fixation, nutrient uptake (NH^+_4 and NO^-_3), nitrate reductase, nitrogenase, glutamine synthetase and alkaline phosphatase activities were affected in both strains following copper-supply. The reduction in all parameters was higher in the wild type than the tolerant strain. The latter produced larger (19.5%) lipid fractions than the wild type even in the absence of added metal. As compared to the wild strains, the tolerant strains showed enhanced enzyme activities, especially alkaline phosphatase (20% higher), low copper uptake and insignificant loss of K^+ and Na^+. The data on lipid production, loss of K^+ and Na^+, and uptake of copper indicate a change in the permeability of the plasma membrane and a possible operation of exclusion mechanism in the tolerant strain, thereby reducing the toxic effect of copper even at higher concentrations.

The genome size of *Chlorella vulgaris* is estimated at 38.8 Mb and comprises 16 chromosomes, the smallest of which is 980 kb in size. Chromosome I includes a terminal sequence (5′-TTTAGGG–3′) repeated 70 times at both termini which are considered to be the telomere (Higashiyama *et al.*, 1995; Songdong, 2008). This telomere is identical to that of higher plants. The subtelomeric sequence, however, is completely different from one arm to the other of chromosome I (Higashiyama *et al.*, 1995). In the present study, the genetic polymorphisms of a copper-tolerant strain of *Chlorella vulgaris* isolated from Al-Asfar Lake, located in the Al-Ahsa province, Saudi Arabia, were compared to the wild type strain utilizing Inter Simple Sequence Repeats PCR (ISSR-PCR) techniques.

Materials and Methods

1. Algal Isolation and Culture: *Chlorella vulgaris* Beyerinck was isolated locally from Al-Asfar Lake, located in the Al-Ahsa province east of Saudi Arabia. The isolation and purification procedures used were similar to previously utilized protocols (Fathi *et al*., 2005; Afkar *et al*., 2010; and Alzahrani *et al*., 2011). Briefly, the alga was grown in Kuhl's medium (1962) and incubated in an illuminated incubator (Precision, USA) at room temperature with a photoperiod of 14L:10 D at 70 μmol m^{-2} s^{-1}. The culture was shaken periodically to prevent clumping.

2. Treatment: Copper-tolerant strains of *Chlorella vulgaris* was isolated by successive culturing on agar plates containing Kuhl's medium with various doses of copper ranging from 0.06 to 0.6 mg dm^{-3} following the protocol of Whitton and Shehata (1982). Colonies that were successfully growing at the threshold copper concentration of 0.5 mg dm^{-3} were further grown repeatedly on plates or in liquid medium containing the same copper concentration, and those colonies were designated as the copper-tolerant strain. When this tolerant strain was subcultured in the basal medium devoid of copper and then transferred to the medium spiked with 0.5 mg l^{-1} copper, a gradual loss of tolerance in the strain was noticed after every successive generation (Rai *et al*. 1991). The wild type and copper-tolerant strains were inoculated equally and cultured in liquid medium (250 cm^3 each) containing 50 cm^3 of nutrient medium supplemented with 1.0 and 300 µg l^{-1} copper, respectively, for seven days. The two cultures were then washed several times with copper free medium before being used for DNA isolation.

3. DNA Isolation: Genomic DNA was isolated using DNAzol®ES (Molecular Research Center, INC., Cincinnati, Ohio, USA) according to the manufacturer's protocol. Briefly, 0.5 g of wild type or tolerant algae was pulverized in 1.5 ml of DNAzol®ES before the DNA was extracted with chloroform. The DNA was then precipitated in 100% ethanol, washed in 75% ethanol, and then dried and solubilized in 8 mM NaOH according to the manufacturer's recommendations. The quality and quantity of the extracted DNA were determined using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, DE, USA).

4. PCR Amplification: For this PCR reaction, 18 primers, reported by Songdong (2008), were used to analyze the variation between the two strains. The reaction volume was 50 µl and included 6 µl 10× reaction buffer (50 mM, Tris-HCl (pH 8.3), 500 µg BSA, 10% Ficoll, 1.0 mM Tartrazine), 2 mM $MgCl_2$, 20 µM primer, 200 µM of dNTPs, 100 ng of the template DNA, and 1.0 unit of Taq DNA polymerase (Takara Inc, Otsu, Shiga, Japan). The thermal profile for the reaction was as follow: 5 minutes at 94°C; 40 cycles of denaturing 20 seconds at 94°C, annealing for 1 minute at 51°C, elongation for 20 seconds at 72°C; and a final extension at 72°C for 6 minutes. The amplified product was resolved electrophoretically on a 1× TAE agarose gel stained with ethidium bromide. The gels were visualized using a BioDoc-It® system (UVP, Upland, California, USA). The reproducibility of the amplifications was confirmed three times. As a control, two primers were used to assure the stability of the produced bands.

The mixed DNA samples of wild type and tolerant strains were used as PCR template to verify the presence or absence of the bands scored with each strain individually.

5. Data analysis: The band patterns for the wild type and tolerant sample were compared to one another and to the band pattern resulting from the mixture of the two samples. The common bands were assigned (1) if present, whereas the other polymorphic bands were marked as absent and assigned (0). The sum number of the scored bands and the percentages of polymorphic bands for the wild type and tolerant samples as well as the sample mixture were respectively calculated. POPGENE version 1.32 (Yeh and Boyle 1997) was used to calculate the Nei's gene diversity (*h*) (Nei, 1973) and Shannon-Weiner diversity indices (*I*) (Lewontin, 1972).

Results

In this study, a total of 18 primers (Table 1) were employed with the wild type, tolerant, and mixture samples. For the wild type sample, the sum of the scored bands was 155, and 64.5% (100 bands) were polymorphic. The fragment sizes ranged between 0.2 kb to 2.5 kb. The average band number per primer was 8.6 for the wild type strain. In contrast, the sum of bands scored for all 18 primers for the tolerant strain was 147, including 92 (62.6%) polymorphic bands. Similarly, the fragment sizes also ranged between 0.2 kb and 2.5 kb, and the average band number per primer for the tolerant sample was 8.17. In the case of the mixed samples of wild type and tolerant strains, the sum number of the produced bands was 117, with an average of 6.5 bands per primer. As expected, when analyzed separately, the numbers of bands in the mixed sample lanes were less than each of the other two samples as more accurate targets are available for the primers.

To ensure the stability of the PCR reaction, primer numbers 24 and 46 were used as a control (Fig. 1). The results of the amplifications using the primers listed in Table 1 are shown in Figures 2-5. The repeatability was confirmed under the same conditions.

The Nei's (*h*) and Shannon-Weiner (*I*) indices of diversity for the wild type and tolerant strains were 0.3891 and 0.5394, respectively.

Discussion

The role of the copper is vital to autotrophic cells as it plays important roles in photosynthesis, namely the function of plastocyanin. It is also a cofactor of many proteins such cytochrome c oxidase, superoxide dismutase, and ascorbate oxidase (Padua *et al.,* 2010). However, higher

concentration of copper leads to impairment of many metabolic pathways such as photosynthesis especially photosystem II (PSII).

Table (1):
Bands summary from ISSR PCR per primer in Wild Type, Tolerant, and Mixed DNA templates.

Primer Sequence		WT		Tolerant		Mixed	
P.#	P. Sequence	No. of bands	No. of polymorphic Bands	No. of bands	No. of the polymorphic bands	No. of bands	No. of the polymorphic Bands
02	$(AC)_8AT$	13	9	11	7	9	0
05	$(AC)_8TG$	5	1	8	4	7	1
08	$(ATG)_6$	6	4	7	5	5	0
24	$(AC)_8TC$	9	5	9	5	10	1
25	$(AC)_8CA$	8	5	10	7	8	0
26	$(AC)_8CC$	7	1	12	6	9	0
34	$(AG)_8AA$	8	4	8	4	5	0
44	$(AC)_8GA$	8	6	8	6	8	0
46	$(AC)_8GG$	7	6	9	8	6	0
55	$(TG)_8GG$	9	6	8	5	0	0
59	$(AG)_8GC$	11	6	10	5	6	0
61	$(AG)_8GT$	10	8	8	6	6	0
62	$(AG)_8CA$	8	7	7	6	8	1
63	$(AG)_8CT$	10	8	9	7	11	1
65	$(AG)_8CC$	13	8	10	5	8	0
67	$(TC)_7CC$	8	5	4	1	2	1
74	$(ACTG)_4$	10	9	5	4	5	0
77	$(ACTC)_4$	5	2	4	1	4	0
Total		155	100	147	92	117	4

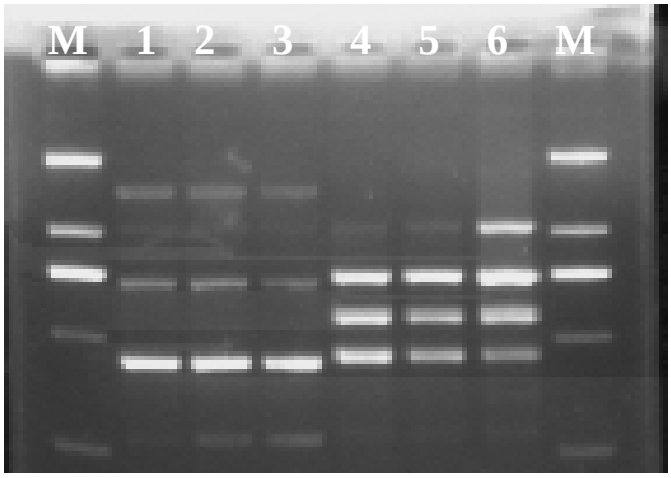

Fig. 1: ISSR PCR products in WT and Tolerant with ISSR control Primer ISSR 24 and 46 as control: products amplified with ISSR 46 using WT as temple (lane 1-3); products amplified with ISSR 24 using T as temple (lane 4-6). M: DNA Marker.

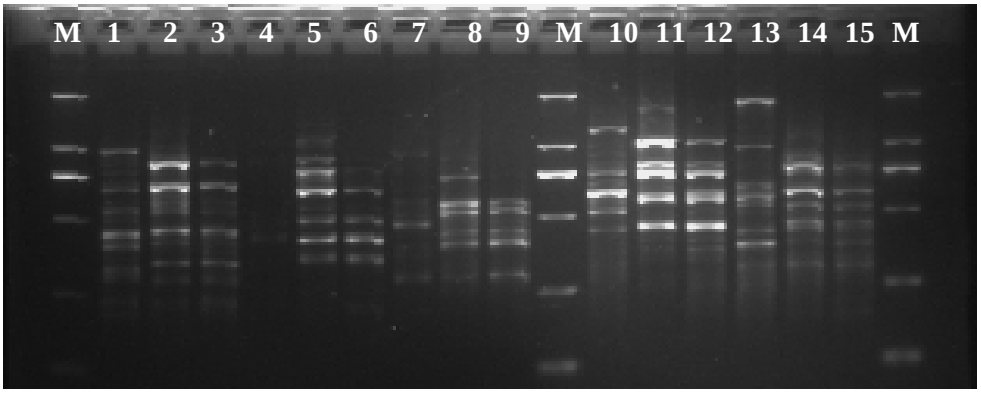

Fig. 2: ISSR PCR products using WT and Tolerant as templates with ISSR Primers 2, 5, 8, 24, and 25.

M: DNA marker DL 2000
1-3: PCR product with primer 02 1, 4, 7, 10, 13：WT
4-6: PCR product with primer 05 2, 5, 8, 11, 14：T
7-9: PCR product with primer 08 3, 6, 9, 12, 15：Mix
10-12: PCR product with primer 24
13-15: PCR product with primer 25

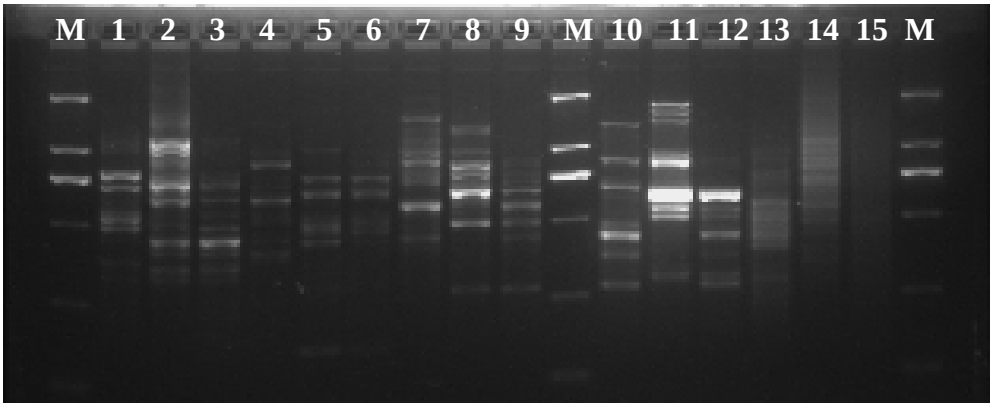

Fig. 3: ISSR PCR products using WT and Tolerant as templates with ISSR Primers 26, 34, 44, 46, and 55.

M: DNA marker DL 2000
1-3: PCR product with primer 26
4-6: PCR product with primer 34
7-9: PCR product with primer 44
10-12: PCR product with primer 46
13-15: PCR product with primer 55

1, 4, 7, 10, 13: WT
2, 5, 8, 11, 14: T
3, 6, 9, 12, 15: Mix

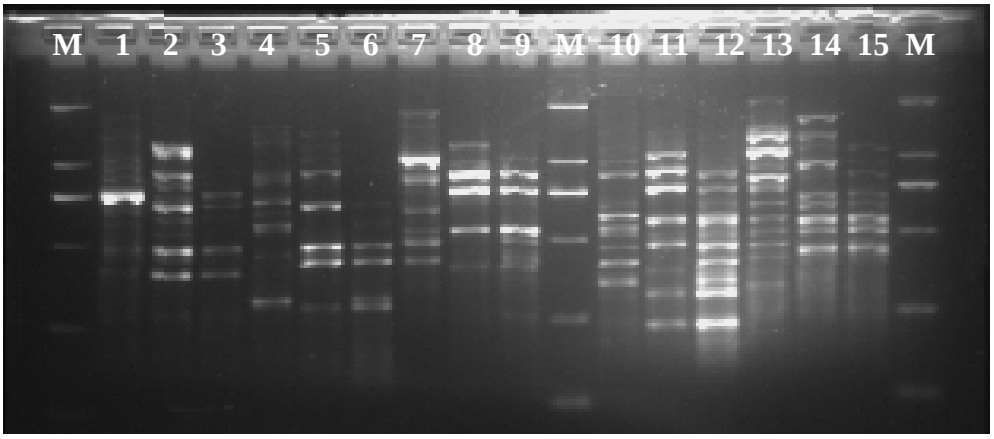

Fig. 4: ISSR PCR products using WT and Tolerant as templates with ISSR Primers 59, 61, 62, 63, and 65.

M: DNA marker DL 2000
1-3: PCR product with primer 59
4-6: PCR product with primer 61
7-9: PCR product with primer 62
10-12: PCR product with primer 63
13-15: PCR product with primer 65

1, 4, 7, 10, 13: WT
2, 5, 8, 11, 14: T
3, 6, 9, 12, 15: Mix

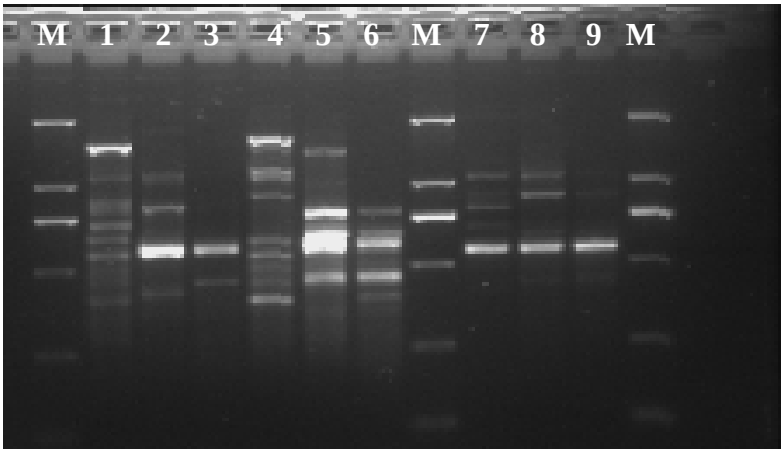

Fig. 5: ISSR PCR products using WT and Tolerant as templates with ISSR Primers 67, 74, and 77.

M: DNA marker DL 2000
1-3: PCR product with primer 67 1, 4, 7, 10, 13：WT
4-6: PCR product with primer 74 2, 5, 8, 11, 14：T
7-9: PCR product with primer 77 3, 6, 9, 12, 15：Mix

Gluathione-S-transferase (GST), polyphenol oxidase, as well as other proteins involved in the oxidative defense mechanism were also among the highly affected molecules under copper stress in *Spinacia oleracea* L (Contreras-Porcia *et al*, 2011).

In this study, all of the measured growth parameters were found to be higher for the tolerant strains in comparison to the wild type strain including total amino acids, total protein, total sugars, proline, K^+, Na^+ under copper stress. Significant decline in the growth rate of the wild type was also reported (Alzahrani *et al*., 2011). The genomic DNA analysis shows that the Nei's gene diversity index (*h*) and Shannon- Weiner diversity index (*I*) for the wild type and tolerant strains were 0.3891 and 0.5394, respectively, clearly indicating a significant difference between the two strains. These results are in agreement with similar treatments of heavy metals using different plant species (Conte, *et al.* 1998; Atienzar *et al*., 2002; Enan, 2006; and Liu *et al*., 2007). Furthermore, the average percentage of polymorphic bands was almost identical (64.5% and 62.5%) for the wild type and tolerant strains, respectively. However, when mixed DNA samples of wild type and tolerant strains were used as the PCR template, the average percentage of polymorphic bands was as low as 3.5%, which is likely due to the fact that the mixed DNA sample was more stable than the individual strains.

The primers containing AG repeats produced a higher number of bands in the wild type strain compared to the remainder of primers with high numbers of polymorphic bands (Table 1), indicating the presence of more AG dinucleotide repeats in the genome of the wild type strain. However, the primers did not show significant differences either in the sum of produced bands or polymorphic bands in the tolerant strain. This result is likely due to the occurrence of a higher mutation rate in their targets throughout the genome of the tolerant strain.

In addition, most of the 18 primers, with the exception of 3 primers, displayed a variation in the number of produced bands as well as the polymorphic bands between the wild type and tolerant strains. Taken together with other biochemical and morphological data (Alzahrani *et al*., 2011), the diversity at the molecular level was significantly high between the two strains. Previous study by Rai *et al*. (1991) has shown that when the tolerant strain of *Anabaena doliolum* was subcultured in the basal medium devoid of copper and then transferred to the medium spiked with 0.5 mg l^{-1} of copper, a gradual loss of tolerance in the strain was noticed after every successive generation. Therefore, the tolerant strain isolated was not a spontaneous mutant but rather the result of adaptation. NMR-based metabolomics and multivariate study on *Chlorella vulgaris* spiked with different concentration of cupric cation as well as cadmium and lead revealed significant diminishing of photosynthesis (Zhang *et al*., 2014). LC-MS/MS analysis also indicated that a high copper-spiked cultures led to oxidative stress. As the redox homeostasis of the copper treated *Chlorella vulgaris* was imbalanced, its growth and development was also affected. However, those parameters were not noticed with the treatment of non-redox active Cd_2^+ and Pb_2^+ (Zhang *et al*., 2014). In the marine alga *Ulva compressa,* the expression of 18 genes coding for many antioxidant enzymes as well as other proteins involved in signal transduction, calcium-dependent-kinase, nucleoside diphosphate kinase, gene expression, protein synthesis and degradation, and electron transport chains (Contreras-Porcia *et al*, 2011). In comparison, *L. nigrescens* under copper stress suffer cellular damage due to the loss of peroxiredoxin (PRX) which belong to the thiol-dependent peroxidase family and is involved in decreasing oxidative stress and reduces hydrogen peroxide (H_2O_2) (Lovazzano *et al*., 2013).

In conclusion, the wild type and copper-tolerant strains of the unicellular alga *Chlorella vulgaris* displayed significant variation at the genetic level as shown by the results of the ISSR-PCR performed on the DNA of the two strains. Further studies using different metal and different concentrations will be required to identify specific markers that would lead

to accurate decipher of the mechanisms underlying *Chlorella vulgaris* adaptation to continually increasing copper concentrations in the environment.

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دراسة التنوع الوراثي في طحلب كلوريلا فولجارس *Chlorella vulgaris* باستخدام تقنية تفاعل البلمرة المتسلسل ISSR-PCR بين سلالتين طبيعية وأخرى مقاومة لتراكيز عالية من النحاس

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الملخص

تم في هذه الدراسة عزل طحلب كلوريلا فولجارس من بحيرة الأصفر الواقعة في منطقة الاحساء الواقعة شرقي المملكة العربية السعودية. لقد تم عزل سلالتين وتربيتهما في المختبر. تم تربية النوع الشائع عند تراكيز طبيعية من النحاس بينما تم تربية النوع الآخر عند تراكيز متزايدة من النحاس. تم عزل الحمض النووي منزوع الأكسجين من كلا العينتين واستخدم كقالب لتفاعلات البلمرة المتسلسلة PCR باستخدام بادئات موجهة لمناطق بينية متكررة ISSR. أنتج التفاعل في حالة النوع الشائع 155 خط موجي band منها 100 (أي ما يعادل 64.5%) متنوعة بينما في حالة النوع المقاوم أنتج التفاعل 147 خط موجي band منها 92 (أي ما يعادل 62.6%) متنوعة. بلغ مجموع الخطوط الموجية في العينة المختلطة (النوع الشائع و المقاوم) 117 منها أربعة خطوط موجية (أي ما يعادل 3.4%) متنوعة. بلغ مجموع التنوع الجيني على مقياس ني (Nei) (*h*) 0.389 بينما بلغ على مقياس شانون (Shannon) (*I*) 0.539. هذه النتائج تشير بشكل واضح إلى أن تكيف طحلب كلوريلا فولجارس لمستويات عالية من النحاس في الوسط البيئي ليست فقط فسيولوجية ولكن على الأرجح بسبب تغيرات على مستوى الجينوم.

الكلمات المفتاحية: تفاعل البلمرة ISSR، التنوع الوراثي، الطحالب الخضراء، كلوريلا فولجارس، مقاومة النحاس.