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THE EFFECT OF SALICYLIC ACID AND TRIACONTANOL ON BIOMASS PRODUCTION AND IMIDACLOPIRID REMOVAL CAPACITY BY CYANOBACTERIA

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ABSTRACT

Pesticide removal capacities of *Synechocystis* sp. and *Phormidium* sp. were investigated in BG11 media. Imidacloprid (IMI) is a widely used systemic insecticide to control plant pests following soil, seed or foliar applications, and is subject to cleaning. Bioremoval is one of the economical water treatment techniques in remediation. Trials were carried out at pH 7.5 for IMI at media with and without triacontanol (TRIA), a naturally occuring plant hormone and Salicylic acid (SA). The removal capacities of *Synechocystis* sp. and *Phormidium* sp. were found higher in media containing TRIA and Salicylic acid. The removal efficiencies were measured at 150 mg L^{-1} concentrations of IMI. *Synechocystis* sp. and *Phormidium* sp. had the maximum values of removal of IMI in the media containing the hormone and Salicylic acid. The results showed that TRIA and Salicylic acid could be considered as a stimulant in pesticide removal by the isolated cyanobacteria cultures.

KEYWORDS: Salicylic acid, triacontanol, cyanobacteria, imidaclopirid, wastewater

INTRODUCTION

As known, ovicidal and larvicidal insecticides are used widely for agricultural, industrial and residential purposes. Their wide use in agriculture has been one of the major factors in the increased productivity in

the last century lead to the publication of famous book Silent Spring by R. Calson in 1962 on their numerous and high environmental impacts, and necessity of balancing the needs with environmental and health issues, when using insecticides (Casida and Quistad 1998).

There are several classifications of insecticides based on their different aspects (Thundiyil et al., 2008). Inorganic ones like heavy metal salts have been used for agricultural pest control (Smith and Secoy 1976). Although organophosphorus insecticides were introduced to replace persistent organochloride insecticides such as DDT due to their low persistence, in time it was realized that their acute toxicity was also quite high (Galloway and Handy 2003). Their impact on freshwater ecosystems by spray drift, leaching, run-off, or accidental spills were presenting potential risks for aquatic flora, such as alterations of the species composition of communities were leading even to changes in structure and functioning of the whole ecosystem (Ma et al. 2005). As summarized by Jeschke and Nauen (2008), IMI was synthesized in 1985, and firstly registered in France in 1991, being introduced as the first representative of a new class of insecticides, namely chloronicotinyls or neonicotinoids, exhibiting a novel mode of action of pest organisms. IMI (ISO 1750, IUPAC:(*E*)-1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine, CAS: (2*E*)-1-[(6-chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine, Reg. No.: 138261-41-3, $C_9H_{10}ClN_5O_2$) is a systemic broad-spectrum contact insecticide, stomach poison as an agonist of the nicotinic acetylcholine receptor leading to paralysis and death of some sucking and biting insects, such as rice hoppers, aphids, thrips, whitefly, termites when applied to seeds, soil and foliage (Baj et al. 1991; Nauen et al. 1998; Schmuck 2003).

Green algae are at the base of most of the aquatic food webs, although blue-green algae are not eaten by other water organisms, they also have essential roles in the nutrient cycling and specially ecologically critical aquatic phosphorus cycle (Sabater and Carrasco 2001). In fact, more recently some valuable information on the ecotoxicological effects of pesticides on green algae has been obtained by Ma et al. (2005). However, little is known about the toxicological aspects of pesticides on cyanobacteria consisting of vast number of species (Ma et al. 2005).

Results of the tests obtained from certain species are limited in applicability in assessing the effects of numerous contaminants on algal communities composed of an array of species with different sensitivities (Ma et al. 2006).

A lot of articles have been published on the comparative sensitivity of mixed various green algae populations towards pesticides Ma et al. 2006; Junghans et al. 2003; Ma 2003).

Yet there are few reports concerning the differential response of various cyanobacteria and green algae species (Ma et al. 2005). In addition, some members of cyanobacteria produce toxins, which have important implications on aquatic organisms and also on human health (An and Kampbell 2003).

On the other hand, cyanobacteria can be used in polluted water treatment, as the literature recently reviewed by Abed et al. (2009) clearly showed. The related literature on the pollutant removal potential of cyanobacteria indicates that, selection of the appropriate composition of the organisms was also related to their population growth in terms of biomass increase (Micheletti et al. 2008).

It is also possible to enhance the growth of microorganisms by stimulation of their growth potential by adding growth stimulators to the culture media. Considering the probability of high stimulation rate and levels at lower concentrations of such a growth promoter, namely triacontanol (TRIA) was reported by Haugstad et al. (1983) for green *Chlamydomonas reinhardtii* and blue-green algae, cyanobacter *Anacystis nidulans* (Haugstad et al. 1983). A similar article was published by Chauan and Singh et al.(1995) on enhancement of growth and productivity of green algae *Spirullina* by eucalyptus kraft black liquor. These are evidences indicating that TRIA could be used as a stimulant to increase efficiency of biological wastewater treatment processes, which can be beneficial in practice, as Chauhan et al. designed their experimental studies, and Langshen et al. (2008) reported TRIA promoted growth of marine photosynthetic bacteria. Thus, we found it relevant to test the hypothesis that mixed *Scenedesmus* cultures could be used in bioremoval of IMI from water and the removal efficiency could be stimulated by addition of TRIA to the culture media. The above mentioned literature implies that, any positive results of the experiments presented here could be leading to trials of water treatment of effluents by TRIA stimulated outdoor mixed cultures of *Scenedesmus* sp.

Triacontanol (TRIA) is a 30-carbon, primary alcohol, of which the plant growth regulating properties discovered in 1977 by Ries et al. Many investigators have reported the influences of TRIA on photosynthesis, nutrient uptake, enzymatic activity and gene regulation, and consequent

increase in producitivity of various crops at its lower concentrations. Even 10 µg liter^{-1} foliar application of TRIA on intact 15 days old rice plants (Chen et al. 2002) or oilseed crops (Ghosh et al. 2008) can be presented here, as the examples of numerous reports suggesting the significance of TRIA in agricultural practices. The most profound effect of TRIA on plants and unicellular green algae is an increase in dry weight (Houtz et al. 1985; Kumaravelu et al. 2000; Malabadi et al. 2005).

Salicylic acid is a phenolic phytohormone and is found in plants with roles in plant growth and development, photosynthesis, transpiration, ion uptake and transport. Salicylic acid also induces specific changes in leaf anatomy and chloroplast structure. Salicylic acid is involved in endogenous signaling, mediating in plant defense against pathogens (Hayat and Ahmad 2007). It plays a role in the resistance to pathogens by inducing the production of pathogenesis-related proteins (Huijsduijnen 2009). It is involved in the systemic acquired resistance (SAR) in which a pathogenic attack on one part of the plant induces resistance in other parts. The signal can also move to nearby plants by salicyclic acid being converted to the volatile ester, methyl salicylate (Taiz and Zeiger 2002). *Synechocystis* sp. and *Phormidium* sp. were selected as test materials, to examine the changes in pesticide removal capacity in the media containing different IMI concentrations. TRIA was used for enhancing the growth of the cyanobacteria used in comparison to the hormone controls. Considering the lack of any reports investigating TRIA effect onto reactive dye removal by *Synechocystis* sp. and *Phormidium* sp., this is the first report investigating dye removal capacity of these test materials. The major objective of this study was to investigate the potential of using cyanobacteria sp. in treating industrial wastewaters containing pesticide, and possibility of increasing their efficiencies.

MATERIALS AND METHODS

Microorganism and culture conditions

Two algal cultures namely *Synechocystis* sp. and *Phormidium* sp. were used in the study, as provided by Ankara University, Faculty of Science Laboratories' from the current culture collection. A series of batch culture experiments were performed, in unshaken flasks, illuminated by cool white

fluorescent lamps emitting 2400 lux of light intensity. The algal cultures were transferred into 100 ml BG 11 medium at a known pesticide concentration in 250 ml Erlenmeyer flasks and incubated at 30 ºC under continuous illumination for 20 days in plant growth chamber (Lab-line® Biotronette) (Rippka 1988).

Triacontanol, Imidaclopirid and Salicylic acid solution

480 ppm TRIA (96% w/v; Aldrich) solution was prepared by dissolving 10 mg of the TRIA in 20 ml chloroform. IMI (35% w/v; Bayer) was supplied by Entomology Unit of Plant Protection Dept. of Faculty of Agriculture, Ankara University. The solubilities of imidacloprid in water, methanol, ethanol, acetone, 2-butanone, dichloromethane, 1,2-dichloroethane, and trichloromethane were measured at temperatures from (293.15 to 353.15) K by a synthetic method at atmospheric pressure. Appropriate volumes of these solutions were added to BG_{11} medium. Salicylic acid solution was prepared by dissolving 50mg of the Salicylic acid in 1000 ml BG_{11} medium.

Effect of Salicylic acid and TRIA on growth of Cyanobacteria

In this set of experiments designed to show the effect of TRIA and Salicylic acid in growth stimulation of *Synechocystis sp.* and *Phormidium* sp. 10mg-1TRIA and 50mg-1 Salicylic acid were transferred into fresh 100 ml BG 11 medium with 1 ml culture solution and control erlenmayers prepared without Salicylic acid and TRIA and without TRIA. Dry weight concentrations were followed between 20 days of incubation.

Effect of Salicylic acid on Removal of IMI by Synechocystis sp. and Phormidium sp.

To examine the effect of Salicylic acid on IMI removal by *Synechocystis* sp. and *Phormidium* sp. cultures, pH of BG 11 with 0 and 50 mg $L^{-1}$of Salicylic acid samples was adjusted to pH 7.5 and 150 mgL^{-1} of IMI were added into these samples before incubation (IMI_{ini}). To acclimatize the cultures to various IMI concentrations, repeated transfers of culture into fresh medium were made by inoculating with 1 ml acclimatized culture solutions and control erlenmayers prepared without Salicylic acid.

Effect of TRIA and Salicylic acid on Removal of IMI by Synechocystis sp. and Phormidium sp.

In this set of experiments to examine the effect of TRIA *and* Salicylic acid on IMI removal by *Synechocystis* sp. and *Phormidium* sp. cultures, 10 mg $L^{-1}$of TRIA, 50 mg $L^{-1}$of Salicylic acid *and* 150 mgL^{-1} of IMI were added into these samples before incubation (IMI_{ini}). Control erlenmayers prepared with TRIA. To acclimatize the cultures to various IMI concentrations, repeated transfers of culture into fresh medium were made by inoculating with 1 ml acclimatized culture solutions and measuring removal at incubation period.

Analytical methods

3 ml samples were taken daily from each of the flasks during the incubation period, and they were centrifuged to precipitate suspended biomass at 3421xg for 5 min. As mentioned before, cell growth of *Synechocystis* sp. and *Phormidium* sp. was determined by measuring dry weight of washed biomass. The dry weight of pellets was obtained at the end of the incubation period by filtering the washed cultures through filter paper, and weighing the biomass after drying to a constant weight at 65 ºC (Aksu et al. 2007 ; Karacakaya et al. 2009).

The concentration of IMI in the supernatant was determined by reading the absorbance of BG 11 media at 600 nm, and IMI at 291 nm, which was predetermined by wavelength scanning of the pesticide sample series, against cell free BG 11 medium blank. Maximum absorbance wavelength was selected by wavelength scanning of 1% IMI solutions in water, ethanol, methanol and aceton within 200 to 300 nm, considering the value reported by Baskaran et al (1997) as 270 nm using a mobile phase of acetonitrile-water (20:80, v/v). All of the measurements were performed at 291 nm considering the IMI spectras held in all of the mentioned solvents. Absorbance measurements for biomass determination were by using Shimadzu® UV 1201V (Japan) and Shimadzu® UV- 1700 model spectrophotometers For centrifugation Hettich® EBA12 (Germany) model centrifuge was used.

Statistical analysis

The experiments were set in a completely randomized design with three replicates. The data were subjected to analysis of variance using Minitab® 14, and significant differences among treatments were compared by descriptive statistics (±S.E.).

RESULTS

IMI_{ini} removal by Synechocystis sp. and Phormidium sp. was investigated in media containing different concentrations of IMI_{ini}, 0 or 10mg L^{-1} TRIA and 50 mg L^{-1} of Salicylic acid. The percentage removal of IMI_{ini} was calculated from equation (1)

$$Removal\ (\%) = (C_o - C_f) / C_o \times 100 \quad \text{(Eq.1)}$$

Insecticide removal capacity is taken as the level of IMI_{ini} removed by the biomass, which was calculated based on the mass balance principle from equation (2).

$$q_m = (C_o - C_f) / X_m \quad \text{(Eq.2)}$$

To calculate the maximum specific insecticide removal values representing the maximum values of IMI per unit dry weight of microbial cells (mg g^{-1}) and, maximum dried cell mass (g l^{-1}), the initial and final concentrations of IMI (mg l^{-1}) were measured respectively. The values of maximum specific IMI_{ini} removal by *Synechocystis* sp. and *Phormidium* sp. were calculated accordingly.

Effect of Salicylic acid and TRIA on growth of Cyanobacteria

In this set of experiments designed to show the effect of TRIA and Salicylic acid in growth stimulation of *Synechocystis sp.* and *Phormidium* sp. 10mg-1TRIA and 50mg-1 Salicylic acid were transferred into fresh 100 ml BG 11 medium with 1 ml culture solution and control erlenmayers prepared without Salicylic acid and TRIA and without TRIA. Dry weight concentrations were followed between 20 days of incubation. Result showed TRIA and Salicylic acid containing samples had a highest dry weight and Salicylic acid containing samples is higher dry weight than Salicylic acid free samples during the experimental period. This range used in the experiments was selected considering the reports on similar applications by Houtz et al. (1985), Kumaravelu et al. (2000), Malabadi et

al. (2005).Effect of Salicylic acid and TRIA on S*ynechosystis* sp. and P*hormidum* sp. dry weight was shown in Table1.The highest dry weight increased in samples containing 10 mg^{-1}TRIA and 50 mg-1 Salicylic acid at all of the *Synechocystis* sp. and *Phormidium* sp. cultures.

Table 1. Effect of SA and TRIA on *phormidum* sp. ve *synechosystis* sp. Dry weight of microbial cells in mg/l (Incubation period: 20 d; T: 30 ºC; illumination: 2400 lx).

Cyanobacteria	without SA (mg/l)	with SA (mg/l)	with SA and TRIA (mg/l)
Synechosystis sp.	2.1	3.6	5.1
Phormidium sp.	1.64	2.8	4.6

Effect of Salicylic acid on IMI Removal by Synechocystis sp. and Phormidium sp.

Salicylic acid with concentration of 50mg^{-1} and150 mg^{-1} IMI separately, transfers of *Synechocystis* sp. and *Phormidium* sp. cultures into fresh 100 ml BG11 medium and control erlenmayers prepared without Salicylic acid .The media was inoculated with 1 ml culture solution and control erlenmayers. Removal during 20 days was examined. Results showed Salicylic acid containing samples had higher removal than Salicylic acid free samples. The effect of Salicylic acid on IMI removal by *Synechosystis* sp. was shown in Figure1.

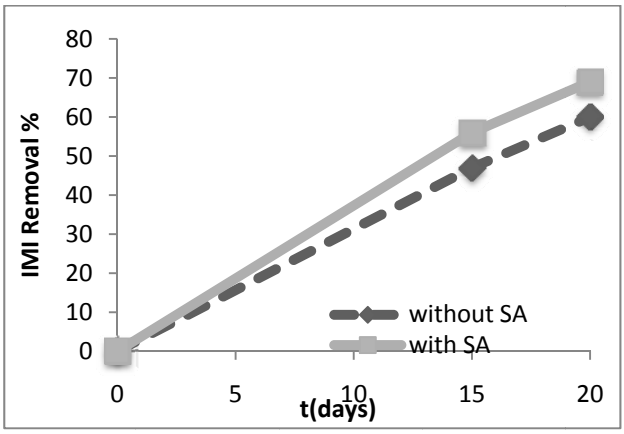

Figure 1. Effect of SA on IMI removal by *Synechosystis* sp. (Incubation period: 20 d; T: 30 ºC; illumination: 2400 lx).

The effect of Salicylic acid on MI removal by *Phormidium sp.* was shown at Figure 2.

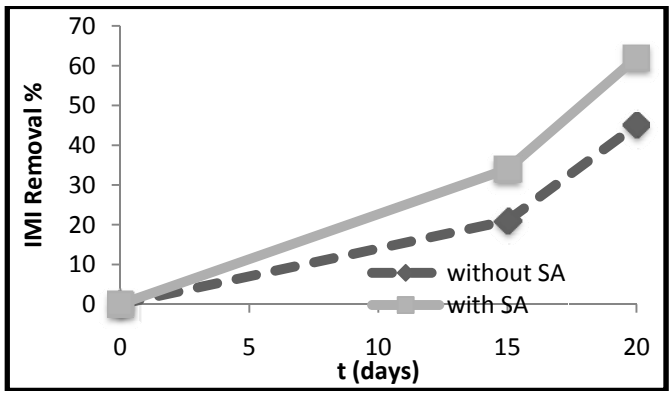

Figure 2. Effect of SA on IMI removal by *Phormidium* sp. (Incubation period: 20 d; T: 30 ºC; illumination: 2400 lx).

Effect of TRIA and Salicylic acid on IMI Removal by Synechocystis sp. and Phormidium sp.

TRIA with 10 mgL^{-1} of concentration, 50 mgL^{-1} of Salicylic acid concentration and 150 mgL^{-1} IMI separately, transfers of culture into fresh 100 ml BG 11 medium and control Erlenmayers prepared with TRIA. The media was inoculated with 1 ml culture solution and control Erlenmayers. Removal during 20 days was examined. TRIA and Salicylic acid containing samples had higher removal than and TRIA containing samples in all days of experiment. Removal of TRIA and Salicylic acid containing and free samples were enhanced in following days. TRIA and Salicylic acid containing samples had the highest removal and Salicylic acid containing samples had the higher removal than Salicylic acid free samples. The effect of TRIA and Salicylic acid on IMI removal by *Synechocystis* sp. cultures was shown in Figure 3.

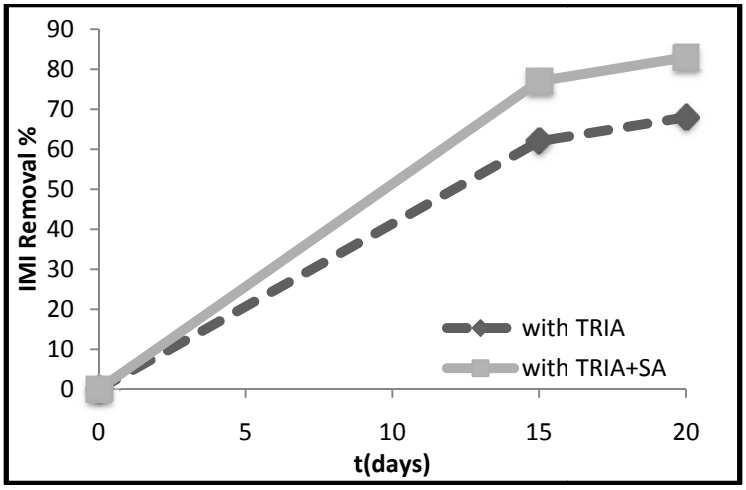

Figure 3. Effect of TRIA and SA on IMI removal by *Synechosystis* sp. (Incubation period: 20 d; T: 30 ºC; illumination: 2400 lx).

The effect of TRIA and Salicylic acid on IMI removal by *Phormidium* sp. cultures was shown in Figure 4.

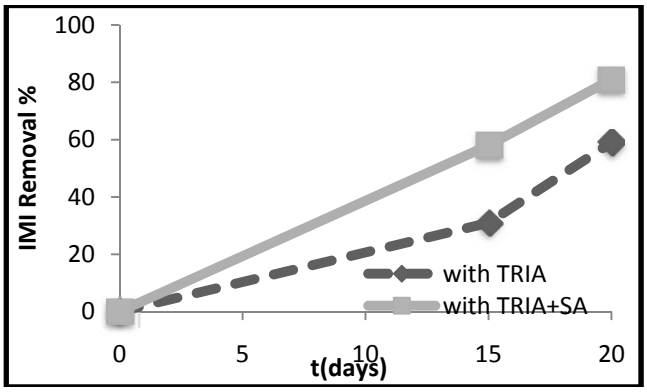

Figure 4. Effect of TRIA and SA on IMI removal by *Phormidium* sp.

The comparison of maximum insecticide removal by *Synechocystis* sp. and *Phormidium* sp. in TRIA and Salicylic acid containing and free samples are shown in Table 2. Removal percentages of the TRIA and Salicylic acid samples were higher than the controls.

Table 2. The comparison of the maximum spesific Imidaclopirid removal per unit dry weight of microbial cells in mg/g (q_m) by *Synechocystis* sp. and *Phormidium* sp. in media TRIA and Salicylic acid, without and with Salicylic acid. (Incubation period: 20 d; T: 30 ºC; illumination: 2400 lx).

Cyanobacteria	without SA (mg/g)	with SA (mg/g)	with SA and TRIA (mg/g)
Synechosystis sp.	4.2	2.5	1.72
Phormidium sp.	5.4	3.21	1.95

DISCUSSION

There are studies in the literature on bioremoval of different types of insecticides by treating the contaminated samples with several algae spp. In

the present study, it was observed that, the tested *Synechocystis* sp. and *Phormidium* sp.*,* removed IMI with higher yields in Salicylic acid and TRIA containing BG11 media than the Salicylic acid and hormone free samples. It was observed that the higher efficiency depended on increased growth rates of the biomass. As a matter of fact, in a previous study, it was reported that, the stimulatory effect of TRIA on growth of *Chlamydomonas* depended on significant increases in cell density, total chlorophyll, CO_2 assimilation and dry weight (Houtz et al. 1985) In another study on *Chlamydomonas reinhardtii* and blue-green algae, cyanobacter *Anacystis nidulans*, and similar article on enhancement of growth and productivity of green algae *Spirullina* by eucalyptus kraft black liquor showed that the growth rate could be increased by further by some treatments Haugstad et al. 1983; Chauhan and Singh 1995) . Our results also support these evidences indicating the potential of Salicylic acid and TRIA in stimulation of growth rate of microalgae, and this potential could be exploited for higher IMI bioremoval efficiency in contaminated water treatment processes, and could be used in practice, as it was mentioned in the article on TRIA promoted growth of marine photosynthetic bacteria (Ries et al. 1977). The results presented here support such indications, by showing total dry weight production and chlorophyll synthesis and also IMI bioremoval by *Synechocystis* sp. and *Phormidium* sp., was increased by addition of TRIA to the culture media. The effects of salicylic acid and Na salicylate in algae (*Scenedesmus subspicatus, Monoraphidium minutum*), in *Lemna minor*, and in *Daphnia magna* were examined (Wang and Lay 1988). Another examination was conducted to investigate the influence of salicylic acid on growth and changes of nucleic acids, protein, photosynthetic pigments, sugar content and photosynthesis levels in green algae (Czerpak et al. 2001).

Although, there are some studies in the literature describing the removal of insecticide by cyanobacteria, there is no report investigating IMI removal capacity of *Synechocystis* sp. and *Phormidium* sp.*,* or removal of pesticide pollutants by adding TRIA hormone into cyanobacteria culture media. Our data indicated that *Phormidium* sp.*,* cultures were potentially suitable for effective treatment of such wastewaters containing IMI and possibly another insecticide, and the removal capacity could be increased by TRIA addition to the media, through stimulation of the biomass production rate. This growth stimulator can be used in practice for IMI, bioremoval at least,

since this natural hormone can be obtained from natural sources economically and easily (Rao et al. 1987).

ÖZET: *Synechocystis* sp. ve *Phormidium* sp. mikroalglerinin pestisit giderim kapasiteleri BG11 besiyerinde araştırılmıştır. Imidacloprid (IMI) toprak, tohum ve yaprak uygulamalarıyla bitki zararlılarının kontrolünde yaygın olarak kullanılan bir insektisittir. Biyogiderim ekonomik bir su arıtım tekniğidir. IMI giderim denemeleri, pH 7.5 da, doğal bir bitki hormonu olan triakontanol (TRIA) ve Salisilik asit (SA) içeren ve içermeyen besiyerlerinde yapılmıştır. *Synechocystis* sp. ve *Phormidium* sp. giderim kapasiteleri TRIA ve SA içeren ortamlarda yüksek olmuştur. Giderim kapasitreleri 150 mg L^{-1} IMI konsantrasyonunda ölçülmüştür. *Synechocystis* sp. ve *Phormidium* sp. TRIA ve SA içeren ortamlarda yüksek kapasite ile IMI giderimi yapmıştır. İzole edilen siyanobakteri kültürleri tarafından TRIA ve SA'in, pestisit gideriminde bir stimülant olarak kullanılabileceği çalışma sonucunda gösterilmiştir.

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