

Influence of glyphosate on photosynthetic properties of wild type and mutant strains of cyanobacterium *Anabaena doliolum*

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Photosynthetic electron transport (Hill activity) and O₂ evolution in both wild type and mutant cells of *Anabaena* were initially stimulated by glyphosate (50–200 µg/ml), but exhibited an inhibition of activity at extremely high glyphosate concentrations (200–400 µg/ml). Results on chlorophyll *a* fluorescence in both the strains exhibited quenching of fluorescence emission at lower doses (20–100 µg/ml) and an increase in fluorescence emission at higher concentrations of glyphosate (100–200 µg/ml). Further results obtained with metabolic inhibitors showed that glyphosate-induced quenching of fluorescence is reversed by the protonophore-CCCP and vanadate—an inhibitor of ATPase. These results suggested an important role of proton gradient across the membrane in mediating the glyphosate toxicity. Results on the phycobilisome (PBsome) fluorescence emission in the presence of high concentration of glyphosate exhibited an increase in the emission intensity together with a blue shift in the emission band at 644 nm, which is indicative of glyphosate-induced loss of energy transmission ability of the PBsomes.

LIGHT-harvesting efficiency and photosynthetic electron transport are the two important processes in photosynthetic organisms which are susceptible to herbicide stress^{1,2}. Glyphosate (*N*-phosphonomethyl glycine) is widely used as a post-emergent, broad-spectrum herbicide and its primary site of action in most of the non-photosynthetic prokaryotes is found to be 5-enol pyruvyl-shikimic acid-3 phosphate (EPSP) synthase enzyme^{3,4}.

Photosynthetic organisms, including plants exposed to lethal concentration of glyphosate, exhibit chlorosis. This is considered to be the primary cause of its weedicide action^{5,6}. Very few reports are available about its inhibitory effect on chlorophyll synthesis⁷. Presumably, earlier workers could not ascertain the inhibitory effect of this herbicide on the photosynthetic processes. Despite a large-scale application of glyphosate as a herbicide, virtually little information is available on the effect of glyphosate on algal photosynthesis^{6,8}. Therefore, glyphosate inhibition of photo system II (PS II) in the algal system is still being disputed⁹. Due to a complex inter-relationship be-

tween the cell metabolism and agro toxicants, it is conjecturable that the herbicidal effect is manifested at sites which are far removed from the original site of action^{10,11}. In view of the above-stated points, the present investigation has been carried out to study the effect of glyphosate on the photosynthetic properties of nitrogen-fixing cyanobacterium *Anabaena doliolum*. Attempts have also been made to compare the wild type and mutant strains to elucidate the mechanism and site of glyphosate action on the photosynthetic processes.

Axenic culture of the N₂-fixing cyanobacterium *A. doliolum* was maintained in Allen and Arnon's medium¹². The cultures were routinely grown in a culture room at a temperature of 24 ± 1°C and were illuminated by cool, white fluorescent tubes with approximate light intensity of 10 W/m² on the surface of culture vessels. The growth of *Anabaena* cells was recorded by measuring the turbidity at 665 nm using a spectrophotometer.

Exponentially growing cells of *A. doliolum* were harvested by centrifugation (5000 g, 10 min) and the pellet was washed twice with the basal medium. The pellet was suspended in 20 ml of the sterilized citrate-buffer (10 mM, pH 6.0) to a final cell density of approximately 10⁶ to 10⁷ cells/ml. An aliquot of 0.2 ml of the cell suspension was diluted to a final volume of 20 ml by adding distilled water and 0.2 ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG; Sigma, USA) stock solution, so as to obtain a final concentration of NTG to 1 mg/ml, as described by Golden¹³. The small aliquots (4 ml) were withdrawn from the NTG-added cell suspension with a time interval of 10 min. Mutagenic treatment of the cells was terminated by 2–3 centrifugal washes (5000 g, 20 min) of the cells with the citrate-buffer (10 mM, pH 6). The rest of the procedure was the same as that described elsewhere¹⁴, except that the colonies were screened using toxic doses of the glyphosate (200 µg/ml).

Anabaena cells were light-incubated for 48 h in the presence of glyphosate (25–300 µg/ml). Thereafter, suitable aliquots were taken out for measurement of the absorption spectra (380–750 nm). The homogenized cell suspension was scanned in a UV-Visible spectrophotometer (UV-1601, Shimadzu, Japan). The quantity of pigments was calculated using the formula given by Astier *et al.*¹⁵. The glyphosate stock solution was neutralized with the alkali solution before using it for further experiments.

O₂ evolution was measured in a Clark-type O₂ electrode (Hansatech, UK) fitted with a circulating water-jacket. The exponential phase cells of *Anabaena* treated with glyphosate (50–500 µg/ml) for 1 h were taken for measurement of O₂ evolution using 2 mM NaHCO₃ as an electron acceptor. The rest of the procedure was the same as that described elsewhere¹⁶. The respiratory O₂ uptake was measured in the presence of photosynthetic inhibitor-DCMU (10 µM). The rate of O₂ evolution was calculated in terms of µmol O₂ evolved/mg protein/min. Protein

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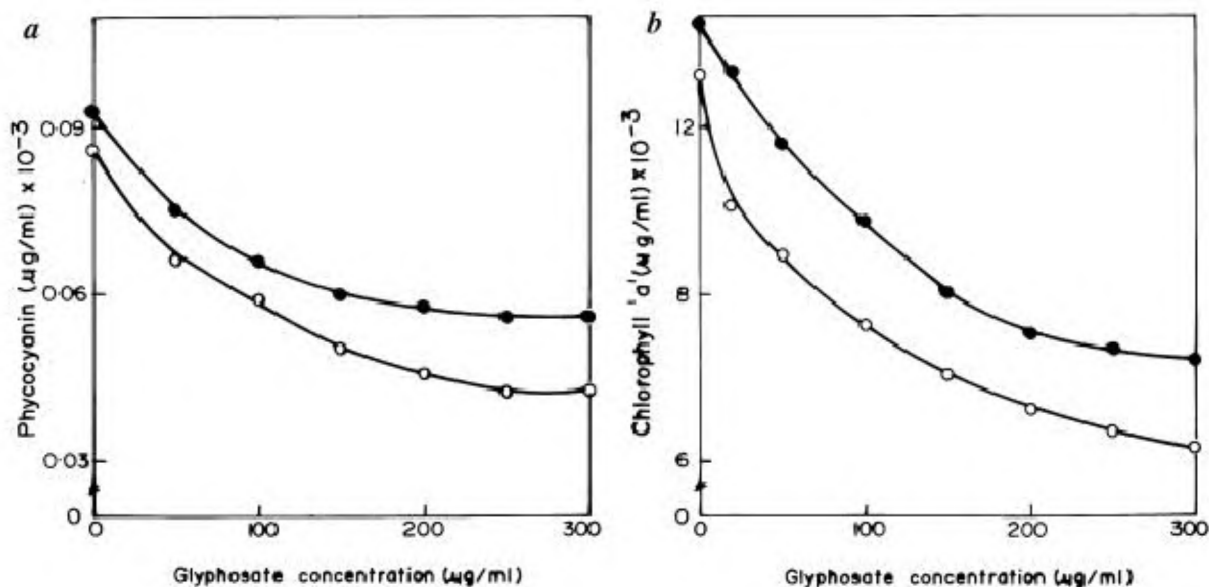


Figure 1. Effect of glyphosate (25–300 µg/ml) on phycocyanin (a) and chlorophyll a (b) in wild type (O—O) and mutant (●—●) cells under nitrate (KNO₃, 5 mM) supplemented condition. Samples were taken out after 48 h of growth in the presence of glyphosate.

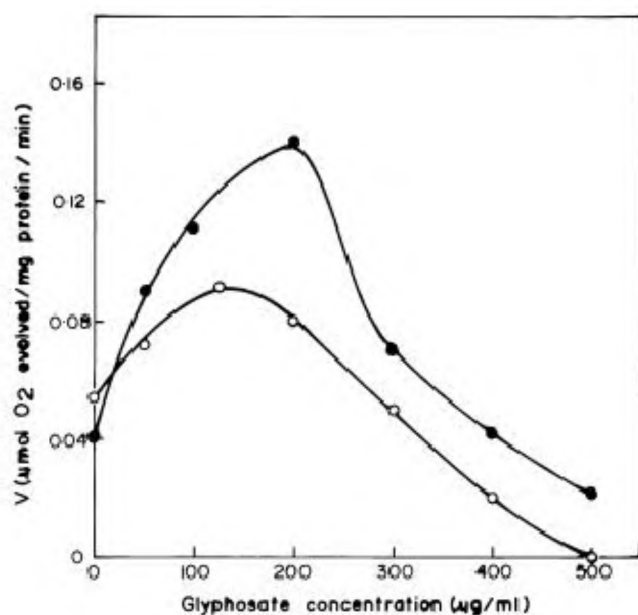


Figure 2. Effect of glyphosate (50–500 µg/ml) on the rate of O₂ evolution (µmol O₂ evolved/mg protein/min) in wild type (O—O) and mutant (●—●) cells of *Anabaena doliolum*. Cells were supplemented with NaHCO₃ as an electron acceptor. Glyphosate was added in experimental flask 1 h before the experiment.

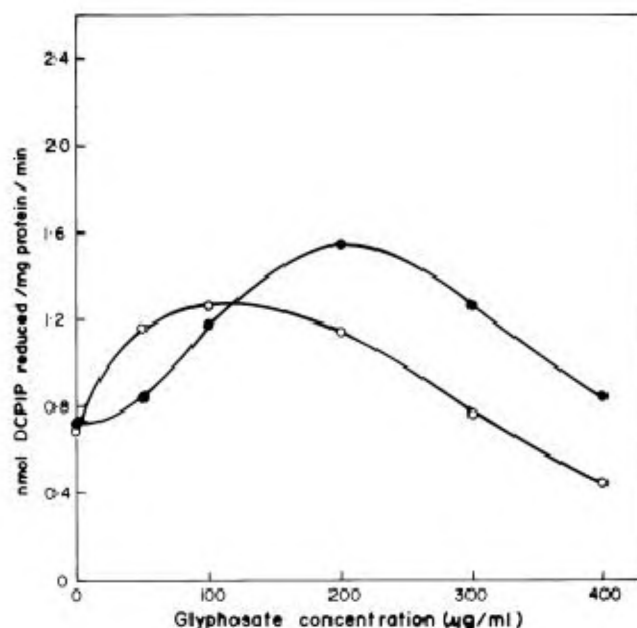


Figure 3. Effect of different concentrations of glyphosate (50–400 µg/ml) on rate of DCPIP reduction (nmol DCPIP reduced/mg protein/min) in wild type (O—O) and mutant (●—●) cells of *A. doliolum*. Glyphosate was added 1 h before the start of DCPIP reduction.

concentration was measured using the method of Lowry *et al.*¹⁷.

Hill activity was measured in the permeaplasts of *Anabaena* as described by Singh and Singh¹⁴. *Anabaena* cells incubated with varying concentration of glyphosate (50–400 µg/ml) for 1 h subsequently used for the measurement

of DCPIP (2,6 dichlorophenol, 1-indophenol) reduction. The reaction was initiated by adding 50 µM of DCPIP. The rate of Hill activity was expressed in terms of nmol DCPIP reduced/mg protein/min. The cells were permeabilized using lysozyme (0.5%, w/v). There was no measurable release of the phycocyanin pigment from the permeaplasts.

Anabaena cells were light-incubated in the presence of graded concentrations of glyphosate (25–250 $\mu\text{g/ml}$) for 24 h. Aliquots of 3.0 ml were withdrawn from each flask and were equalized in terms of chlorophyll *a* content. Prior to the measurement of fluorescence emission spectrum, the cells were homogenized using glass beads and were suspended in glycerol solution (25%, v/v) in order to avoid settling in the cell suspension. The room-temperature fluorescence emission spectra (600–730 nm) of chlorophyll *a* and phycobilisomes were recorded in a UV-Vis spectrofluorimeter 3010 (Hitachi, Japan) using the excitation wavelengths of 435 and 570 nm respectively. All the spectra were measured using a slit width of 5 nm and light path of 1.0 cm.

Metabolic inhibitors were dissolved in ethanol solution in such a way that the final concentration of ethanol in the assay mixture did not exceed 0.1% (v/v). *Anabaena* cells were light-incubated in the presence of glyphosate (40 $\mu\text{g/ml}$) for 24 h. The same cells were supplemented with metabolic inhibitors like carbonyl cyanide *m*-chloro-

phenylhydrazone (CCCP, 10 μM) and vanadate (100 μM), 1 h prior to the measurement of room-temperature emission spectra. All data are mean of three independent experiments.

The results on glyphosate-induced changes in the pigmentation of wild type and mutant strains showed a concentration-dependent decline in level of all the major pigments with inhibition maxima at 300 $\mu\text{g/ml}$ (Figure 1). Glyphosate-induced loss of pigments was higher in the wild type than the mutant strain. Higher concentrations of glyphosate (100–250 $\mu\text{g/ml}$) showed a faster downfall in chlorophyll *a* content than phycocyanin. The chlorophyll *a* pigment in both the strains was relatively more susceptible to glyphosate toxicity than phycocyanin and carotenoids. Inhibition of chlorophyll synthesis by glyphosate is a well-known phenomenon in photosynthetic organisms, leading to an induction of chlorosis^{5,18}.

The rate of oxygen evolution in wild type and mutant cells was stimulated by glyphosate up to 100 and 185 $\mu\text{g/ml}$ respectively. Thereafter, a concentration-dependent de-

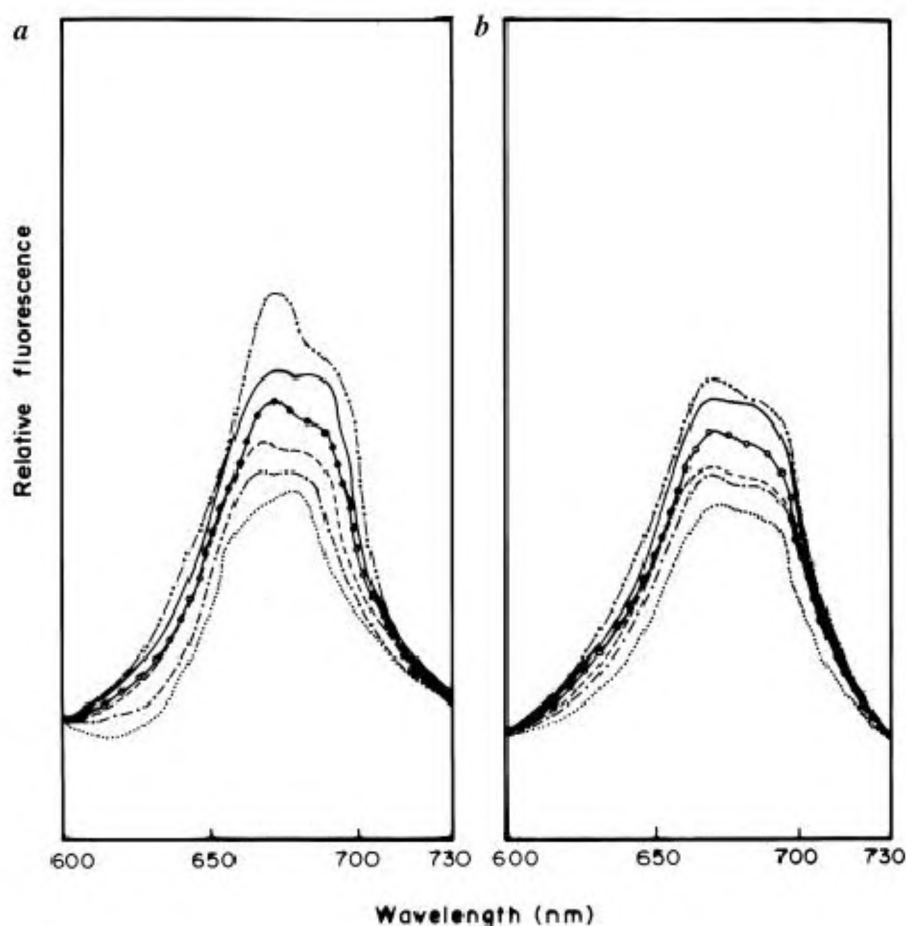


Figure 4. Room-temperature chlorophyll *a* fluorescence emission spectra (600–730 nm) of the wild type (*a*) and mutant (*b*) cells treated with graded concentrations (0–250 $\mu\text{g/ml}$) of glyphosate for 24 h. 0 $\mu\text{g/ml}$ (—); 25 $\mu\text{g/ml}$ (---); 50 $\mu\text{g/ml}$ (.....); 100 $\mu\text{g/ml}$ (-·-·-); 200 $\mu\text{g/ml}$ (— · — · —); 250 $\mu\text{g/ml}$ (○—○).

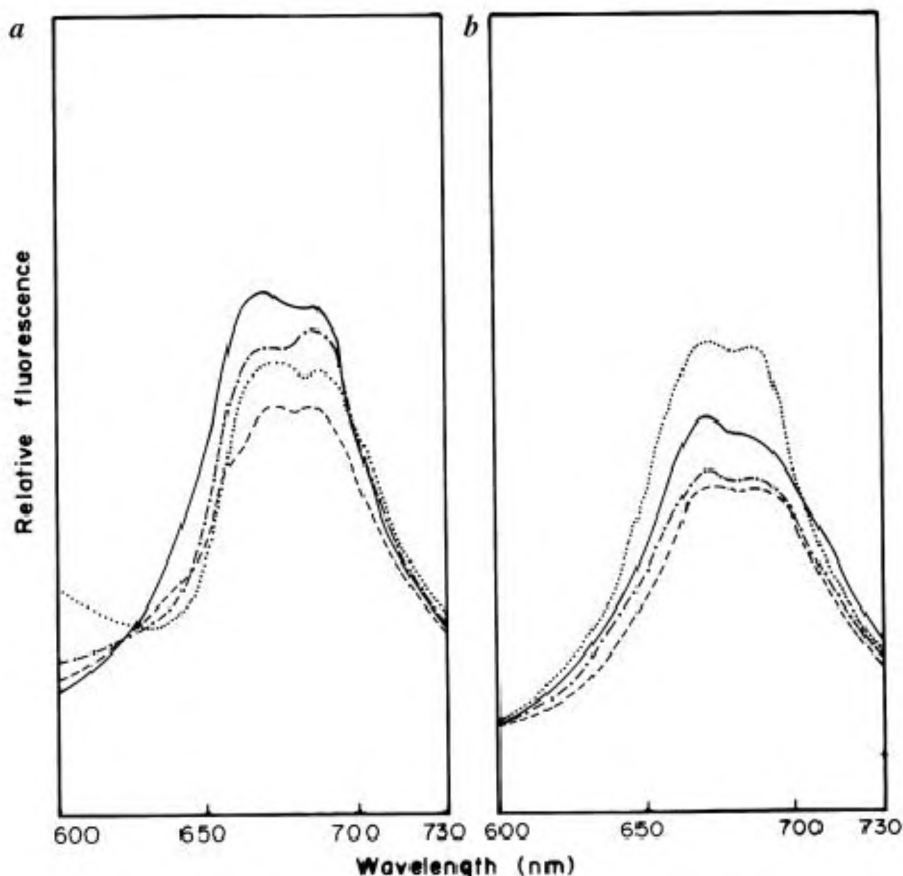


Figure 5. Effect of metabolic inhibitors on chlorophyll *a* fluorescence emission spectra (600–730 nm) of wild type (*a*) and mutant (*b*) cells treated without (—) and with glyphosate (40 µg/ml) (---) for 24 h; glyphosate (40 µg/ml) + CCCP (.....); glyphosate (40 µg/ml) + vanadate (— · — · —). Metabolic inhibitors were added 2 h before measurement of emission spectra.

crease in the rate of O_2 evolution was observed (Figure 2). The overall rate of O_2 evolution in the mutant strain was found to be relatively higher than the wild type throughout the concentration range of glyphosate.

The results on Hill activity revealed a glyphosate-dependent biphasic pattern in both strains (Figure 3). The initial phase of glyphosate-induced stimulation of Hill activity in both the wild type and mutant strains showed the rate maxima at 100 and 200 µg/ml respectively, which exhibited approximately twofold increase in the activity compared to the control (without glyphosate). Beyond the rate-saturating doses, a concentration-dependent inhibition of the Hill activity was observed in both the strains. Photosynthetic electron transport in the mutant cells was found to be less susceptible to glyphosate toxicity than the wild type. Overall results indicated that the Hill activity in both the strains was relatively less sensitive to glyphosate toxicity than the O_2 evolution. The present results are in conformity with the findings that have demonstrated that a threshold level of glyphosate is required for direct inhibition of the photosynthesis¹⁹. A 50% reduction in the rate of O_2 evolution in the green alga *Scenedesmus*

sp. is recorded at a very high concentration of glyphosate⁸.

Chlorophyll *a* emission spectra obtained for both the wild type and mutant strains exhibited two characteristic emission peaks at 672 and 687 nm. A glyphosate-induced quenching of the emission peaks was observed up to 100 µg/ml of glyphosate (Figure 4). A further increase in glyphosate concentration resulted in concentration-dependent stimulation in the emission intensity in both the strains. Glyphosate-induced changes in the emission intensity indicated that fluorescence emission in the wild type strain was relatively more susceptible to glyphosate than the mutant. Glyphosate-induced quenching of the chlorophyll fluorescence at lower concentrations may be interpreted in terms of non-photochemical quenching of chlorophyll emission²⁰. As such glyphosate-induced structural damage to the pigment antenna can be ruled out on the ground that higher concentrations of glyphosate do not inhibit fluorescence emission, but result in stimulation of emission intensity.

Effect of metabolic inhibitors on chlorophyll *a* fluorescence emission showed that addition of CCCP to the gly-

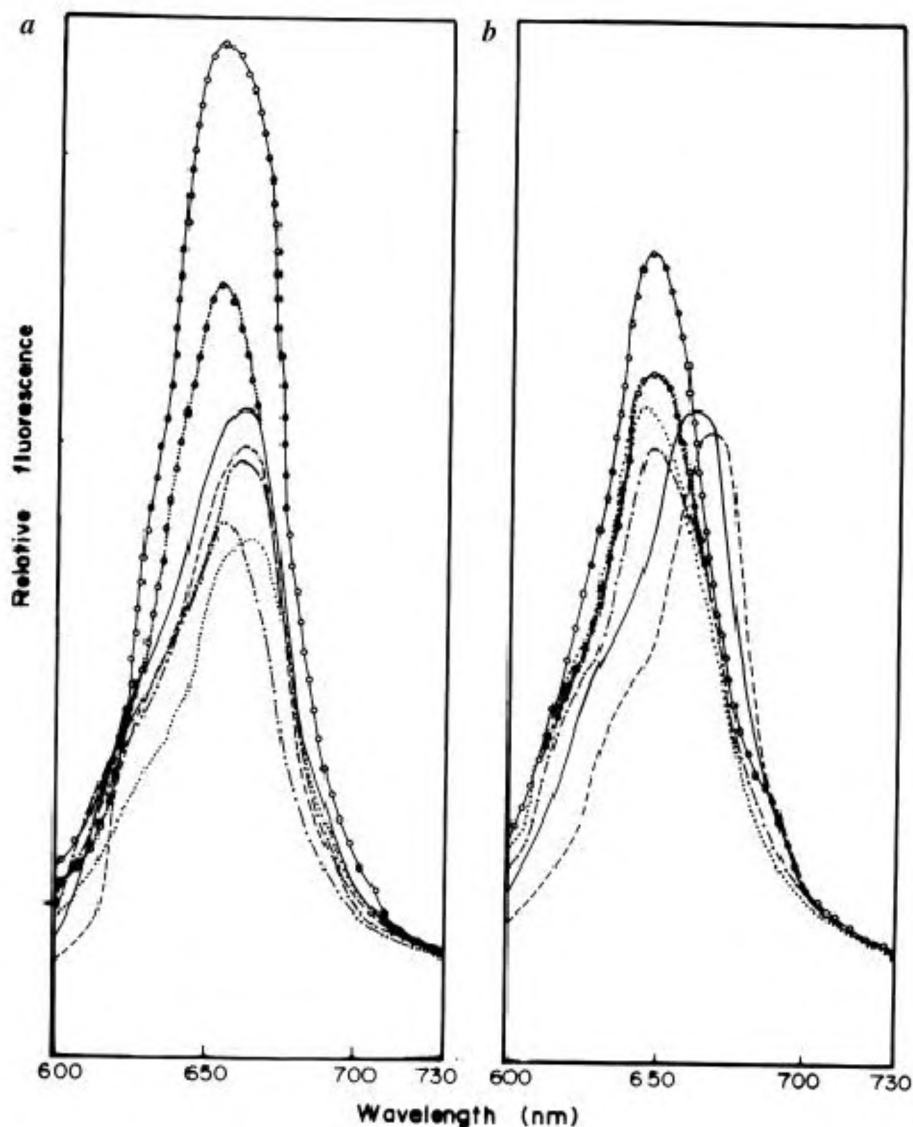


Figure 6. Room-temperature (25°C) phycobilisome fluorescence emission spectra (600–730 nm) of wild type (*a*) and mutant (*b*) cells of *A. doliolum* recorded in arbitrary units. Cells were treated for 24 h with different concentrations (0–250 µg/ml) of glyphosate. 0 µg/ml (—); 25 µg/ml (---); 50 µg/ml (----); 100 µg/ml (.....); 150 µg/ml (.....); 200 µg/ml (O---O) and 250 µg/ml (O—O).

phosphate (40 µg/ml)-treated wild type cells could restore the level of fluorescence emission by 19.5% (Figure 5). Glyphosate-induced fluorescence quenching at lower doses of glyphosate might be mediated by the build-up of proton gradient (ΔpH) across the membrane²¹. However, the mutant cells did not show any significant alteration in the emission intensity probably due to the lesser susceptibility of the mutant strain to this dose of glyphosate. Addition of vanadate—an ATPase inhibitor, could also revert the glyphosate-induced quenching of chlorophyll *a* fluorescence. In wild type cells, vanadate-induced reversal was approximately 14% compared to the control (without vanadate). However, addition of vanadate to mutant cells not only restored glyphosate-induced decline in the emission intensity, but also resulted into significant

rise in emission intensity when compared with the control. However, reversal of glyphosate effect on fluorescence emission in the presence of vanadate may be due to inhibition of ATPase²², which results in accumulation of inorganic phosphate—a known antagonist of glyphosate toxicity²³.

The room-temperature emission spectra of phycobilisomes (PBsomes) obtained for both the wild type and mutant strains exhibited a characteristic emission peak at 664 nm, which showed a glyphosate-dependent declining pattern up to 50 and 100 µg/ml of glyphosate respectively (Figure 6). A further increase in the concentration of glyphosate resulted in a concentration-dependent rise in the emission intensity accompanied by a blue shift in the emission peak at 640 nm. The glyphosate-dependent de-

cline in the emission spectra of PBsomes at lower concentrations might not be attributable to structural damage, as the intensity of PBsome emission could be stimulated by higher doses of glyphosate. Earlier, it has been demonstrated that an increase in the PBsome fluorescence accompanied by blue-shift in the emission peak (644 nm), encompasses conformational change in the PBsome resulting into impairment in the energy transfer from PBsomes to PS II reaction centre²⁴.

A corollary of these results suggests that glyphosate-induced impairment in energy transfer in the pigment antenna and build-up of the proton gradient (high energy state) across the membrane account for impairment in the photosynthetic property of *Anabaena* cells.

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Novel mechanism of distance-oriented bee movement, secondary pollen dispersal and reticulate pollination – a case study in *Melipona panamica*

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The efficiency of secondary pollen dispersal, effect of distance isolation, and possible consequence of reticulate pollination were assessed specifically by movements of stingless bees (*Melipona panamica*) in lowland Neotropical forest vegetation in Panama, because this insect is one of the predominant pollen vectors there. The trial consisted of two identical strips

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