Studies of Photosynthesis Inhibition by Phytoluminography

R. Blaich, O. Bachmann, and Iris Baumberger

Bundesforschungsanstalt für Rebenzüchtung, Geilweilerhof, D-6741 Siebeldingen

Z. Naturforsch. **37 c**, 452–457 (1982); received January 18, 1982

Chlorophyll Luminescence, Photosynthesis Inhibition, Simazine, Phytoluminography

Inhibition of photosynthesis by simazine or diuron, as well as disturbance brought about by darkness, fungal infections, heat, high concentrations or lack of CO_2, lead to a luminescence of chloroplasts after irradiation with photosynthetically active light. This effect is the basis of phytoluminography, a new method which may replace in many cases the tedious procedures used at present to determine the photosynthetic capacity of living plant tissue. Both qualitative analyses of luminescence images produced by an image intensifier as well as quantitative measurements using a photomultiplier are possible.

Autoradiographic studies have shown that luminescence images of herbicide-damaged leaves are identical with autoradiographs of the same leaf obtained after labelling with radioactive CO_2. In contrast to autoradiography, phytoluminography does not damage the tested parts of plants nor is there danger of contamination. There is no need for expensive chemicals and skilled technicans, and immediate results are obtained.

Quantitative measurements of phytoluminescence after the application of simazine and diuron (DCMU) to *Elodea canadensis* in submersed culture showed that approximately 1 M of inhibitor per 20 M of chlorophyll ($a + b$) is needed to obtain maximal luminescence, which indicated total inhibition of photosynthesis.

Both luminescence and inhibition are reversible if the inhibitor is removed by dialysis.

Introduction

The luminescence (delayed fluorescence) of chloroplasts after illumination with photosynthetic active light was described in 1951 by Strehler and Arnold [1]. The intensity is very weak, and not visible to the naked eye, necessitating the use of a photomultiplier for its measurement. Chloroplast luminescence is due to a partial reversal of the energy flow of photosynthesis (for a detailed explanation see [2, 3]).

In 1979 Björn and Forsberg [4] described a device based on the principle of a Bequerel typ phosphoroscope equipped with a light amplifier, by which they were able to produce and photograph luminescence images of green parts of plants. They demonstrated that damage to the photosynthetic system – although not visible otherwise – changed the intensity of the luminescence, and they proposed the application of luminography for the early diagnosis of such damage.

Although phytoluminography might be a valuable tool for basic research in photosynthesis, our aim was to make the method suitable for applied work. In particular the relationship between luminescence and photosynthetic activity had to be established and facilities for quantitative measurements had to be added to the apparatus, to support the qualitative observations of luminescence images.

Reprint requests to Dr. R. Blaich.

0341-0382/82/0500-0452 $ 01.30/0

Materials and Methods

The *analysis of luminescence* was carried out with a device based on the apparatus of Björn and Forsberg [4]. By the use of a new type of high resolution light amplifier (Proxitronic type BV 2542 QG 35) the image quality could be improved considerably. The relatively low amplification (7500 max.) of this type is compensated by its fiber glass screen, which allows contact prints of the screen without loss of light. The gain in sensitivity is at least fivty-fold as compared with normal macrophotography of the screen. Photographs are taken with a special camera that allows the film to press on the fiber screen. For a 400 ASA film an exposure time of 5–10 seconds is needed.

The image of the specimen projected onto the photocathode of the amplifier by means of a SLR lens (35 mm/1:2,0) equipped with a red filter (< 600 nm).

For *quantitative measurements* the image amplifier is replaced by a photomultiplier (Proxitronic FV 38800/2). The data is recorded by micro-com-

puter graphics. The intensity of the luminescence was calculated from the specifications of the photomultiplier and the optical conditions of our device.

For *excitation and observation of luminescence*, a rotating slot disc allows alternating illumination of the specimen by means of a fiber optic and observation with the light amplifier.

At present the intensity of the light (3800 °K) on the specimen is limited to 15 W/m² for technical reasons.

The rotating diaphragm normally turns at the rate of 4 revs/sec, so that the specimen is illuminated for 0.08 sec and observed after a pause of 0.04 sec for another 0.08 sec. After a further pause of 0.04 sec the cycle is completed and illumination of the sepcimen begins again.

For a single observation of both relative intensity and duration of the luminescence, the light path must be switched manually after a continuous illumination of the specimen for 5 sec. Due to the poor reproducibility of the switching speed, this method allows only qualitative observations.

For *fluorescence observations*, the specimen is lighted with a weak source of blue light consisting of a 6 V/2,5 W tungsten bulb, run with 10 V and equipped with a blue filter (< 450 nm).

The photosynthetic activity of leaves was monitored by *autoradiography*. Green parts of plants treated with photosynthesis inhibitors were observed under the luminoscope. At the appropriate stage they were incubated at 20 000 lux for 5 min in an atmosphere containing 0.03% of $^{14}CO_2$ (4 μCi/l), then fixed by heat or liquid nitrogen and transferred to an x-ray film. Untreated plant parts were also labeled and served as a control, as well as autoradiographs obtained after application of labeled simazine. For this purpose the leaf stem was put into a suspension of ^{14}C simazine (100 ppm, 0.2 μCi/ml) in water.

The induction of luminescence by different amounts of simazine was measured with the photomultiplier. In a petri dish 2–3 g (wet weight) of fresh sprouts of *Elodea canadensis* were submersed in 30 ml water. Simazine or DMCU was added in amounts of 50 nM. The increasing luminescence was measured continuously with the photomultiplier. After 30 min, luminescence had reached a constant level and more inhibitor was added. This procedure was repeated until no further increase of luminescence could be obtained.

The chlorophyll content of plant parts was determined according to the method of Hiscox and Israelstam [7]. All experiments were carried out at room temperature (20–24 °C).

Results and Discussion

1) Qualitative observations

Healthy green parts of plants pre-illuminated with strong light (100 W/m²) for at least 3 min, show a faint luminescence which does not change after prolonged continuous observation.

If the same specimes are kept in the dark for at least 15 min, they show a much stronger luminescence which, after switching off the light, disappears within a few seconds. If the observation under pulse light is continued for some minutes, the luminescence becomes weaker and eventually reaches the level of that of a pre-illuminated specimen. This effect obviously is due to the influence of the exciting light. Leaves or other green parts of all plants studied so far behaved in the same manner.

A disturbance of the photosynthetic system as provoked by inhibitors like diuron or by incubation of the leaf in an atmosphere with a high content of CO_2 (over 20%) led to a strong luminescence in damaged areas, and this had – according to our photographic record – the same intensity as that of leaves kept in the dark. However, this luminescence was not diminished by the exciting light. Complete abolishment of luminescence was obtained only by severe damage, leading to disintegration of the photosynthetic system (*e.g.* heat or other protein-denaturing agents).

Among others the method was used to follow the penetration of simazine into leaves of *Vitis* (Fig. 1) and cotyledons of *Raphanus* (Fig. 2). If the inhibitor is applied via the petiole (as a 10 ppm aqueous suspension) visual damage to the leaf (yellowing veins) appears only after several days. Luminescence, however, begins to show up after some minutes. It spreads along the veins and eventually covers the whole leaf. Tests with radioactively labeled simazine showed that the areas of luminescence coincided with the areas penetrated by the inhibitor (Fig. 2 e).

The significance of luminescence in relation to photosynthesis was tested by autoradiography of leaves labeled with $^{14}CO_2$. For this purpose the

Fig. 1. a) Phytoluminograph of a leaf of *Vitis vinifera* after 6 h treatment with diuron. b) Autoradiograph of the same leaf after 5 min incubation in $^{14}CO_2$.

penetration of unlabeled simazine via the petiole into the leaf was checked with the luminoscope. At an appropriate stage the leaf was incubated for 5 min in an atmosphere containing labeled CO_2.

The autoradiographs obtained show that *leaf areas with a persisting luminescence are completely devoid of photosynthesis.*

2) Quantitative measurements

Whole leaves of *Hibiscus sinensis* and *Vitis vinifera* were treated with simazine. The course of luminescence during prolonged observation, as measured with a photomultiplier and recorded with micro-computer graphics, is shown in Fig. 3. A leaf kept in the dark overnight shows a relatively strong luminescence, the halflife time of which is 2–3 seconds according to one-shot measurements. During continuous observation in pulse light, the luminescence diminishes progressively (Fig. 3a, e) and eventually reaches the level of that of a pre-illuminated leaf (Fig. 3b).

If the whole leaf area is poisoned with simazine, the luminescence intensity constantly remains on its high initial value whether the leaf was pre-illuminated or kept in the dark (Fig. 3c).

A leaf with only a part of its surface influenced by simazine (corresponding to the leaf in Fig. 1a) behaves in an intermediate manner (Fig. 3d).

Relations between luminescence intensity of leaves completely blocked with simazine and the intensity of the exciting pulse light are given in Fig. 4. So far technical limits allow low light intensities only (up to 15 W/m²). Within this range doubling of the exciting light adds a constant amount to luminescence.

The intensity of luminescence is fairly well reproducible if the leaf samples are selected carefully (identical age, chlorophyll content etc.). Measurements were normally repeated on ten different leaves, where the standard deviation did not exceed 16%. The absolute values of luminescence depend on the properties of the leaf. A typical value for a healthy leaf from the middle of the vegetation

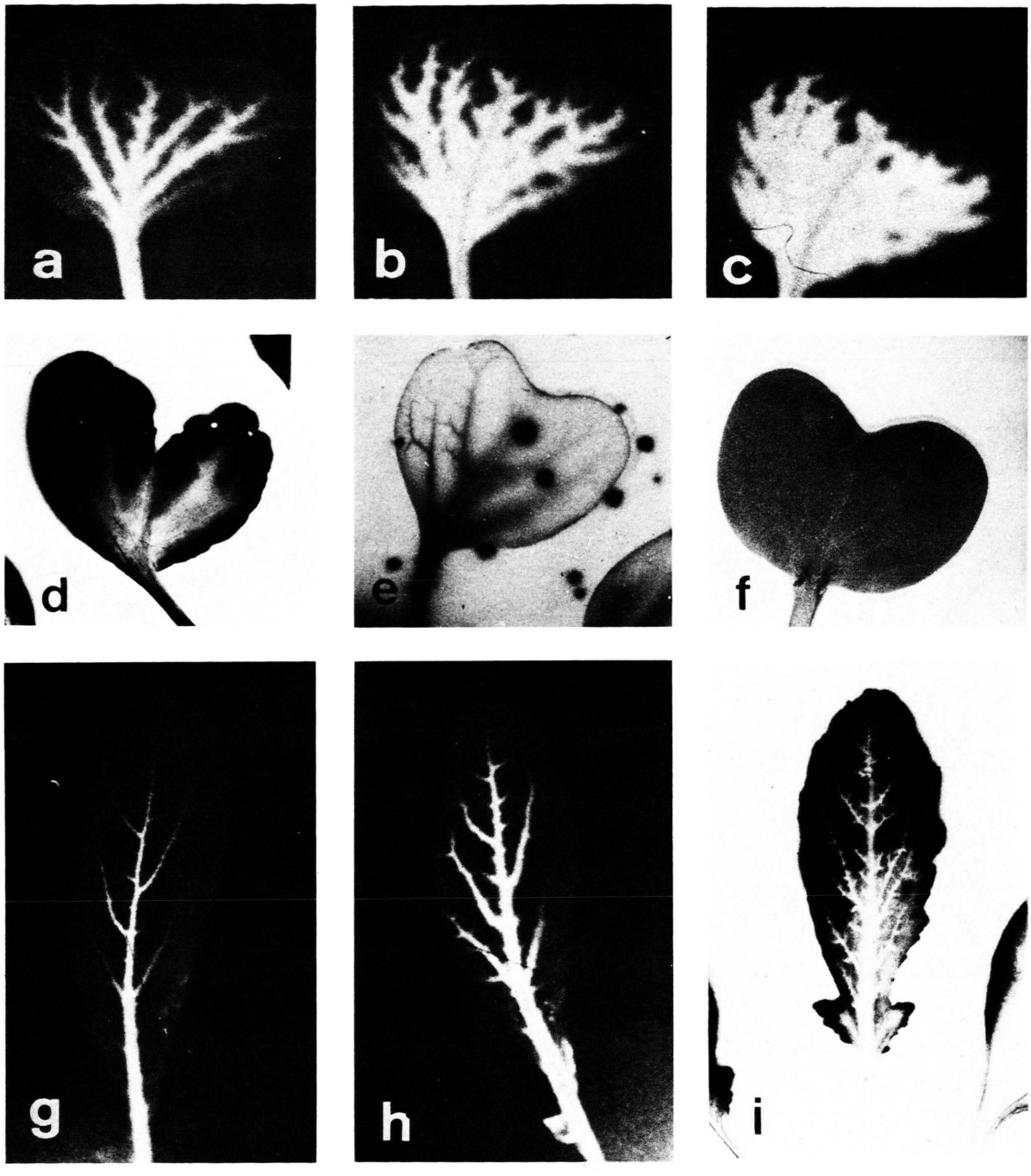

Fig. 2. Cotyledons and secondary leaves of *Raphanus sativus* after the uptake of simazine via the petiole. a)–c) Phytoluminograph after 5, 10 and 20 min treatment. d) Autoradiograph after a 10 min treatment with simazine an labelling of the leaf with $^{14}CO_2$ (5 min); there is no CO_2-fixation and hence no photosynthesis near the veins. e) Autoradiograph after a 10 min incubation in radioactive simazine. f) Control: normal visual appearance of a cotyledon after 2 d treatment with simazine. g) Luminograph of a secondary leaf after a 5 min and after a 10 min treatment. i) Autoradiograph of a leaf treated 15 min and labelled with $^{14}CO_2$.

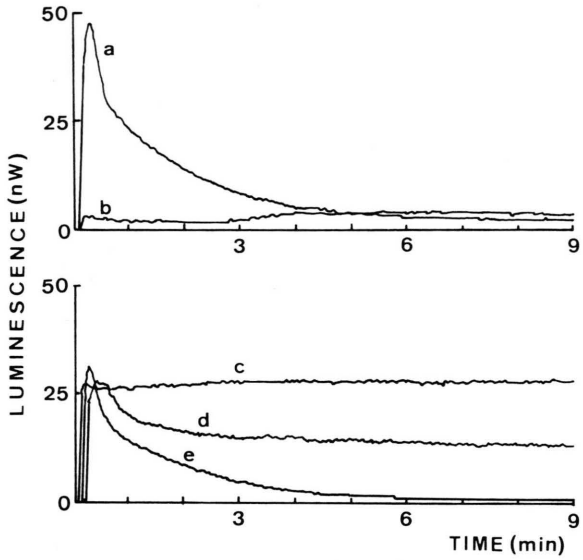

Fig. 3. Course of luminescence of *Vitis* leaves under white pulse light. The leaves were illuminated with four pulses per second (length of pulse = 0.08 sec) and luminescence was measured between the pulses. Its maximal intensity corresponds to 30 μW/m² of leaf surface approximately. a) Well developed leaf kept in the dark for 30 min; b) same leaf preincubated in light (100 W/m²) for 30 min; c) young leaf after 24 h application of simazine via the petiole; d) same leaf as c) after 12 h treatment. For its image under the luminoscope see Fig. 1; e) control: same leaf before treatment.

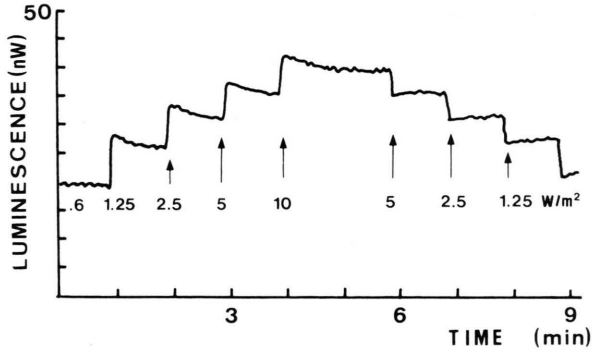

Fig. 4. Luminescence of *Elodea* sprouts (2 g wet weight = 2.3 mg of chlorophyll $a + b$) submersed in a saturated simazine solution (4 mg/l). The intensity of the exciting pulse light was increased stepwise from 0.6 W/m² to 10 W/m² in one minute steps. Doubling of the light intensity adds constant amounts to luminescence values.

period is 20 μW/m² of surface as estimated from the sensitivity of our photomultiplier and the optical conditions of our device. This corresponds to one or two ppm of the exciting light.

Since the application of simazine via the petiole or the roots of terrestrial plants leads to unknown local concentrations within the leaf, further studies were carried out with *Elodea canadense*. Due to the submersed growth of this species the inhibitors could be applied in defined amounts and penetrated quickly. Fig. 5 shows the effect of different amounts of simazine on the luminescence of submersed *Elodea* sprouds. At low concentrations there exists a linear correlation between the amount of inhibitor added and luminescence, indicating a high affinity of the inhibitor for its binding site in the photosynthesis system. The diagram allows estimation of the maximum number of binding sites. The experiment was repeated ten times and in all cases the result indicated a relation of 4–8 binding sites per 100 molecules of chlorophyll.

3) Comparative fluorescence analyses

At the beginning of our experiments we looked for relations between fluorescence and luminescence. The phenomena seem rather similar. However, the difference of fluorescence intensity in inhibited vs. normal areas of a leaf (Kautsky effect) did not exceed 50%. Photographs of such leaves show a rather low contrast between damaged zones and their surroundings. As a consequence quantitative measurements of different leaves were difficult to compare. Therefore fluorescence analysis was abandoned in further studies.

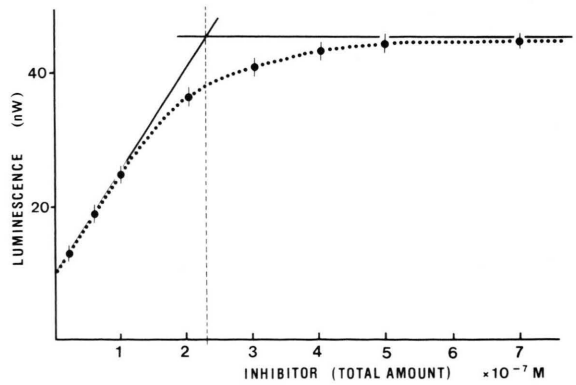

Fig. 5. Induction of luminescence of *Elodea* sprouts (same conditions as in Fig. 4) by increasing amounts of simazine. The plants were continuously illuminated with pulse light. The amount of inhibitor was increased every 30 min. This time was sufficient for luminescence to reach a constant level depending on the amount of inhibitor added. DCMU and simazine give identical results.

Conclusions

The luminescence phenomena described may be considered as a special case of the Kautsky effect, which shows up as a fluorescence increase in leaf areas where photosynthesis is blocked somehow. Sometimes even a secondary rise of the luminescence after its first peak was obtained, as described by Walker [10] for fluorescence induction in spinach leaves. This effect could, however, not be reproduced regularly, and so far the conditions under which it occurs remain unclear.

Although the Kautsky effect has been used successfully to study photosynthesis inhibition [8, 9] we feel that luminography instead of fluorescence analysis offers advantages which out-weight the more elaborate apparatus needed.

1) The difference between damaged and control areas is nearly 100% as compared to a difference of 50% obtained with fluorescence measurements.

2) Fluorescence is also present in dead parts of green plants, allowing no distinction between parts of a leaf where the energy flow of photosynthesis is disturbed reversibly and parts where the photosynthetic structures are disintegrated irreversibly due to non-specific damage like freezing or heat. These two levels of damage are easily differentiated by phytoluminography:

a) healthy areas of leaves pre-incubated in the dark show a luminescence which disappears within some minutes due to the observation light. If the leaf is pre-illuminated, there is only a very faint luminescence from the beginning.
b) leaf areas with blocked or otherwise disturbed photosynthesis always show a strong luminescence which does not diminish during prolonged observations.
c) areas with disintegrated photosynthetic structures show no luminescence at all, though the chlorophyll may still be present.

There is still a lack of knowledge on photosynthetic activity during rise and fall of luminescence. Although we know that maximal luminescence indicates a total lack of photosynthesis, we were not able to get reliable values for these transitional stages. The results of Laasch *et al.* [11] who find 300–500 chlorophyll molecules per inhibitor binding site indicate that photosynthesis is inhibited before luminescence has reached its maximum.

Although the analysis of transitional stages remains to be done, we are already using phytoluminography as a valuable tool for applied work to monitor activity of herbicides or other photosynthesis inhibitors such as fungal toxins or root exudates.

Acknowledgements

We are most grateful to Dr. H. Steffan for the isotope labelling experiments, to Professor H. K. Lichtenthaler and Professor A. Wild for critical discussions and for the skilled technical assistance of Margit Schneider, Doris Schneider, and R. Wind.

[1] B. L. Strehler and W. Arnold, J. Gen. Physiol. **34,** 809 (1951).
[2] S. Malkin, in: Photosynthesis I (A. Trebst and M. Avron, eds.), Springer Verlag Berlin Heidelberg New York 1977.
[3] J. Lavorel, Bioenergetics of Photosynthesis (Govindjee, ed.), 223, Academic Press New York 1975.
[4] L. O. Björn and A. S. Forsberg, Physiol Plant **47,** 215 (1979).
[5] P. V. Sane, T. S. Desai, and V. G. Tatake, Z. Naturforsch. **35,** 289 (1980).
[6] W. F. Bertsch and J. R. Azzi, Biochim. Biophys. Acta **94,** 15 (1965).
[7] J. D. Hiscox and G. F. Israelstam, Can. J. Bot. **57,** 1332 (1979).
[8] K. Pfister and H. K. Lichtenthaler, Z. Naturforsch **36,** 645 (1981).
[9] N. R. Baker and M. Bradbury, in: Plants and the Daylight Spectrum (H. Smith, ed.), Academic Press New York (in press).
[10] D. A. Walker, Planta **153,** 273 (1981).
[11] H. Laasch, K. Pfister, and W. Urbach, Z. Naturforsch. **36 c,** 1041–1049 (1981).
[12] J. L. Ellenson and R. G. Amundson, Science **215,** 1004 (1982).

Note added in proof:

After the conclusion of our manuscript Ellenson and Amundson [12] published a paper on the application of phytoluminography (DLE imagery) for the detection of SO_2-stress in soybean leaves, which supports and enlargens our results.