Plant Cell Reports (1989) 8:399–402

Plant Cell Reports

A microtest system for the serial assay of phytotoxic compounds using photoautotrophic cell suspension cultures of *Chenopodium rubrum*

Jutta Thiemann, Arnold Nieswandt, and Wolfgang Barz

Lehrstuhl für Biochemie der Pflanzen, Westfälische Wilhelms-Universität, D-4400 Münster, Federal Republic of Germany

Received August 11, 1989 – Communicated by E. W. Weiler

ABSTRACT

Chenopodium rubrum photoautotrophic cell suspensions were grown in plastic tissue culture dishes under photoautotrophic conditions. Growth was monitored by measuring cell number, packed cell volume, chlorophyll content and oxygen production. Such microtiter dishes are suitable systems for the serial assay of growth inhibition and various physiological effects (i.e. chlorophyll fluorescence, cell viability, oxygen production) of photoautotrophic cells as caused by herbicides and fungal phytotoxins. The applicability of the test system is discussed.

Abbreviations

pcv = packed cell volume; fr.w. = fresh weight; rpm = revol. per minute; DMSO = dimethyl sulfoxide; PMS = phenazine methosulfate; NBT = nitro-blue tetrazolium chloride;

INTRODUCTION

Heterotrophic plant cell cultures are suitable systems for testing the biological activity and the cellular metabolism of nutrients, toxins, or environmental and agrochemical compounds (Gressel 1984; Grossmann et al. 1982; Mumma and Davidonis 1983; Sandermann et al. 1984). Several assay systems have been devised allowing the serial monitoring of numerous compounds or different concentrations in small cell culture volumes in a short period of time (Potrykus et al.1979; Grossmann 1988).

Photoautotrophic plant cell suspension cultures which greatly resemble mesophyll cells (Hüsemann 1985) can well be used for the assay of photosynthesis-inhibiting herbicides (Sato et al. 1987). Such cells are routinely grown in Erlenmeyer or double-tier flasks (Hüsemann and Barz 1977) so that serial assays require considerable effort in time, labour, and shaker space. To further promote the applicability of these green cells for the serial determination of phytotoxic compounds we have now established a microtest system using Chenopodium rubrum photoautotrophic cell suspensions kept in transparent microtiter dishes. Our investigations were designed

1) to demonstrate the efficient growth of the photoautotrophic cells in small volumes as monitored by cell number, pcv, chlorophyll content, and oxygen production, and

2) to measure the inhibitory effects of various herbicides and a fungal phytotoxin on cellular growth and several physiological parameters of the green cells.

Offprint requests to: W. Barz

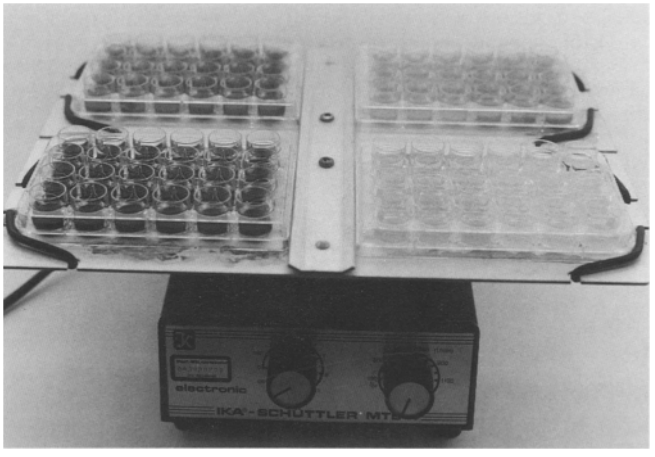

Fig.1: Photograph of transparent microtiter dishes used as a microtest system for photoautotrophic growth of Chenopodium rubrum cell suspensions; dishes are shown without lid

MATERIAL AND METHODS

Cell culture. Photoautotrophic Chenopodium rubrum cell suspension cultures (Hüsemann and Barz 1977) were used at the early stage of stationary growth. Cells (100 mg fr.w. x 1.5 ml^{-1}) were suspended in fresh culture medium.

Loading of microtiter dishes. Plastic microtiter dishes (8.5 x 12.5 cm, Greiner, Nürtingen, FRG) containing 24 wells with 3 ml volume each were loaded under aseptic conditions with 1.5 ml cell suspension per well. Prior to the application of cells to the dishes the cell suspensions were mixed with the herbicides and fusaric acid which were applied in sterile-filtered methanol solutions so that the organic solvent in the cell culture medium was set at 0.25 % (v/v). The dishes further contain 15 additional compartments (volume 1 ml) which were each filled with 750 µl $K_2CO_3/KHCO_3$-buffer generating a 2% CO_2 partial pressure (Hüsemann and Barz 1977) in the gas sphere of the dishes. Four such microtiter dishes tightly sealed with parafilm were incubated on a MTS4 shaker (Jahnke und Kunkel, Staufen, FRG) at 350 rpm under continuous white light (80µE·m^{-2}·s^{-1}) at 25°C.

Determination of growth parameters. For the determination of cellular growth in the microtiter dishes the cell suspensions from an appropriate number of wells were removed under sterile conditions after various time intervals. For each value the cells of four wells were separately measured and the standard deviation calculated.

PCV was determined by centrifugation (1400 rpm/10 min, Sigma 2KD-Centrifuge) of cells in calibrated, conical special tubes (volume 2 ml, Fa. Schlee, Witten, FRG).

Cell number was determined by resuspending the cells from the pcv assays and a 200 µl aliquot was withdrawn. The cells were mixed with 4.8 ml chromic acid (10% v/v), incubated at 70°C for 10 min and counted in a Fuchs-Rosenthal chamber under a microscope.

Chlorophyll content was measured photometrically (Ziegler and Egle 1965) using acetone extracts of the residual cell suspensions from the pcv assays.

Production of oxygen of the cells was assayed with a Hansatech (Bachofer, Reutlingen, FRG) oxygen meter equipped with a Clark electrode. After a short preincubation period and determination of the dark respiration the net oxygen production was measured using the cells from one well. Light intensity was set at 1050 µE x m^{-2} x sec^{-1}.

Assay of herbicide tolerance. The inhibitory effect of herbicides on the photoautotrophic cells was measured after 8 days of incubation. For each herbicide concentration one dish was used; the cells from 12 wells were assayed for measuring pcv and chlorophyll content. The cells from the other 12 wells were separately taken for recording oxygen production, chlorophyll fluorescence and cell viability; the standard deviations were calculated.

Chlorophyll fluorescence was recorded using the same cell batch as used for the measurements of oxygen production. White light was filtered through a Corning 4-96 filter to give blue actinic light. The filter (Wratten 88A) used in the detector probe transmits red light (λ>700 nm). The cells were kept in darkness for 5 min, illuminated for 1 min and during this time period fluorescence intensity was recorded. The initial maximum signal (F_P) and the terminal signal (F_T) of fluorescence were used to calculate the R_{fd}-values according to the equation $R_{fd} = (F_P - F_T)/F_T$.

Cell viability is represented by the ability of cells to generate reducing equivalents as measured by the reduction of NBT. 1 ml cell suspension from the determination of the oxygen production was thoroughly mixed with 1500 µl ethylacetate, 10 µl DMSO and 200 µl test reagent (1 mM NAD^+, 1 mM PMS, 1 mM NBT, 50 mM TRIS-HCl, pH 7.5) on a vibromixer for 30 sec. After 1 hour at 20°C the violet ethylacetate phase was photometrically assayed at 520 nm against an ethylacetate blank. This procedure had previously been checked for linearity of pigment production with different concentrations of NADH from a standard solution (data not shown).

Chemicals. Herbicides were obtained from Bayer AG (Leverkusen), fusaric acid came from Sigma Corp., and NBT and PMS were purchased from Serva.

RESULTS

Growth of photoautotrophic cells in microtiter dishes

The growth of photoautotrophic Chenopodium rubrum cell suspensions in the wells of transparent microtiter dishes (Fig.1) under photoautotrophic conditions proceeds essentially with the same rate as previously observed using double-tier Erlenmeyer flasks (Hüsemann 1985). As documented by the data for cell number, chlorophyll content and oxygen production (Fig.2), a short lag-phase of 2-3 days is followed by a linear growth rate up to day 10. During the subsequent stationary phase cell expansion occurs as shown by a substantial increase in pcv (Fig.2) up to day 18. Beyond this time gradual cellular decay is observed as shown by the decrease in chlorophyll content, oxygen formation and cell number (Fig.2). Increase and decrease of total chlorophyll content per well are significantly correlated with the course of total oxygen production per well showing maximum photosynthetic activity of the cells around days 8-12. Beyond days 16-18 the cells increasingly suffer from evaporation of water from the culture medium thus leading to rather viscous suspensions.

In general, the satisfactory growth of photoautotrophic Ch. rubrum cells in the microtiter dishes leads to the assumption that this set up will provide a versatile system for the serial assay of biologically active compounds.

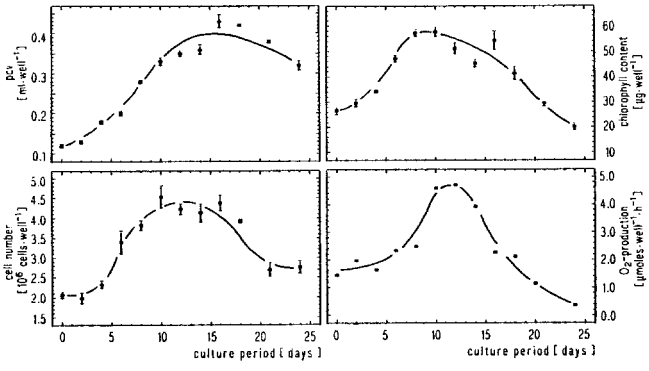

Fig.2: Growth of photoautotrophic Chenopodium rubrum cell suspensions in plastic microtiter dishes.

Effect of herbicides on photoautotrophic cells in microtiter dishes. To demonstrate the application of the microtiter system the herbicides metribuzin (an asymmetric triazinone), diuron (an urea derivative) propanil (a carboxyanilide) and dinoseb (a nitrophenol derivative) were incubated with photoautotrophic Ch. rubrum cells in a concentration range of 10^{-7} - 10^{-5}M. The mode of action of these herbicides on photosynthetic electron transport (relevant for all herbicides tested), uncoupling of ATP-production in photosynthesis and respiration (dinoseb) are described in the literature (Fedtke 1982).

The herbicide assays were evaluated for pcv, chlorophyll content, oxygen production and cell viability after 8 days of incubation. Furthermore, slow fluorescence kinetics during a dark to light transition were recorded. The initial and the terminal values for chlorophyll fluorescence during binding of herbicides to thylakoids are supposed to be indicative of the photosynthetic capacity of photosystem II (Havaux and Lannoye 1985; Klosson and Krause 1981; Kitajima and Butler 1975). Therefore, the relative fluorescence F_P and the R_{fd}-values allow an estimation of the photosynthetic capacity of herbicide-affected green cells.

For the determination of cell viability an extraction procedure for the water insoluble dye was developed which in comparison to earlier reports (Dixon 1985; Withers 1980) allowed the analyses of numerous assays in a much shorter time.

Efficacy of herbicide action is normally recorded in terms of the pI50-values (Fedtke 1982) which are appr. 6.5-6.7 for metribuzin. The data measured for this herbicide in the microtest system with Ch.rubrum cells show (Fig.3) that 10^{-7}M metribuzin does not alter pcv and chlorophyll content during the experimental period. A small increase in chlorophyll fluores-

cence, reduced values for oxygen production and the R_{fd} value indicate that this herbicide concentration moderately reduces photosynthetic electron transport. 10^{-6}M metribuzin represents a lethal concentration best demonstrated by the data for oxygen production, cell viability and the R_{fd}-value. The decrease in chlorophyll content is reflected by a similarly reduced value for its fluorescence. The residual values for pcv, chlorophyll content and fluorescence (Fig.3) even found at 10^{-5}M herbicide after 8 days further decrease upon longer incubation periods; photobleaching of chlorophyll then becomes more important.

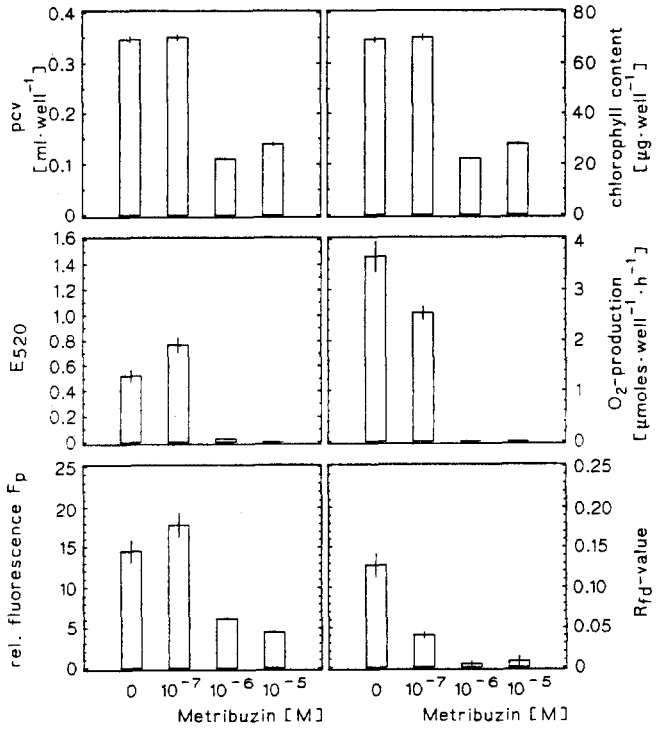

Fig.3: Concentration dependent effect of the herbicide metribuzin on growth, photosynthetic capacity and cell viability of photoautotrophic Chenopodium rubrum cell suspensions

The data in Fig.3 indicate that the lethal concentration of metribuzin for Ch. rubrum photoautotrophic cells appears to be between 10^{-7} and 10^{-6}M. Additional experiments with the microtest system and metribuzin concentrations stepwise increased from 10^{-7}M to 10^{-6}M allowed the lethal concentration to be set at 2×10^{-7}M (data not shown). An identical value for the lethal metribuzin concentration was later confirmed using our photoautotrophic Ch.rubrum cells grown in double-tier flasks (Hüsemann and Barz 1977) and the same methods for evaluating herbicide action(data not shown).

The herbicide diuron (pI50:6.7-7.5; Fedtke 1982) significantly affects Ch. rubrum cells at 10^{-7}M (Fig. 4) as shown by the values for pcv,chlorophyll content, cell viability and oxygen production. Electron transport (decrease in the R_{fd}-value) also appears to be reduced though a slight increase in chlorophyll fluorescence (F_p-value) indicates that the binding of diuron to the Q_B-protein of the thylakoid membranes (Fedtke 1982) has not yet irreversibly damaged the membranes. Diuron at 10^{-6}M and higher concentrations (Fig.4) led to cell death essentially as described for metribuzin.

Propanil (pI50:5.6-6.8; Fedtke 1982) also tested at 10^{-7} to 10^{-5}M showed moderate herbicide effects only at 10^{-6}M as demonstrated by an increased F_p-value for chlorophyll fluorescence. Oxygen production, cell viability and the R_{fd}-value, however, decreased to 0 at a herbicide concentration of 10^{-5}M (data for propanil not shown).

In case of the uncoupler dinoseb (pI50:5.1-5.8; Fedtke 1982) the half maximum inhibition of the Hill reaction can be expected to occur between 2 and 8 x 10^{-6}M. Evaluation of this herbicide in the microtest system using the same procedures showed the lethal concentration to be between 10^{-7} and 10^{-6}M (data for dinoseb are not shown). However, in contrast to the other herbicides dinoseb led to a more rapid decrease in chlorophyll content (complete decay within 3 days at 10^{-5}M).

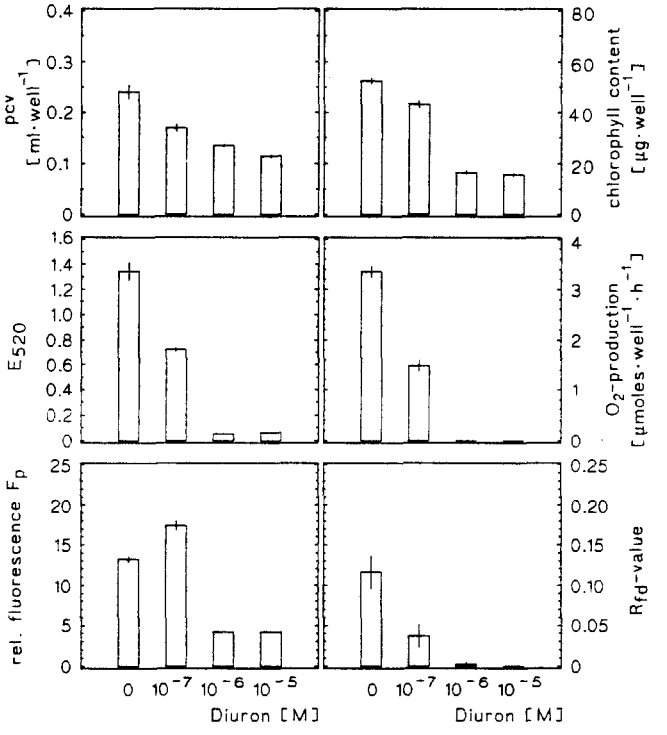

Fig.4: Concentration dependent effect of the herbicide diuron on growth, photosynthetic capacity and cell viability of photoautotrophic Chenopodium rubrum cell suspensions

Effect of fusaric acid on photoautotrophic cells in microtiter dishes. Fusaric acid (5-n-butyl-2-carboxypyridine) a powerful membrane-active phytotoxin produced by several Fusarium species (Mutert et al.1981) strongly inhibits photosynthesis of Ch.rubrum cells and leads to substantial photobleaching of chlorophyll (Barz 1981). When tested in the microtiter dishes with our cells either under white light or in darkness (Fig. 5) 50 µM fusaric acid completely blocked photosynthetic oxygen production accompanied by significant chlorophyll destruction. This decay of the photosynthesis pigments is much less obvious in the cells cultured in darkness,though cellular growth (pcv) will practically not occur under these conditions.

DISCUSSION

Exact measurements of easy-to-determine parameters for the accumulation of biomass and photosynthetic activity of photoautotrophic cells have demonstrated that such cell suspensions readily grow in small volumes (appr. 1-1.5 ml) under photoautotrophic conditions. The growth curves shown in Fig. 2 are essentially identical to the data obtained with Ch.rubrum cells kept

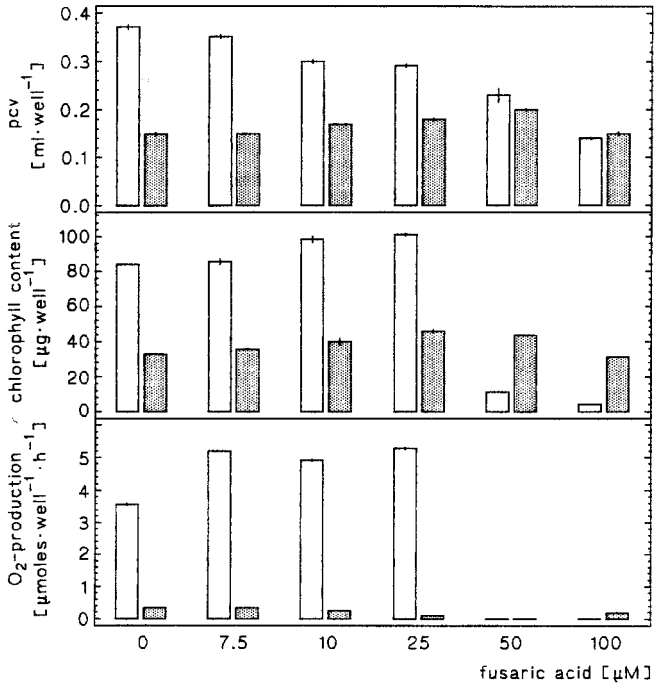

Fig.5: Concentration dependent effect of the phytotoxin fusaric acid on growth and photosynthetic capacity of photoautotrophic Chenopodium rubrum cell suspensions kept in light (open bars) and darkness (shaded bars)

in Erlenmeyer or double-tier flasks (Hüsemann and Barz 1977). The only noteworthy difference between the two culture methods concerns the much shorter stationary growth phase with subsequent rapid cellular decay (Fig.2) in the plastic dishes due to evaporation of water from the growth medium. In general, however, the microtiter dishes can well be applied for screening purposes with photoautotrophic cells.

The comparatively small values for the standard deviation in Fig.2-5 indicate that cells from a few wells only will be sufficient for each quantitative assay. This, however, requires that the loading of the microtiter dishes with the cells and the various measurements are precisely and carefully conducted under well established standard conditions.

In addition to the parameters determined in our experiment for cell growth and physiological activity other, non-destructive methods could also be applied. Using special micro-electrodes determinations of 1) changes in the conductivity of the nutrient medium (Hahlbrock und Kuhlen,1972), 2) nutrient uptake (i.e. NH_4^+, NO_3^-) or 3) pH value of the growth medium are possible and such data will also provide important information on the performance of a cell suspension (Grossmann 1988).

The data obtained with the herbicides (Fig.3 and 4) indicate that the procedure described represents a reliable technique to measure inhibitory or lethal concentrations which are essentially identical to literature reports (Fedtke 1982) which had been obtained with different methods.

One shaker-load of four microtiter dishes contains 96 wells so that numerous different concentrations or a substantial number of parallels can be measured in one experiment with a minimum amount of time, biomass and labour. It is herewith recommended that our microtest system using photoautotrophic cells allows the serial screening of compounds such as phytohormones, growth retardants, macro- or micronutrients, herbicides, phytotoxins or any other material with a biological activity affecting green plant cells.

Furthermore, the microtest system will be useful for either the analyses of elicitor- and sucrose-induced secondary compounds (Hüsemann et al.1989) or biotransformation reactions of xenobiochemicals in photoautotrophic cells if rapid and sensitive analyses (i.e. GC, HPLC) are available for the subsequent serial determination of compounds in small volumes of nutrient medium or cell extracts.

Acknowledgement

Financial support by Bundesminister für Forschung und Technologie, Bonn, and Fonds der Chemischen Industrie is gratefully acknowledged.

REFERENCES

Barz W (1981) Chemische Rundschau 34: 3-6
Dixon RA (1985) in: Dixon RA (ed) Plant Cell Culture, a practical approach. Practical Approach Services, IRL Press,Oxford Washington DC, pp 18-19.
Fedtke C (1982) Biochemistry and Physiology of Herbicide Action. Springer, Berlin Heidelberg New York, pp23-85.
Gressel J (1984) In: Maramorosch K (ed) Advances in Cell Culture Vol 3, Academic Press, London New York San Francisco, pp 93-181.
Grossmann K, Rademacher W, Jung J (1982) Plant Cell Reports 1:281-284.
Grossmann K (1988) In: Maramorosch K (ed) Advances in Cell Culture Vol 6. Academic Press, Orlando San Francisco, pp 89-136.
Hahlbrock K, Kuhlen E (1972) Planta 108:271-278.
Havaux M, Lannoye R (1985) Z.Pflanzenzüch. 95:1-13.
Hüsemann W, Barz W (1977) Physiol. Plant.40:77-81.
Hüsemann W (1985) in: Vasil IK (ed) Cell culture and Somatic Cell Genetics of Plants,Vol 2. Academic Press, Orlando San Diego New York, pp 213-252.
Hüsemann W, Fischer K, Mittelbach I, Hübner S, Richter G, Barz W (1989) In: Kurz WGW (ed) Primary and Secondary Metabolism of Plant Cell Cultures II, Springer, Heidelberg, pp 35-46.
Kitajima M, Butler WL (1975) Biochim.Biophys.Acta 376: 105-115.
Klosson RJ, Krause GH (1981) Planta 151:347-352.
Mumma RO, Davidonis GH (1983) Prog. Pestic.Biochem.3: 255-278.
Mutert WU, Lütfring H, Barz W, Strack U (1981) Z. Naturforsch. 36c:338-339.
Potrykus I, Harms CT, Lörz H (1979) Plant Sci. Lett.14: 231-235.
Sandermann H, Scheel D, Trenck Tvd (1984) Ecotoxicol. Environm. Safety 8:176-182.
Sato F, Takeda S, Yamada Y (1987) Plant Cell Reports 6:401-404.
Withers LA (1980) in: Ingram DS, Helgeson JP (eds) Tissue Culture Methods for Plant Pathologists, pp 63-70.
Ziegler R, Egle K (1965) Beitr. Biol.Planz 41:11-63.