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Triacontanol hormone stimulates population, growth and Brilliant Blue R dye removal by common duckweed from culture media

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ABSTRACT

This work is focussed on assessing the potentialities of *Lemna minor* (L.) for the treatment of reactive dyes polluted wastewaters and investigating the possibility of bioremoval performance stimulation by adding triacontanol hormone to the cultures. In the vast literature describing removal of reactive dyes, considering the lack of reports using of common duckweed in wastewater treatment apparently due to the inadequate efficiency. In the present study, the experiments showed that 1 mg l^{-1} triacontanol stimulated duckweed growth. The effect of different dye types (Reactive Orange 14, Reactive Red 120, Reactive Black 5, Brilliant Blue R, and Reactive Brilliant Blue R) onto duckweed growth was tested. Plants grew at most in media with Brilliant Blue R. The highest biomass, in terms of frond number (87 ± 1.5) were accompanied with 59.6% maximum dye removal were found in samples containing 2.5 mg l^{-1} initial Brilliant Blue R and 1 mg l^{-1} triacontanol, indicating hormonal stimulation of both activities. The results presented here that *L. minor* (L.) could be used effectively to treat wastewaters containing dye.

1. Introduction

Attention attracted to the number of varieties and the total volume of synthetic colorant production, their extensive use and the environmental impacts of at least 80,000 tons of commercially available dyes discharged in the wastewater effluents of various manufacturing industries was described in a literature about bioremoval of Remazol Brilliant Blue R dye [1]. Such effluents contain reactive chemicals like Brilliant Blue R, affecting aquatic life significantly by forming some soluble toxic, mutagenic and carcinogenic components and changing light absorption properties which interact with potential toxicity [2]. Another issue is the aromatic rings in their structure, increasing the resistance to natural biodegradation which are resulting the requirements for the treatment of effluents by procedures physical or chemical methods [3]. Since these processes are methodologically demanding, time and energy consuming and cost ineffective, many procedures combining physical, physicochemical, chemical techniques with biological ones have been developed to remove reactive dyes [3–5]. Numerous reports described using of biomaterials such as bacteria, cyanobacteria, fungi, floating algae, duckweeds, submergible plants and agricultural wastes in removing reactive dyes from aqueous environment [5–8]. The review article on the methods that have been and being used recently for this purpose included adsorption onto various sorbents, chemical decomposition by oxidation, photodegradation and microbiological decoloration, employment of activated sludge, pure cultures and microbe consortiums is worth to mention here [9]. Although it is well known that common duckweed, *Lemna minor* have long been used for testing ecotoxicity in environmental hazard assessment, including studies on the reactive dye remediation, bioremoval of reactive dyes by duckweeds was not mentioned in the articles referred above. Some other microorganisms and *Lemna* spp., on the other hand, have long been used in phytoremediation of several pollutants [9–14]. In addition, there are lots of studies related to removal of dyes by algae [15,16]. The report on comparison of decolorization of polymeric dye (R-478) and Remazol Brilliant Blue R (RBBR) by 103 wood-rotting *Irpex lacteus* and *Pleurotus ostreatus* fungal strains, or article describing a combination of advanced treatment techniques for reactive dye removal from wastewater are good examples of tedious attempts to solve the problem [17]. In an investigation on Remazol Brilliant Blue R and Orange G removal by the white rot fungus *Dichomitus squalens*, *L. minor* growth inhibition bioassay was used, and the number of fronds and their weight were used as two endpoints of the bioassay [18]. Although some results presented showing comparative advantage of *L. minor* over nine other hydrophyte species in scavenging of textile dye effluents [19], *Lemna* spp. have been very rarely used in studies focussing on bioremoval of dyes. In a study on the comparison of the complementary *Lemna* duckweed and algal growth, it was found that 10 mg l^{-1} of "Brilliant Blue R spezial" inhibited average *Lemna* growth rate about 22% on white surfaces, but did not inhibit on black surfaces under the test beakers [20].

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This conclusion can also be considered as the reason of the lack of the interest in the literature of bioremoval of reactive dyes by *Lemna* spp., in spite of the vast literature on bioaccumulation of several other pollutants.

Great variability from one biomass source to another in bioremoval capabilities related with toxic ions was observed and explained in terms of the exhibited specificity of the organisms towards the subject parameters as pH, other ions, and temperature [21]. It is well known that the quantity of total biomass and its growth rate in the polluted media was one of the important parameters determining the performance in bioremoval performance [18]. It has long been known that natural hormones take a very important part in growth and development of plants [22], as of all organisms [23]. Plant hormones, growth regulators and inhibitors have been used in practice for numerous purposes [24]. New natural hormones are still being discovered, and their interrelationships, action mechanisms, relations with metabolic developments are being enlightened [25–27]. Triacontanol (TRIA) is also a relatively newly discovered plant hormone; this can exert its stimulatory effects even at its considerably low foliar concentrations applied to higher plants [28]. It is a 30-carbon primary alcohol, influencing gene regulation, membrane integrity and selective permeability, regulation of activities of several enzymes, photosynthesis, nutrient uptake, mineral nutrition, stress tolerance and consequent increase in productivity of various crops at femtomolar doses, growth responses in plants were found to have a rapidity not shown for other plant hormones or growth regulators. Isolation of TRIA-regulated photosynthetic and photorespiratory proteins and related genes in rice, the complex mechanisms involved in the regulation of photosynthesis and its stimulation, and the evaluation of the results as an indication of suppressed stresses are worth to mention here [29–34]. As it was found in higher plants, the most profound effect of TRIA on marine photosynthetic bacteria [35] was on the growth slope and rate. Similar findings were reported for two species of cyanobacteria on dry weight basis, and were also correlated with bioremoved yields of two reactive dyes by these species [36]. Considering all of these reports, it can be hypothesized that TRIA can stimulate the population growth of the vascular hydrophytic plant species *L. minor* at its lower concentrations and to test the stimulatory effects of this economically extractable hormone on the stress tolerance and reactive dye bioremoval capacity, by adding into the culture media.

The major aim of the study was to define the effect of different TRIA concentrations and dye type onto *Lemna* sp. growth, and to determine the removal rates of this duckweed at different dye concentrations and pH levels.

2. Materials and methods

2.1. Plant and growth conditions

Commercially purified from a goldfish supplier, *L. minor* L. was used to investigate the growth pattern and the changes in toxic dye removal capacity of the plant population in media containing TRIA and the results were compared with the hormone control samples. As in many reports on the same subject, the purity of the cultured material was checked by using the morphological information per Natural Resources Conservation Services, United States Dept. of Agriculture [37], taking the article describing the systematics of *Lemnaceae* as a formidable challenge to systematic investigations, as a comprehensive phylogenetic analysis of all currently recognized species of Lemnaceae had been carried out using more than 4700 characters including data from morphology and anatomy, flavonoids, allozymes, and DNA sequences from chloroplast genes and introns [38,39].

A series of batch culture experiments in unshaken flasks, illuminated by cool white fluorescent lamps were carried out at 2400 lx light intensity. The cultures were transferred into 100 ml E medium. The content of the medium is (mg l^{-1}) KH_2PO_4:680, KNO_3:1515, $Ca(NO_3)\cdot 4H_2O$:1180, $MgSO_4\cdot 7H_2O$:492, H_3BO_3:286, $MnCl_2\cdot 4H_2O$:3.62, $FeCl_3\cdot 6H_2O$:5.40, $ZnSO_4\cdot 7H_2O$:0.22, $CuSO_4\cdot 5H_2O$:0.22, EDTA:11.2 [40]. Erlenmeyer flasks (250 ml) at containing known dye concentrations were incubated at room temperature (25–30 °C) under continuous illumination for 7 d [41].

2.2. Triacontanol and dye solutions

TRIA (96% w/v; Aldrich) solution was prepared by dissolving 0.5 g of the chemical in (1 l) chloroform. 2% (w/v) and was diluted with water to the concentrations required for the experiments. Reactive Orange 14, Reactive Red 120, Reactive Black 5, Brilliant Blue R, and Remazol Brilliant Blue R dye stock solutions were prepared by dissolving the chemicals obtained from Sigma-Aldrich® in distilled water. Selected volumes of the stock dye solutions were added to E media.

2.3. Experimental methods

Uniform, healthy duckweeds with 2–3 fronds per colony were selected and washed with 0.5% NaOCI for 1 min, then with E media before placing into the series of media prepared for the experiments. Twenty of these plants were placed into E media (pH 6) to determine the effect of TRIA on growth of them. For this purpose, *L. minor* was cultivated in media containing 0; 1; 5; 10 and 20 mg l^{-1} TRIA for 7 d. At the end of the incubation period, number of fronds was counted.

To investigate the effect different dyes onto growth of *L. minor*, 50 of plants were put into media containing nearly 10 mg l^{-1} dye and incubated for 7 d. The population growth was determined by counting of the plants after the incubation. For the examination of the effect of initial pH on dye removal by the plants, pH value of E media containing 0 or 1 mg l^{-1} TRIA was adjusted to 6, 7, and 8. Then, 10 mg l^{-1} of Brilliant Blue R, in which the plants grew much more vigourously than other dyes, was added into the media to determine dye removal at various pH levels.

For the experiments on the effect of initial Brilliant Blue R concentrations on the removal rate, 50 plants were placed onto media with 1 mg l^{-1} TRIA and the controls, which contained 2.5, 4.9, 7.0, and 10.1 mg l^{-1} Brilliant Blue R. Removal rate of the dye was followed during the whole incubation period. Control flasks including only media and dye were also prepared to observe any reaction occurs between media and dye.

2.4. Analytical methods

3 ml Samples were taken daily from each flask throughout the incubation period. Growth of the plants was determined by counting the fronds [12]. The concentration of the dye in the supernatant was determined by reading the absorbance at 435 nm for Reactive Orange 14, 520 nm for Reactive Red 120, 600 nm for Reactive Black 5, 560 nm for Brilliant Blue R, and 590 nm for Remazol Brilliant Blue R, depending on the preliminary experiments performed with their suitable concentrations in E media. Cell free E medium was used as the blank and spectral absorbance measurements were done by using a Shimadzu® UV 2001 (Japan) model spectrophotometer.

2.5. Dye removal efficiency

Removal of the dyes by the duckweed was investigated as a function of initial pH and initial dye concentrations in media with TRIA

Table 1
Effect of TRIA onto growth of *Lemna minor* (initial frond number: 20; incubation period: 7 d; illumination: 2400 lx).

TRIA concentration (mg l^{-1})	Number of fronds
0	60
1	68
5	52
10	49
20	44

Table 2
Effect of different dyes onto growth of *Lemna minor* (initial frond number: 50; incubation period: 7 d; illumination: 2400 lx; TRIA concentration: 1 mg l^{-1}).

Dye	C_0 (mg l^{-1})	Growth
Reactive Orange 14	10.2	–
Reactive Red 120	10.3	+
Reactive Black 5	10.1	–
Brilliant Blue R	10.1	++
Remazol Brilliant Blue R	11.2	–

and the controls. The percentage removal of dye was calculated from Eq. (1)

$$\text{removal}\,\% = \frac{C_0 - C_f}{C_0} \times 100 \quad (1)$$

In the equation, C_0 and C_f the initial and final concentrations of the pollutant (mg l^{-1}), respectively.

2.6. Statistical analysis

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

3. Results

3.1. Effect of triacontanol on growth of L. minor

Results of the experiments, which were carried out in media with 0, 1, 5, 10, and 20 mg l^{-1} TRIA, to find the most stimulatory TRIA concentration is shown in Table 1. As seen in the table, the highest frond number was found in 1 mg l^{-1} TRIA samples. As the numbers decreased at higher hormone concentrations, further experiments were performed by using 0 and 1 mg l^{-1} TRIA samples only.

3.2. Effect of dye type on to growth of L. minor

To find the effect of different dyes on the population growth of duckweed, nearly 10 mg l^{-1} five dyes, Reactive Orange 14, Reactive Red 120, Reactive Black 5, Brilliant Blue R, and Remazol Brilliant Blue R were added into E media containing nearly 1 mg l^{-1} TRIA. As the results presented in Table 2 show, *L. minor* could not grow in the presence of Reactive Orange 14, Reactive Black 5, or Remazol Brilliant Blue R dyes, but in media with the tested concentrations of Brilliant Blue R or Reactive Red 120, plants were not affected by the toxicity of these dyes. In addition, the highest surviving rate was observed in the media containing 10.1 mg l^{-1} Brilliant Blue R. Due to these results, further experiments were carried out with media including this dye only.

Table 4
Effect of pH onto Brilliant Blue R removal by *Lemna minor* effect (initial frond number: 50; incubation period: 7 d; illumination: 2400 lx).

pH	C_0 (mg l^{-1})	*Y*%
5	11.0	0
6	10.1	27.7
7	11.8	0

3.3. Effect of initial dye concentration onto growth of L. minor

Initial dye concentrations affected growth rate of the duckweed, as shown by the data presented in Table 3. To see the effect of initial dye concentration on growth of *L. minor*, initial Brilliant Blue R concentrations added into media were increased from 2.5 to 10.1 mg l^{-1}. In all of the hormone samples, the number of fronds was higher than the hormone controls at all dye concentrations tested in the study. Number of fronds increased with the increase in dye concentrations up to 7.0 mg l^{-1}, and the maximum growth in terms of frond numbers was 112 at this dye concentration.

3.4. Effect of pH on dye removal

To find a suitable pH value for the most effective dye removal, trials were performed at three initial pH values in the media containing nearly 10 mg l^{-1} dye and 1 mg l^{-1} TRIA. The effect of initial pH on the dye removal is summarized in Table 4. As shown, *L. minor* removed the dye with the highest rate of 27.7% at pH 6. Since the plants could not grow in E media at pH 5 or 7, the subsequent experiments for effective dye removals were done at pH 6 only.

3.5. Effect of initial dye concentration on the dye removal

In control flasks (media without plant, but with dye) there was no reaction between dye and media. This result proved that, dye was removed only by the duckweed tested in the study. Dye removal rate was investigated at different initial Brilliant Blue R concentrations varied between 2.5 and 10.1 mg l^{-1} in media with 1 mg l^{-1} TRIA and the hormone controls (Fig. 1 and Fig. 2). Although the removal rates at the 7th day decreased with increasing initial dye concentrations at all tested conditions, comparatively higher rates were obtained in TRIA samples. The maximum yield obtained in the media with the 2.5 mg l^{-1} Brilliant Blue R, which was the lowest of the tested initial dye concentrations.

At the end of the incubation period, the removed dye rate was 58.0% in the hormone controls, and 59.6% in TRIA samples. At the 3rd day of the incubation period however, the difference between the hormone samples and their corresponding controls were significantly higher, which can be taken as indication of a saturation mechanism related with the bioremoving activity irrespective of the hormonal stimulation. Still, the significant difference between the 13.3% removal rates corresponds to 38.3% efficiency of hormone

Table 3
Effect of initial Brilliant Blue R concentration onto growth of *Lemna minor* (initial frond number: 50; incubation period: 7 d; illumination: 2400 lx).

Brilliant Blue R concentration (mg l^{-1})	Number of fronds		Removal %	
	Without TRIA	1 mg l^{-1} TRIA	Without TRIA	1 mg l^{-1} TRIA
2.5	86 ± 2.0	87 ± 1.5	58.0 ± 2.0	59.6 ± 0.4
4.9	106 ± 4.5	107 ± 6.5	38.3 ± 4.1	44.9 ± 3.1
7.0	98 ± 2.0	112 ± 2.0	34.6 ± 0.4	36.5 ± 2.2
10.1	98 ± 1.5	106 ± 2.0	22.8 ± 1.0	27.7 ± 1.9

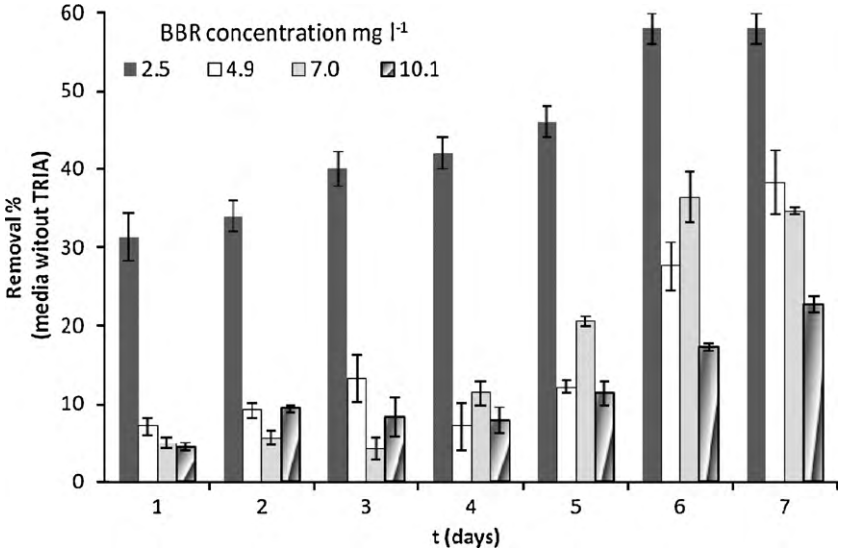

Fig. 1. Effect of initial Brilliant Blue R concentrations onto removal of Brilliant Blue R (initial frond number: 50; incubation period: 7 d; illumination: 2400 lx).

controls and the efficiency of 4.9 mg l^{-1} dye containing samples and considerably lowers than at the same dye concentration. In 4.9 mg l^{-1} dye samples, the efficiency was 38.3% in hormone controls, considerably lower than 44.9% removal by TRIA samples. But, there was not a significant difference between the highest removals by the controls and hormone samples at 7.0 mg l^{-1} initial dye containing samples. Removal rate (34.6%) in the controls was increased by the presence of the hormone only to 36.5%. However, at the highest 10.1 mg l^{-1} dye concentration tested, the difference in the measured removal efficiency at the 6th day of the incubation period was considerably higher again. The efficiency was 17.3% in the controls, and it was 27.2% in TRIA samples. Although the removal rate in hormone samples decreased approximately 50%, in comparison with the lower dye concentrations tested, the efficiency in terms of the quantity of the dye removed was still at a satisfactory level. It can be concluded at this point that, *L. minor* exerted considerably higher removal rates and yields in the presence of stimulatory TRIA concentration up to certain initial dye concentrations.

In summary, the increase of dye concentration decreased the population growth in the hormone control samples effectively and the differences in the corresponding control and hormone samples were increased by to a certain level by the stimulatory effect of TRIA on the population growth under the toxic stress caused by the dye.

4. Discussion

The hydrophytes took up large class of compounds including several derivatives of aromatic compounds such as phenols quickly, at rates faster than bacteria would degrade and sequester the contaminants at varying rates [42]. It was added that, since duckweed could not degrade the persistent organics, accumulate them in their tissues within a certain time instead. Hence, they had evolved a defence mechanism to deal with the threat. Referring to the Ph.D. work by J. Tront in the same group, it was also stated that overall uptake rate was highly dependent on plant metabolic rates. The results presented above also indicated that bioremoval rate and efficiencies of reactive dyes by *L. minor* were considerably different, and uptake rate was highly dependent on the metabolic rate. In our study, *L. minor* had a high ability to remove low concentrations of the applied dye with a rate of 59.6%, thus with an increase in Brilliant Blue R concentration the removal rate decreased (27.7%), as well.

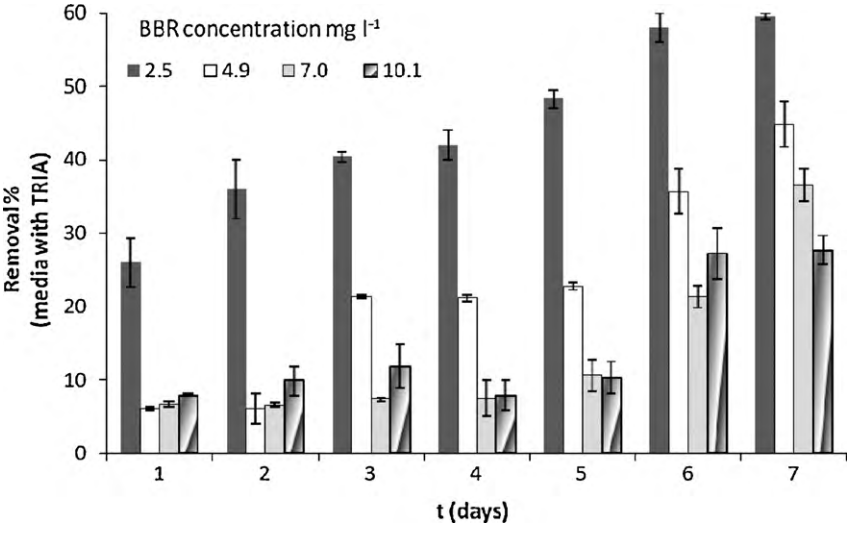

Fig. 2. Effect of initial Brilliant Blue R concentrations onto removal of Brilliant Blue R (initial frond number: 50; incubation period: 7 d; illumination: 2400 lx).

Toxic pollutant removal process by *Lemna* sp. might be included adsorption and degradation [43]. In our study, when plants were exposed to toxic dye, primarily, dye mechanically attached to the duckweed with a fast rate. After this mechanic attachment, *L. minor* might degrade the exposed dye by an enzymatic pathway. Thus, it can be observed that the dye removal started from the 1st day via mechanic attachment. Removal of the pollutant slowly got saturated in the 5th day of the incubation; however, there was a sudden increase in dye removal in the 6th and 7th day of the incubation which might be an enzymatic degradation process (Figs. 1 and 2).

In the present study, *L. minor* removed the applied dye with higher rates in media with TRIA rather than hormone control samples at all tested dye concentrations. This result might be related to the positive effect of TRIA on plant growth. On the other hand, our results showed that with an addition of just 1 mg l^{-1} TRIA into the media, frond number increased from 60 to 68. As a growth hormone, TRIA stimulates growth through higher metabolic rates of plants, algae and cyanobacteria at its certain concentrations [28–35]. This property of TRIA can be considered as the indications of the potential of TRIA to increase the performance of the common duckweed in bioremediation of numerous pollutants. It was shown that peroxidase, glutathione S-transferase and antioxidant enzyme activities in the *L. minor* were significantly higher than the controls following 1, 2 and 7 d of the exposure to hexachlorobenzene, a persistent environmental contaminant [44,45]. In another study it was also found that guaiacol peroxidase and glutathione S-transferase were involved in the xenobiotic metabolism and antioxidative system, which took part in the xenobiotic metabolism and antioxidative system responses of *L. minor* to propanil herbicide [46]. On the other hand, it was concluded that duckweed species developed mechanisms to cope with metal toxicity basing on the protective mechanism limiting the metal uptake rather than on an enhancement of the antioxidative metabolism [47]. Similar to these previous studies, in our study, with an increase in pollutant concentration from 2.5 to 4.9 mg l^{-1}, frond number increased in both hormone and control samples to cope with the stress condition.

There are sustainable, economical and practical lagoon-based technologies and floating cover systems developed for the bioremoval of various pollutants by *Lemna* spp. including common duckweed [48]. In our investigation considering easy cultivation and collection of floating hydrophytes in such systems, it can be concluded that TRIA hormone can be used as an effective and economical agent, which can be obtained from sources such as active fraction of tea waste, sugarcane press mud or alfalfa meal, for stimulation of bioremoval of some reactive dyes at least. The results presented here may lead to the exploitation of the potentials offered by duckweed and this natural hormone, also in some other treatment practices.

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