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Detection of Reactive Oxygen Species (ROS) in Cyanobacteria Using the Oxidant-sensing Probe 2',7'-Dichlorodihydrofluorescein Diacetate (DCFH-DA)

Rajneesh, Jainendra Pathak, Ananya Chatterjee, Shailendra P. Singh and Rajeshwar P. Sinha*

Laboratory of Photobiology and Molecular Microbiology, Centre of Advanced Study in Botany, Institute of Science, Banaras Hindu University, Varanasi, India
*For correspondence: r.p.sinha@gmx.net or rpsinhabhu@gmail.com

[Abstract] Reactive oxygen species (ROS) are cell signaling molecules synthesized inside the cells as a response to routine metabolic processes. In stress conditions such as ultraviolet radiation (UVR), ROS concentration increases several folds in the cells that become toxic for the cell survival. Here we present the method for *in vivo* detection of ROS by using an oxidant-sensing probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in cyanobacteria. This method provides reliable, simple, rapid and cost effective means for detection of ROS in cyanobacteria.

Keywords: Reactive oxygen species, 2',7'-Dichlorodihydrofluorescein diacetate, Cyanobacteria, Ultraviolet radiation, Oxidative damage

[Background] Cyanobacteria are the most ancient oxygenic photoautotrophs; they play an important role in the biomass production in both aquatic and terrestrial ecosystems and serve as source of various value-added products (Vaishampayan *et al.*, 2001; Häder *et al.*, 2007; Fischer, 2008). In recent years the depletion of the ozone layer has resulted in an increase in solar ultraviolet radiation (UVR) influx, which is harmful to all organisms residing on Earth including cyanobacteria (Holzinger and Lutz, 2006). The UVR harms cyanobacteria directly by acting on DNA/proteins or indirectly through oxidative damage from reactive oxygen species (ROS) (He and Häder, 2002). In plants, algal and mammalian cells various fluorescence and chemiluminescence methods have been used for detecting ROS (Crow, 1997; He and Häder, 2002; Soh, 2006; Wu *et al.*, 2007; Palomero *et al.*, 2008).

2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) is a non-fluorescent, cell-permeable dye which is hydrolyzed intracellularly into its polar, but non-fluorescent form DCFH on the action of cellular esterases and thus is retained in the cell. Oxidation of DCFH by the action of intracellular ROS and other peroxides turns the molecule into its highly fluorescent form 2',7'-dichlorofluorescein (DCF) that can be detected by various fluorescent methods (He and Häder, 2002; Rastogi *et al.*, 2010; Singh *et al.*, 2014) (Figure 1). Although DCFH-DA is widely used for the detection of ROS, it should be noted, however, that the dye cannot be used as an indicator for a specific form of ROS (Marchesi *et al.*, 1999).

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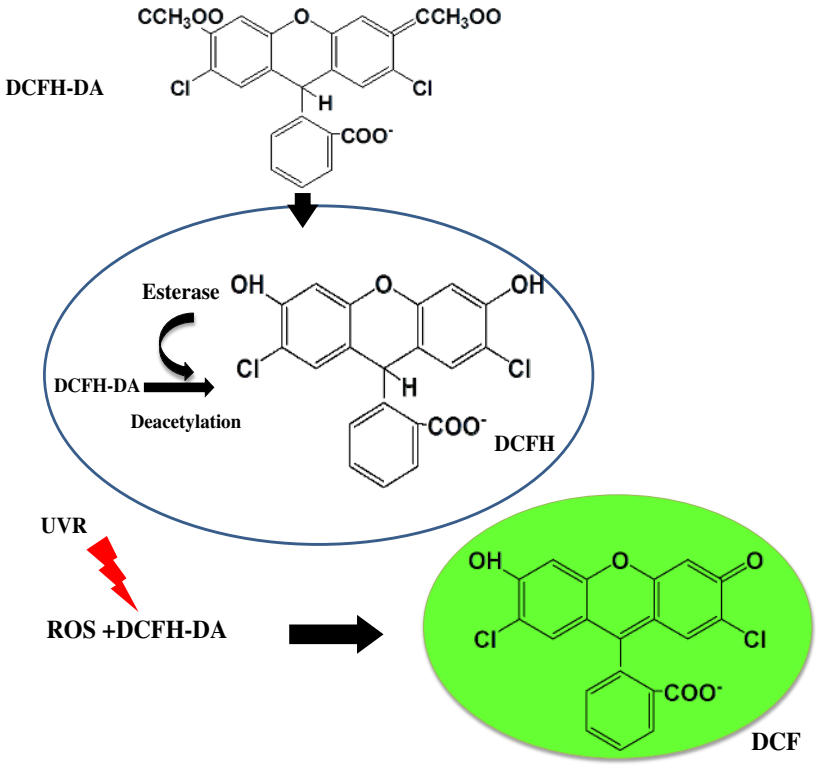

Figure 1. Mechanism of action of DCFH-DA probe inside the cell (Adapted from He and Häder, 2002)

Materials and Reagents

1. 2 ml RNase, DNase free microcentrifuge tube (Thermo Fisher Scientific, Invitrogen™, catalog number: AM12425)
2. Glass microscope slides (Fisher Scientific, catalog number: 12-544-4)
3. Glass microscope coverslips (Fisher Scientific, catalog number: S17525B)
4. Millipore membrane filter (EMD Millipore, catalog number: HAWP04700)
5. Cuvette (fluorescence spectroscopy) 3 ml (Hellma, catalog number: 101-QS)
6. Cyanobacterial cells *e.g.*, *Nostoc* sp. strain HKAR-2
   Note: Nostoc sp. strain HKAR-2, an autotrophic, filamentous and heterocystous cyanobacterium, was grown under axenic conditions in nitrogen-free liquid BGA medium (Safferman and Morris, 1964) at 20 ± 2 °C under continuous white light (12 ± 2 Wm^{-2}) to an OD_{750} of 0.8 to 0.9 (exponential growth phase) which was measured using quartz cuvette in a spectrophotometer.
7. Nail varnish (Lakme)
8. Potassium phosphate dibasic anhydrous (K_2HPO_4)
9. Potassium phosphate monobasic (KH_2PO_4)
10. 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, catalog number: D6883)
11. 100% ethanol (Sigma-Aldrich, catalog number: 459836)

12. 50 mM phosphate buffer (see Recipes)
13. 2 mM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) stock solution (see Recipes)

Equipment

1. Glass Petri-dishes (Corning, catalog number: 3160-102)
2. Measuring cylinder
3. 295 nm UV cut-off filter (Ultraphan, Digefra, Munich, Germany) to facilitate the desired wavebands of UV-B (280-315 nm), UV-A (315-400 nm) and PAR (400-700 nm)
4. UV-treatment chamber fitted with UV-B (Philips Ultraviolet-B TL 40 W: 12, Philips Lighting, model: TL 40W/12 RS SLV/25), UV-A (Philips Ultraviolet-A TL 40 W: 12, Philips Lighting, model: TL-K 40W/10-R UV-A) and PAR (55.08 ± 9.18 µmol m^{-2} sec^{-1}) (OSRAM L 36 W: 32 Lumilux de luxe warm white and Radium NL 36 W: 26 Universal white, Germany) lamps
5. Glass rod (Fisher Scientific, catalog number: 11-380A)
6. Magnetic stirrer (REMI ELECTROTECHNIK, model: 2 MLH)
7. Refrigerated centrifuge (REMI ELECTROTECHNIK, model: CM-12 PLUS)
8. Shaker
9. Fluorescence microscope (Nikon eclipse Ni fluorescence microscope processed by NIS Elements (BR))
10. Fluorescence spectrophotometer (Agilent Technologies, model: Cary Eclipse)
11. Spectrophotometer (Hitachi High-Technologies, model: U-2900, Double beam spectrophotometer)
12. Quartz cuvette (3.5 ml) (Cole-Parmer, JENWAY, catalog number: 035 028)

Software

1. NIS-Elements (BR) imaging software (Nikon)
2. SigmaPlot 11 software

Procedure

A flow chart of the sample preparation is shown in Figure 2.

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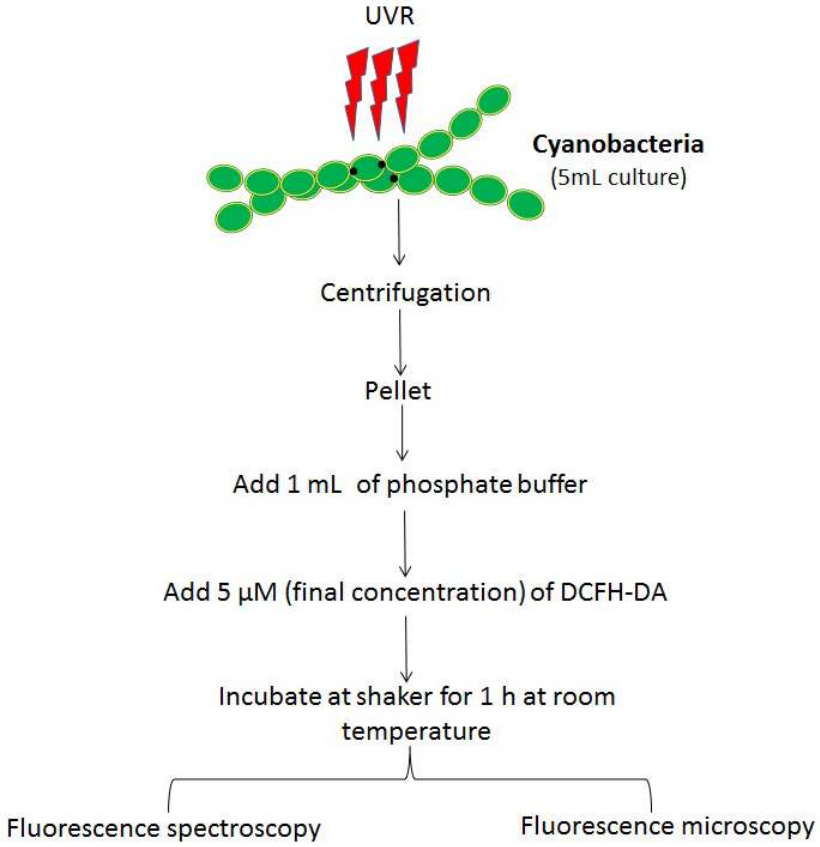

Figure 2. Flow chart showing various steps involved in the protocol

1. Transfer 100 ml of cyanobacterial culture to sterile glass Petri-dishes with the help of a measuring cylinder. Cover the Petri dishes with a 295 nm UV cut-off filter and transfer them to a UV light treatment chamber.
2. Irradiate the cyanobacteria with UV-A, UV-B and PAR and maintain the temperature of the chamber at 25 ± 2 °C to avoid a heating effect. Mix the culture with a glass rod at regular intervals or use magnetic stirrer (15-20 rpm) to avoid self-shading of the cells.
3. After desired time intervals (hereafter 12 h and 24 h), take 5 ml of sample and harvest cells by centrifugation at 9,050 *x g* for 20 min at room temperature.
4. Resuspend the cell pellet in 1 ml phosphate buffer (see Recipes).
5. Add 2.5 µl of 2 mM 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) solubilized in ethanol (see Recipes) to the sample mixture.
6. Incubate the sample mixture on a shaker (15 rpm) at room temperature in the dark for 1 h.
7. After 1 h incubation
    a. For fluorescence microscopy: Take a clean glass slide and add 30 µl of culture. Cover the cells with a glass cover slip. Seal the slide with nail varnish to avoid drying out. Cells were visualized under a fluorescence microscope using an excitation wavelength of 488 nm and emission was detected in the range of 500-600 nm (Figure 3).

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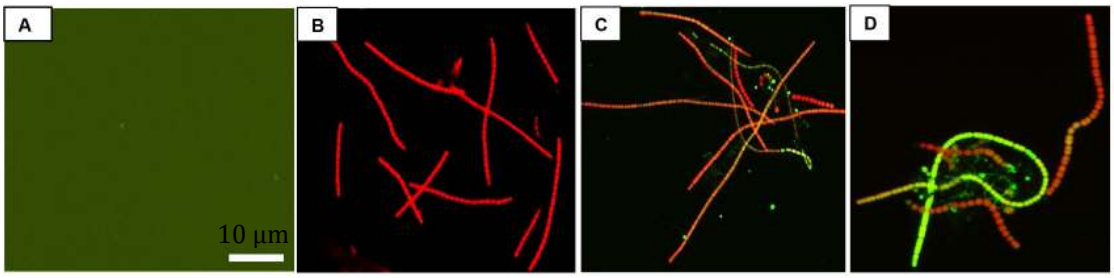

Figure 3. Fluorescence images of UV-A + UV-B + PAR exposed *Nostoc* sp. strain HKAR-2 showing green DCF fluorescence after reaction with ROS. Negative control (containing DCFH-DA only; showing the basal level of fluorescence) (A); fluorescence at 0 h (B); 12 h UV-A + UV-B + PAR exposure (C); 24 h UV-A + UV-B + PAR exposure (D). PAR: Photosynthetically active radiation. Scale bar =10 µm.

b. For fluorescence spectroscopy: 3 ml of liquid sample was added to a cuvette for fluorescence spectrophotometric analysis (Figure 4). The cuvette was placed in fluorescence spectrophotometer and the sample was excited at 485 nm. Emission was recorded in the range of 500-600 nm. The exposure time was limited to 600 msec to reduce the damage of cells. Fluorescence was measured in terms of emitted fluorescence intensity after different durations of stress exposure.

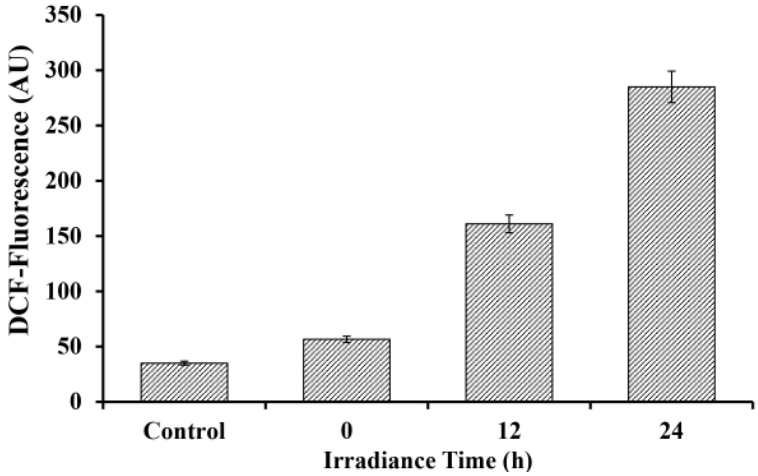

Figure 4. Fluorescence intensity of DCF after reaction with ROS generated due to varying duration of UV-A + UV-B + PAR exposure in *Nostoc* sp. strain HKAR-2 (means ± SD, n = 3). Control: Untreated sample. PAR: Photosynthetically active radiation.

Data analysis

The UVR irradiated cells were analyzed using a Nikon Eclipse Ni fluorescence microscope processed by NIS-Elements (BR) imaging software. The microscope was equipped with the following filter set: UV: (DAPI) EX 340 nm EM 488 nm, blue: (FITC) EX 495 nm EM 510 nm and

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green: (PI 550) EX 550 nm EM 650 nm. Cells were imaged in the epifluorescence mode with a 20x objective lens. The image analysis was performed by NIS-Elements (BR) imaging software provided by Nikon and images were saved in JPEG format. In addition, the fluorescence of the samples was measured by a fluorescence spectrophotometer (Cary Eclipse, Agilent Technologies) with an excitation wavelength of 485 nm and an emission band between 500 and 600 nm. The data of the fluorescence spectra were exported to excel and the fluorescence intensity values at 525 nm were extracted. A bar diagram was plotted with SigmaPlot 11 software. All fluorescence measurements were performed at room temperature. All results are presented as mean values of three replicates for fluorescence spectrophotometer analysis and random sites of filaments were used for fluorescence microscopy. All data were analysed by one-way analysis of variance (Brown, 2005). Once a significant difference was detected post hoc multiple comparisons were made by using the Tukey test. The level of significance was set at 0.05 for all tests. All statistical analyses were performed by using SigmaPlot 11 software.

Notes

1. Direct exposure of light to UV treated samples should be avoided.
2. The stock solution of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) once prepared, was kept in -20 °C for further use and direct exposure to light should be avoided (it remains stable for more than 3 months).
3. All solutions were filtered through 0.25 µm size Millipore membrane filter before use.

Recipes

1. 50 mM phosphate buffer (400 ml)
   8.7 g K_2HPO_4
   6.8 g KH_2PO_4
   Adjust the pH to 7.00
   Filter through 0.25 µm size Millipore membrane filter before use
2. 2 mM (w/v) 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) stock solution
   Dissolve 0.974 mg of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) in 1 ml of absolute ethanol

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