

Role of Photosynthetically Active Radiation and Dark Conditions on the Repair of Ultraviolet-B Radiation-induced Damages in the Cyanobacterium *Nostoc* sp. Strain HKAR-2

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Abstract

Introduction: We have investigated the effects of ultraviolet-B (UV-B; 280-315 nm) radiation on Chl *a*, phycocyanin, allophycocyanin, photosynthetic efficiency and reactive oxygen species (ROS) generation in *Nostoc* sp. strain HKAR-2 and the role of photosynthetically active radiation (PAR) and dark on the repair processes.

Methods: Samples were exposed to UV-B for 14h followed by PAR and dark treatment for 10h. Chl *a*, phycocyanin and allophycocyanin concentration and photosynthetic efficiency i.e., the photosynthetic quantum yield of PS II (Fv/Fm) were estimated. *In vivo* detection of ROS by fluorescence microscopy and spectroscopy was done using the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH).

Results: Progressive decrease of 50% and 41% in survival rates of cyanobacterium was observed after 12 and 14h of exposure respectively and a significant increase in survival was observed during the recovery process in PAR and dark. Chl *a*, phycocyanin and allophycocyanin concentration decreased after 12h of UV-B exposure. Significant recovery of phycocyanin and allophycocyanin was observed in the samples placed on PAR. Fv/Fm decreased with time and 42% decrease was observed after 14h of exposure. Pronounced fluorescence intensity, due to produced ROS was observed after 14h of UV-B exposure. The significant decrease in DCF fluorescence was observed in the samples placed in PAR and dark.

Conclusions: UV-B exposed cyanobacteria kept on PAR showed speedy recovery in comparison to those kept in dark. This indicates the possible role of repair processes such as photoreactivation in the repair of damage caused by UV-B to cyanobacterium. Hence, further studies are needed for the thorough understanding of repair processes in cyanobacteria in response to UVR.

INTRODUCTION

Cyanobacteria are cosmopolitan prokaryotes that can survive in almost every habitat, hence are the most dominant group of photoautotrophic, photolysis mediated oxygen evolving, Gram-negative microorganisms. Cyanobacteria possess an ability to fix nitrogen and therefore play important role in maintaining the fertility of rice fields [1] and are the most important biomass producers [2]. Depletion in the ozone layer has resulted in an increased influx of deleterious ultraviolet

radiation (UVR; 280-400nm) on the Earth's surface. UVR is absorbed by biomolecules such as proteins and nucleic acids which ultimately results in lethal effects on all organisms ranging from prokaryotes to mammals [3, 4]. In their natural habitats, cyanobacteria are exposed to harmful doses of UV-A (315-400nm) and UV-B (280-315nm) radiation while harvesting solar radiation for photosynthesis [5]. The high energetic UV-B directly affects DNA and proteins and indi-

rectly affects the cells *via* the production of reactive oxygen species (ROS) [6]. Lipids, proteins, and DNA are the main targets for the potentially toxic ROS and it also damages the photosynthetic apparatus resulting in inhibition of photosynthesis [7]. UV-B causes chemical modification in DNA and formation of dimers result in a change in its molecular structure. UV-A radiation is not directly absorbed by the DNA, but still can induce DNA damage by producing a secondary photoreaction of existing DNA photoproducts or *via* indirect photosensitizing reactions [8]. Cell differentiation, morphology, growth, survival, N_2 metabolism, pigmentation, orientation and motility, protein profile, phycobiliprotein composition, DNA and $^{14}CO_2$ uptake are affected by UVR [9, 10]. Cyanobacteria have developed various defense mechanisms such as avoidance, scavenging, synthesis of screening compounds, repair and programmed cell death against damaging effects of UVR [5]. Certain UV-absorbing pigments such as scytonemin and mycosporine-like amino acids (MAAs) are produced by various organisms including cyanobacteria as the first line of defense [11]. Enzymes such as catalase (CAT), superoxide dismutase (SOD), peroxidase (POD), and scavengers such as vitamin B, C, and E, glutathione, and cysteine play a crucial role in defense against UVR [12]. Photoreactivation, mismatch repair (MMR), excision repair and certain other mechanisms like SOS (save our soul) response, damage tolerance (dimer bypass), checkpoint activation and programmed cell death (PCD) or apoptosis efficiently remove DNA lesions, hence ensuring the genomic integrity [13]. Photoreactivation is a repair mechanism that involves photolyase enzyme and repair occurs in presence of light [14, 15]. These repair mechanisms are found in both prokaryotes as well as eukaryotes. Considering this information, we attempted to study the damage caused by continuous UV-B exposure to cyanobacteria in terms of pigmentation, proteins content, photosynthetic efficiency and ROS generation and comparative account of repair processes taking place in cyanobacteria after keeping it for recovery under PAR and dark.

METHODS

Test Organism

Nostoc sp. strain HKAR-2 (Fig. 1), characterized by Rastogi et al. [16], and was taken for the present study. Cultures were grown axenically in the BG-11 medium [17] without nitrogen source in a culture room at 28 ± 2 °C and illuminated with fluorescent light of 10 ± 2 Wm^{-2} .

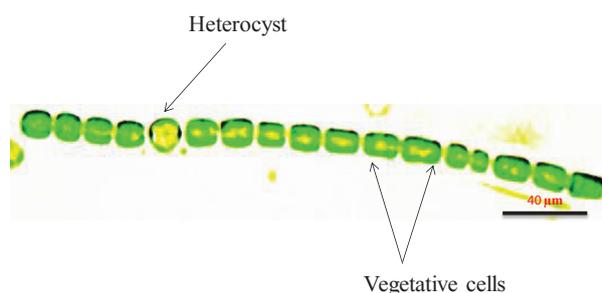


Figure 1: A Filament of the Cyanobacterium *Nostoc* sp. Strain HKAR-2 Containing Vegetative Cells and Heterocyst. Scale bar = 40 μm .

Source and Mode of UV -B Irradiation

The cut-off filter foil of 295 nm (Ultraplan, Digepra, and Munich, Germany) was used to obtain the desired radiation regimes of UV-B. Cultures were irradiated with UV-B in a specially fabricated chamber fitted with UV-B lamps (Philips Ultraviolet-B TL 40 W: 12, Holland) whose distance to the sample was adjusted to have a UV intensity of ~ 0.6 Wm^{-2} .

Percent Survival

The effect of UV-B radiation on the survival of *Nostoc* sp. strain HKAR-2 was determined by measuring the percent survival after exposing the cultures to UV-B radiation for 2, 4, 6, 8, 10, 12, and 14h. After 14h sample was divided into two parts and one set was kept in PAR and another set was kept in dark for recovery for 10h. Untreated samples served as control. Aliquots (100 μL) were withdrawn at desired time intervals after UV-B exposure and plated on agar plates. Plates were kept in the dark for 48h and thereafter transferred to light in the culture room. Colonies appearing after 15 days of growth were counted in a colony counter and percent survival was calculated.

Estimation of Chlorophyll *a* (Chl *a*)

Chl *a* was estimated by the method following Dere et al. [18]. Cyanobacterial sample (5 mL) was centrifuged at 5,000 rpm for 10 min. Pellet was dissolved in 2 mL of methanol (100%) in a test tube. It was incubated for 24h at 4 °C and then centrifuged at 10,000 rpm for 5 min. Spectrum was taken at 250-700 nm against methanol as a blank using spectrophotometer.

Estimation of Phycocyanin

A known volume of culture suspension was taken and centrifuged at 10,000 x g for 10 min to obtain the pellet. Phycobilins were extracted from pellet using 0.05 M phosphate buffer by repeated freezing and thawing followed by sonication. The absorbance was taken at 562, 615 and 652 nm against phosphate buffer as blank. Estimation of phycocyanin was done as per the method of Bennett and Bogorad [19].

Determination of Photosynthetic Activity

Determination of Photosynthetic Activity of PSII (F_v/F_m)

The treated cyanobacterial samples of *Nostoc* sp. strain HKAR-2 were dark-adapted for 30 min to allow complete oxidation of PSII reaction centers and the minimum (F_0) and maximum (F_m) fluorescent yields of PSII in the dark-adapted state were determined. The yields of both F_0 and F_m were used to calculate the photosynthetic activity of PSII (F_v/F_m) by the formula described by Chen et al. [20]. The value of F_v/F_m was determined by using PAM fluorometer (PAM-2500, Heinz Walz GmbH, 2008, Effeltrich, Germany).

Detection of ROS Production using 2', 7'-Dichlorodihydrofluorescein Diacetate (DCFH-DA)

To detect the production of ROS after exposure of UV-B radiation, 5 μM (final concentration) of DCFH-DA solubilized in

ethanol was added to the cultures and incubated the samples on a shaker at room temperature in the dark for 1h. UV irradiation rapidly auto-oxidizes DCFH-DA or hydrolyzes DCFH [7, 21]] hence, DCFH-DA is added only after irradiation. After 1h of incubation samples were subjected to fluorescence microscopy and fluorescence spectrophotometric analysis [7].

Statistical Analysis

Results were statistically analyzed by one-way analysis of variance (ANOVA) and values shown are mean of three replicates.

RESULTS

In the present study, no significant decrease in survival of the cyanobacterium *Nostoc* sp. strain HKAR-2 was observed up to 6h of UV-B exposure. However, a progressive decrease upto 50 and 41% in survival rates was observed after 12 and 14h of UV-B exposure respectively. The significant increase in survival of the cyanobacterium was observed during the recovery process in PAR as well in the dark, but recovery was more in the samples which were placed in PAR (Fig. 2).

Effects on pigmentation and biliproteins were determined by estimating Chl *a*, phycocyanin and allophycocyanin concentrations. It was observed that Chl *a* concentration decreased continuously with increasing duration of UV-B exposure and the maximum decrease of approximately 52 and 50% was observed after 12 and 14h of UV-B exposure respectively. It was observed that Chl *a* concentration was maintained upto 8h of UV-B exposure. Thereafter decrease of approximately 52 and 50% was observed after 12 and 14h of UV-B exposure respectively. Significant recovery was observed in both samples i.e. samples kept in PAR and dark (Fig. 3a). However, in case of phycocyanin and allophycocyanin pronounced decrease of 42 and 54% was observed after 14h of UV-B exposure respectively. Samples placed in PAR showed recovery of phycocyanin to some extent while samples kept in dark showed no recovery at all (Fig. 3 b). While in case of allophycocyanin significant recovery was observed in both PAR as well as in dark conditions (Fig. 3c).

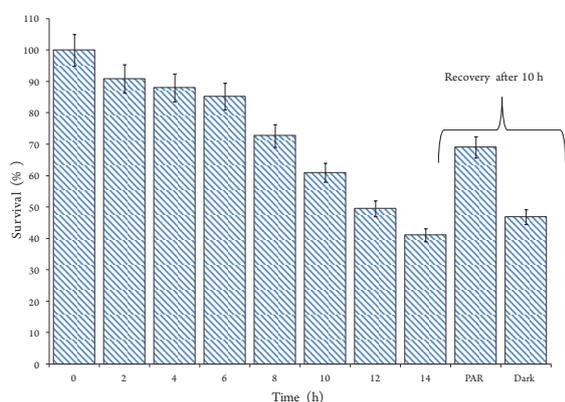


Figure 2: Effects of UV-B Radiation and Recovery of Damage in PAR and Dark on Percent Survival of *Nostoc* sp. Strain HKAR-2. Results are expressed as Means of Three Replicates. Vertical Bars indicate the Standard Deviation of the Means.

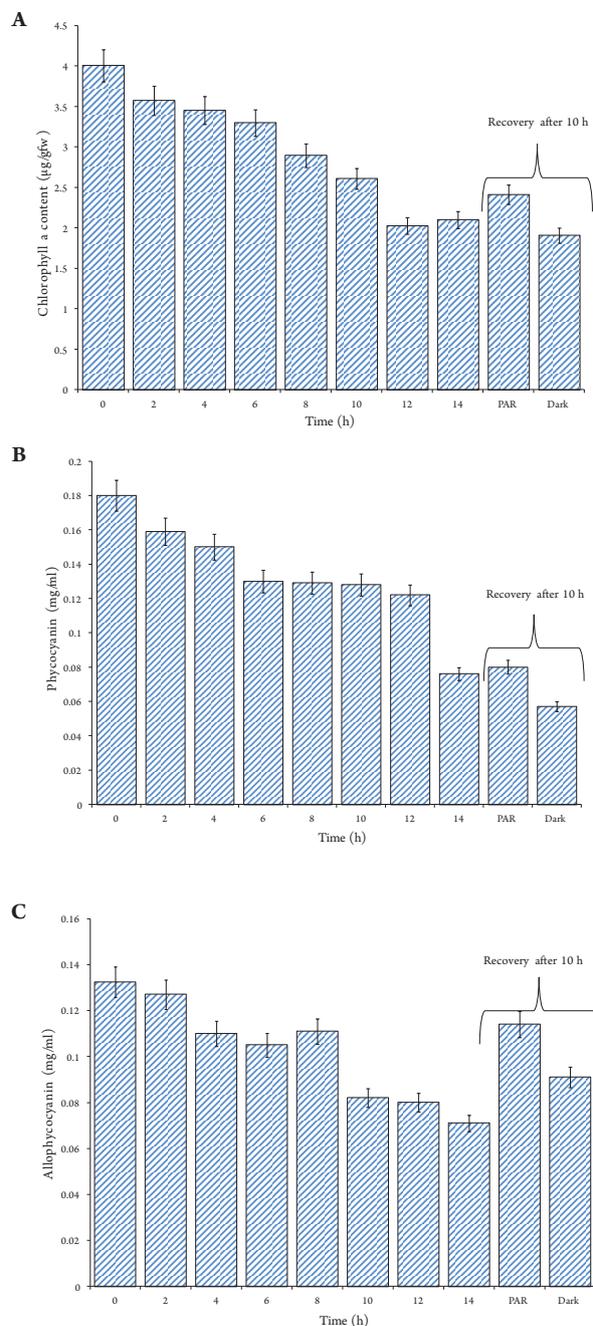


Figure 3: Effect of UV-B Radiation and Recovery of Damage in PAR and Dark on Chl *a* (a), Phycocyanin (b) and Allophycocyanin (c) Content of *Nostoc* sp. Strain HKAR-2. Results are expressed as Means of Three Replicates. Vertical Bars Indicate the Standard Deviation of the Means.

The photosynthetic efficiency of the cyanobacterium was measured by the photosynthetic quantum yield of PS II (F/F_m) under UV-B exposure and it was found that as the exposure of UV-B increases, photosynthetic efficiency decreases. After 14h of continuous UV-B exposure, 50% decrease in photosynthetic efficiency was observed (Fig. 4). After 14h of UV-B exposure cyanobacterial samples were placed in PAR and dark for recovery for 10h and speedy recovery was observed in the cyanobacterial samples which were kept under PAR in comparison to those which were kept in dark.

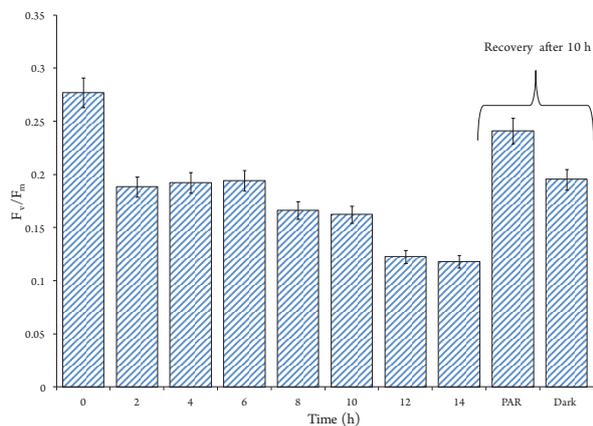


Figure 4: Variance of Fv/Fm in Nostoc sp. Strain HKAR-2 in Response to 0.6 W/m² UV-B Exposure and after Recovery in PAR and Dark. Results are expressed as Means of Three Replicates. Vertical Bars indicate the Standard Deviation of the Means.

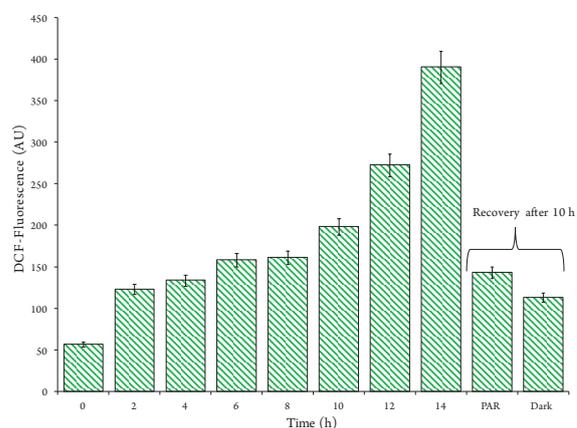


Figure 5: Emission Spectra of DCF Fluorescence intensities from Nostoc sp. Strain HKAR-2 Cells at 0 to 14 h of UV-B Exposure as measured by a Spectrofluorophotometer followed by Recovery in PAR and Dark for 10h. Results are expressed as Means of Three Replicates. Vertical Bars indicate the Standard Deviation of the Means.

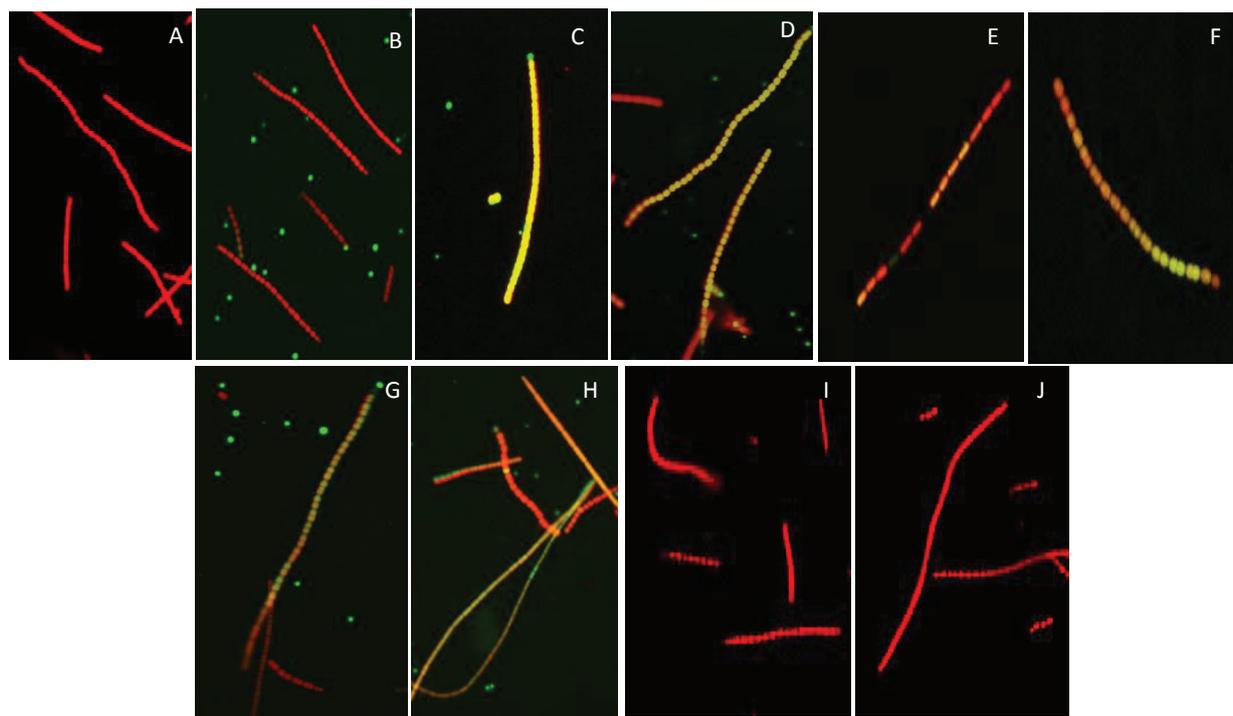


Figure 6: Fluorescence Images of UV-B exposed Nostoc sp. Strain HKAR-2 showing Intracellular Green DCF Fluorescence produced at Different Time Intervals as a Result of ROS Production (a) Control, (b) 2h, (c) 4h, (d) 6h, (e) 8h, (f) 10h, (g) 12h, (h) 14h, (i) after Recovery in PAR for 10h, (j) after Recovery in Dark for 10h.

DCFH-DA exhibited widespread background green fluorescence due to auto-oxidation and noticeable increasing fluorescence in the ROS generating cells. As the duration of UV-B increased, ROS also increased in same proportion, which is very well evident in Fig. 5 and Fig. 6. The most pronounced fluorescence intensity was observed after 14h UV-B irradiated cyanobacterial samples (Fig. 5), indicating a significant increase in ROS level under UV-B stress (Fig. 6). A

significant decrease in DCF fluorescence was observed after keeping the samples under PAR and dark.

DISCUSSION

Cyanobacteria are sensitive to UV-B radiation and increased levels of UV-B flux might lead to the death of these microorganisms. Damage/inactivation of certain biochem-

ical and physiological processes might lead to inhibition of growth or absolute loss of cell's viability [5]. Usually, cyanobacteria succeed in mitigating the damage and survive under moderate UV-B radiation in natural conditions due to the active role of photoreactivation and other defense mechanisms [22]. These results suggest that UV-B affects the electron transport system *via* the quenching analysis of tested cyanobacteria. The photoreduction of protochlorophyllide to chlorophyllide under UV-B stress attributing to the decrease in Chl *a* content has been reported earlier [23]. UV-B radiation induced bleaching of photosynthetic pigments particularly the accessory light harvesting complex phycobiliproteins has been reported by several workers [8, 23]. Strong inhibition of phycocyanin by UV-B radiation has also been reported and it seems that proteinaceous pigments rich in aromatic amino acids are the primary target of UV-B radiation [23]. The damage of PSII proteins such as D1 may be a possible explanation for the damage by UV exposure in test organism *Nostoc* sp. strain HKAR-2. The decrease in optimum photosynthetic yields (Fv/Fm) under UV radiation has also been seen in several other cyanobacterial species [24]. DCFH is basically nonfluorescent but turned into highly fluorescent form, when oxidized to DCF by intracellular ROS such as peroxide (O₂²⁻), superoxide anion radical (O²⁻), hydroxyl radicals (OH[·]), hydrogen peroxide (H₂O₂), hypochlorous acid (HOCl), peroxy (RO²⁻), alkoxyl (RO[·]), hydroperoxyl (H²), singlet oxygen (¹O²) and peroxynitrite (ONOO⁻) [25]. ROS generated in response to UV-B, has been reported to affect the nucleic acids, lipids, as well as the photosynthetic machinery, ultimately resulting in the death of the organisms [7]. In the present study, ROS was generated in response to UV-B radiation as indicated by high fluorescence DCF. The fluorescence at 0h (control) occurs due to ROS which is generated during normal metabolism in photosynthetic organisms [26]. The photosynthetic electron transport system in an oxygen atmosphere serves as the major source of ROS. The over-reduction of electron transport chain in chloroplasts that results in non-photoelectron leakage of oxygen by the photosystem (PS) machinery leads to the formation of ROS in the light [25]. A ROS-induced oxidative modification of the D1 as well D2 proteins of PS-II was observed in the cyanobacterium *Synechocystis* 6803 by Lupínková and Komenda [27]. Although UV-B radiation is detrimental to all organisms, it is more detrimental to photosynthetic organisms such as cyanobacteria. Photoreactivation utilizes the enzyme photolyases is thought to be major DNA repair machinery in several higher plants such as rice, wheat, maize and *Arabidopsis* [28, 29]. Photolyases bind to Pyr <> Pyr, in absence of photoreactivating light and stimulate the NER system *in vitro* or *in vivo* [14]. Similar findings were also reported in *Microcoleus vaginatus* and *Phormidium tenue* [30]. These results suggest that the light is essential than dark for repairing damages caused by UVR and presence of light repair in cyanobacteria increase their survival ability in regions exposed to higher doses of UVR irradiance.

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CONFLICTS OF INTERESTS

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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AUTHORS' CONTRIBUTIONS

R.P. Sinha, Rajneesh and J. Pathak designed the experiments. Rajneesh and D. Srivastava performed the experiments. H. Ahmed and V. Singh analyzed the data. J. Pathak and Rajneesh wrote the paper and S.P. Singh and R.P. Sinha edited the manuscript.

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