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Effect of lycorine on the green algae *Chlamydomonas reinhardtii* under UV-C irradiation

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Abstract: Lycorin (LYC) is an inhibitor of the growth in higher plants through the inhibition of the last step of ascorbic acid biosynthesis. Ascorbates are mainly involved in regulation of intracellular levels reactive oxygen species. There is still limit information about its green microalgal toxicity and specificity. Therefore, present experiment was focused on the LYC toxicity on the green microalgae, *Chlamydomonas reinhardtii,* moreover the cells of microalgae were exposed to UV-C irradiation to increase ROS. The attention was primary given to the antioxidant response. The higher concentrations than 25 µM LYC with 30 min UV-C (250 nm) exposure absolutely inhibited the growth. 10 µM LYC and 25 µM LYC treatment together with UV-C irradiation suppress the growth caused oxidation stress and enhances antioxidant response.

Key Words: Chlamydomonas reinhardtii, UV irradiation, lycorine

INTRODUCTION

Lycorine (LYC) is Amaryllidaceae alkaloid which suppress the growth in the higher plants (Deleo et al. 1973). It is considered to inhibit the last step in ascorbic acid (AA) biosynthesis (Onofri et al. 2003). AA is important plants and algal substance. Among it's the most important roles are antioxidant substance, enzyme cofactor and a precursor for oxalate and tartrate synthesis. Later, it was discovered that it also participates in growth and resistance to environmental stress. AA is pivotal antioxidant in scavenging reactive oxygen species (ROS), its synthesis increases during oxidative stress. (Lin et al. 2016; Smirnoff and Wheeler 2000). LYC use in microalgae research has been poorly studied. Some studies using LYC reported that AA participates in cooper stress tolerance in *Ulva compressa* or cadmium in *Scenedesmus quadricauda* (Kovacik et al. 2017; Mellado et al. 2012). However, the results of LYC treatments are controversial, some investigation showed only its toxic effect (Loewus 2000).

Algae are photosynthetic organisms. Apart from photosynthetically active radiation (400–700 nm), algae are also exposed to UV light (Tao et al. 2010). Because UV-A and UV-B are passed though atmosphere, their toxicity is widely observed in the different living organisms (Holzinger and Lutz 2006, Tao et al. 2010). UV-C is electromagnetic radiation that includes wavelengths shorter than 280 nm. It has the highest energy from UV radiation. It is efficiently filtered by the ozone layer in the stratosphere. Therefore, its toxicity insufficiently studied (Muller-Xing et al. 2014). Owing to the ozone depletion, there is a risk of UV-C radiation penetration on Earth. Accordingly, it is important to understand how plants respond to UV-C in terms of stress resistance, growth and development. The protective mechanisms of plants to UV-C stress could include the formation of antioxidant compounds (Castronuovo et al. 2014).

Because there is limit information related to LYC and UV-C irradiation toxicity, green algae, *Chlamydomonas reinhardtii,* was exposed to LYC treatment and 10 or 30 min UV-C (250 nm) irradiation. UV is generally to be toxic because it triggers oxidative stress and enhance the accumulation of ROS (Castronuovo et al. 2014, Holzinger and Lutz 2006, Tao et al. 2010). Therefore, the attention of present experiment was given to growth and antioxidant response.

MATERIAL AND METHODS

Cultivation of algae

LYC and UV irradiation toxicity was tested by cultivating *Chlamydomonas reinhardtii* in the presence of 10, 25, 50 and 100 µM LYC and 10 and 30 min/day UV-C exposure. *Chlamydomonas reinhardtii* was cultivated under sterile conditions in Tris-Acetate-Phosphate medium (TAP medium) at 21 ± 1 °C and illuminated at 130 µmol/m/s with a 12 h light/12 h dark photoperiod. Then, algae (700 mg of fresh biomass) were inoculated into the Erlenmayer flasks containing 150 ml of liquid TAP medium supplemented with the corresponding LYC concentration. Thereafter, the selected samples were exposed to UV-C (250 nm) for 10 and 30 min per day. After 5 days, *Chlamydomonas reinhardtii* were harvested and lyophilized.

Optical density

Optical density was measured every day. 200 µl of fresh sample was added into the wells of UV transparent spectrophotometric desk and left to stand for 20 min at room temperature. Thereafter, the absorbance of sample was measured at 750 nm against a blank. The result was expressed as optical density value (OD750); (Chioccioli et al. 2014).

Growth rate

Biomass increase was evaluated in zero and harvesting day. The samples were rinsed 3 times to remove residual medium. Thereafter, samples were lyophilized and weighed. Growth rate was calculated as $\mu = \ln (x1/x0)/t1\text{-}t0$ where x1 and x0 are weight of algae (g/L) and the culture time t1 and t0 days (Krzeminska et al. 2014).

Extraction methodology

Three replicates were performed for each sample. The weighing of lyophilized sample was homogenized by the homogenizer in 1 ml of extraction solvents which were 80% methanol, 96% ethanol and acetone. Incubation was followed for 2 hours at 55 °C (Hynstova et al. 2018).

Estimation of total antioxidant capacity

The total antioxidant capacity of extracts was investigated by phosphomolybdenum assay (Alam et al. 2013). Trolox was used as the standard.

Determination of polyphenols

The total phenolic content was detected by FC assay (Folin-Ciocalteu assay), based on the reduction of a phosphowolframate – phosphomolybdate complex by phenolic compounds (Kosar et al. 2005). Gallic acid was used as the standard. The result was expressed as an equivalent in mg gallic acid on 1 g dry weight.

Determination of flavonoids

The flavonoids were determined by colorimetric method (Jia et al. 1999). Rutin was used as the standard. The result was expressed as an equivalent in mg rutin on 1 g dry weight.

DPPH assay

α, α-diphenyl-β-picrylhydrazyl (DPPH) free radical scavenging assay was used to determinate antioxidant activity (Brandwilliams et al. 1995). The result was expressed as a value EC50 in mg/ml. EC50 is extract's concentrations to obtain 50% antioxidant effect.

Determination of chlorophylls (a and b), total carotenoids and pheophytins

Spectrophotometric determination of chlorophylls (a and b) and total carotenoids were measured at 665, 649 and 479 nm. Weighing of lyophilized sample was extracted in 96% ethanol. Extract was pipetted into the holes of UV transparent spectrophotometric plate. Thereafter, the chlorophyll a (Ca), chlorophyll *b* (Cb), and total carotenoids (Cx + c) were calculated as: $C_a = 13.95 * A665 – 6.88 * A649$ (µg/ml), $C_b = 24.96 * A649 – 7.32 * A665$ (µg/ml), $C_{x+c} = (1000 * A479 – 2.05 * C_a – 114.8 * C_b) / 245$ (µg/ml); (Hynstova et al. 2018).

Statistical analysis

Each sample had 3 biological and 2 technical repetitions. All data was expressed as a mean of standard deviation. The data were determined by one-way ANOVA variance test followed by T-test at $p < 0.05$.

RESULTS AND DISCUSSION

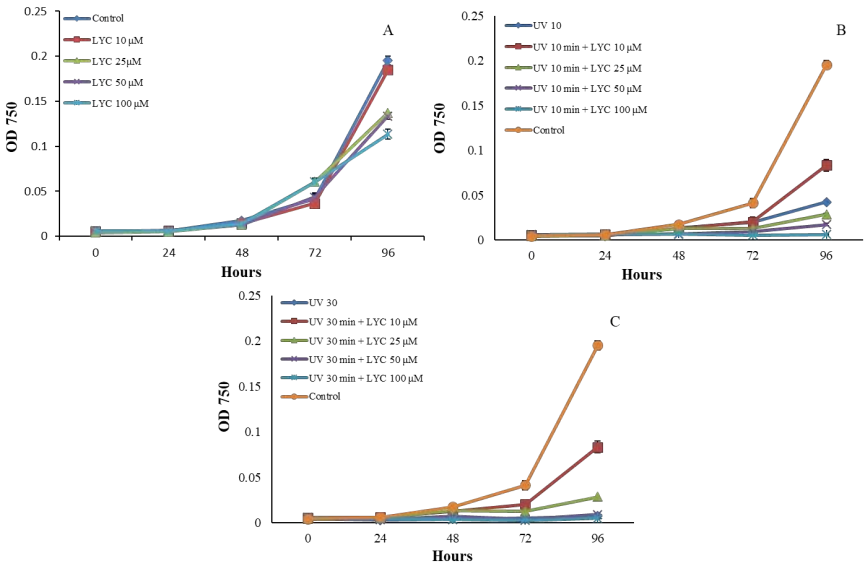
Effect of toxicant on the growth

Preliminary screening for potential concentrations was done by growth study based on daily spectrophotometric reading of absorbance at 750 nm. Different concentrations of LYC (10 µM, 25µM, 50 µM and 100 µM) and time of UV-C irradiation (10 and 30 min) were used for pre-experiment.

Growth was inhibited by an increasing concentration of the toxicant. 50 and 100 µM LYC inhibited the growth. But their combination with UV-C irradiation was too toxic for *C. reinhardtii* (Figure 1A). Other treatment also suppressed the growth compared to control, combination of LYC and UV had synergistic effect (Figure 1B, C). Therefor, 10 µM LYC, 25 µM LYC, 10 min of UV-C irradiation, 30 of UV-C irradiation and their different combination were chosen for sequential analysis. There was no change between control and 10 µM LYC.

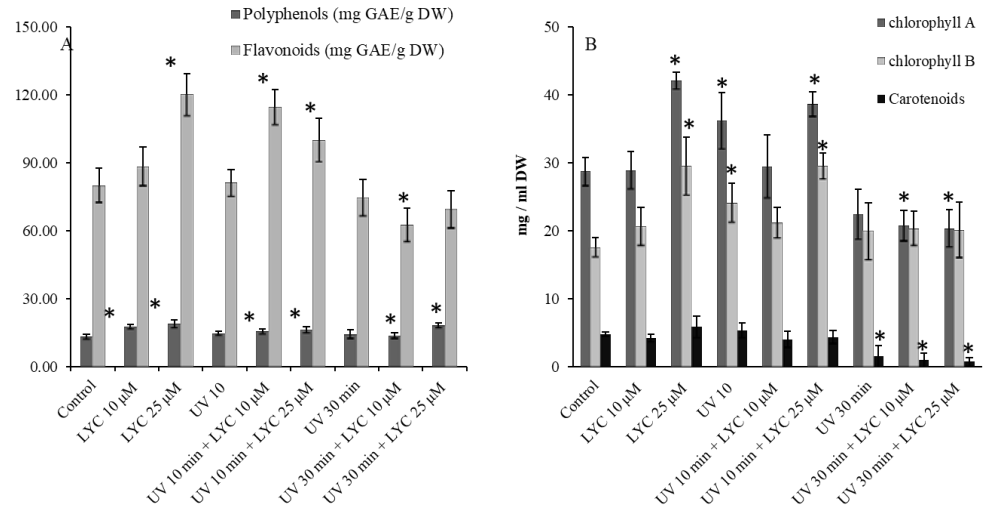
Previous study reported that 100 µM decreased the growth and at very low concentration 10^{-6} suppressed the cell division in higher plant, red algae and yeast (Arrigoni et al. 1975). Beyond, *Ulva compressa* was treated up to 100 µM concentrations, despite there was no mention about growth inhibition (Mellado et al. 2012). Most of yeasts strains haven't showed sensitivity to LYC (Onofri et al. 2003). Different organisms, even within order, are differently sensitive to LYC (Arrigoni et al. 1975, Onofri et al. 2003).

Figure 1 Influence of Lycorine treatments and UV-C irradiation on the growth of Chlamydomonas reinhardtii. Error bars correspond to standard error of mean. A, B, C) Optical density in value OD 750 during 96 hours.



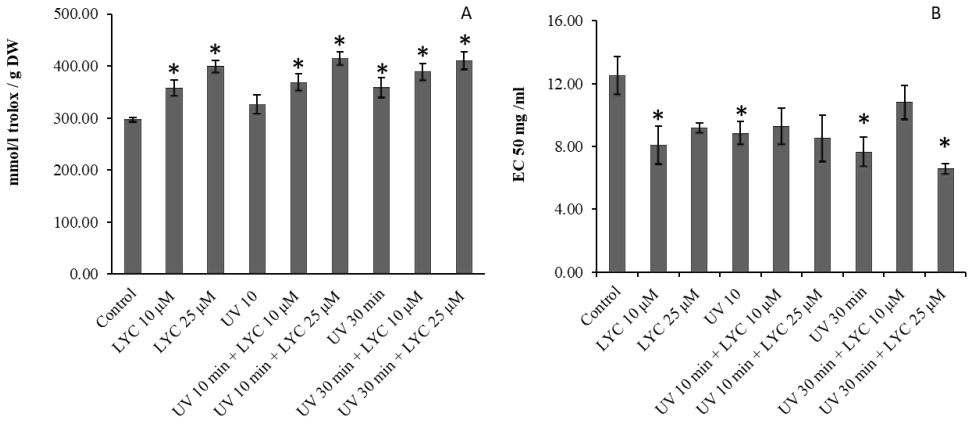
Polyphenols and flavonoids are secondary metabolites with antioxidant properties, their syntheses often increase during stress. Due to the structure, flavonoids are consider for protective compounds during UV irradiation (Holzinger and Lutz 2006). In our case, significant increase of flavonoids was observed only in 10 µM LYC, 25µM LYC and 25µM LYC + 30 min UV irradiation. No shift was monitored in UV-C exposure (Figure 2). Carotenoids can be overproduction in response to light stress in *Duneliella salina* (Fu et al. 2013). In our experiment, there was no enhancement. Reduced chlorophyll and carotenoids level was also observed in *Bellerochea yucatanensis, Biddulphia sinensis, Ditylum brightwellii, Lauderia annulata* and *Thalassiosira rotula* under UV-B irradiation (Dohler 1985).

Figure 2 A) Polyphenols contain expressed as mg GAE g DW, Flavonoids contain expressed as mg RE g DW (dry weight). B) Amount of chlorophyll a, chlorophyll b and carotenoids expressed as mg/ml. Error bars correspond to standard error of mean. (*) indicate significant differences compared to the control ($p < 0.05$, $n = 6$) according to T-test.



Antioxidant testing was completed by total antioxidant capacity and DPPH assay. Total antioxidant capacity was significant increased in all treatment except 10 µM LYC (Figure 3 A). DPPH assay was expressed as value EC 50. It means what concentration you need to reduce 50 % of DPPH solution. The control samples achieved the highest values. All treatments samples responded to stress increased antioxidant response (Figure 3).

Figure 3 A) Total antioxidant capacity expressed as mmol/l Trolox g DW (dry weight). B) DDPH assay expressed as a value EC 50. Error bars correspond to standard error of mean. (*) indicate significant differences compared to the control ($p < 0.05$, $n = 6$) according to T-test.



CONCLUSION

Strategies for UV experiment and substance toxicity are complicated. Their toxicity depends on many circumstances as a concentration, time of exposure, wavelength, distance from radiation etc. In our conditions, 30 min UV-C (250 nm) irradiation exposure with higher than 25 µM LYC treatment were highly toxic and absolutely inhibited the growth. All treatment, apart from 10 µM LYC, had negative impact on the growth and redox state. They suppressed the growth caused oxidation stress and enhanced antioxidant response. Next step is going to target on molecular level of toxicity focused mainly on enzymatic and non-enzymatic antioxidant substances. Moreover, it could be interesting to find optimal doses and use hormesis to increase beneficial compounds.

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