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RESEARCH ARTICLE

Evaluation of Azadirachtin on *Arthrospira plantensis* Gomont growth parameters and antioxidant enzymes

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Abstract – Azadirachtin (Aza) used as insecticide due to inhibiting growth of insects and preventing them from feeding on plants. To understand the effects of contamination of this insecticide on phototrophs, and to determine the responses of these organisms against these insecticides are extremely important in understanding how the ecosystem is affected. In this study, chlorophyll-*a* amount, OD 560 and antioxidant parameters (total SOD, APX, GR, Proline, MDA and H_2O_2) were determined in order to understand the effect of Aza on *Arthrospira platensis* Gomont. Aza was applied between 0–20 $\mu g\,mL^{-1}$ concentrations for 7 days in the study. Enzyme analysis was conducted at the end of the 7th day. There was a statistically significant decrease in the absorbance of OD560 and the chlorophyll-*a* content in *A. platensis* cultures exposed to the Aza (0–20 $\mu g\,mL^{-1}$) during 7 days due to the increase in pesticide levels. SOD activity decreased at 8, 16 and 20 $\mu g\,mL^{-1}$ concentrations; GR enzyme activity showed a significant decrease compared to the control at a concentration of 20 $\mu g\,mL^{-1}$. APX activity did not change significantly compared to control. The MDA content increased significantly at 16 and 20 $\mu g\,mL^{-1}$ concentrations. The H_2O_2 content significantly increased at 12, 16 and 20 $\mu g\,mL^{-1}$ concentrations ($p < 0.05$) while the free proline content decreased at 4 $\mu g\,mL^{-1}$ concentration ($p < 0.05$). As a result, regarding the Aza concentrations used in this study may be a step to prevent pesticide pollution in the environment.

Keywords: oxidative stress / antioxidant / *Arthrospira platensis* / azadirachtin

1 Introduction

Azadirachtin (Aza) is a pesticide obtained from solvent extract of powdered *Azadiracta indica* seeds and its chemical structure is very complex as triterponoid in the limonoid class (Schmutterer, 1990; Burt, 1990; Mordue and Blackwell, 1993; Morgan, 2009). This pesticide contaminates water bodies by the raining and air circulation after forming cloud during the application in forest areas. In addition, the insecticide accumulation in terrestrial areas is prone to enter aquatic environments through erosion and surface runoff. In the aquatic ecosystem, the pesticide taken up by different organisms tend to accumulate (Sundaram, 1996; Sundaram *et al.*, 1996). Even if this insecticide is known to be nontoxic to vertebrate animals, it have detrimental effetcs for most aquatic organisms (Morgan, 2009). Aza blocked cell proliferation by inhibiting RNA synthesis in the study tested on freshwater protozoa (Fritzsche and Cleffmann, 1987). In addition, the negative effects of this insecticide on microtubule formation were found (Bilker *et al.*, 2002; Salehzadeh *et al.*, 2003). Therefore it may display algicidal acitivities due to these features. Since Aza can be an alternative to synthetic pesticides, the reliability of this natural product on algae must be demonstrated.

Algae and cyanobacteria are primary producers that can colonize aquatic ecosystems including rivers and ponds. As a food source for zooplankton and some fish, these organisms are effective in transporting xenobiotics to higher trophic levels through the food chain. Therefore, a situation that is effective on cyanobacteria and algae, affects the structure and function of the aquatic ecosystem (Sundaram, 1997).

A. platensis is used as a nutritional supplement for animals and humans due to its high component content such as protein, carbohydrates, carotene, phycocyanin, chlorophyll, minerals, essential fatty acids, vitamins. Being high ecological and economical features, have led most scientific research to focus on these organisms (Ciferri, 1983; Ali and Saleh, 2012).

Pesticide toxicity may effect the production rate of reactive oxygen species (ROS) by interfering with electron transport reactions in algal cells (Liu *et al.*, 2015), and thus may cause oxidation of proteins, fatty acids and nucleic acids

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(Cho and Park, 2000; Cargnelutti *et al.*, 2006; Chen *et al.*, 2009). The antioxidant defense systems in the organisms which can be enzymatic or non-enzymatic detoxify the ROS molecules such as superoxide radical ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). There are three enzymes that play a key role in enzymatic antioxidant systems. Superoxide dismutase (SOD) catalyzes that $O^{\cdot-}$ convert to molecular oxygen and hydrogen peroxide (H_2O_2) thus it is the first cell defense line against the reactive oxygen species (ROS) species (Hassan and Scandalios, 1990). Ascorbate peroxidase (APX) converts H_2O_2 to molecular oxygen and H_2O, Glutathione reductase (GR) reduces the oxidized glutathione by using NADPH as a substrate (Foyer *et al.*, 1994; Urso and Clarkson, 2003). Malondialdehyde (MDA) formed by the peroxidation of lipids contain three or more than double bonds and this metabolite shows the oxidation levels in cells (Altınısık, 2000). Proline is an iminoacid that its free levels rise under stress factors (Bassi and Sharma, 1993; Delauney and Verma, 1993) and protects cell parts and cell contents such as enzymes, membranes and polyribosomes (Arakawa and Timasheff, 1985; Kadpal and Rao, 1985; Rudolph *et al.*, 1986). Recent studies have demonstrated that pesticide toxicity could differently affect the activity of some antioxidant enzymes in plant cells as well as in algae and cyanobacteria. Saladin and Clement Magne (2003) found that the pesticide treatment caused the proline accumulation in *Vitis vinifera*. Fatma *et al.* (2007) observed that heavy metals, pesticides and salt stresses induced enhancement of intracellular proline content in the *Westiellopsis prolifica*.

There are a few studies investigating the negative effects of Aza on freshwater algae. Sundaram (1997) observed Aza reduced the chlorophyll-*a* and protein content in indoor aquatic microcosms. Prasad *et al.* (2007) found that low extract concentrations of *Azadirachta indica* (1% and 2%) had a positive effect on *Nostoc muscorum* growth and increased the amount of photosynthetic pigments, but that high concentrations (4 and 8%) inhibited cyanobacterial biomass. Chia *et al.* (2016) found that the high doses of extract obtained from the *A. indica* decrease the growth of *Scenedesmus quadricauda*. Although these studies in literature are not known the oxidative stress caused by this pesticide on aquatic phototrophs. According to our knowledge, this is the first study which aimed to uncover the effects of Aza application on *A. platensis*. This study aims to investigate the effects of Aza on *Arthrospira platensis* growth and antioxidant parameters.

2 Material and Methods

2.1 Algae culture and treatment

A. platensis-M2 was obtained from the Soley Microalgae Institute (California, USA) (Culture collection No, SLSP01). Algae were grown in Spirulina Medium (Aiba and Ogawa, 1977) under axenic conditions. 20 mL algal culture were inoculated to 180 mL culture medium in Erlenmeyer flask and were allowed to grow under the conditions of 93 µmol photons m^{-2} s^{-1} photosynthetically available radiation in 12:12 h light/dark cycle at 30±1 °C during 10 days. At the end of 10 days, cultures were renewed, and all the flasks contained 50 mL algal culture. A commercial formulation of Aza (NeemAzal-T/S, Trifolio-M GmbH, Germany, 10 g/L) was used for experimental analysis and prepared in distilled water. Various concentrations of Aza (0, 4, 8, 12, 16, 20 µg mL^{-1}) were added to the culture medium. The range of concentrations was determined with preliminary range-finding bioassays.

2.2 Cell growth and chlorophyll-*a* assay

Optic density (OD) of microalgae was measured spectrophotometrically over a period of 7 days under control and stressed conditions taking absorbance at 560 nm and 750 nm. Although 560 nm and 750 nm gave similar results, OD_{560} was selected for measuring *A. platensis* M2 growth (*e.g.*; Rangsayatorn *et al.*, 2002; Tang *et al.*, 2003; Arunakumara *et al.*, 2008). Chlorophyll-*a* content was estimated by methanol extraction and measured spectrophotometrically during 7 days (MacKinney, 1941).

2.3 Antioxidant assays

On the 7th day of the study, 2 mL culture solutions from Aza concentrations exposed algae medium that were centrifuged at 14.000 rpm for 20 min at 4 °C and resulting pellets were kept at −20 °C until enzyme activity measurements. Pellets were grounded with liquid nitrogen and suspended in specific buffers with proper pH values for each enzyme. The protein concentrations of algal cell extracts were determined according to Bradford (1976), using bovine serum albumin (BSA) as a standard.

Superoxide dismutase (SOD; EC 1. 15. 1. 1) activity was determined by the method of Beyer and Fridovich (1987), based on the photoreduction of NBT (nitroblue tetrazolium). Extraction of pellets (0.2 g) was performed in 1.5 mL homogenization buffer containing 100 mM K_2HPO_4 buffer (pH 7.0), 2% PVP and 1 mM Na_2EDTA. After centrifugation at 14.000 rpm for 20 min at 4 °C, the resulting supernatants were used to measure SOD activity. The reaction mixture consisted of 100 mM K_2HPO_4 buffer (pH 7.8) containing 9.9×10^{-3} M methionine, 5.7×10^{-5} M NBT, %1 triton X-100 and enzyme extract. Reaction was started by the addition of 0.9 µM riboflavin and mixture was exposed to light with an intensity of 375 µmole m^{-2} s^{-1}. After 15 min, reaction was stopped by switching off the light and absorbance was read at 560 nm. SOD activity was calculated by a standard graphic and expressed as unit mg^{-1} protein.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined according to Wang *et al.*, (1991) by estimating the decreasing rate of ascorbate oxidation at 290 nm. APX extraction was performed in 50 mM Tris–HCl (pH 7.2), 2% PVP, 1 mM Na_2EDTA, and 2 mM ascorbate. The reaction mixture consisted of 50 mM KH_2PO_4 buffer (pH 6.6), 2.5 mM ascorbate, 10 mM H_2O_2, and enzyme containing 100 µg protein in a final volume of 1 mL. The enzyme activity was calculated from initial rate of the reaction using the extinction coefficient of ascorbate (E = 2.8 mM cm^{-1} at 290 nm).

Glutathione reductase (GR; EC 1. 6. 4. 2) activity was measured with the method of Sgherri *et al.*, (1994). Extraction was performed in 1.5 mL of suspension solution containing 100 mM KH_2PO_4 buffer (pH 7.0), 1 mM Na_2EDTA, and 2% PVP. The reaction mixture (total volume of 1 mL) contained 100 mM KH_2PO_4 buffer (pH 7.8), 2 mM Na_2EDTA, 0.5 mM

oxidised glutathione (GSSG), 0.2 mM NADPH and enzyme extract containing 100 µg protein. Decrease in absorbance at 340 nm was recorded. Correction was made for the non-enzymatic oxidation of NADPH by recording the decrease at 340 nm without adding GSSG to assay mixture. The enzyme activity was calculated from the initial rate of the reaction after subtracting the non-enzymatic oxidation using the extinction coefficient of NADPH (E = 6.2 mM cm^{-1} at 340 nm).

The malondialdehyde content was determined by the method of Heath and Packer (1968). 0.2 g of pelet was homogenized in 3 mL of 0.1% TCA (4°C) and centrifuged at 4100 rpm for 15 min and the supernatant was used in the subsequent determination. 0.5 mL of 0.1 M Tris–HCl pH 7.6 and 1 mL of TCA–TBA–HCl reagent (15% w/v) (trichloro-acetic acid–0.375% w/v thiobarbituric acid–0.25 N hydro-chloric acid) were added into the 0.5 mL of the supernatant. The mixture was heated at 95 °C for 30 min and then quickly cooled in the ice bath. To remove suspended turbidity, the mixture centrifugated at 4100 rpm for 15 min, then the absorbance of supernatant at 532 nm was recorded. Non-specific absorbance at 600 nm was measured and subtracted from the readings recorded at 532 nm. The MDA content was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1}. For determination of the hydrogen peroxide content, 0.5 mL of 0.1 M Tris–HCl (pH 7.6) and 1 mL of 1 M KI were added to 0.5 mL of supernatant. After 90 min, the absorbance was recorded at 390 nm.

The proline content was determined by the method of Weimberg *et al.* (1982). 0.1 g of pelet was homogenized in 10 mL of 3% aqueous sulphosalicylic acid and the homoge-nates were incubated in the hot water bath at 95 °C for 30 minutes. The samples were cooled and centrifuged at 4100 rpm for 10 min. Two milliters of the extract reacted with 2 mL of acid–ninhydrine and 2 mL of glacial acetic acid for 1 h at 100 °C. The reaction mixture was extracted with 4 mL toluene. The chromophore containing toluene was separated and the absorbance was recorded at 520 nm.

2.4 Statistical analysis

The differences between the control and treated samples were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to LSD. Three replicate cultures were used for each treatment. The mean values ± SE were given in figures.

3 Results

Azadirachtin displayed growth-enhancing effect for OD560 absorbance in the early days. There was a significant decrease in OD560 absorbance and chlorophyll-*a* in Aza-exposed *A. platensis* cultures according to the days and concentrations comparison with each other ($p < 0,05$) (Fig. 1a,b).

While the total SOD activity decreased significantly at 8, 16 and 20 µg mL^{-1} concentrations ($p < 0.05$) (Fig. 2a), the total APX activity did not show a significant change at all concentrations (Fig. 2b). GR enzyme activity displayed a significant decrease at 20 µg mL^{-1} Aza concentration compared to control ($p < 0.05$) (Fig. 2c).

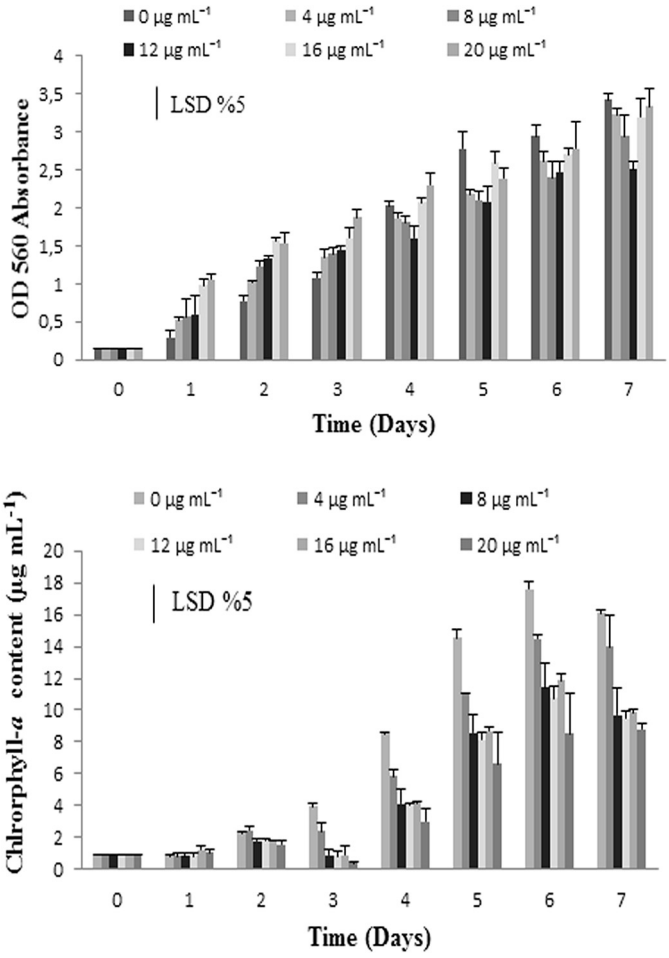

Fig. 1. Biomass values (a) and (b) chlorophyll-*a* content of *Arthrospira platensis* supplemented with 0–20 µg mL^{-1} Aza concentrations during 7 days. Data are the means ± SE of three replicates.

MDA content increased significantly in *A. platensis* at 16 and 20 µg mL^{-1} Aza concentrations compared to control ($p < 0.05$) (Fig. 3a). Similarly, the amount of H_2O_2 increased significantly at 12, 16 and 20 µg mL^{-1} Aza concentrations ($p < 0.05$) (Fig. 3b). The free proline content of *A. platensis* cultures exposed to Aza showed a significant decrease at 4 µg mL^{-1} concentration compared to control ($p < 0.05$) (Fig. 3c).

4 Discussion

In this study, Aza, which is insecticidal and is obtained from *Azadirachta indica*, has been investigated in some parameters such as OD560, chlorophyll-*a* amount, SOD, GR and APX activities and H_2O_2, malondialdehyde and proline content to evaluate oxidative stress of non-target aquatic cyanobacteria.

Sundaram (1997) observed the effects of Aza indoor aquatic microcosms for 20 days. Accordingly, 3 and 4.5 µg mL^{-1} Aza concentrations reduced the chlorophyll-*a* and protein content, whereas it was stimulated at 1.5 µg mL^{-1} Aza concentration. Consequently, it was indicated that the

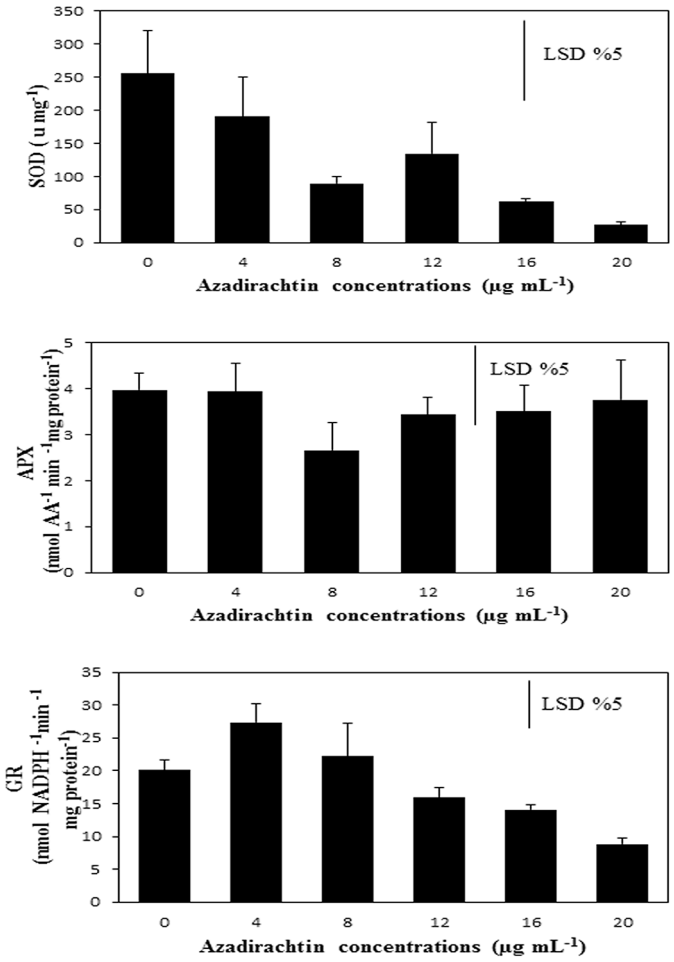

Fig. 2. Total superoxide dismutase (SOD) (a), ascorbate peroxidase (APX) (b) and glutathione reductase (GR) (c) activities of *A. platensis* supplemented with 0–20 µg mL^{-1} Aza concentrationS. Data are the means ± SE of three replicates. Mean values in columns are significantly different at the 5% level according to the least significant differences (LSD) Test.

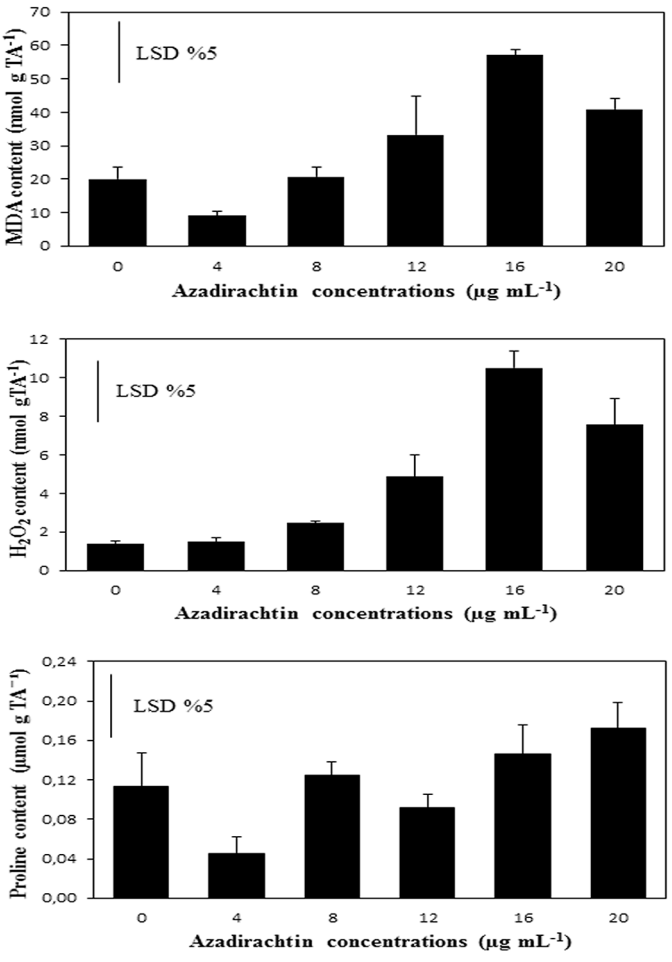

Fig. 3. Malondialdehyde (a), hydrogen peroxide (b) and proline (c) contents of *A. platensis* supplemented with 0–20 µg mL^{-1} Aza concentrations Data are the means ± SE of three replicates. Mean values in columns are significantly different at the 5% level according to the least significant differences (LSD) Test.

decrease of chlorophyll content might be due to the growth inhibitory feature of the pesticide. Prasad *et al.* (2007) applied the aqueous extracts of *Azadirachta indica* on *Nostoc muscorum*. They found that low-extract concentrations (1% and 2%) had a positive effect on algae growth and increased the amount of photosynthetic pigments, but that high concentrations (4 and 8%) reduced cyanobacteria biomass. Chia *et al.* (2016) found that the high doses of extract obtained from the *A. indica* inhibited the growth of *Scenedesmus quadricauda* and reported that the raw extract had reduced chlorophyll-*a* concentration, dry weight and cell density of microalgae depending on the concentration. In the same study, it was observed that the application of 1000 mg L^{-1} extract completely stopped the algal growth at the end of the third day and caused the lysis of the cells. This situation explained as the decrease in the amount of chlorophyll-*a* reduces photosynthesis and it caused the inhibition of CO_2 assimilation adversely effecting the cell division. In our study, it was determined that high Aza concentrations decreased both growth rate and chlorophyll-*a* in *A. platensis*. The growth-enhancing effect of Aza in the early days is supported with low concentrations, enhancing the growth rate and chlorophyll-*a* in previous studies. The studies in the literature support our study revealing the toxic effects of this pesticide.

SOD is an antioxidant enzyme responsible for detoxifying superoxide radicals' production in cells under stress conditions (Elstner *et al.*, 1988). Kong *et al.* (1999) showed that SOD enzyme is a key enzyme that eliminates active oxygen in algae cells. Total SOD activity decreased at 8, 12, 16 and 20 µg mL^{-1} concentrations compared to control in Aza treated *A. platensis* cultures. Wang *et al.* (2011) have reported that Cypermethrin pesticide inhibits SOD activity at high concentrations (>50 µg L^{-1}) on *Skeletonema costatum*, *Scrippsiella trochoidea* ve *Chattonella marina* and suggested that inactivation of SOD activity is caused by Cypermethrin and thus inhibits algal growth. Lee and Shin (2003) found that cadmium applications reduced the activity of SOD enzyme in *Nannochloropsis oculata*. They reported that this decrease is related with the inactivation of the enzyme by H_2O_2 production in different compartments (Vitoria *et al.*, 2001). Therefore, the excessive H_2O_2 accumulation may inactivate the enzyme.

Cao *et al.*, (2011) investigated the effects of manganese deficiency on SOD enzyme activity in *Amphidinium* sp. and suggested that it reduced SOD enzyme activity, which may be caused by some reasons such as a decrease in active oxygen production, loss of photosynthetic functions and oxygen release. Hollnagel *et al.* (1996) studied the effect of light on *Gonyaulax polyedra* and observed that the activity of SOD decreased 2-3 times depending on the lack of photosynthesis during the night phase. AZA displayed to cause significant reductions in the amount of chlorophyll-*a* with increasing concentrations. Loss of photosynthetic metabolism may result in significant reductions in SOD enzyme activity, or increase in the amount of superoxide resulting from a decrease in SOD enzyme activity may have reduced chlorophyll-*a*.

GR enzyme is an enzyme found in different plants, animals and microorganisms (Flohe and Gunzler, 1976). GR and glutathione are effective in inactivating H_2O_2 in plant cells due to the functions in the Haliwell-Asada pathway (Bray *et al.*, 2000). GR catalyzes the last step of the ascorbate-glutathione pathway. In Aza application, GR enzyme activity decreased at 20 $\mu g\,mL^{-1}$ concentration. Lee and Shin (2003) reported that the GR activity of *N. oculata* decreased as a result of Cd^{+2} applications in algae. Sáenz *et al.* (2012) found that Cypermethrin concentrations causing algicidal effects have inhibitory effects on GR activity since they cause oxidative stress damages on *Pseudokirchneriella subcapitata*. Bailly *et al.* (1996) attributed that the decrease in GR activity may relate with the loss of seed viability, when the moisture and temperature applied to sunflower seeds. Schickler and Caspi (1999) specified that the high concentrations of Cd^{+2} application cause the reduction of GR enzyme activity on *Alyssum* sp. via direct reactions of sulfidril groups interfering with the glutathione ring and metals. According to these results, it can be deduced that GR activity decreased due to the loss of viability of the cells, degradation of the enzyme structure or the effect of the reactions on the enzyme.

APX uses ascorbic acid as an electron donor to eliminate harmful H_2O_2 (Verma and Dubey, 2003). GR is required for regeneration of ascorbate (Broadbent *et al.*, 1995). As a result of Aza applications, there was no change in GR activity except 20 $\mu g\,mL^{-1}$, which supports the absence of changes in the amount of APX enzyme at similar concentrations in these pesticide applications. Because the ascorbate pool is balanced by GR, it has been reported in previous studies that GR activity is associated with APX activity (Teisseire and Vernet, 2001; Mallick and Rai, 1998).

The MDA content increased at 16 and 20 $\mu g\,mL^{-1}$ Aza concentrations while H_2O_2 content increased at 12, 16, 20 $\mu g\,mL^{-1}$ Aza concentrations in *A. platensis* cultures. In our study, changes in the MDA content are parallel to the changes in the H_2O_2 content. The increasing H_2O_2 content leads to formation of OH^- radicals by Haber-Weis reaction and thus lipid peroxidation was increased. (Bowler *et al.*, 1992; Goel and Sheoran, 2003). The MDA content, an indicator of lipid peroxidation, was increased by the Endosulfan concentrations (Kumar *et al.*, 2008). Wang *et al.* (2011) reported that Cypermethrin increased in the MDA contents of *S. costatum*, *S. trochoidea* and *C. marina*. In addition, non-functional superoxide dismutase caused the accumulation of O_2^- in cells at this application. It is known that lipid peroxidation is associated with the O_2^- content in the cell (Choudhary *et al.*, 2007).

Moreover, in our study, SOD activity decreased but H_2O_2 increased with rised Aza concentration application. However, the H_2O_2 content may be increased due to increased activity of oxidases such as glycolate oxidase, glucose oxidase, amino-acid oxidase and sulfite oxidase found in plants (Asada and Takahashi, 1987; Asada, 1999). In addition, unchanged APX which is capable of detoxifying H_2O_2 enzyme activity detoxifing H_2O_2 from the medium causes this molecule to accumulate in the cells (Morita *et al.*, 1999).

The free proline content decreased at 4 $\mu g\,mL^{-1}$ Aza concentration compared to the control in *A. platensis*. Most of the studies in the literature suggest that the proline content increases with stress conditions. However, proline decreases were observed under stress conditions in some studies. Ewald and Schlee (1983) found that sulfide reduces the free proline content because it inhibits proline synthesis on *Trebouxia* sp. The free proline content reduced at Aza intermediate concentrations may be due to the use of free proline by free radicals.

In conclusion, the decreases in biomass and chlorophyll-*a* are related with the increases in Aza concentrations. The changes in antioxidant enzyme activities and other parameters differed according to the used concentrations. Aza, which is known to have low toxic effects in vertebrates and humans, may cause dangerous consequences for the lake ecosystem.

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