A Simple Agar Plate Method, Using Micro-algae, for Herbicide Bio-assay or Detection

by S. J. L. WRIGHT
School of Biological Sciences
University of Bath
Bath BA2 7AY
England

INTRODUCTION

There is growing interest in the interactions of microbes with the pesticides which enter soil and aquatic environments. In view of their ubiquity, ecological importance and affinities with plants, it is appropriate to examine the interactions of the micro-algae with pesticides, especially herbicides.

Inhibitory effects of herbicides on micro-algae, especially green algae, have been widely reported and algal bio-assays for herbicides proposed (WRIGHT, 1974). Most algal bio-assay systems utilize liquid cultures. However, THOMAS et al. (1973) examined the effects of herbicides on algal growth using a method (IKAWA et al., 1969) in which the algae were dispersed in an agar medium.

In this paper a simple method is described for determining the inhibitory effect of herbicides on the growth of micro-algae on agar medium. The technique, which is analogous to the agar diffusion method for antibiotic assay, may be used for rapid bio-assay of herbicides or detection of toxic residues. It is more convenient to use, especially with multiple samples, than a corresponding liquid culture assay (WRIGHT, 1972; 1974).

MATERIALS AND METHODS

Organisms and growth medium

Chlorella 211/8h (formerly Chlorella pyrenoidosa strain Emerson 3) was obtained from the Culture Centre of Algae and Protozoa, Cambridge, England. Another unicellular green alga, a Pleurococcus sp., was isolated in our laboratory from a pond wall. The algae were grown on a medium containing (g/l): KNO_3, 1.25; KH_2PO_4, 1.25; $MgSO_4.7H_2O$, 2.5; $Fe_2(SO_4)_3$, 0.004; EDTA, 0.05; to which was added 1ml of a solution containing (g/l): H_3BO_3, 2.9; $MnCl_2.4H_2O$, 1.8; $ZnSO_4.7H_2O$, 0.22; MoO_3, 0.018; $CuSO_4.5H_2O$, 0.08. The complete medium, at pH6.0, was solidified with 1% Ion Agar No.2 (Oxoid). Stock cultures were maintained at room temperature on agar slopes in daylight or fluorescent light and sub-cultured fortnightly. Cultures may be stored effectively in the dark at 5° for several months.

Bulletin of Environmental Contamination & Toxicology,
Vol. 14, No. 1 © 1975 by Springer-Verlag New York Inc.

Preparation of inoculum

Algal cultures were grown in liquid medium of the same composition under controlled conditions by the method previously described (WRIGHT, 1972; 1974). Cultures were removed after 2½ days, when in late exponential phase, and diluted with an equal volume of sterile medium to provide a homogeneous cell suspension of suitable density for inoculating agar plates. As a simple alternative to the use of closely controlled growth conditions, the algae may be grown to a suitable cell density in 4 days by bubbling filtered air into cultures standing at room temperature (ca. 22°) near a fluorescent light (65/80W).

Experimental procedure

An aerosol spray (Universal type) was used to uniformly inoculate the dry surface of the agar medium (15ml) in standard size Petri dishes. This was done by passing the spray over the medium surface in a zig - zag fashion under a protective canopy, taking 2 sec per plate. This method was quicker than using dropping pipettes and glass spreaders and gave consistently even inoculation of plates. The inoculum was absorbed by the agar surface which then had only a very faint green colour. Plates were normally used on the day of inoculation, although it was possible to use them some days later when stored in polythene bags in a cool cabinet.

Pure herbicides were dissolved in a suitable solvent, usually ethanol, and applied in different concentrations to 6.0mm diameter Antibiotic Assay Discs (Whatman). Discs were impregnated with herbicide solution either by addition, in 5 or 10µl aliquots, from micro-pipettes (method A), or, by dipping the discs into the solution (method B). In either case an equivalent volume of the solvent only was applied to control discs. Using method A the discs can be accurately loaded with the required amounts of herbicides by allowing the solvent to evaporate in between application of further aliquots. A standard procedure was adopted for impregnating discs by method B. The discs were held in forceps and a small sector immersed into the solution. The herbicide solution was quickly taken up by the whole disc, whereupon it was immediately removed and touched once onto the side of the glass vessel. This method gave surprisingly reproducible impregnation of the discs. By comparison of the inhibition of Chlorella caused by the herbicide barban applied by the methods A and B, it was estimated that the mean volume of solution absorbed per 6mm disc using method B was approx. 45µl, actual values ranging from 42 to 48µl. Although it is less rigidly standardized, Method B has the advantage of speed and simplicity. Herbicide impregnated discs were dried on metal gauze at room temperature or in a current of warm air before being placed on the inoculated agar surface. Standard size Petri dishes accommodate up to 4 discs, but in order to avoid merging of inhibition zones, no more than 2 discs per plate should be used.

Plates were incubated, agar surface uppermost, at room temperature (mean, 25°) on a white background 15 cm beneath twin 65/80W white fluorescent strip lights, providing 7,700 lux. Both Chlorella and Pleurococcus formed a dense mat of growth in 2 to 3 days, with clear zones around discs showing the extent of growth inhibition. Zone diameters on replicate plates were measured, using vernier calipers, after 2 or 3 days.

RESULTS AND DISCUSSION

Evaporation of the solvent from discs prior to placing on the agar ensured that only herbicide diffused into the medium. There was no growth inhibition around discs to which solvent only was added.

Using application method A, several phenylamide herbicides were tested against Chlorella. In all cases it was possible to establish a range over which growth inhibition, as determined by clear zone diameter, related directly to quantity of herbicide per disc. Inhibition by phenylcarbamates was in the order: barban > chlorpropham > propham, thus supporting previous observations with a liquid culture assay (WRIGHT, 1972). The results for chlorpropham and propham agreed with the relative toxicity of these herbicides to a sensitive plant sp. (CLARK & WRIGHT, 1970a). Zone boundaries, which varied in clarity with different herbicides, were well defined for barban and growth inhibition was linearly related to barban in the range 1 to 20µg per disc. Chlorella was markedly sensitive to inhibition by the triazine herbicide cyanazine, with a linear response in the range 0.1 to 0.8µg per disc. Corresponding ranges for the phenylurea herbicides monuron and fenuron were 0.4 to 1µg and 1 to 10µg per disc respectively. Other workers, using Chlorella liquid cultures assays, also found monuron to be more inhibitory than fenuron (GEOGHEGAN, 1957; KRATKY & WARREN, 1971). The lowest quantities of some herbicides causing inhibition of Chlorella growth, representing the limits for detection by the agar plate method, were (µg per disc): propham, 5; chlorpropham, 1; barban, 1; monuron, 0.4; fenuron, 1; propanil, 2; cyanazine, 0.1.

Aniline and 3-chloroaniline, microbial metabolites of propham and chlorpropham respectively, did not inhibit Chlorella growth even at levels as high as 50µg per disc; agreeing with previous observations on the non-phytotoxicity of these herbicide degradation intermediates (CLARK & WRIGHT, 1970b).

Application method B was used in studies on the comparative sensitivity of Chlorella and Pleurococcus to phenylamide herbicides. In each case examined Chlorella was the more sensitive and this was particularly evident with barban (Fig. 1). An assay for barban, applied to discs by method B, is also shown (Fig. 2). In cases where inhibition zone boundaries were indistinct (e.g. chlorpropham), the definition was improved by allowing overnight diffusion of herbicide from the discs into the agar prior to

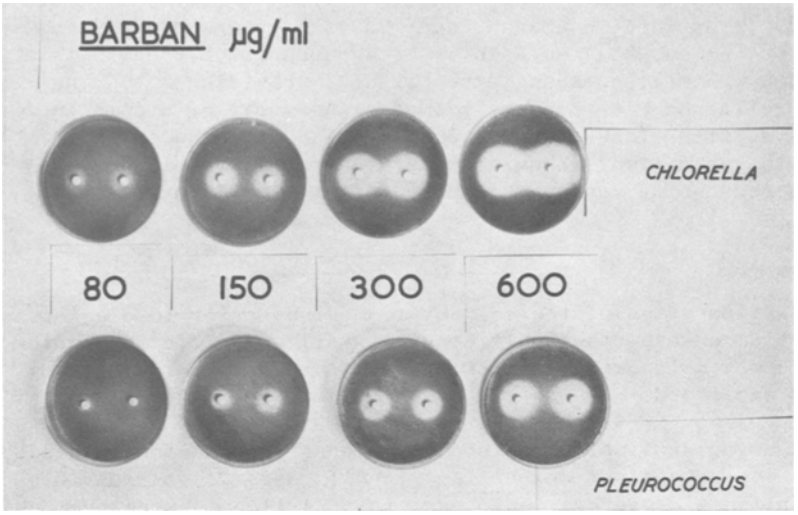

Fig.1. Comparison of the inhibition of growth of Chlorella and Pleurococcus, by barban. Discs were dipped in ethanol solutions of barban at the concentrations indicated (application method B).

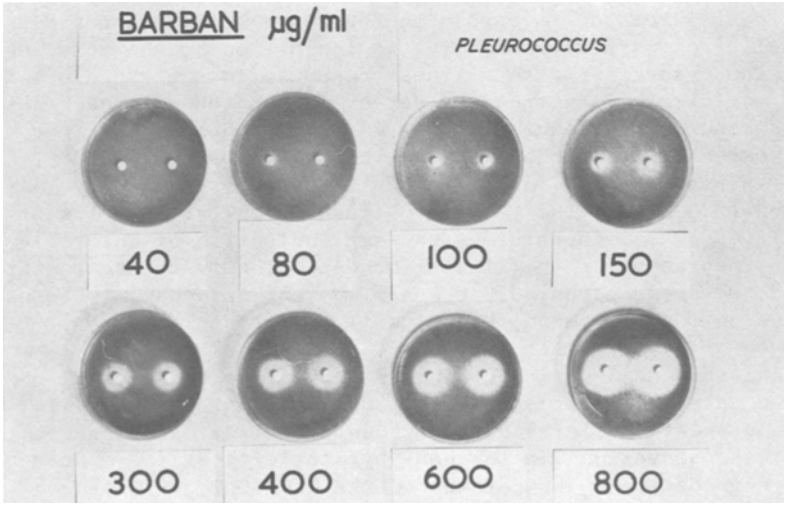

Fig.2. Barban bio-assay using Pleurococcus, showing some plates from the assay range. Discs were dipped in ethanol solutions of the herbicide at concentrations indicated (method B).

inoculation.

Spray - inoculation of the agar surface gave a rapid and even distribution of algal cells and hence uniformity among plates and between experiments. Basic precautions should be taken to prevent spread of the aerosol. The use of a large population of unicellular organisms for herbicide bio-assay ensures that individual variations in response are less influential than might be the case with higher plants.

Differences in potency among herbicides of the same chemical type are detectable by the algal assay, with indications that inhibition of algal growth parallels the order of relative phytotoxicity. The sensitivity of different algal spp. to individual herbicides may be compared. However, in attempting to compare the inhibitory action of different types of herbicide it might be necessary to take other factors (e.g. diffusion characteristics) into consideration. The readily available and easily cultivated Chlorella, which responds to the inhibitory action of diverse compounds, is in many respects suitable for general herbicide bio-detection. However, it seems possible that a wide screening of the micro-algae might provide species or strains with exceptional sensitivity to specific herbicides. Such cultures, if genetically stable, would be ideal for bio-assay purposes. The algal types screened need not be restricted to the unicellular green algae. We have observed that even filamentous blue-green algae, such as Anabaena spp., can be grown evenly in or on agar and that herbicide-inhibition zones are well defined. This, together with our observations (unpublished) on the high order of sensitivity of blue-green algae to some herbicides, indicates the breadth of scope in screening micro-algae for sensitive types.

The paper disc - agar diffusion technique is suitable for detection of herbicide (and possibly other pesticide) residues in soil or water, from which they are extractable into volatile organic solvents. Extracts could be stored as such, or alternatively, paper discs impregnated with the extracts could be stored dry, as could known standards, pending application to inoculated agar plates when convenient.

The method might find application in: primary screening for herbicidal or algicidal activity; evaluation of algicides; toxicity tests on known herbicide breakdown intermediates or products; studies on the action of pollutants and toxicants on growth and physiology of micro-algae. The use of large agar dishes, which are readily spray - inoculated, facilitates the testing of multiple samples.

SUMMARY

A simple, inexpensive method is described for the bio-assay of herbicides using micro-algae growing on agar plates. A result is obtainable in 2 days and the method is suitable for bio-detection of herbicide residues, or toxicity studies on soil or aquatic pollutants.

ACKNOWLEDGEMENTS

The assistance of Mrs. A. Forey and Mrs. J. Little (technical) and Mr.C. Wilson (photography) is gratefully acknowledged. The work was supported by a grant from the Agricultural Research Council.

REFERENCES

CLARK, C.G. and S.J.L. WRIGHT: Soil Biol. Biochem. 2, 19 (1970a).

CLARK, C.G. and S.J.L. WRIGHT: Soil Biol. Biochem, 2,217 (1970b).

GEOGHEGAN, M.J.: New Phytol. 56, 71 (1957).

IKAWA, M., D.S. MA, G.B. MEEKER, and R.P. DAVIS: J. agr. Fd Chem. 17, 425 (1969).

KRATKY, B.A., and G.F. WARREN: Weed Sci. 19, 658 (1971).

THOMAS, V.M., L.J. BUCKLEY, J.D. SULLIVAN, and M. IKAWA: Weed Sci. 21, 449 (1973).

WRIGHT, S.J.L: Chemosphere. 1, 11 (1972).

WRIGHT, S.J.L: In, Industrial Aspects of Biochemistry, p. 495., ed. B. Spencer. Amsterdam: North Holland, 1974.