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Atrazine and Methyl Viologen Effects on Chlorophyll-*a* Fluorescence Revisited—Implications in Photosystems Emission and Ecotoxicity Assessment

Analia Iriel[1], Johanna M. Novo[2], Gabriela B. Cordon[3] and María G. Lagorio*[2]

[1]Centro de Estudios Transdisciplinarios del Agua, Facultad de Ciencias Veterinarias, Universidad de Buenos Aires, Buenos Aires, Argentina
[2]INQUIMAE/ Dpto. de Química Inorgánica, Analítica y Química Física, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Buenos Aires, Argentina
[3]LART-IFEVA/ Dpto. de Métodos Cuantitativos y Sistemas de Información, Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina

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ABSTRACT

In this work, we use the effect of herbicides that affect the photosynthetic chain at defined sites in the photosynthetic reaction steps to derive information about the fluorescence emission of photosystems. The interpretation of spectral data from treated and control plants, after correction for light reabsorption processes, allowed us to elucidate current controversies in the subject. Results were compatible with the fact that a nonnegligible Photosystem I contribution to chlorophyll fluorescence in plants at room temperature does exist. In another aspect, variable and nonvariable chlorophyll fluorescence were comparatively tested as bioindicators for detection of both herbicides in aquatic environment. Both methodologies were appropriate tools for this purpose. However, they showed better sensitivity for pollutants disconnecting Photosystem II–Photosystem I by blocking the electron transport between them as Atrazine. Specifically, changes in the (experimental and corrected by light reabsorption) red to far red fluorescence ratio, in the maximum photochemical quantum yield and in the quantum efficiency of Photosytem II for increasing concentrations of herbicides have been measured and compared. The most sensitive bioindicator for both herbicides was the quantum efficiency of Photosystem II.

INTRODUCTION

Plant leaves emit chlorophyll-*a* fluorescence displaying a spectral distribution with two maxima: one in the red (around 685 nm) and the other in the far red (around 735 nm) (1–3). The ratio between the fluorescence emission in the red and in the far red called the "fluorescence ratio" has been extensively analyzed in connection with changes in the photosynthetic apparatus, stress situations and nutrient starvation in plants (4–7).

As the energy absorbed by chlorophyll-*a* in plants can undergo three deactivation pathways: (1) start of the electron transfer to perform photosynthesis, (2) excess energy dissipation as heat or (3) excess energy emission as fluorescence; the last process takes place in competition with the other two (8). A consequence of this competition is the increase in the yield of chlorophyll fluorescence that takes place when a photosynthetic tissue is transferred from the dark to the light. In fact, when chlorophyll-*a* in Photosystem II (PSII) is excited, it transfers electrons to the primary acceptors (Quinone A [QA]) in the photosynthetic chain. Once the QA has accepted an electron, it is not able to accept another until it has been transferred to the next acceptor plastoquinone B (QB). During this time (closed reaction centers) the fluorescence emission increases from an initial value F_0 up to a maximum value F_m (Fig. 1). This period of time is usually in the order of 1 s. Later, fluorescence starts to fall (fluorescence quenching) for several minutes to finally reach a stationary state (F_s). The fluorescence quenching has a photochemical and a nonphotochemical contribution. The photochemical quenching (q_p) is due to activation of enzymes involved in the carbon metabolism induced by light and the opening of stomata. The nonphotochemical quenching (q_{Np}) is due to an increase in the yields of heat dissipation (8,9). The variation in chlorophyll fluorescence quantum yield as a function of time is called Kautsky kinetics.

The spectral distribution of chlorophyll fluorescence and more specifically, the fluorescence ratio, changes during the Kautsky kinetics. Working in conditions of initial (F_0) or steady-state (F_s) fluorescence, a constant spectral distribution may be recorded.

Both fluorescence ratios obtained from the spectral distribution of the initial fluorescence and the photosynthetic parameters derived from Kautsky kinetics are normally used for ecotoxicity assessment in plants (5).

Although chlorophyll-*a* fluorescence ratio in leaves is widely used in literature, controversional manuscripts about the origin of the red and far-red bands have been published. Some authors argue that at room temperature, both peaks are due mainly to the emission of PSII (2,10).

Other authors claim that PSI contribution to the far-red band is not negligible, depending its contribution on the studied plant species and on the irradiation intensity (3,4,11,12). According to these researchers, room temperature emission in the red is due exclusively to PSII whereas in the far red it is due to both

*Corresponding author email: mgl@qi.fcen.uba.ar (María G. Lagorio)

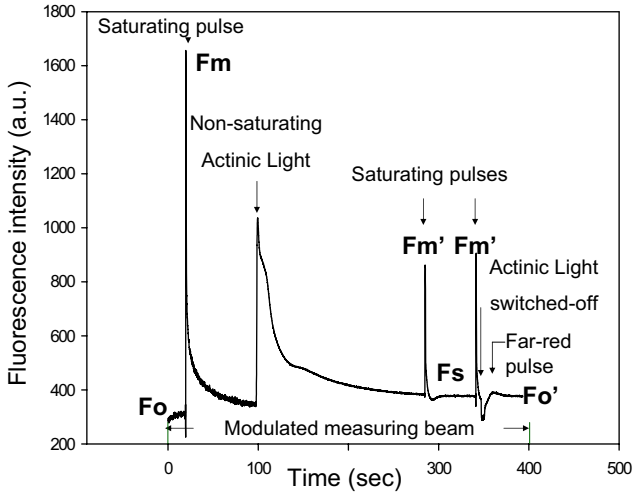

Figure 1. Variable chlorophyll fluorescence recorded with a pulse-modulated fluorometer for a typical plant leaf of *Spathiphyllum wallisi*.

photosystems. In a relative new manuscript, Pédros *et al.* (13) affirm that "…it was usually assumed that at room temperature most of this fluorescence is emanated by PSII. Nevertheless, the PSI contribution is around 5% at 683 nm and 30–35% in the far-red shoulder."

Herbicides that affect the photosynthetic processes at different specific points may be good tools to infer information about photosystems fluorescence emission and they may help to elucidate controversies on this subject. In fact, if a substance affects the emission of one of the photosystems and does not affect the other, two possible situations may arise: either the peaks ratio in the fluorescence spectra does not change, meaning that there is only one emitting species or the peaks ratio does change implicating the involvement of two emitting species. This simple hypothesis is, however, obscured by the presence of light reabsorption processes in leaves that introduce important distortions in spectra (14,15). Actually, assuming only one emitting species, it is also possible to have apparent variations in peaks ratio, if the herbicide affects the magnitude of the light reabsorption processes (*e.g.* due to chlorophyll destruction). That is why, to achieve the robustness of the above hypothesis, working with fluorescence spectra free from light reabsorption artifacts is essential.

Both Atrazine and Methyl viologen (MV) are widely used herbicides to protect crop plants. They are, however, dangerous because they accumulate into aquatic environments resulting in significant environmental damage. Atrazine presents high water mobility and strong persistence in the environment (16). Atrazine has been banned in the European Union since 2004, but it continues to be used in many countries including those which are large grain producers such as the United States of America, Argentina and Brazil. The herbicide has action as an endocrine disruptor, deteriorates the immune systems and leads to birth defects. It was also proved to reduce sperm and steroid production affecting the reproductive systems in humans (17). In fact, Atrazine reduces androgen production and stability and it is reasonable to anticipate a demasculinization effect in all vertebrates through ingestion (18). MV, a banned herbicide in the European Union, is also harmful to humans and its toxicity has been associated with suicides (19).

Using photosynthetic organisms to monitor pollutants in the environment is significant because they allow not only the detection of xenobiotics but they can also assess toxicity, making a substantial contribution in ecotoxicology. Furthermore, these techniques allow the monitoring of large areas at the same time. In this framework, one of the most important challenges is to identify those plants that present changes in their photophysical behavior when being in the presence of pollutants.

Several authors have already studied changes in the yield of chlorophyll fluorescence in the presence of herbicides (20–22). Conrad *et al.* found that some triazines and derivatives of phenylurea produced variations in the yield of the *in vivo* chlorophyll-*a* fluorescence of PSII (20). These changes were successfully correlated with the concentration of the herbicides, laying the basis for a monitoring system. Ralph studied the response of a seagrass to four herbicides (Diuron, Atrazine, Simazine and Glyphosate) (21). He found that the variation in the sites where the herbicides attacked, their absorption rates and whether the photosynthetic effect was direct or indirect, influenced the chlorophyll-*a* fluorescence response. Fan *et al.* reported a study of the mechanism of action of MV on the electron flow through PSII in spinach-leaf disks (22). They suggested that the presence of this herbicide accelerated several electron-transfer steps in PSII.

In the first part of the present work, we took advantage of the herbicides actions on specific points in the photosynthetic chain and we have studied the effect of Atrazine and MV (Paraquat) on F_0 chlorophyll fluorescence (corrected for light reabsorption processes) reinterpreting spectra in terms of photosystems contributions.

In the second part of the research, we have explored the changes in the variable fluorescence, experimented by herbicide-treated plants in relation to untreated controls. Data from Kautsky kinetics were then compared with the corresponding changes in the fluorescence ratio from F_0 spectra with and without correction for light reabsorption, with the aim of finding the best herbicide indicator. The study was performed on the species *Ficus benjamina*, *Hedera helix* and *Spathiphyllum wallisi*.

MATERIALS AND METHODS

Herbicide treatments. Atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine) (Sigma Aldrich analytical standard) and MV dichloride hydrate or Paraquat (1,1′-dimethyl-4,4′-bipyridinium dichloride-856177 Aldrich, 98% purity) were used as herbicides in the present research.

In a first stage, herbicide treatment was performed on two plant species: *F. benjamina* and *H. helix.* Healthy leaves from these species were washed with distilled water and disks of 30 mm diameter were cut and immersed in high concentrated herbicide solutions for 24 h. The concentration of each herbicide treatment was chosen as to match the average application rates used in the field (in spray) (23). Atrazine solution was 500 ppm whereas paraquat concentration was 250 ppm.

As a second step, aquatic plants of *S. wallisi* were purchased at an Aquarium store and they were kept in a nutrient solution prepared by adding 1.0 mL of FloraPride (FloraPride Tetra Holding (US), Inc., Blacksburg, VA; made in Germany) to each 3.8 L of water and kept until they were use. The plant nutrient FloraPride contained 3.00% of soluble potash as K_2O and 0.19% water soluble iron. The aquatic plants were immersed for 72 h in different solutions (with a constant nutrient concentration) containing increasing concentrations of each herbicide. Atrazine contents were varied from 0 to 2300 ppm, and MV contents from 0 to 100 ppm. Control samples were prepared by immersion in free-herbicide solutions containing only the nutrients.

Reflectance. Diffuse reflectance spectra were obtained for groups of stacked leaves using a Shimadzu UV3101 spectrophotometer equipped

with an integrating sphere. $BaSO_4$ was used as reference for the 100% reflectance. The obtained data were used in the application of the model to account for light reabsorption processes (14,15).

Fluorescence. Spectral distribution of initial fluorescence. Experiments were carried out on both control and herbicide-treated leaves that have been previously dark adapted for 15 min. Emission spectra of the initial fluorescence (F_0) of leaves were determined using a Photon Technology International steady-state fluorometer. The excitation wavelength was fixed at 460 nm and the emission wavelength was varied from 600 to 800 nm. The irradiation photon flux was kept below 20 μmol cm^{-2} as to avoid induction of Kautsky kinetics (9,14,15).

Experimental fluorescence spectra were then corrected for the detector sensitivity and finally for light reabsorption artifacts according to a physical model previously developed (14,15). This correction was performed by dividing the experimental fluorescence spectra ($I_f^m[\lambda]$) by the gamma function (Eqs. [1]–[3]).

$$I_f^c(\lambda) = \frac{I_f^m(\lambda)}{\gamma(\lambda, \lambda_0)} \quad (1)$$

$$\gamma(\lambda, \lambda_0) = \frac{1}{1 + \sqrt{\frac{F(R_\lambda)}{F(R_\lambda)+2}}} \frac{1}{1 + \sqrt{\frac{F(R_\lambda)\,(F(R_\lambda)+2)}{F(R_{\lambda_0})\,(F(R_{\lambda_0})+2)}}} \quad (2)$$

$$F(R) = \frac{(1 - R_\infty(\lambda))^2}{2R_\infty(\lambda)} \quad (3)$$

where $I_f^c(\lambda)$ is the corrected fluorescence emission, γ (λ, λ_0) is the correcting function and $F(R)$ is the remission function that is obtained from reflectance values ($R_\infty[\lambda]$) according to Eq. (3). In the equations above, λ and λ_0 denote emission and excitation wavelengths respectively.

The ratio of maxima, placed at about 685 and 735 nm ($F_{red}/F_{far\text{-}red}$), was calculated for both corrected and noncorrected spectra.

Variable fluorescence. Photosynthetic parameters were obtained with a pulse amplitude modulated (PAM) fluorometer Hansatech, FMS1 (24). Leaves were dark adapted for 15 min before the start of fluorescence measurements in all the cases. To record the variable chlorophyll fluorescence, the leaves were then illuminated with a modulated beam of low intensity to measure the initial fluorescence F_0. Then, a saturating pulse was applied to determine F_m. From these data, the maximum photochemical quantum yield (F_v/F_m where $F_v = F_m - F_0$) was calculated (see Fig. 1). Subsequently, the samples were exposed to the actinic light until a steady-state fluorescence value (F_s) was reached, and a new saturating pulse was applied to record the maximum fluorescence for light-adapted leaves (F_m'). The quantum efficiency of PSII ($\Phi_{PSII} = (F_{m'} - F_s)/F_{m'}$) was then obtained (9). From the values of Φ_{PSII}, measured as a function of the herbicide concentration, the apparent inhibition constants were obtained following the mathematical approach described by Preuss and Hall (25).

All the measurements were performed at room temperature (25°C) on the adaxial face of leaves.

RESULTS

Values of $F_{red}/F_{far\text{-}red}$, F_v/F_m and Φ_{PSII} for *F. benjamina* and *H. helix* treated with atrazine 500 ppm are shown in Table 1.

In the case of *F. benjamina* leaves treated with Atrazine, $F_{red}/F_{far\text{-}red}$ values increased 27–28% (for both corrected and noncorrected spectra) in relation to their respective controls. For *H. helix* treated with atrazine the obtained values increased about 14–15% (with and without correction). Observed differences between control and treated leaves were statistically significant (Student test $P < 0.05$).

Data presented in Table 1 are the means ± standard deviation resulting from five measurements. Significant differences between controls and herbicides-treated samples were determined by the paired *t*-test and *P* values <0.05 were considered significant.

The photosynthetic parameters decreased appreciably with Atrazine treatment (Table 1). The maximum quantum yield, F_v/F_m, diminished about 51% for *F. benjamina* and 44% for *H. helix* and the value for Φ_{PSII} declined very sharply (91–92%) in both cases. The differences were statistically significant (Student test $P < 0.05$).

Values of $F_{red}/F_{far\text{-}red}$, F_v/F_m and Φ_{PSII} for *F. benjamina* and *H. helix* treated with MV 250 ppm are shown in Table 2.

Average fluorescence ratios for MV-treated leaves were slightly lower than those for the controls, but differences were not statistically significant.

The photosynthetic parameters decreased with MV treatment (Table 2). The maximum quantum yield, F_v/F_m, diminished about 16% for *F. benjamina* and 15% for *H. helix* and the value for Φ_{PSII} decreased 39% and 55% respectively. The differences were statistically significant (Student test $P < 0.05$) for the Φ_{PSII} parameter.

Table 1. Fluorescence ratio and photosynthetic parameters for control and Atrazine-treated leaves of *Ficus benjamina* and *Hedera helix*.

Plant species	Treatment	$F_{red}/F_{far\text{-}red}$ without correction for light reabsorption	$F_{red}/F_{far\text{-}red}$ with correction for light reabsorption	F_v/F_m	Φ_{PSII}
F. benjamina	Control	0.66 ± 0.03	1.91 ± 0.09	0.83 ± 0.02	0.76 ± 0.01
	Atrazine	0.84 ± 0.03	2.45 ± 0.09	0.41 ± 0.02	0.07 ± 0.02
H. helix	Control	0.61 ± 0.03	1.80 ± 0.09	0.81 ± 0.01	0.75 ± 0.02
	Atrazine	0.70 ± 0.03	2.06 ± 0.09	0.45 ± 0.03	0.06 ± 0.01

Table 2. Fluorescence ratio and photosynthetic parameters for control and Methyl viologen-treated leaves of *Ficus benjamina* and *Hedera helix*.

Plant species	Treatment	$F_{red}/F_{far\text{-}red}$ without correction for light re-absorption	$F_{red}/F_{far\text{-}red}$ with correction for light re-absorption	F_v/F_m	Φ_{PSII}
F. benjamina	Control	0.63 ± 0.06	1.90 ± 0.18	0.83 ± 0.02	0.76 ± 0.01
	MV	0.52 ± 0.03*	1.60 ± 0.09*	0.7 ± 0.1*	0.44 ± 0.07
H. helix	Control	0.61 ± 0.09	1.80 ± 0.27	0.81 ± 0.01	0.75 ± 0.02
	MV	0.55 ± 0.06*	1.60 ± 0.18*	0.69 ± 0.03	0.34 ± 0.05

*Means not significantly different ($P > 0.05$) from the control.

This first set of experiments showed that the fluorescence ratio $F_{red}/F_{far\text{-}red}$ increases in the presence of Atrazine while it is not considerably affected by the presence of MV. In addition, the photosynthetic parameters resulted very responsive to stress, in particular Φ_{PSII}. This parameter was more sensitive to Atrazine than to MV.

In the second set of experiments, the fluorescence of an aquatic plant, *S. wallisi*, was tested as a function of the herbicide concentration. The fluorescence ratio $F_{red}/F_{far\text{-}red}$ (with and without correction for light reabsorption) as a function of the logarithm of herbicide concentration is shown in Figs. 2 and 3 for Atrazine and for MV respectively.

In the case of Atrazine, the fluorescence ratio increased as atrazine concentration augmented from 1 to 2300 ppm. The slope of this rise for data corrected for light reabsorption processes resulted about 2.6 times the slope for noncorrected data.

On the other hand, no significant variation was found in the fluorescence ratio, for increasing MV concentrations (Fig. 3).

Changes for the photosynthetic parameters F_v/F_m and Φ_{PSII} as a function of herbicide concentration are shown in Figs. 4 and 5 for atrazine and for MV respectively.

Both parameters F_v/F_m and Φ_{PSII} decreased with herbicides concentration. However, the decline in photosynthesis activity was better shown by Φ_{PSII} variation. Atrazine stress resulted more efficient than MV effect in affecting the quantum yield of PSII. The apparent inhibition constants, calculated from the values of Φ_{PSII} shown in Figs. 4 and 5, were 65 ppm for Atrazine and 70 ppm for MV (higher the apparent inhibition constant, less potent the herbicide).

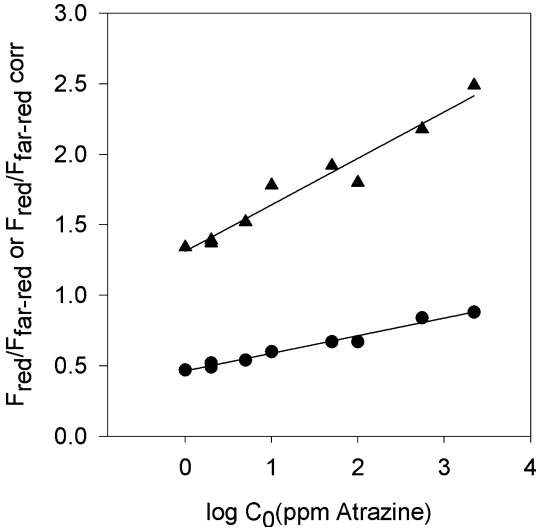

Figure 2. *Spathiphyllum wallisi* fluorescence ratio, (●) without correction for light reabsorption ($y = 1.25 \times 10^{-1}x + 4.66 \times 10^{-1}$) and (▲) corrected for light reabsorption ($y = 3.29 \times 10^{-1}x + 1.31$), as a function of the logarithm of atrazine concentration.

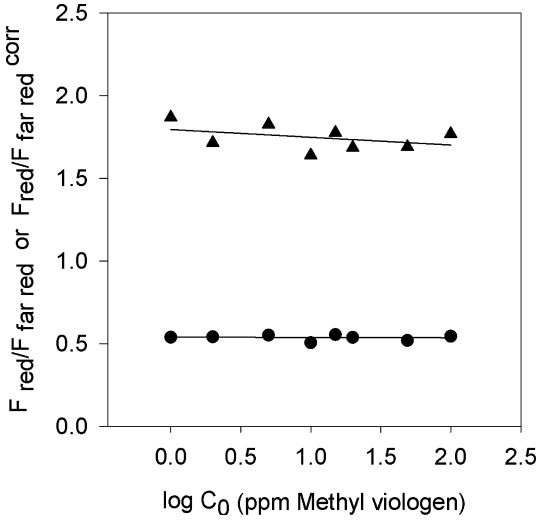

Figure 3. *Spathiphyllum wallisi* fluorescence ratio, (●) without correction for light reabsorption ($y = -1.92 \times 10^{-3}\ x + 5.39 \times 10^{-1}$) and (▲) corrected for light reabsorption ($y = -4.67 \times 10^{-2}x + 1.80$), as a function of the logarithm of methyl viologen concentration.

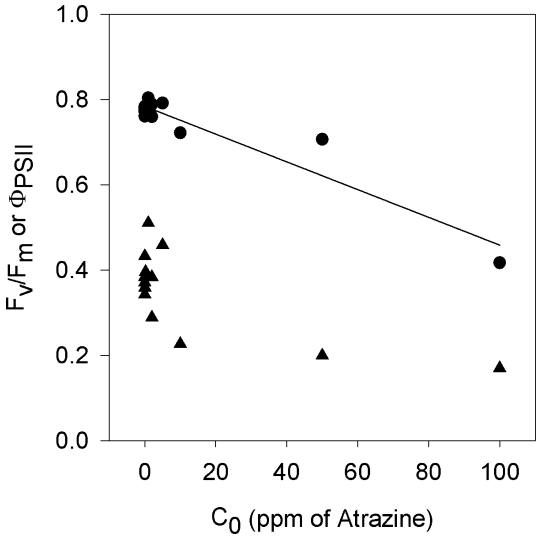

Figure 4. Photosynthetic parameters for *Spathiphyllum wallisi* treated with different atrazine concentrations. F_v/F_m (●) ($y = -3.26 \times 10^{-3}\ x + 7.84.10^{-2}$) and Φ_{PSII} (▲).

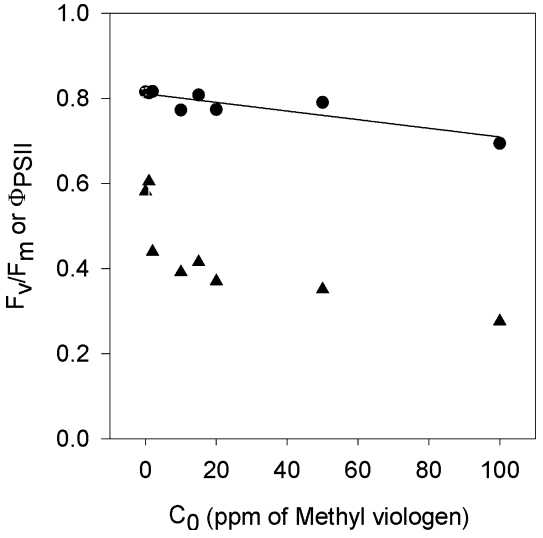

Figure 5. Photosynthetic parameters for *Spathiphyllum wallisi* treated with different methyl viologen concentrations. F_v/F_m (●) ($y = -1.01 \times 10^{-3}\ x + 8.11 \times 10^{-1}$) and Φ_{PSII} (▲).

DISCUSSION

Atrazine treatment augmented the observed fluorescence ratio ($F_{Red}/F_{Far\text{-}red}$) for the studied species. This enhancement cannot be attributed to changes in light reabsorption processes during the herbicide treatment because a significative increase is still present after the correction for light reabsorption. It is known that Atrazine produces a blockage of electron transfer between PSII and PSI as represented in Fig. 6 (26). The impediment imposed by the herbicide to the electron transfer leads to an increase in the radiative decay pathway for the excited chlorophyll dimer P_{680} in PSII. Specifically, Atrazine competes with the binding site on the D1 protein with the second electron acceptor QB, in the photosynthetic chain (26,27). It should be noted, then, that the increase in the fluorescence ratio (corrected by light reabsorption) caused by Atrazine can only be interpreted assuming a contribution of PSI emission to the far-red band. If the two bands were due only to PSII emission, Atrazine would increase both bands but the corrected fluorescence ratio should not present any change from control values.

MV did not affect appreciably the fluorescence ratio for the studied species. The slight decrease in the fluorescence ratio for MV treatment in the aquatic plant may be related, however, to an indirect destructive action on PSII. In fact, MV accepts the electrons from PSI instead of ferrodoxine (see MV action site in Fig. 6) preventing the formation of NADPH and causing the production of reactive oxygen species. The reactive species like superoxide anion, hydrogen peroxide and hydroxy radicals are responsible for the degradation of PSII proteins (26,28,29).

As it was justified above, the effect of herbicides on the plant fluorescence spectra corrected by reabsorption is consistent with the contribution of the two photosystems to the emission at room temperature.

From the experiments performed with the PAM fluorometer on variable chlorophyll fluorescence a linear decrease in F_v/F_m as a function of concentration was obtained for both herbicides. The slope of the linear fitting is higher for Atrazine than for MV. The parameter Φ_{PSII} also decreased with herbicides concentration but with a different behavior. In fact, for Φ_{PSII} the rate of the decline was very high for low concentrations—lower than about 20 μM—, remaining almost constant above this concentration. Again, the parameter change was more sensitive to Atrazine than to MV. When the raw data are analyzed (data not shown), it is observed that the presence of increasing Atrazine concentrations increases F_0 appreciably while F_m augments slightly. So in this case, the main responsible for the decrease in F_v/F_m ($=1 - F_0/F_m$) is the rise in F_0. In fact, Atrazine produces disconnection between PSII and PSI leading to an increase in the initial fluorescence as it has been observed in the nonvariable fluorescence measured with the steady-state fluorometer.

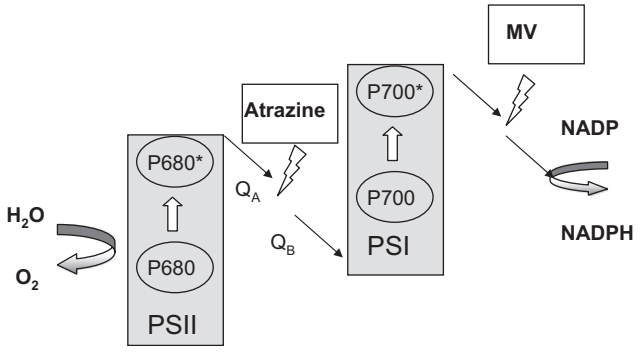

Figure 6. Scheme of the action sites for Atrazine and for methyl viologen in the photosynthetic transport chain. (P680: reaction center for PSII, P700: reaction center for PSI, QA: plastoquinone A, QB: plastoquinone B).

Ralph (21) has previously observed an increase in the maximum fluorescence (F_m) and in the initial fluorescence (F_0) and a decline in the maximum quantum yield ($F_m - F_0/F_m$) from photosynthetic organisms treated with triazine herbicides. Our results with Atrazine on *F. benjamina*, *H. helix* and *S. wallisi* strongly agreed with those reported by Ralph to assess herbicide toxicity of *Halophila ovalis* (21).

MV acted differently than Atrazine: at increasing concentrations, a slight decrease in F_0 was detected and an important reduction in F_m and in $F_{m'}$ (probably due to the destruction of PSII) was observed. Fan *et al.* (22) found that low MV concentrations decreased the chlorophyll-*a* fluorescence yield corresponding to dark-adapted reaction centers (F_0) and closed reaction centers (F_m) and also diminished the light-adapted photochemical efficiency of PSII. Our results with MV on *F. benjamina*, *H. helix* and *S. wallisi* agreed with the previous study published by Fan *et al.* using variable chlorophyll fluorescence on spinach leaves (22).

CONCLUSIONS

The effect of Atrazine and MV on plant chlorophyll fluorescence has been revisited performing analysis on corrected spectra to account for light reabsorption processes. At room temperature, the results definitively reinforce the assignment of the red band to PSII emission and the far-red band to both PSII and PSI contributions. In the present work, low photon flux excitation was used and the analysis was so performed on F_0 (where the contribution of PSI is expected to be more important). In our opinion, most of the bibliographic controversies on the contribution of PSI to plant fluorescence at room temperature comes from the different working conditions of the experiments. It should be noted that PSI emission may be negligible when working at high photon fluxes (F_m in Kautsky curve) but not when working at fluxes low enough as not to induce the Kautsky kinetics (9).

Both initial chlorophyll fluorescence (F_0) spectral distribution and variable chlorophyll fluorescence are sensitive, nondestructive tools to detect pollutants affecting the photosynthetic process in plants. In this work, it has been shown that different plant species respond similar to this kind of stress. However, this behavior cannot be directly extended to other species, so the choice of ordinary and abundant plants with high responses (strong photosynthetic damage) is relevant for using this methodology. Aquatic monitoring of herbicides is envisaged by the use of aquatic plants like *S. wallisi*.

The fluorescence ratio corrected by reabsorption was really good to monitor Atrazine concentration while it turned worthless to monitor MV in water. On the other hand, variable chlorophyll fluorescence proved to be adequate for detection of both herbicides, showing however, better performance as bioindicator for pollutants disconnecting PSII–PSI by blocking the electron transport between them as Atrazine.

Finally, the most sensitive bioindicator for both herbicides turned out to be Φ_{PSII}, a parameter that can be easily calculated in field with portable fluorometers.

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