Volume 52(1):85-88, 2008
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Photosynthesis inhibition by exogenously generated singlet oxygen – a note of caution

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ABSTRACT Two commonly applied exogenous singlet oxygen sensitizers, rose bengal (RB) and methylene blue (MB) were studied in terms of toxicity and photodynamic efficacy in green leaves. Their effects on photosynthesis with and without the singlet oxygen generating illumination were measured as changes in Photosystem II photochemical yield. Although the two photosensitizers caused the same, concentration-dependent weak inhibition in the dark, RB was more efficient to promote photodynamic injury to *Nicotiana tabacum* leaves in the presence of visible light. Results show that RB is more advantageous for leaf studies than MB. RB, however, should be used in moderation, as it may cause an additional, singlet oxygen independent inhibition of photosynthesis when applied at high concentrations. The application limit was about 2 mM using youngest fully expanded leaves of 4-week-old, green-house grown tobacco plants.
Acta Biol Szeged 52(1):85-88 (2008)

KEY WORDS

singlet oxygen
Rose bengal
methylene blue
photosynthesis
photochemical yield

In plants, mitochondrial and chloroplast electron transport, peroxisomes and cell-wall enzymes are the main sites reactive oxygen species (ROS) (for reviews see Foyer and Noctor 2003; Apel and Hirt 2004; Asada 2006). Singlet oxygen ($^1O_2$), a non-radical form of ROS is mainly produced by Photosystem (PS) II through energy transfer from excited chlorophyll to oxygen. This $^1O_2$ is mainly quenched by PS II reaction centre, presumably by oxidation of the D1 protein, although tocopherols and carotenoids also participate. *In vivo*, oxidized D1 is replaced at relatively high turnover rate to preserve PS II from photoinactivation (Barber and Andersson 1992; Trebst 2003; Telfer et al. 2004; Krieger-Liszkay 2005; Telfer 2005; Krieger-Liszkay and Trebst 2006). Apart from well detectable physicochemical damage, $^1O_2$ may also activate genetically determined stress-response pathways (op den Camp et al. 2003; Fischer et al. 2007; Kim et al. 2008). Although $^1O_2$ is generally assumed to be produced and reactive within the hydrophobic interior of PS II and initiate signalling through its products only, recent experiments suggests that a small fraction may leave the thylakoid membrane (Fischer et al 2007).

The various roles of $^1O_2$ in photosynthesis has been extensively studied by direct detection of this ROS (Macpherson et al. 1993; Hideg et al. 1994; 1998, Telfer et al. 1994) and by studying effects of artificially generated $^1O_2$. The latter is an established experimental practice, based on comparing responses to high irradiation and to the external $^1O_2$. It was utilized in a variety of studies, including chlorophyll and protein oxidation in the PS II reaction center (Telfer et al. 1994; Mishra 1994; Hideg et al. 2007), the role of various chloroplast antioxidants and energy dissipation pathways in oxidative stress tolerance (Knox and Dodge 1984; Jimenez and Pick 1993; Baroli et al. 2003); as well as gene expression responses (Green and Fluhr 1995; A-H-Mackerness 1998; Fischer et al. 2005). Rose bengal (RB) and methylene blue (MB) are two typical type-II photosensitizers (Foote 1968), known to generate $^1O_2$ when excited by visible light (Tomita et al. 1969; Lee and Rodgers 1987; Lambert et al. 1996). Their respective $^1O_2$ quantum yields (the number of $^1O_2$ molecules generated for each photon absorbed by one photosensitizer molecule) in aqueous media are relatively high, 0.79 (RB) (DeRosa and Crutchley 2002) and 0.52 (MB; Usui and Kamogawa 1974). Photosensitizers are applied in a wide range of concentrations, sometimes as high as 40 mM (A-H-Mackerness 1998). To ensure the reliability of experiments with exogenous photosensitizers, it is essential that these chemicals do not interfere with plant metabolism themselves. The aim of the present work was to study the effect of RB and MB on photosynthesis, to establish the concentration range where their effect is only through $^1O_2$ generation.

Materials and Methods

Youngest fully expanded leaves of 4-week-old tobacco (*Nicotiana tabacum* L.) plants were infiltrated locally with various concentrations of RB or MB in water solution as described earlier (Hideg et al. 2002). After infiltration, leaves were kept in darkness for 20 min, in a well-ventilated space in order to let excess water to evaporate. This period also served as dark adaptation before measuring initial photochemical param-

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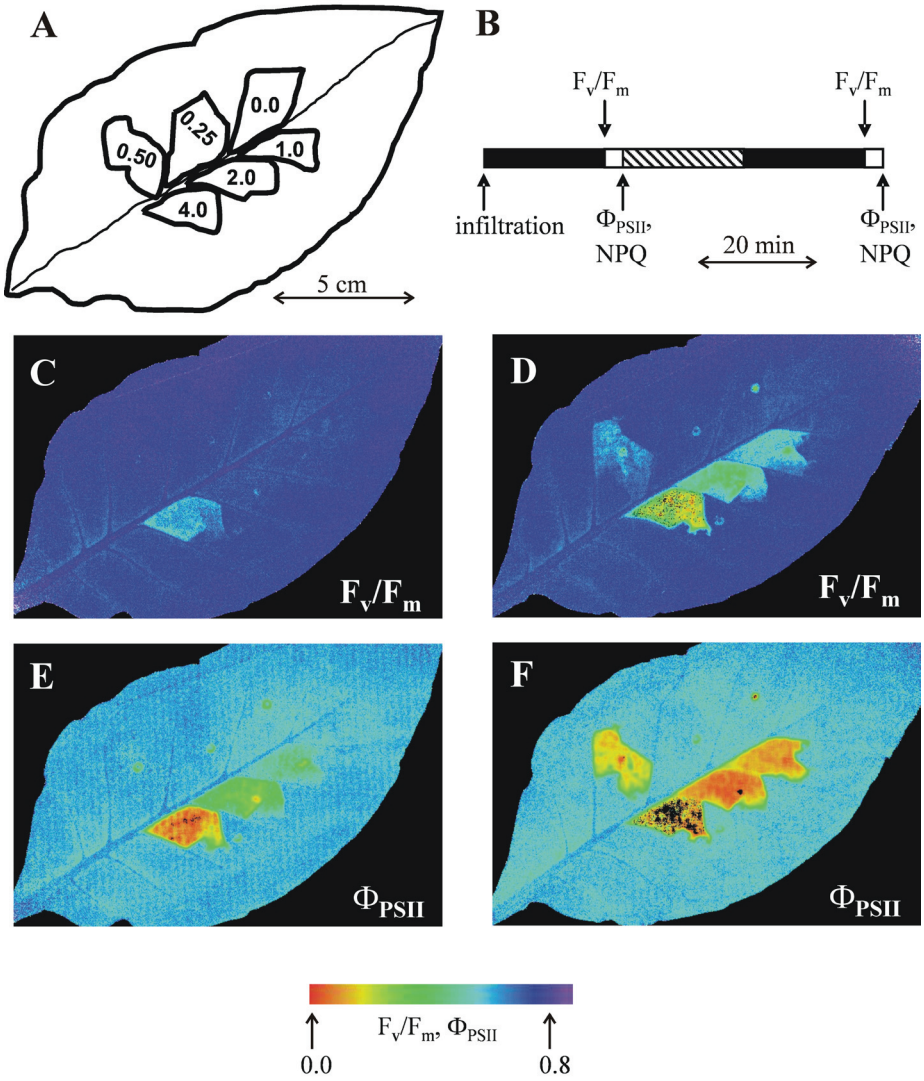

Figure 1. Effects of rose bengal on photochemical yield. (A) Outline of infiltrated areas and rose bengal concentrations (mM in water) in a tobacco leaf. (B) Scheme of the experiments, timing of photosynthesis measurements and light conditions. Black areas on the time bar represent dark adaptation, white areas show 45 µmol m^{-2} s^{-1} actinic light applied during photosynthesis measurements and the shaded part refers to 20 min illumination with 35 µmol m^{-2} s^{-1} PAR. (C-F) Colour-coded images of maximum (F_v/F_m, images C, D) and effective (Φ_{PSII}, images E, F) PS II quantum yield measured before (C, E) or after (D, F) the 20 min illumination with 35 µmol m^{-2} s^{-1} PAR.

eters. After this measurement, leaves were first kept under 35 µmol m^{-2} s^{-1} PAR for 20 min in order to activate photosensitizers, then in darkness for an other 20 min before a second measurement (see Fig. 1B for an outline of the experimental protocol). Photochemical yield of PS II electron transport (Φ_{PSII}) was calculated from variable chlorophyll fluorescence parameters, measured with the MAXI-version of the Imaging-PAM (Heinz Walz GmbH, Effeltrich, Germany). First F_o, the minimal fluorescence yield of dark adapted samples was imaged at low pulse frequency modulated measuring light, while images of the maximal fluorescence yield, F_m, were obtained with the help of a saturation (8000 µmol m^{-2} s^{-1} PAR) pulse. Based on F_o and F_m, the images of potential PS II quantum yield, F_v/F_m, were derived. Then samples were illuminated with 45 µmol m^{-2} s^{-1} PAR for 3 minutes, which resulted in a non-photochemical quenching of the maximal fluorescence yield, F_m', is with respect to F_m. The effective PS II quantum yield of illuminated samples was calculated from the expression $\Phi_{PSII} = (F_m' - F)/F_m'$. (Schreiber et al. 1986). Photosynthetic parameters are shown either as colour-coded images of the whole leaf (Hideg and Schreiber 2007) or as values averaged from indicated leaf areas. The latter are mean values calculated from four experiments and are presented with standard deviations. When averaging photochemical parameters, the infiltration site itself (a ca. 2 mm diameter area around the pinhole) was not included.

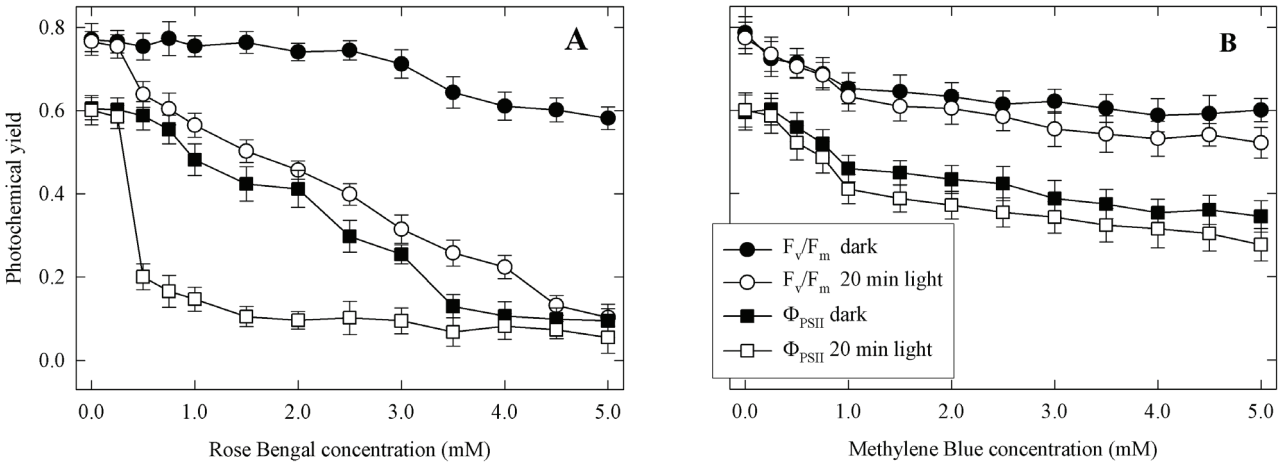

Figure 2. Decrease in maximum (circles) and effective (squares) PS II quantum yield in tobacco leaf areas infiltrated with various concentrations of rose bengal (A) or methylene blue (B) before (full symbols) or after (empty symbols) 20 min illumination with 35 μmol m^{-2} s^{-1} PAR.

Results and Discussion

Figure 1. shows colour coded images of maximum (F_v/F_m) and effective (Φ_{PSII}) PS II quantum yield of a tobacco leaf infiltrated with various concentrations of RB locally. Because leaf vasculars limit the spreading of the infiltrating solution inside the leaf tissue, it was possible to study the effect of various concentrations on the same leaf. The arrangement of various infiltration spots on the leaf is shown in Figure 1A. A fluorescence image taken before infiltration (data not shown) as well as comparison of non-infiltrated areas in Figure 1. confirmed that there were no marked differences in photosynthetic parameters recorded from various parts (for example between left vs. right, tip vs. base, etc.). Image of the area infiltrated with water only (marked as 0.0 mM RB in the image) demonstrates that the infiltration process itself did not affect PS II electron transport. Photochemical parameters were measured before and after illuminating the leaf with 35 μmol m^{-2} s^{-1} PAR for 20 min, according to the experimental protocol shown in Figure 1B. Without light activation, the presence of RB itself had no effect on F_v/F_m up to 3 mM infiltrating concentration (Fig. 1C, 2A). The actual PS II photochemical yield Φ_{PSII} measured after 3 min acclimation to 45 μmol m^{-2} s^{-1} PAR actinic light was affected by the presence of RB in the leaf (Fig. 2E) in a concentration dependent way (Fig. 2A). Because measurement of Φ_{PSII} requires light, it is not possible to conclude whether the decrease in Φ_{PSII} was due to $^1O_2$ formed during the 3 min exposure to actinic light or it was caused by the presence of RB in the leaf. Photoactivation of RB by a prolonged, 20 min exposure to 35 μmol m^{-2} s^{-1} PAR clearly showed that $^1O_2$ produced by the exogenous photosensitizer damaged PS II. This was visible not only as Φ_{PSII} but also as F_v/F_m limitation (Figs. 1D, 1F and 2A). There is no evidence how the RB which was forced into the leaf tissue penetrates into the cells, whether it is delivered inside the chloroplasts or not. Nevertheless, it is unlikely that this water soluble molecule would reach inside the thylakoid membranes. Therefore the observed irreversible (data not shown) loss of photochemical yield is very likely a secondary effect of $^1O_2$ photodamage.

The other water soluble exogenous photosensitizer, MB had much smaller effect on photosynthesis than RB. Figure 2. shows that when applied at the same concentrations as RB, its effect was different in two main aspects. One, without the 20 min photoactivation MB limited Φ_{PSII} to a smaller extent that RB. Two, MB was practically not photoactivated in the leaf: photosynthetic parameters were only slightly lower (at most concentrations in a statistically not significant way) after the 20 min illumination than before (Fig. 2B). The latter can not be explained by differences in $^1O_2$ quantum yields, which is only 1.5-times higher for RB than for MB, and can not be entirely due to differences in the absorption properties of the two photosensitizers either. RB has a maximal absorption at 559 nm which is not shaded by chlorophylls, and its molar extinction coefficient (90,400 $M^{-1}cm^{-1}$, Seybold et al. 1969) is about 10-times higher than MB at the same wavelength. The absorption spectrum of MB, which is maximal at 664 nm in water solution, overlaps with that of chlorophyll in the leaf tissue, leading to less efficient excitation of MB and consequently lower photodynamic effect. However, MB has a second absorption peak at 610 nm, with ca 37,400 $M^{-1}cm^{-1}$ molar extinction coefficient, which is about 2/3 of the value at its 664 nm absolute maximum (Ion et al. 2003). Because this lower wavelength peak does not overlap with chlorophyll, the lack of MB photodynamic effect in the leaf can not be ascribed to insufficient excitation. Although both photosensitizers dissolve in water and are therefore expected

to be equally hydrophilic, differences in their localization within the leaf tissue may contribute to differences in their photo-toxicity.

Due to their unknown micro-localization, the functioning of exogenous $^1O_2$ photosensitizers in photosynthetic tissue is still not fully understood. Nevertheless, striking similarities between their effects and those of stress-inducible, apparently internal $^1O_2$ production (Knox and Dodge 1984; Telfer et al. 1994; Mishra 1994. Fischer et al. 2005; Hideg et al. 2007 and references therein) make this topic interesting and well worth exploring, specially *in vivo*. Data presented here showed that exogenous photosensitizers should be chosen and applied with care. When applied at the same concentrations, MB was found to be less phototoxic then RB. The latter is an excellent photosensitizer for leaf studies, but should not be applied at high concentrations to avoid additional, non-photodynamic effects.

Acknowledgements

Experiments were supported by grants from the Hungarian National Research Foundation (OTKA T49438 and OTKA-NKTH K67597).

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