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Effect of Herbicides on Chlorophyll-a, β- Caroten, Phycocyanin and Allophycocyanin Content of *Anabaena* sp.

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Abstract

Pigments are frequently use in medicine, food, pharmacology, cosmetic, ink and textile preparations. Cyanobacteria have a great potential for use as pigment producer, because they are easy to grow with simple nutrient requirements, unlike other microbial systems and additionally have a large number of pigment. Cyanobacteria produce some pigments such as chlorophyll-a, carotenoids, phycobiliproteins.

In addition, cyanobacteria can fix free atmospheric nitrogen. Environmental conditions affect the cyanobacterial growth and pigment production. Herbicides use commonly in paddy fields. Because of this, herbicides can affect to non-target microorganisms such as cyanobacteria. In this study, production of some pigments by *Anabaena* sp. GO10 were studied using different herbicides in BG-11 medium. As herbicide, fenoxaprop-p-ethyl and cyhalofop- butyl used in this study.

In this work, different herbicide concentrations were applied on pigment production. Initial fenoxaprop-p-ethyl concentrations (6,25 mg/L) stimulated chlorophyll-a, β- caroten, phycocyanin and allophycocyanin contents. But increasing herbicide concentrations suppressed to the all of pigment contents. The all of pigment contents of *Anabaena* sp. GO10 completely suppressed by 100 mg/L fenoxaprop-p-ethyl concentration. The other herbicide is cyhalofop-butyl. Initial cyhalofop-butyl concentrations partly stimulated the pigment contents. But, the pigment contents of *Anabaena* sp. GO10 increased sharply by 25 mg/L cyhalofop-butyl concentrations. The all of pigment contents of *Anabaena* sp. GO10 completely repressed by 400 mg/L cyhalofop-butyl concentrations.

Keywords: *Anabaena*, chlorophyll-a, β- caroten, phycocyanin, allophycocyanin, herbicide

INTRODUCTION

Weeds have always been recognized as one of the major constraints on yield and quality of rice and a significant pest problem in temperate rice culture [1] which can reduce rice yields by competing for moisture, nutrients, and light during the growing season. Weed seed contamination of rice grain lowers grain quality and may lower the cash value of the rice crop. Effective weed control is one of the major requirements to ensure a successful wet land rice production [2].

Modern sustainable paddy cultivation worldwide involves extensive use of agrochemicals such as insecticides, fungicides but especially herbicides. Herbicide demand has unique characteristics compared with other common productive inputs in rice culture systems such as land, labour, seeds and chemical fertilizers [3]. The goal of herbicide use is to kill or stunt weed infestation allowing the rice to grow and gain a competitive advantage. The use of rice herbicides has been expanding enormously worldwide over the past 20- 40 years [4].

Herbicides become incorporated in soil directly, during plant treatment, and indirectly, via water or residues of plant and animal origin. After application, herbicides may evaporate, may be washed away through surface run-off, may leach into deep soil strata and ground water, may be inactivated by plants, or may be adsorbed in soil in which case they become subject to chemical or microbiological degradation. Herbicides are specific regarding their toxic level. However, the application of several chemicals may lead to synergy and development of toxic effects hazardous for humans and the ecosystem. Herbicides may cause acute and genetic toxicity which are perilous for the biota inhabiting the ecosystem [5].

Cyhalofop butyl is intended to provide postemergent control of selected grassy weeds in rice. It is to be used for the control of barnyard grass (*Echinochloa spp*) and silver top (*Lepthochloa fusca*) in rice. This herbicide will be applied at 0.5-1.0 L/ha (142-285 g ac/ha) as a foliar spray together with spraying oil at 1 L/ha. The main effects associated with repeat dose toxicity of cyhalofop-butyl in animals were hepatocellular proliferation, inflammation and gross enlargement of liver and bile duct hyperplasia. Macroscopic and microscopic abnormalities in liver and kidneys were also consistently observed [61].

Fenoxaprop- P-ethyl {ethyl (*R*)-2-[4-[(6-chloro-2-benzoxazolyl) oxy] phenoxy] propanoate} is an aryloxyphenoxypropionate postemergence herbicide inhibiting fatty acid synthesis in grasses through inhibition of acetyl CoA carboxylase [62]. The herbicide can be used

on several crops: Gelmini *et al*. [6] reported the use of fenoxaprop-P-ethyl (FPE) on onion, Nisha and Chopra [7] on wheat and McMullan [8] described its use on barley. The use of fenoxaprop-P-ethyl against annual and perennial grasses in rice is well documented by many authors [9-12].

Traditionally, paddy fields are home-ecosystems to many species [17]. The nitrogen-fixing cyanobacteria form a prominent component of microbial population in rice paddy fields, since they significantly contribute to fertility as natural biofertilizers [43, 49]. Their contribution to the maintenance of soil fertility, by fixing atmospheric N_2 (diazotrophy), is particularly important in rice field soils [50]. Otherwise, cyanobacteria are characterized by the production of various pigments on natural or synthetic media. These pigments are usually described in terms of various shades of blue, violet, red, yellow, and green.

As against widespread use of synthetic dyes not known to be environment friendly, demand for natural pigments for coloring fabrics, foods/feeds, cosmetics and printing inks are increasing [51]. Natural colours are generally extracted from fruits, vegetables, roots and microorganisms and are often called "biocolours" because of their biological origin [52]. Microbial pigments are a promising alternative to other colour additives extracted from vegetables or animals because they are considered natural, pose no seasonal production problems and show high productivity [51].

Pigment producing microorganisms are yeast, fungi, bacteria, micro algae and are quite common in nature. Carotenoids are yellow to orange-red pigments present in a wide variety of bacteria, algae, fungi and plants (53) having the functions of food colorants, absorbers of light energy, oxygen transporters, provitamin A, scavengers of active oxygen, antitumor and enhancers of *in vitro* antibody production [54- 58]. Besides this, carotenoids protect the pigment–protein complexes and the chloroplast against photooxidation (59). A number of natural carotenoids pigments produced by plants also contribute to enhanced immune system and reduced risk of degenerative diseases, such as cancer, cardiovascular diseases, macular degeneration and cataract by scavenging reactive oxygen radicals and acting as anti-aging agent (60].

The influence of herbicides on cyanobacteria has been extensively reviewed in many studies [14, 15]. Generally, cyanobacteria are quite sensitive to herbicides, because they share many of the physiological features of higher plants, which form the site of herbicide action [13]. Many reports available indicate interaction between cyanobacteria and herbicides, including effects of herbicides on algal growth, photosynthesis, nitrogen fixation, biochemical composition and metabolic activities as well as degradation and removal of herbicides by algae and cyanobacteria [16 - 23]. Furthermore, an ideal biofertilizer strain of cyanobacteria must have the ability to tolerate or even resist to toxic actions of herbicides [24]. High bensulfuron-methyl concentrations (8- 10 ppm) inhibited the growth and photosynthesis of over 50% in *A. variabilis* and *Nostoc commune* rice field isolated; nitrogenase activity decreased by 94-98% in *A. variabilis* and by 85-86% in *N. commune* after 24 hours' incubation with 10 ppm and 20 ppm of the herbicide, respectively [25]. Ahluwalia et al. (26) proved that the incorporation of relatively higher doses (> 5 $\mu g.ml^{-1}$) of diquat into *N. muscorum* and *Cylindrospermum* sp. cultures could be highly toxic, thereby reducing their chlorophyll *a* content and contributing to a progressive decrease in growth which culminates in complete lysis of the cells with the increasing level of the herbicide. Okmen and Ugur (27) reported that bispyribac-sodium (100 $\mu g.ml^{-1}$) partly supressed the growths and nitrogenase activities of ten cyanobacteria.

Production of pigments by cyanobacteria have been utilized as an important cultural characteristic in describing the organisms. Nevertheless, very little is known about the effect of herbicides on pigment production, because the formation of pigment is influenced by the pH of the medium, aeration, temperature of the growth and carbon and nitrogen sources. Most reports demonstrated that the sensivity of cyanobacteria toward herbicides and their metabolic activities behavior changed in the presence of herbicides. Until now, a work has not been done on the effects of cyhalofop butyl, and fenoxaprop-P-ethyl on pigment contents of cyanobacteria.

In this work, we report the experimental findings obtained on the effect of a rice herbicides cyhalofop butyl, and fenoxaprop-P-ethyl on the chlorophyll- a, β-caroten, phycocyanin and allophycocyanin contents of *Anabaena* sp. These parameters may be of great relevance to determine the toxicity of cyhalofop butyl, and fenoxaprop-P-ethyl on *Anabaena* sp. and, the studies carried out provide a preliminary idea about the inhibitory or stimulatory effect of cyhalofop butyl, and fenoxaprop-P-ethyl on photosynthetic activities in cyanobacteria.

MATERIALS AND METHODS

Test organisms and cultivation

Cyanobacterial culture obtained from previous studies by Dr. Gulten Okmen, Mugla Sıtkı Kocman University, TURKEY. This including; *Anabaena* sp. GO10. Stock cultures were grown in the N-free BG- 11 medium as previously described [28]. Temperature was maintained at 25 ± 2°C and cultures were grown under a cool white light. Cells in the logarithmic phase of growth were collected from cultures and used as inocula for experiments. Experiments were conducted in batch cultures by using 10 ml of inoculated medium flasks in 25ml. Culture media were adjusted accordingly pH 8 with 1N NaOH and 1N HCl. Illumination was supplied with 600 lux cool white light [29, 30].

Influence of cyhalofop butyl and fenoxaprop-P-ethyl on pigment contents

The influence of different concentrations of cyhalofop butyl, (6.25- 400mg/L) and fenoxaprop-P-ethyl (6.25-100mg/L) on the chlorophyll- a, β-caroten, phycocyanin and allophycocyanin contents were also tested on *Anabaena* sp. GO10. The experimental cultures were grown in 25ml flasks containing 10ml N-free BG-11 medium under the same conditions as described below. According to Rippka (30), the cultures were grown in a liquid sterilized medium at 25 ± 2°C under cool white light (600 lux) for 30 days. At the end of 30 days, chlorophyll *a*, β- caroten, phycocyanin and allophycocyanin contents of the cultures were determined as described below techniques.

Appropriate control systems containing no solvent and herbicide were included in each experiment. Control and treated cultures were grown under the same temperature and light intensity as mentioned above. All experiments were performed in triplicate and the average values were presented.

ANALYTIC METHODS

Determination of dry weight

The pellets of centrifuged cultures were washed with distilled water three times, then dried to a constant weight at 70°C for 12h and dry weights were measured [28, 31].

Determination of chlorophyll a content

The spectrophotometric method (Shimadzu, UV-1201V, Japan) recommended by Porra et al., (32) was used for determination. Chlorophyll *a* contents were calculated on wet weights. All pigment extractions were subsequently repeated until no more pigment was extracted.

Determination of β-caroten content

The β- caroten contents was determined spectrophotometrically at 436 nm against a heptane blank (Anonymous, 2002). The quantities of β- caroten in the extracts were calculated from the measurement of absorbance at 436 nm using the equations. β- caroten contents were calculated on dry weights. All pigment extractions were subsequently repeated until no more pigment was extracted [33].

Determination of phycocyanin and allophycocyanin contents

The spectrophotometric method recommended by Boussiba and Richmond (1979) was used for determination. Samples were centrifuged, ultrasonicated and the pigment contents were estimated in the supernatant according to Boussiba and Richmond (1979). Pigment contents were determined at 615nm and 652nm. The quantities of phycocyanin and allophycocyanin in the extracts were calculated from the measurement of absorbance at 615 and 652nm using the equations. Phycocyanin and allophycocyanin contents were calculated on dry weights. All pigment extractions were subsequently repeated until no more pigment was extracted [34].

Statistical treatment

All experiments were performed in 3 replicates. Data presented in this study are presented in means ± standard deviation (SD).

RESULT AND DISCUSSION

In this study, we had been determined the effects of different concentrations of fenoxaprop-P-ethyl and cyhalofop butyl on pigment contents of *Anabaena* sp. GO10.When *Anabaena* sp. was cultured in the presence of various fenoxaprop-P-ethyl and cyhalofop butyl concentrations, distinct effects were seen on pigment contents. The pigment contents of cyanobacterium treated with different concentrations of fenoxaprop-P-ethyl under 600 lux light intensity are listed in Figure 1, 2, 3, and 4.

The nitrogen- fixing cyanobacteria are known to dominate the water- logged paddy fields and help in the nitrogen economy of rice agriculture [35- 37]. Although the use of the herbicide is aimed at eliminating weeds, a major portion is deposited on the surface of the soil and might adversely affect the non-target soil microflora. Information on resistance to herbicides, and for these herbicides in particular, are lacking. In Turkey, fenoxaprop-P-ethyl and cyhalofop butyl are mostly used for eliminating weeds in paddy fields [38]. For this reason, the herbicides were chosen for this study.

The maximum chlorophyll *a* content was determined in *Anabaena* sp. GO10 (0.003 mg/ml) at 6.25 mg/L fenoxaprop-P-ethyl concentration whereas, the lowest chlorophyll *a* content of *Anabaena* sp. GO10 was shown at 50 mg/L fenoxaprop-P-ethyl concentration. The chlorophyll-a contents of this strain were completely repressed during the 100 mg/L fenoxaprop-P-ethyl concentrations (Figure 1). Most reports have demonstrated that the inhibitory effect of herbicide became greater with an increase in herbicide concentration and suggested that the reduction in the dry matter of algae may be due to a decrease in algal photosynthesis caused by the inhibition of synthesis of chlorophyll, which is the most important pigment in algal cells for collecting solar energy for photosynthesis [20, 39].

Similarly, the highest β- caroten content (0.26 mg/L) was determined by 6.25 mg/L fenoxaprop-P-ethyl concentration in *Anabaena* sp. GO10. The β- caroten contents of this strain were partly repressed during the 50 mg/L fenoxaprop-P-ethyl concentrations, whereas β-caroten contents of this strain were completely repressed during the 100 mg/L fenoxaprop-P-ethyl concentrations (Figure 2).

Otherwise, the highest phycocyanin content was determined in *Anabaena* sp. GO10 (0.006 mg/ml) at 6.25 mg/L fenoxaprop-P-ethyl concentration. The phycocyanin contents of this strain were partly repressed during the 50 mg/L fenoxaprop-P-ethyl concentrations, whereas phycocyanin contents of this strain were completely repressed during the 100 mg/L fenoxaprop-P-ethyl concentrations (Figure 3). Allophycocyanin contents of *Anabaena* sp. GO10 were partly inhibited up to 25 mg/L fenoxaprop-P-ethyl concentration whereas allophycocyanin contents of strain were completely inhibited up to 100 mg/L fenoxaprop-P-ethyl concentration (Figure 4). It is similar to the previous report by Marco et al. (1990), who found that the organophosphorus insecticide trichlorfon (at concentrations ranging from 20 to 300 μgml^{-1}) decreased biliprotein content in *Anabaena* PCC 7119 [42].

In this study, the maximum chlorophyll *a* content was determined in *Anabaena* sp. GO10 (0.004 mg/ml) at 25 mg/L cyhalofop butyl concentration whereas, the lowest chlorophyll *a* content of this strain was shown at 200 mg/L cyhalofop butyl concentration (Figure 5). Growth studies showed that the cyanobacterial strains were capable of growing both photoautotrophically and photoheterotrophically [44, 45]. In *N. muscorum*, a concentration of 25 ppm carbofuran was observed to be stimulatory under most of the experimental conditions established by Kar and Singh (1978) [46].

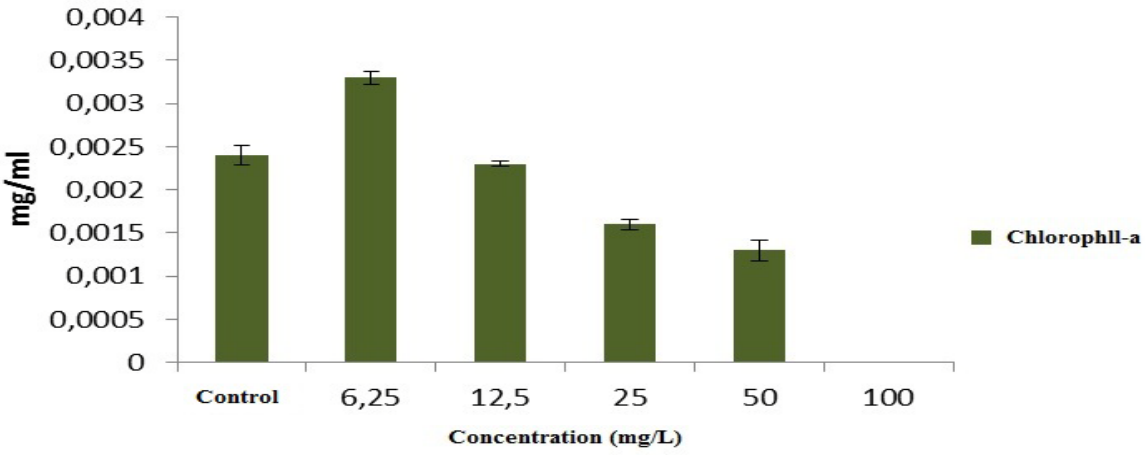

Figure 1. Influence of fenoxaprop -P-ethyl on chlorophyll *a* contents in *Anabaena* sp. GO10

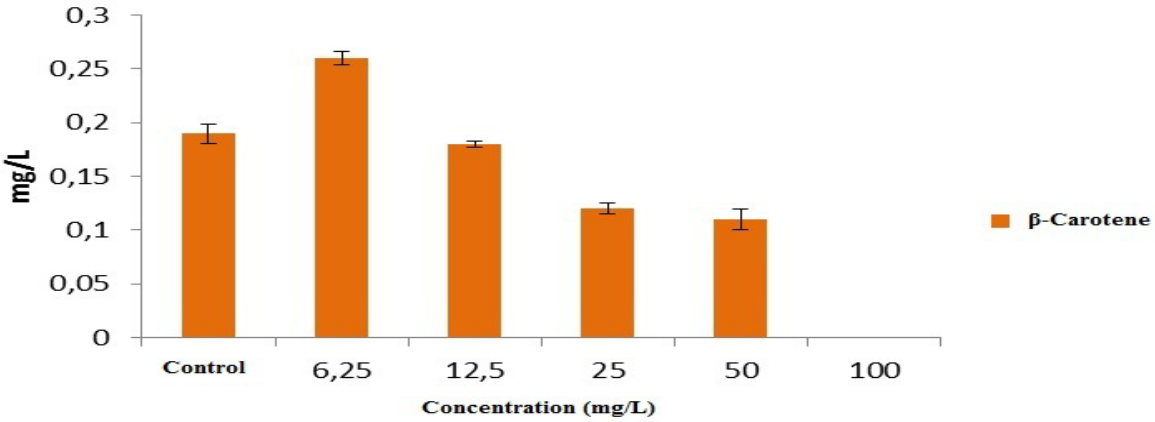

Figure 2. Influence of fenoxaprop-P-ethyl on β- caroten contents in *Anabaena* sp. GO10

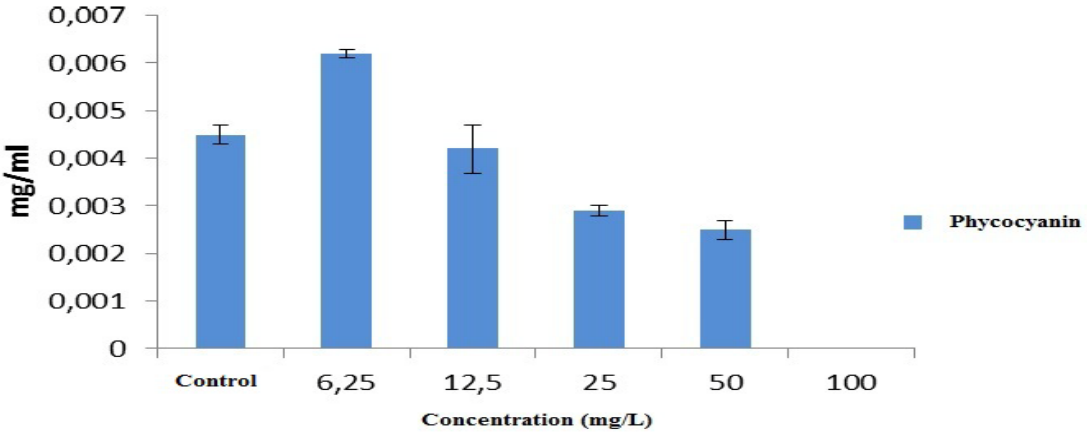

Figure 3. Influence of fenoxaprop-P-ethyl on phycocyanin contents in *Anabaena* sp. GO10

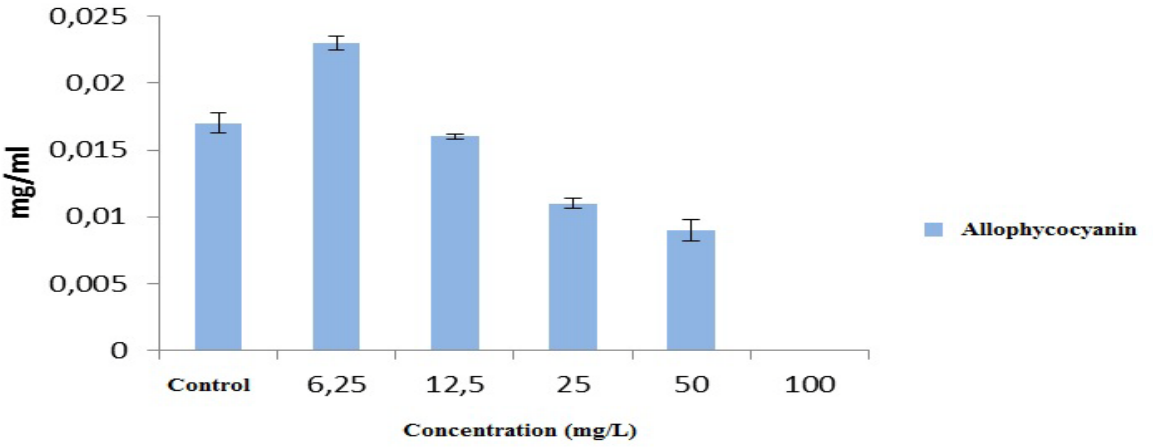

Figure 4. Influence of fenoxaprop-P-ethyl on allophycocyanin contents in *Anabaena* sp. GO10

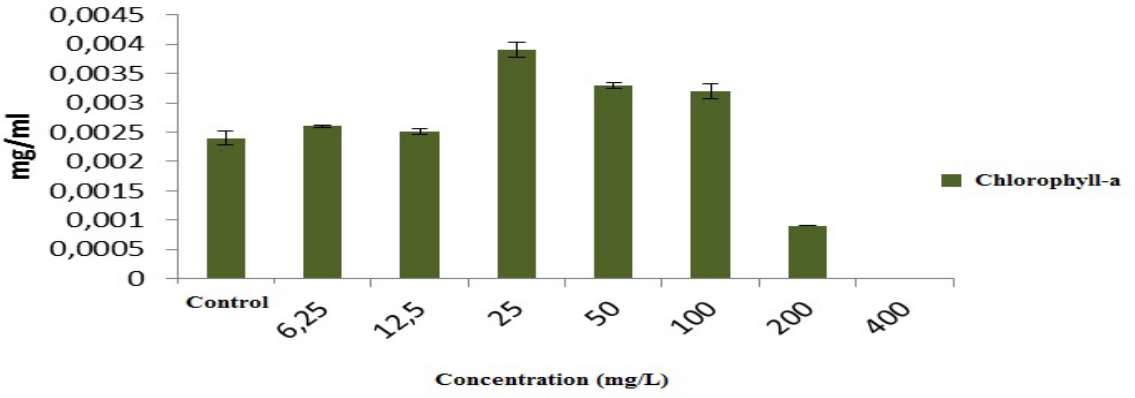

Figure 5. Influence of cyhalofop butyl on chlorophyll *a* contents in *Anabaena* sp. GO10

The chlorophyll *a* content of this strain was completely repressed by 400 mg/L cyhalofop butyl concentration. Therefore, chlorophyll *a* contents of *Anabaena* sp. GO10 were partly inhibited up to 100 mg/L cyhalofop butyl concentration whereas chlorophyll *a* contents of the strain were sharply inhibited up to 200 mg/L cyhalofop butyl concentration (Figure 5). Pandey reported that (1985) chlorophyll *a* synthesis was reduced after exposure to propanil however phycocyanin / chlorophyll *a* ratio showed a positive correlation [40]. Allen and Arnold (1969) suggested that the photoautotrophic growth of *Nostoc calcicola* was due to inhibition of CO_2 fixation and not because of nitrogen, since phycocyanin serves as a ready-made source of fixed nitrogen [41].

Similarly, the highest β- caroten (Figure 6), phycocyanin (Figure 7), and allophycocyanin (Figure 8) contents were determined by 25 mg/L cyhalofop butyl concentration in *Anabaena* sp. GO10. However, the all of pigment contents of this strain were partly repressed up to100 mg/L cyhalofop butyl concentrations, whereas all of pigment contents of this strain were sharply repressed by 200 mg/L cyhalofop butyl concentration. In addition to, the all of pigment contents were completely supressed by 400 mg/L cyhalofop butyl concentration (Figure 6, 7 and 8). Gonzalez-Barreiro et al. (2006) showed that the serious effects on growth for microalgae by herbicide added to culture medium [47]. The main characteristic of cell death or decrease of cell viability, whether from senescence, acute stress, or aging, seems to be the loss of the ability of cells to maintain homeostasis [48].

The data obtained in this study provide information about the inhibitory or stimulatory effect of these herbicides on pigment contents of cyanobacteria, which exhibits different sensitivity to the herbicide. These findings suggest a limit or avoidance of the use of these herbicides in paddy fields, due to its inhibitory effect on biological nitrogen fixation or photosynthesis and hence a possible reduction in rice crop yields.

In this study, we have shown a clear physiologic distinction in *Anabaena* sp. Generally *Anabaena* sp. GO10 had the best optimal performance of pigment contents in cyhalofop butyl concentrations, so it is thought that it is a suitable genus for biofertilizer. A better understanding of the mechanism of action of the herbicide on pigment contents requires further study of the biochemical targets of the herbicide in cyanobacteria.

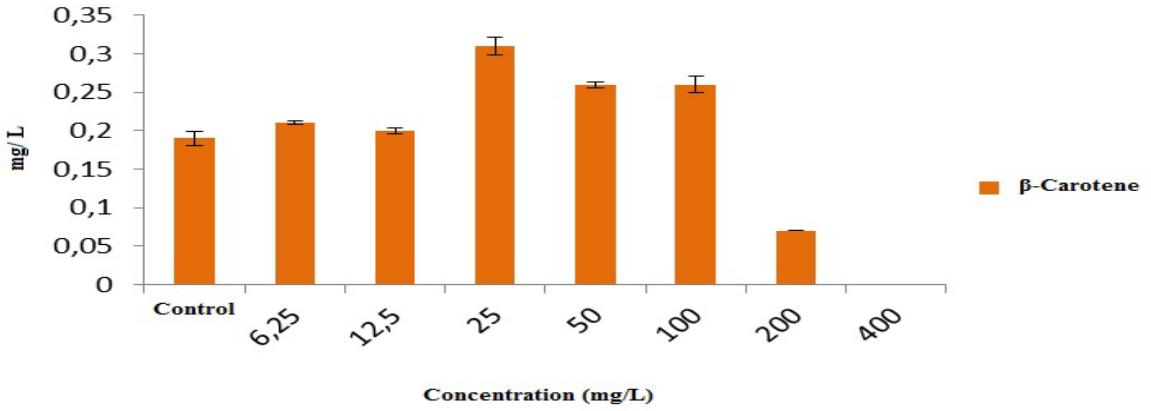

Figure 6. Influence of cyhalofop butyl on β- caroten contents in *Anabaena* sp. GO10

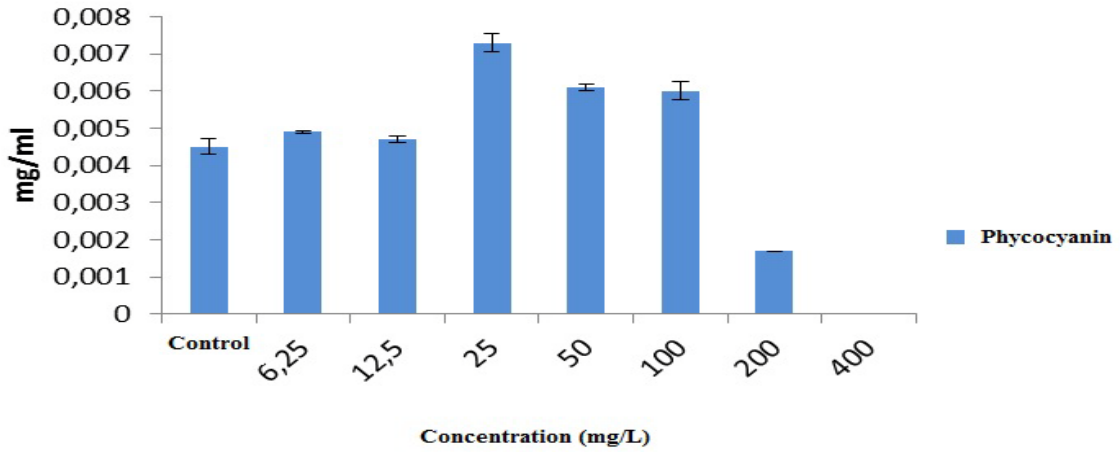

Figure 7. Influence of cyhalofop butyl on phycocyanin contents in *Anabaena* sp. GO10

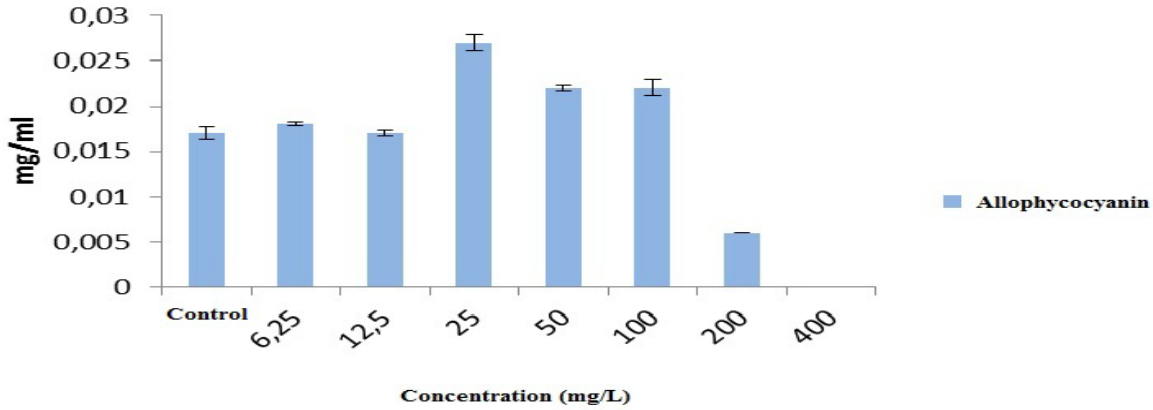

Figure 8. Influence of cyhalofop butyl on allophycocyanin contents in *Anabaena* sp. GO10

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