Biological Systems to Assay Herbicidal Bleaching

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Two strains of *Scenedesmus acutus* were found useful to study the influence of bleaching agents on either the greening process or the fully pigmented algal cell during growth. Both physiological conditions exhibit high sensitivity to bleaching herbicides. With this new assay, contrasting bleaching effects with the same compound can be found allowing differentiation of the herbicidal action of bleaching agents which apparently is a multifunctional one. Furthermore, the I_{50} can be determined rather rapidly in a simple graphical method by a Dixon plot. A subsequent application of bleaching herbicides to cultures of the fungus *Phycomyces blakesleeanus* rules out a possible specific action of the compounds assayed on chlorophyll or photosynthetic redox carriers. This latter assay can show whether or not the herbicides tested perform a direct inhibition of carotene synthesis as is the case with difunon or SAN 9789.

Introduction

Microalgae are a convenient tool for the study of herbicides affecting the photosynthetic apparatus. Although not yet fully recognized, they exhibit several advantages as compared to higher plants:

1. Suitable species can be easily cultured under semi-sterile conditions in well defined liquid and simple media with high growth rates under continuous light.
2. Controlled growth conditions can be maintained.
3. Herbicides can be exactly applied via the culture medium.
4. Inhibitor effects develop quickly after application [1].
5. Absence of long-distance transport allows for small substance concentrations to be tested.
6. Absolute quantitative changes of cellular compounds can be rapidly and reproducibly determined when referred to the (constant) culture volume.
7. Determination of uptake, accumulation and excretion of herbicides (and metabolites) can be followed [2].
8. Handling and determination of gas exchange can be done rather conveniently.
9. Direct comparison of cellular and cell-free photosynthetic systems is possible with some species. For some algae, *e. g. Bumilleriopis filiformis*, chloroplasts with high photosynthetic activity can be obtained and a direct comparison of herbicidal influence on the cell and its cell-free photosynthetic system can be made (see *e. g.* [13]).
10. In connection with the above-mentioned criteria herbicidal activity can be checked on redox carriers and pigment inventory during autotrophic growth and during the development of the photosynthetic apparatus as well.

This paper reports some progress on the last point. Most microalgae (except for *Euglena*, which is a rather unique species) build up a green chloroplast when grown in the dark in the presence of glucose. We succeeded, however, to isolate a mutant strain WDG of *Scenedesmus acutus* which stays pale during heterotrophic growth and turns green after transfer to autotrophic conditions.

Material and Methods

Assays were made in a greening system with a strain of *Scenedesmus acutus* WDG having no chlorophyll and no carotenoids when grown in the dark. After having been placed into autotrophic medium and illuminated as described below, herbicides were added and chlorophyll content was determined after 5 days (for methods see [4]). With the wild strain of *Scenedesmus acutus* (276-3a, Algae Collection of the University of Göttingen) the bleaching effect of herbicides on growing cells with full pigment inventory was measured two days after application.

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Abbreviations: Chl, chlorophyll a+b; pcv, packed cell volume; see Table I for herbicide trade names.

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Cultivation of algae was carried out in a growth apparatus (Kniese, Marburg, Germany) at 22–23 °C with 250 ml vessels provided with sterile air enriched with 5% CO_2, v/v. The autotrophic cultures were illuminated continuously by a bank of fluorescent lamps (Osram L 6 W/32 and L 65 W/25) with an intensity of 10,000 lux (equivalent to 35 W/m²; Yellow Springs, Radiometer, model 65 A). For autotrophic growth a sterile mineral medium according to Kunert *et al.* [5] was used. For heterotrophic growth in the dark 0.5% glucose and 0.25% Difco yeast extract were added to the autotrophic medium.

The strain 1+ of *Phycomyces blakesleeanus* of the Halbsguth collection (University of Frankfurt) was cultivated in Erlenmeyer flasks on a glucose/asparagine liquid nutrient medium at 23 °C in the dark as described [6].

β-carotene extraction and quantification was carried out according to Sandmann and Hilgenberg [7].

Results

During greening in the light, strain WDG of *Scenedesmus* precultured in the dark shows high sensitivity during the greening process against the pyridazinone herbicide SAN 9789 at concentrations of about 10^{-8} M (Fig. 1 A). In this system linearity between reciprocal value of chlorophyll content and herbicide concentration is established and the Dixon plot [8] can be employed for cellular systems determining the I_{50} as 8.8×10^{-9} M. The same figure can be arbitrarily estimated directly from the graph of Fig. 1 A. With concentrations of 5×10^{-8} M SAN 9789 and higher greening is inhibited completely. Due to the good linearity of the Dixon plot the assay using the greening process allows for the

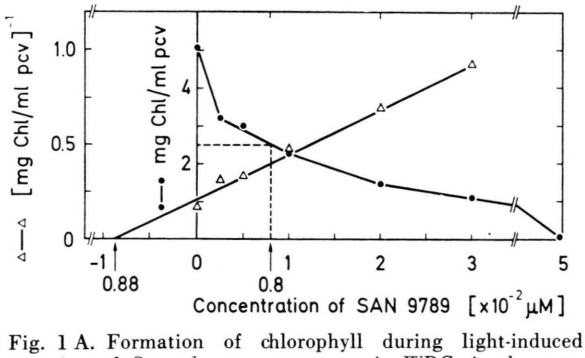

Fig. 1 A. Formation of chlorophyll during light-induced greening of *Scenedesmus acutus*, strain WDG, in the presence of SAN 9789.

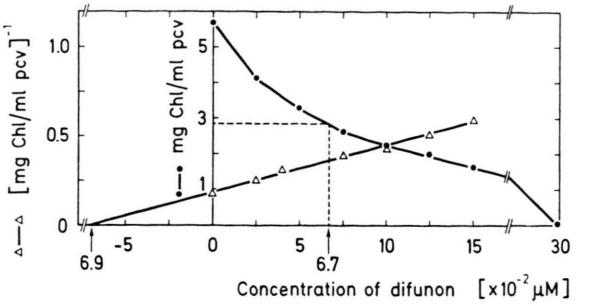

Fig. 1 B. Formation of chlorophyll during greening of *Scenedesmus acutus*, strain WDG, in the presence of difunon (EMD-IT 5914).

Table I. The influence of some herbicides on the pigment apparatus.

Herbicide added	(A) mg Chl/ml pcv with 0.1 µM herbicide in greening experiment (strain WDG)	% of control	(B) mg Chl/ml pcv with 1.0 µM herbicide in growth experiment (wild type)	% of control
Control	4.08	100	13.81	100
Difunon	1.61	39	2.07	15
SAN 9789	0	0	1.38	10
SAN 9774	1.36	33	13.12	95
BAS 44521	2.98	73	7.04	51
DRW 320	3.68	90	13.40	97
Bentazon	3.26	80	13.21	95
Nitrofen	2.78	68	4.83	35
Diuron	1.25	31	12.43	90

(A) Herbicides present during a 5 days greening time of *Scenedesmus acutus*, strain WDG.

(B) Herbicides present during 2 days of cultivation of fully green *Scenedesmus acutus*, wild type.

In both assays, cell density was 0.3–0.6 µl packed cell volume per ml algae suspension. No growth occurred during the experimental time used here. Greening in (A) started on the 4th and 5th cultivation day only.

Herbicides:

Difunon (EMD-IT-5914): 5-dimethylamino-methylene-2-oxo-4-phenyl-2,5-dihydrofurane-carbonitrile-(3); [from Celamerck];

SAN 9789: 4-chloro-5-methylamino-2-(3-trifluoromethylphenyl) pyridazin (2 H)-one; [from Sandoz AG];

SAN 9774: 4-chloro-5-amino-2-(3-trifluoromethylphenyl)-pyridazin-3 (2 H)-one; [from Sandoz AG];

BAS 44521: 4-chloro-5-methoxy-2-(3-trifluoromethylphenyl)-pyridazin-3-(2 H)-one; [from BASF AG],

DRW 320: α-*tert*.butyl-α-cyano-N (2-trifluoromethyl-4-chloro)-phenylhydrazone; [from Bayer AG];

Bentazon: 3-isopropyl-2,1,3-benzothiadiazin-4-one-2,2-dioxide; [from BASF AG];

Nitrofen (TOK): 2,4-dichlorophenyl-*p*-nitrophenyl ether; [from Rohm and Haas];

Diuron: N'-(3,4-dichlorophenyl)-N,N-dimethylurea; [from Riedel de Haen].

determination of the I_{50} with 2 to 3 data, which is not possible with direct plotting. Further, the herbicide concentrations applied must not be close to the expected I_{50} which is necessary when the herbicides are tested for their effect on pigments during growth.

Difunon acts in the same way as SAN 9789 in the greening experiment (Fig. 1 B). The I_{50} from the Dixon plot is 6.9×10^{-8} M. Concentrations smaller than 3×10^{-8} M do not exhibit a bleaching effect.

In Table I the action of different herbicides on the greening process and on chlorophyll content of the wild strain is compared. A strong inhibition of chlorophyll formation during greening is seen with SAN 9789, SAN 9774, difunon and diuron, to a minor extent with BAS 44521 and nitrofen (part A). In the growth experiment of the wild type, however, all herbicides mentioned except SAN 9774 and diuron show an inhibitory effect on the chlorophyll content of the developed photosynthetic apparatus (part B). Nitrofen is more active in the growth experiments, the chlorophyll level is markedly affected by SAN 9789 or BAS 44521, which have either a methylated amino or a methoxy group while SAN 9774 – with a non-methylated amino group – is inactive. In the greening experiment, however, the latter compound is more active than BAS 44521. The dimethylamino derivative (SAN 6706) was shown to be an effective bleaching agent in heterotrophically grown *Chlorella fusca* [9].

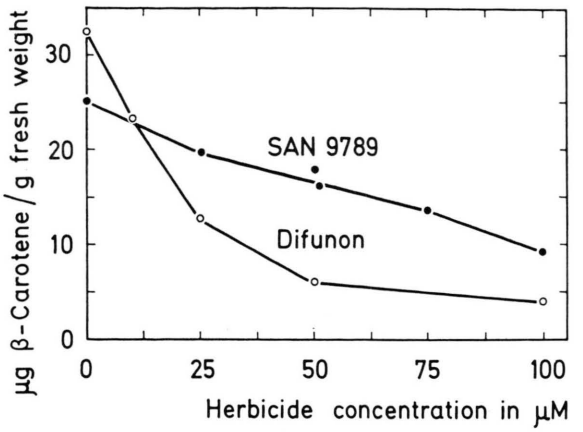

Fig. 2. Inhibition of β-carotene formation of *Phycomyces blakesleeanus* in the presence of difunon or SAN 9789.

Both difunon and SAN 9789 can decrease β-carotene formation when added to the fungus *Phycomyces* from 10 to 100 μM in the dark (Fig. 2). As in this non-photosynthetic organism the conversion of carotene into other terpenoids is very small in contrast to carotene formation [10] this experiment indicates that SAN 9789 as well as difunon directly inhibit carotene formation. This was already suggested as the primary herbicidal action for pyridazinones (see *e. g.* [11]) and difunon [12] using higher plants and algae.

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