TRIACONTANOL COMPENSATES FOR CADMIUM TOXICITY EFFECTS ON GROWTH AND PHOTOSYNTHESIS

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Abstract. Efficiency of bioremediation of polluted aquatic environments with the use of microalgae can be substantially increased if tolerance of algae to stress factors is enhanced with application of very small concentrations of natural bioactive compounds. Such a substance, with a yet unknown action mechanism, but already used to improve crop quality and quantity under adverse growth conditions, is triacontanol (TRIA), a wax constituent of the plant cuticle. The present study investigates the benefic influence of 5 µM TRIA on growth and photosynthetic performance of the *Tetradesmus obliquus* green microalga (a test organism for bioindication of water quality) exposed for one week to the adverse influence of 50 µM cadmium chloride in the nutrient medium of axenic cell cultures. Results reveal that inhibitory influences of Cd on cell divisions, biomass production, photosynthetic pigment content and photochemical performance of photosynthesis are efficiently alleviated by TRIA. Thus, TRIA can be used to achieve a higher cell density and an enhanced stress tolerance of algal populations, in order to optimize biological purification of aquatic habitats polluted with Cd.

Keywords: bioactive plant metabolite; heavy metal stress; photosynthetic performance; *Tetradesmus obliquus*

INTRODUCTION

Water pollution can be reduced with environmental-friendly approaches which use different aquatic organisms for both bioindication of water quality and remediation of adversely affected aquatic environments. Planktonic microalgae combine several useful characteristics of micro-organisms and plants. They have a high rate of multiplication and a pronounced adaptability, as well as a large metabolic plasticity and a capacity to bioaccumulate, sequester and biotransform polluting agents [9, 18]. Water-soluble forms of heavy metals are frequent polluting chemicals especially in anthropically affected aquatic habitats. Some of them are essential mineral micronutrients for algae and higher plants, but others have no physiological functions and exhibit toxicity even at very small concentrations. Cadmium is such a non-essential heavy metal, which may enter in the aquatic ecosystems mainly from mining and industrial activities [14]. In algal cells the most important effects of Cd toxicity include inhibition of several enzymes, membrane transporters, transcription factors and other proteins, induction of deficiency symptoms for iron, manganese, zinc and copper due to uptake antagonism through biological membranes, disturbance of cell division, as well as secondary effects concerning oxidative and osmotic stress [4, 6, 12, 25].

Planktonic microalgae are able to accumulate significant amounts of Cd ion from the surrounding water, with a high bioconcentration factor. This depends mainly on the concentration of external heavy metal, on the exposure period and on the nutritional status of the algal populations. The bioaccumulated Cd ions are immobilized and sequestered by binding to phytochelatins (protective peptides produced in response to increased heavy metal concentrations in the cytosol) [7, 14]. Due to the abiotic stress caused by water pollution, the algal cells allocate less energy for growth and multiplication, more resources being fueled in the synthesis of protective metabolites and in other antistress reactions which ensure the survival under adverse conditions. This is the reason why the cell density of algal populations declines and biomass production decreases under the influence of water pollution, the most sensitive individuals are eliminated and the others get hardened through specific metabolic modulation processes which lead to increased stress tolerance. Small concentrations of several bioactive compounds may stimulate this process of hardening under stress conditions, by increasing vitality and protective capacity, or by inducing a faster or more intense defense during the so-called priming process [26, 29]. Some of these bioactive compounds, which contribute to a higher biomass production, to a more efficient anti-stress protection and to an enhanced remediation capacity, are natural or synthetic growth regulators, while others are elicitors or signal-transducers which may be detected by plant cells and induce specific metabolic and developmental changes [1, 10, 22]. Triacontanol (TRIA) is a natural bioactive compounds with several stimulating effects on plant growth and on the production of specific secondary metabolites [20]. It is a wax constituent of the plant cuticle, and when it penetrates into plant cells it triggers several metabolic modifications related to defense and increased stress tolerance. At present, its action mechanism is unknown, but it is used as a foliar spray to improve crop production under unfavorable growth conditions [8, 21, 27]. In a study conducted in 2020, Islam et al. concluded that TRIA interacts with phytohormones in counteracting stress-induced physio-biochemical damages in plants [11]. Concerning interaction of TRIA with heavy metal toxicity, Karam et al. established that in canola plantlets foliar application of 20 µM TRIA reduced the oxidative stress caused by treatment for 7 days with 1.5 mM Cd [13]. In contrast with higher plants, the influence of TRIA on algal stress tolerance is largely unknown.

The aim of the present work is to reveal beneficial physiological influences of triacontanol on the alleviation of Cd toxicity in a green microalgal species

which is largely used in bioindication and remediation of water pollution, as well as in the production of biodiesel. The specific objectives are to reveal the influence of TRIA on adverse effects of Cd on algal cell division rate, on dry biomass production, on light-harvesting pigment content, and on the efficiency of photosynthetic use of light energy. The main hypothesis is that in analogy with its stimulative effects on metabolic processes of medicinal and crop plants, TRIA intensifies defensive processes of algal cells, which lead to a more successful protection against Cd toxicity. A better knowledge of the interactions of TRIA with Cd toxicity in algae would enable a more efficient biological purification of aquatic environments polluted with heavy metals.

MATERIAL AND METHODS

The biological material used in the experiments consisted of axenic monoalgal cultures of *Tetradesmus obliquus* (Turpin) Wynn [31], originating from the algal culture collection of the University of Las Palmas de Gran Canaria (Spanish Bank of Algae), grown in Bold's basal nutrient medium [3], in an incubation-shaking algal growth chamber (Certomat BS-1, Sartorius, Germany), at a constant temperature of 22 °C. Illumination was provided for 14 hours per day by red and blue LEDs (SolarStorm 110 VegMaster, California Lightworks, USA) which ensured a photosynthetically active photon flux density of 360 µM photons m^{-2} s^{-1}, the horizontal shaker of the above-mentioned growth chamber being set to 90 rpm. Similar amounts (10 mL) of the same initial algal culture were inoculated under sterile conditions in Erlenmeyer flasks containing 200 mL sterile nutrient media. The control cultures were grown in the unmodified Bold's medium, while in the other experimental variants 5 µM triacontanol (TRIA), 50 µM $CdCl_2$, or a combination of 50 µM $CdCl_2$ and 5 µM TRIA were provided in Bold's nutrient solution (from stock solutions of 5 mM TRIA and 50 mM $CdCl_2$). The concentrations were chosen after preliminary experiments with different series of TRIA and Cd quantities. The treatments were applied for seven days in statical algal cell cultures.

The dynamics of the cell density in the algal cultures (reflecting the difference between cell divisions and cell death) was determined cytometrically with Bürker's cytometer, under a light microscope. At the end of experiments, 100 mL of each algal culture were filtered and dry biomass was determined by dehydrating the fresh algal biomass in an oven at 80 °C for three days (until the dry weight remained constant) [23].

Photosynthetic pigments of the algal cells were extracted with N,N-dimethylformamide after centrifugation of homogenized cultures for 10 min at 3000g. From the extracts, chlorophyll *a*, chlorophyll *b* and total carotenoid pigment contents were determined spectrophotometrically according to [30] and expressed on a fresh weight basis.

Parameters of the induced chlorophyll fluorescence were determined *in situ* in homogenized algal cell suspensions brought to similar cell densities, using a photosynthetic efficiency analyzer (FMS-2, Hansa-tech, United Kingdom). In algal cultures dark-adapted for 15 min the ground fluorescence (Fo) and the maximal fluorescence (Fm) were measured, and the potential quantum yield of photosystem II (PSII) was calculated as the Fv/Fm ratio, where Fv is the difference between Fm and Fo [17]. In algal cultures exposed to a constant background illumination (the so-called actinic light), the pulse amplification modulated parameters of chlorophyll fluorescence were measured, such as the steady state level of fluorescence (Fs) and the modulated maximal fluorescence (Fm'). With their use, the effective quantum yield of PSII: ΦPSII = (Fm' – Fs) / Fm', and the non-photochemical quenching of the singlet excited state of chlorophyll *a*: NPQ = (Fm – Fm') / Fm' were determined, immediately before the determination of final dry biomass and of photosynthetic pigment content of the same algal cultures [15, 16].

Every experimental variant was set in five repetitions, while measurements with every sample were performed in triplicate, at the end of the seven days treatment period. Statistical analysis of the experimental data was performed with the RStudio 1.3 "car" and "ggplot2" statistical packages. The Shapiro-Wilk test was used for analysis of normality of data distribution, while the homogenity of variances was determined with Bartlett's test. All the data followed the normal distribution and were homogenous. Significance of differences was determined with the one-way ANOVA, followed by the post-hoc Tukey HSD test. Differences were considered significant at $p < 0.05$.

RESULTS

The final cell density of seven days old algal cultures was moderately, but significantly decreased by the presence of 5 µM TRIA in the culture medium (from 1258 ± 51 x 10^6 cells mL^{-1} to 995 ± 80 x 10^6 cells mL^{-1}). Water pollution with 50 µM $CdCl_2$ resulted in a very low cell density of algal populations upon exposure to the heavy metal for seven days (236 ± 32 x 10^6 cells mL^{-1}). But when the same concentration of Cd ions was supplied to the nutrient medium together with 5 µM of TRIA, the reduction in cell density was less pronounced (621 ± 56 x 10^6 cells mL^{-1}) than in the presence of Cd without TRIA. If Cd alone decreased cell density by more than four times as compared to the control, in the presence of TRIA the adverse effect of Cd on cell divisions resulted in a reduction by only two times of the final cell density (Fig. 1a).

The final dry biomass of seven days old algal cultures was not influenced significantly by TRIA (94 ± 7 mg L^{-1}, as compared to 81 ± 3 mg L^{-1} in the

control), but was reduced by more than two times by Cd (40 ± 8 mg L^{-1}). This effect of the heavy metal on biomass accumulation was completely reversed by TRIA, as the biomass of algal cultures treated simultaneously with Cd and TRIA was similar with the one registered in the control cultures (81 ± 5 mg L^{-1}, Fig. 1b).

Concerning the photosynthetic pigment content of algal cells in the experimental variants, one may observe that the amount of chlorophyll *a* (as well as the quantity of chlorophyll *b*, data not shown) was not significantly modified by TRIA (2980 ± 533 µg g^{-1} instead of 2829 ± 201 µg g^{-1} in the control), but was extremely reduced by the exposure of algal cultures for seven days to 50 µM of water-soluble Cd ions (237 ± 41 µg g^{-1}). When the same concentration of Cd was combined with 5 µM TRIA, the decreasing effect of Cd was significantly less pronounced, even though the chlorophyll *a* content remained well below the one measured in the control cultures (983 ± 317 µg g^{-1}, Fig. 2a). Because Cd toxicity decreased with different degrees the amount of chlorophylls and of carotenoid pigments in the algal cells, the ratio between total chlorophylls and total carotenoids exhibited a more moderate decrement that the chlorophyll *a* content (0.764 ± 0.210, as compared to 1.635 ± 0.296 in the control). TRIA also moderated this effect of Cd on the pigment ratio (1.451 ± 0.090), without exerting any significant influence on this biochemical stress marker in the absence of Cd (1.940 ± 0.242, Fig. 2b).

From among the several conventional and pulse amplification modulatied parameters of the induced chlorophyll fluorescence, related to the functioning of photosystem II (PSII) in the harvesting and the photochemical conversion of light energy, the effective quantum yield of PSII and the non-photochemical quenching of the singlet excited state of chlorophyll *a* were selected for presentation and interpretation. The effective quantum yield of PSII (ΦPSII) was around 0.7 in the control cultures (0.752 ± 0.012), it was not influenced by the presence of 5 µM TRIA (0.729 ± 0.011), but it was reduced to values around 0.5 (0.548 ± 0.053) in the cultures exposed for seven days to 50 µM $CdCl_2$. The decreasing effect of Cd was completely annihilated by the presence of TRIA (0.736 ± 0.022) in the algal cultures treated with the heavy metal (Fig. 3a). The non-photochemical quenching of the singlet excited state of chlorophyll *a* (NPQ) was significantly increased (with more than 100%, from 0.652 ± 0.046 in the control to 1.552 ± 0.242) by the exposure of algal cells to Cd toxicity. This increment was moderated by the simultaneous presence of TRIA (0.825 ± 0.021), which by itself, in the absence of Cd, did not cause any significant change of this parameter (0.657 ± 0.113) in comparison with the control cultures (Fig. 3b).

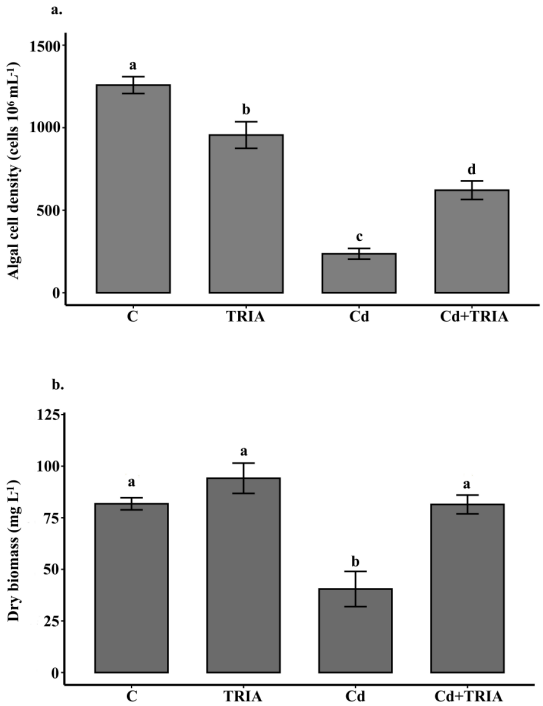

Figure 1. Cell density (a.) and dry biomass (b.) of *Tetradesmus obliquus* cultures treated for seven days with 5 µM triacontanol (TRIA), 50 µM cadmium chloride (Cd) or a combination of 50 µM cadmium chloride and 5 µM triacontanol (Cd + TRIA). C – control. Vertical bars represent ±SE from means (n = 5), different letters represent significant differences at $p<0.05$

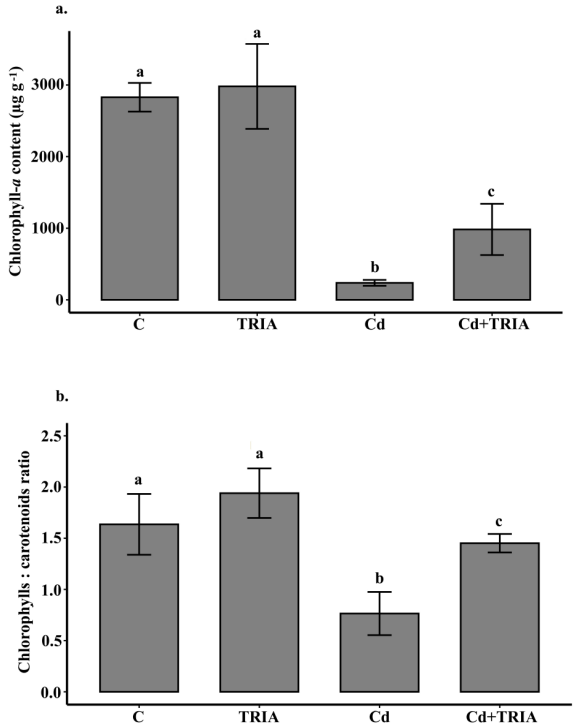

Figure 2. Chlorophyll *a* content (a.) and chlorophylls to carotenoids ratio (b.) of *Tetradesmus obliquus* cells treated for seven days with 5 µM triacontanol (TRIA), 50 µM cadmium chloride (Cd) or a combination of 50 µM cadmium chloride and 5 µM triacontanol (Cd + TRIA). C – control. Vertical bars represent ±SE from means (n = 5), different letters represent significant differences at $p<0.05$

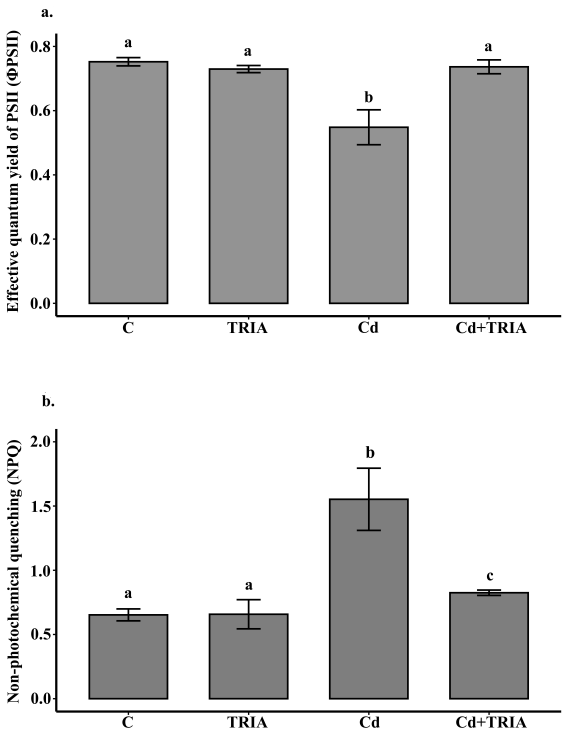

Figure 3. Effective quantum yield of photosystem II (a.) and non-photochemical quenching of the singlet excited state of chlorophyll *a* (b.) in *Tetradesmus obliquus* cells treated for seven days with 5 µM triacontanol (TRIA), 50 µM cadmium chloride (Cd) or a combination of 50 µM cadmium chloride and 5 µM triacontanol (Cd + TRIA). C – control. Vertical bars represent ±SE from means (n = 5), different letters represent significant differences at $p<0.05$

DISCUSSION

TRIA decreased the cell density of algal cultures upon exposure for seven days, most probably because it disturbed processes involved in cell division. No such effect of this plant-derived bioactive molecule was reported previously, and the uptake of this lipophylic substance by plant cells, as well as its action mechanisms on molecular level remain unknown at present. Reduction of cell density due to inhibition of cell divisions by cadmium was demonstrated for several plants. When cell cultures of the green alga *Chlamydomonas reinhardtii* were treated for several days with 5 µM and 100 µM Cd, a severe inhibition of cell divisions was observed after the second day of exposure [12]. The fact that the presence of 5 µM TRIA significantly reduced the negative effects of 50 µM Cd on the cell density of algal populations suggests that, beside its individual disturbing influence on cell divisions, it had a more pronounced antagonistic interaction with Cd toxicity, attenuating the negative effect of this heavy metal on the dynamics of algal cell density. In contrast with its decreasing influence on cell density, TRIA had no negative effect on growth of the algal biomass by the end of the seven days treatment period. An enhancement of algal biomass production by TRIA was reported by Tastan et al. for the green microalga *Chlorella vulgaris* [28]. For some higher plant species (energy willow, several medicinal herbs and some vegetables) it was demonstrated that low concentrations of TRIA may even stimulate biomass production, especially under non-optimal growth conditions [1, 8, 10]. E.g. in seedlings of *Erythrina variegata* exposed to 10 µM, 100 µM and 1 mM Cd, exogenously applied TRIA stimulated root and shoot growth [19]. Ali et al. have demonstrated in 2020 that foliar spraying with 1 µM TRIA reduced As toxicity effects on growth of wheat seedlings [2]. The fact that TRIA lowered cell density but did not impair biomass production of algal cultures suggests that it had a stimulative influence on metabolic processes which resulted in an enhanced cell growth, without stimulating cell divisions. Thus, the algal cells became bigger, but their number remained smaller. If more organic nutrients are accumulated, the chances for survival under environmental stress conditions may increase [21]. The fact that upon several days of exposure to millimolar concentrations of water-soluble Cd salts reduced biomass production was demonstrated for several crop plants, and even for green and red algae [6, 15, 25]. In the presence of TRIA, Cd could not exert its negative influence on algal biomass growth, thus interaction of these two substances in the metabolic processes resulted in a sustained primary production in Cd-polluted aquatic environments. Based on these results, micromolar amounts of TRIA may ensure a higher algal biomass for a more efficient bioremediation of waters polluted with Cd.

Photosynthetic pigments are responsible for the harvesting, unidirectional transmission and photochemical conversion of light energy. Primary photochemical reactions can be performed only by some chlorophyll *a* molecules which constitute the reaction centres of photosystems. Other chlorophyll *a* molecules, together with chlorophyll *b* and carotenoid pigments (carotenes and xanthophylls) are organized in light-harvesting complexes within the thylakoid membranes of the chloroplasts. Carotenoids also have an important photoprotective function, being able to dissipate excess light energy as infrared radiation, and to annihilate the harmful singlet oxygen generated by photooxidative processes. From a hydrobiological point of view, the chlorophyll *a* content of water samples is a largely used parameter for estimation of primary production of phytoplankton. In the present experiments, the fact that Cd reduced drastically the chlorophyll *a* content of the algal cells is due to its inhibitory effect on specific steps of the chlorophyll biosynthesis pathway, and also to stimulation by Cd of processes leading to chlorophyll degradation, as the structure of thylakoid membranes is also affected [4, 29]. Similar decreasing effect of Cd on chlorophyll content was also reported for higher plants and for other algal species [1, 6, 7]. E.g. in the red alga *Gracilaria domingensis* a significant reduction of chlorophyll *a* content was registered only at Cd concentrations which reached 300 µM [25], reflecting that the treshold of Cd concentration which causes

lowered chlorophyll contents depends highly on species characteristics, as well as on exposure time and growth conditions. In the present experiments TRIA did not cause significant changes in the chlorophyll content of algal cells, although previous reports evidenced an increased chlorophyll quantity in leaves of higher plants treated with this bioactive compound [1]. Even if it did not modify the chlorophyll content of algal cells, TRIA had a significant ameliorative influence on the decrement of chlorophyll content caused by Cd toxicity. This alleviative effect is probably due to its interaction with metabolic processes involved in chlorophyll synthesis and disturbed by Cd. Because chlorophylls and carotenoids are synthesized in different metabolic pathways, Cd acts differently on the production of the two types of photosynthetic pigments. This is why after seven days of exposure 50 µM Cd decreased more moderately the amount of carotenoids than the quantity of chlorophylls, which resulted in a reduction to the chlorophylls to carotenoids ratio. By exerting a partial compensatory influence on the inhibition of photosynthetic pigment synthesis by Cd, TRIA reduced the difference between the value of the above-mentioned pigment ratio registered in the Cd-treated and in the control algal cultures. Because it did not influence directly the metabolism of photosynthetic pigments, TRIA did not modify the chlorophylls to carotenoids ratio under normal developmental conditions. Changes in the ratio between the two types of photosynthetic pigments were found to be early and sensitive biochemical markers of several environmental stresses, including certain heavy metals [9, 18, 23]. A positive compensatory influence on carotenoid pigment content, similar to TRIA, was reported for maize leaves, when Cd treatment (5 µM and 50 µM) was combined with 50 mM silicon used as a priming agent [29].

Induced chlorophyll fluorescence analysis has become a very useful technique to investigate photosynthetic performance of photoautotrophic organisms under various stress conditions. One of the most useful parameters is the effective quantum yield of photosystem II, which reflects the proportion of the light energy absorbed by chlorophylls associated with PSII that is used in photochemical processes of the light reactions of photosynthesis [17]. TRIA does not interfere with these processes, but it annihilates the decreasing effect of Cd on light use efficiency, suggesting the protective role of this bioactive compound against functional disturbances caused by Cd in PSII. Similar negative influences of Cd on photochemical processes were also reported for crop plant species, while in algae a decreased effective quantum yiels of PSII was also caused by water pollution with different other heavy metals [7, 9, 16]. Another useful parameter of the induced chlorophyll fluorescence is the non-photochemical quenching of the single excited state of chlorophyll *a* (NPQ), which was proved to efficiently protect plants from photooxidative damage, by dissipating the excess absorbed light energy as heat. NPQ increases when stress conditions impair the use of light energy for carbon dioxide assimilation. Under these conditions, protective mechanisms tend to convert the unused light energy into harmless infrared radiations, instead of being used for overproduction of harmful reactive oxigen species which may lead to a generalized photooxidative damage [24]. This is the reason why algae exposed to Cd toxicity exhibited a significantly increased value of the non-photochemical chlorophyll fluorescence quenching. Because TRIA exerted a protective role for the photosynthetic apparatus against this Cd toxicity, in the algal cultures treated simultaneously with Cd and TRIA, NPQ was much smaller that in the cultures exposed only to Cd, being almost as low as in control and as in the cultures provided with TRIA but not treated with Cd. Similar results with moderation by protective agents of the increased NPQ were reported for other heavy metals which may be present in industrially polluted aquatic environments [9, 22, 26]. Aziz et al. found that application of 50 µM and 100 µM of TRIA did not alleviate effects of salt stress (150 mM NaCl) on NPQ in sunflower leaves, but stimulated root and shoot growth under elevated soil salinity [5].

In conclusion, 5 µM triacontanol dissolved in the aquatic medium significantly enhances the tolerance of *Tetradesmus obliquus* to water pollution caused by 50 µM $CdCl_2$. Cell divisions are severely inhibited by Cd, and the simultaneous presence of TRIA compensates only partially for this effect. When biomass production is reduced by Cd, this reduction is completely annihilated by TRIA, thus a higher algal mass (with less, but bigger cells) will be able to bioaccumulate and to sequester Cd from the polluted water. Protective mechanisms against the photooxidative damage intensified by Cd, as revealed by changes in non-photochemical quenching of chlorophyll fluorescence, are more efficient in the presence of TRIA, revealing its contribution to an enhanced stress tolerance of the photosynthetic apparatus. Enhancement of algal tolerance to water-polluting heavy metals by provision with small amounts of TRIA may be an efficient and cost-effective procedure to optimize biological purification of polluted aquatic environments. More investigations have to be performed to elucidate the action mechanism of TRIA in the plant metabolism and its interrelation with environmental stress factors.

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