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The effects of some selected herbicides and plant hormones on the growth of Prototheca wickerhamii

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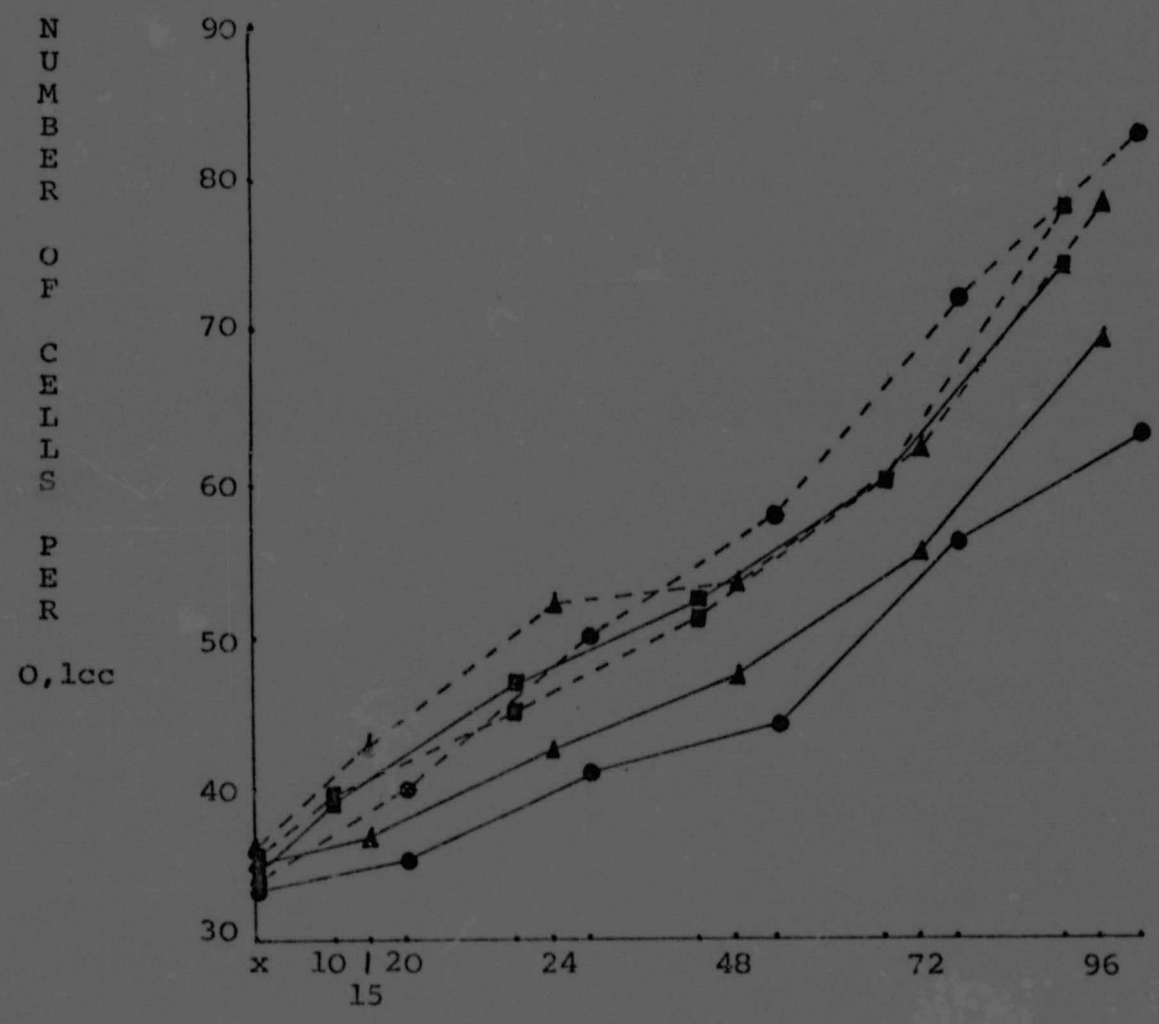


FIGURE 13 SAMPLING OF PROTOTHECA WICKERHAMII TREATED WITH HERBICIDE FOR VARIED TIME INTERVALS FOLLOWED BY REPLACEMENT WITH NUTRIENT BROTH

LEGEND

-●- = 10 hour treatment
-▲- = 15 hour treatment
-●- = 20 hour treatment
-●- = 10 hour control
-▲- = 15 hour control
-●- = 20 hour control

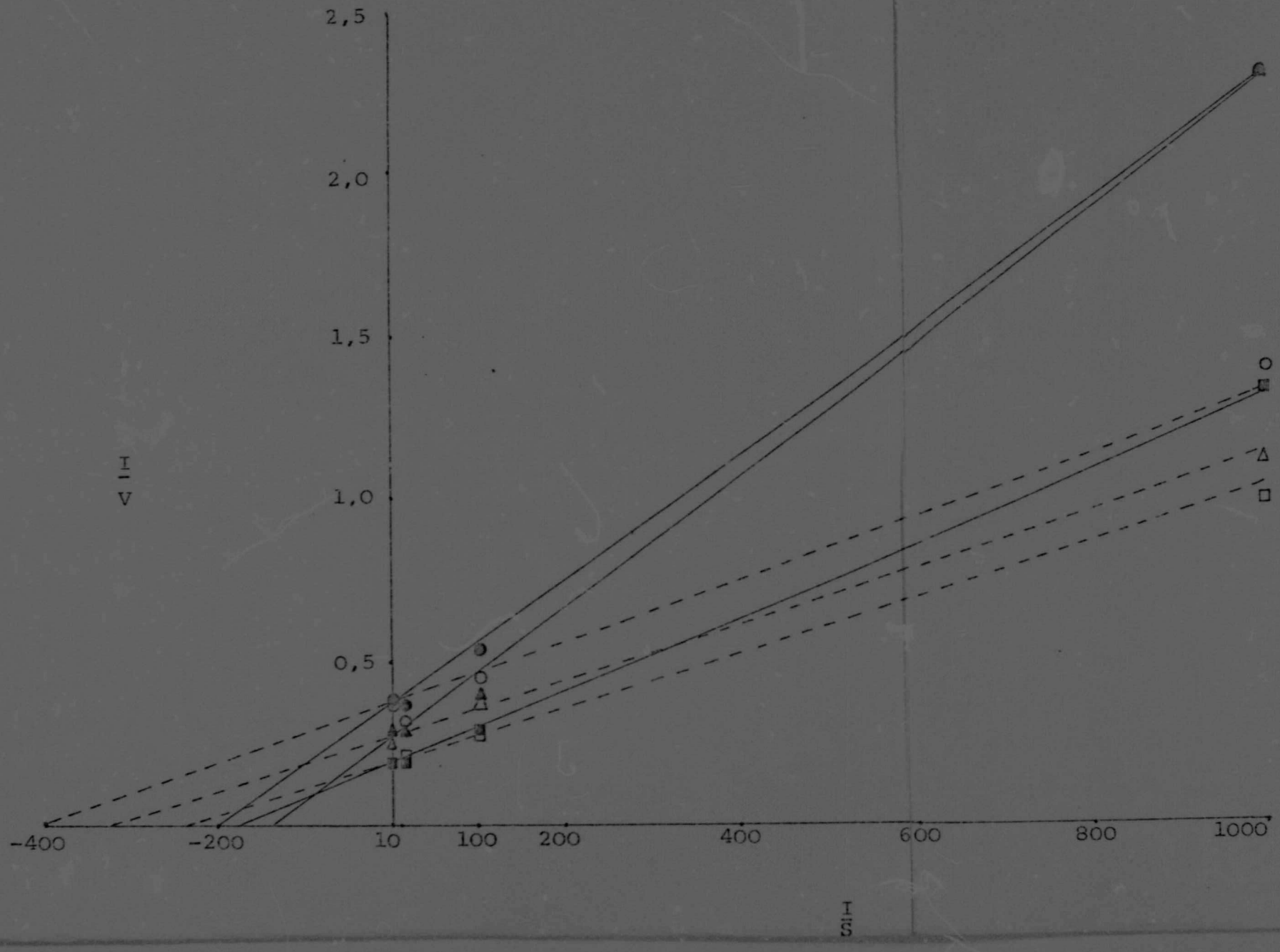

2,5
2,0
1,5
1,0
0,5
I/V
-400
-200
10
100
200
400
600
800
1000
I/S

FIGURE 14 EFFECT OF HERBICIDE ON PROTOTHECA WICKERHAMII AT VARIED SUBSTRATE LEVELS FOR DIFFERENT TIME PERIODS

LEGEND

-●- = 10 hours with herbicide
-□- = 10 hours without herbicide
-▲- = 15 hours with herbicide
-△- = 15 hours without herbicide
-◉- = 20 hours with herbicide
-○- = 20 hours without herbicide

DISCUSSION

a) Culturing techniques

From figure 1 in the results it can be seen that the cells grown on a laboratory bench, where the room temperature was 20°C and the daylight received was 500 Lux, showed a slow increase in number. These conditions were considered unsuitable for further experimental studies. The cultures were then grown in an incubator where the temperature was maintained at 32°C (Barker, 1935; Davies et al, 1964; Cooke, 1968a; Klintworth et al, 1968; Emmons et al, 1970). Since 32°C approaches the temperature of the skin of the host, the use of this temperature will be consistent with the environment of this organism.

The provision of a twelve hour light/dark supply at 2000 Lux produced a growth rate similar to that obtained when no light was supplied (figure 1). Epel and Krauss (1965; 1966) showed that light in the blue end and near U.V. region of the spectrum was inhibitory to cell division of Prototheca zopfii. Their light source was supplied at 1200 ft.c. whereas the 2000 Lux used in these experiments corresponds to 200 ft.c. Cool, white, fluorescent light at this intensity causes only 4,6% inhibition of Prototheca zopfii cell division (Epel and Krauss, 1965; 1966). It was decided to culture the cells under a twelve hour light/dark cycle at 2000 Lux as this more closely approximates to natural conditions.

Figures 2 and 3 show the growth rates obtained in different nutrient media. Casselton and Stacey's (1969) solution (figure 2) shows a marked difference, in the growth rates of the cells, from the nutrient broth solution. This difference is probably due to the pH of the media used. After autoclaving the nutrient broth has a pH of 7,2 while that of Casselton and Stacey's solution is 6,2. Prototheca cells are reported to have a maximum pH requirement of 7 (Callely and Lloyd, 1964; Klintworth

et al, 1968; Emmons et al, 1970).

Barker (1935) reported that Prototheca zopfii was unable to utilise sucrose. Figure 2 however shows the production of the greatest number of cells in the shortest period of time when sucrose is added to the nutrient solution. Examination of these sucrose grown cells shows that they are translucent and have a diameter of 2µm whereas the cells in glucose are 7µm to 10µm in size. The translucence and small size of these cells suggests that they do not utilize sucrose but storage products for the normal functions of growth and cell division. Figure 3 shows that the samples of cells grown in nutrient solution containing sucrose have the lowest absorption of light in the spectrophotometer. Translucence and small size, due to utilisation of reserves, of these numerous cells will explain the low absorbance obtained.

Statistical analysis (table 5) of minimum sample size required showed that the larger number of samples needed for the spectrophotometer made this method of determination unsuitable. It was found that the glutaraldehyde used to fix the cells before analysis degenerated with time and on exposure to light changed from a pale yellow to a dark orange colour. This necessitated a control for every sample taken, to counteract the colour changes, which made the spectrophotometer method cumbersome to work with. The results thus indicate that the cells grown in nutrient broth, although they show a slower growth rate, are more consistent and require fewer samples. Cultures grown in the log phase, up to ninety six hours, on hemacytometer examination, require only five samples (table 4).

b) Herbicides

The effects of herbicides and plant growth hormones on the host were considered when selection for study was undertaken. Chemicals with little or no effect

on mammalian cells were chosen (Stecher, 1968; Muzik, 1970). The addition of 5ml of herbicides to 5ml of nutrient broth (table 6) produced a constant pH of 7,3. This is probably due to a buffering action by the nutrient broth (pH 7,2). Coconut milk was not neutralised as the effect of sodium hydroxide on the constituents was not known. Addition of the milk, in its natural form, to the nutrient broth produced a neutral solution (pH 6,6).

Figure 4 shows that MH; NAA; 2,4,5-T; simazine; Na gold salt and CA_3 do not affect the number of cells produced when added to the cultures. IAA, IBA and IPA all affect cell growth with maximum effect being obtained at 400µg/ml. Figure 5 shows that kinetin has no effect on cell growth. Coconut milk however, causes stimulation of growth at the 50% v/v level. Steward (1968) and Caplin in 1952 suggested that 15% by volume of coconut milk in a nutrient solution be used as a stimulant. The reason for the higher percentage utilised here is not known. IAA, IBA and IPA studied at the lower concentrations show an effect on cell growth at 40µg/ml which is accentuated with increase in concentration. Figure 6 shows the overall effect of IAA, IBA and IPA on the growth rate of Prototheca wickerhamii. There is a steady decline in cell number from 40µg/ml to 400µg/ml herbicide, where inhibition appears complete.

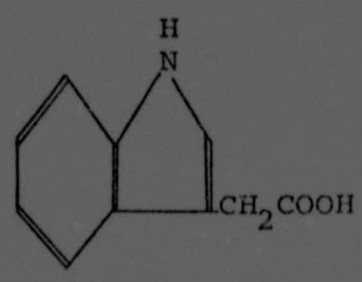
It was expected that if these chemicals are inhibitory, the mean number of cells obtained at inhibition should be the same as the forty eight hours control i.e. 15 cells per 0,1cc. Instead of which, approximately 21 cells per 0,1cc was recorded. This is indicative of a delay either in absorption and/or effectivity of the herbicides before the cells are inhibited. Time course studies (figures 7 and 8) with IAA, IBA and IPA show a levelling off in cell number increase after ten hours. Statistical analysis (table 4) indicated that only one sample need be taken at twenty four hours but it was decided to take three samples for accuracy. Cell concentrations were increased to facilitate easier detection

of changes in the cell number over these shorter periods of time. Changing the light/dark cycle so that the herbicides were added nine hours (figure 7) and three hours (figure 8) before dark, had no effect on the delay period. Prototheca wickerhamii thus appears to lack a circadian rhythm. The delay of ten hours for absorption and/or effectivity of these herbicides explains the 21 cells per 0,1cc obtained at inhibition.

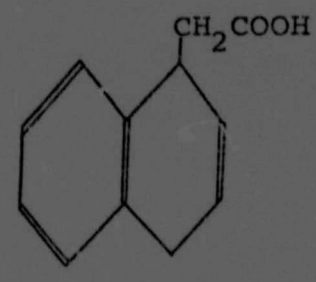
Percentage inhibition of cell growth for the herbicides tested is shown in figures 9 and 10. Coconut milk was the only compound tested which stimulated growth. IAA, IBA and IPA had an inhibitory effect on Prototheca wickerhamii from 40µg/ml to a maximum 400µg/ml. None of the other chemicals, even at the higher concentrations used, produced any effects on Prototheca wickerhamii.

c) Radioactive herbicide studies

The results obtained in the isotope experiments indicate that both IAA-C14 and NAA-C14 are actively taken up by the Prototheca wickerhamii cells. As these two compounds are similar in structure (Stecher, 1968), it is possible that they may both be taken up by the active uptake mechanisms operating in the cells.



IAA



NAA

As IAA-C14 is utilized in the cell while NAA-C14 probably accumulates without entering any metabolic pathways, it would appear that the active uptake mechanism is non selective. This study should have included one of the other herbicides, with a different structure e.g. 2,4,5-T to elucidate the uptake mechanism operative in Prototheca wickerhamii.

The 2,4 DNP used in control b) prevents active phosporylation taking place by interference with cytochrome b in the cytochrome system (James, 1953; Sutcliffe, 1962). Calcium chloride was added to the solution to preserve membrane integrity (Sutcliffe, 1962; White and Taniguchi, 1972). Metabolic inhibitors or cold treatment prevent active uptake mechanisms from functioning in the cells of the control cultures. The results obtained using controls b) and c) are similar with control c) being the less laborious method to use.

d) Electron microscopy

Comparison of herbicide treated Prototheca cells (plates 2 and 3) with the normal cells (plate 1) shows no difference in ultrastructure. Nuclei, mitochondria, storage products and ground material appear similar. Measurements of cell size, nucleus diameter and mitochondria length show no significant difference due to treatment (table 23). Examination of the grids however showed more dead cells present in the herbicide treatments (plate 4). The untreated culture had significantly fewer degenerating cells (table 25). As there were no visable structural changes in the treated cells, this suggests that the site of action of the herbicides is at a molecular level. The inhibition probably involves the mitochondria rather than the nucleus as the division rate of the treated cells remained constant whereas the rate of death increased.

e) Inhibition mechanism studies

Figure 13 shows the growth curves obtained when IAA is left in the cultures for varying time intervals and is then replaced with fresh nutrient broth. The control cultures follow a similar growth pattern, as is expected. Treatment of the cells with IAA for ten hours shows that there is no change in the number of cells produced in this culture. In the fifteen hour and twenty hour experiments,

the number of cells produced was considerably lower than that obtained in the control cultures for the same time periods (table 26). This indicates that cell number increase has been inhibited by the IAA. Replacement of the herbicide with new nutrient broth resulted in cell number increase at the original growth rate. The type of inhibition involved here is of a competative nature as this inhibition may be reversed by removal of the inhibitor and replacement with new substrate (Fruton and Simmonds, 1959; Patton, 1965).

Further work using a modified "enzyme-kinetic" type of experiment showed that varied substrate concentrations in the range of 1,0 to 0,25 (table 27) had no influence on the number of cells produced. The nutrient broth concentrations at these levels are non "limiting". Figure 14 shows the results obtained with substrate concentrations varied from 1,0 to 0,001. The linear Lineweaver-Burke type graphs obtained for the different time periods, with and without inhibitor appear to cut commonly on the y axis. This is the type of figure expected for competative inhibition in an "enzyme-kinetic" reaction (Fruton and Simmonds, 1959; Patton, 1965). These results correlate with those obtained in the former experiment.

The results have shown that IAA, IBA and IPA inhibit the growth of Prototheca wickerhamii. Indications are that these herbicides are actively taken up and competatively inhibit metabolic activity of these cells. The site of inhibition is most probably the mitochondrion. Prototheca cells have been reported to utilise acetate, propionate and butyrate as assimilates (Lloyd and Callely, 1965). These compounds are converted to acetyl CoA and enter the Krebs cycle where they are oxidised to intermediary metabolites (Callely and Lloyd, 1964a and b; Lloyd and Callely, 1965).

It is suggested that the IAA, IBA and IPA taken up

by Prototheca wickerhamii cells undergo the chemical transformations, as suggested by Callely and Lloyd, and probably enter the T.C.A. cycle as indolyl-acetyl CoA. Inhibition could be brought about by interference in chemical reactions by the indole group of the compound. This inhibition is reversed or removed on supply of new substrate to the system.

Evidence for this argument is obtained from the results where the time taken for inhibition by IBA is the same as that for IAA and IPA (figures 7 and 8). Lloyd and Callely (1965) suggest that butyric acid utilised by Prototheca cells is broken down to two C_2 units whereas acetic and propionic acids form only one C_2 unit. Butyrate is thus oxidised at twice the rate of acetate or propionate (Lloyd and Callely, 1965). Supply of IBA to Prototheca wickerhamii cells will yield only one C_2 unit with an attached indole group, if it is transformed by the process suggested by Lloyd and Callely (1965). The rate of inhibition with IAA, IPA and IBA, each with one indolyl C_2 unit, should thus be the same.

As the site of inhibition of Prototheca wickerhamii appears to be the T.C.A. cycle in the mitochondrion, this poses a problem when dealing with the host tissues. Anderson et al (1936) found the LD_{50} of mice injected intraperitoneally to be 25mg/kg while the LD_{50} of IBA and IPA was 100mg/kg. The inhibitory concentration of IBA and IPA for Prototheca wickerhamii at 400µg/ml is thus four times greater than the LD_{50} for mice injected intraperitoneally. It is not known if, with cutaneous application, the LD_{50} of these herbicides would apply to man. It is probably that combined or sequential applications of these chemicals, to build up a 400µg/ml dose, might prove inhibitory to Prototheca cells and be non harmful to the host cells. Further experimental work using human tissue cultures may elucidate the LD_{50} of these herbicides in cutaneous cells.

It is known that IAA is present in human urine as a normal secretion product from tryptophan (Anderson et al, 1936; Armstrong and Robinson, 1954; Jepson, 1956; Fruton and Simmonds, 1959). In mice the LD_{50} requires a lower concentration of IAA than of IBA or IPA. (Anderson et al, 1936). The presence of IAA in the tissues as a product from tryptophan would account for a lower additional requirement of IAA to attain the LD_{50} concentration. As the IAA content of urine from patients suffering from protothecosis has not been determined, it is not known if it is normal or not. It is, however, suggested that a disorder in the tryptophan-IAA conversion might produce a low concentration of IAA in the tissues which would decrease resistance of the patient to protothecal invasion. This is a possible explanation for the low number of confirmed cases of protothecosis in humans, but would have to be examined further. Increasing the IAA concentration of these patients may cure this disease.

CONCLUSION

To date, no cure for the disease protothecosis has been reported. Of the herbicides and plant hormones selected for study, only IAA, IBA and IPA had an inhibitory effect on the growth of Prototheca wickerhamii. Evidence indicates that the mitochondrion is most probably affected and it is suggested that the site of action is the Kreb's cycle. This poses a problem if these chemicals are to be used, as the enzymes of the T.C.A. cycle are common to pathogen and host. Also, the inhibitory concentration of IBA and IPA for Prototheca wickerhamii at 400µg/ml is four times greater than the LD_{50} for mice injected intraperitoneally. The LD_{50} of these compounds in cutaneous tissue of man will have to be established before treatment can be recommended. It is suggested that the IAA content of urine from patients suffering from protothecosis be examined. Knowledge of the amount of IAA present in the host should give an indication if it is possible to use any of these herbicides, either in conjunction or sequentially, to build up a 400µg/ml dose. Further studies are thus essential before a cure can be recommended for protothecosis.

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APPENDICES

APPENDIX i

DEFINITION OF TERMS USED

MH	= maleic hydrazide
2,4,5-T	= 2,4,5-trichlorophenoxyacetic acid
Simazine	= 2-chloro-4,6 bis (ethlyamino)-s-triazine
Na gold salt	= sodium chloroauric acid
IAA	= indolyl-3-acetic acid
IBA	= indolyl-3-butyric acid
IPA	= indolyl-3-propionic acid
GA_3	= gibberellic acid
2,4-D	= 2,4-dichlorophenoxyacetic acid
µg	= 10^{-6} g
µm	= 10^{-6} m
µg/ml	= ppm

APPENDIX ii

BRAY'S SCINTILLATION MIXTURE

Solvents	1,4-dioxane	88%
	methanol	10%
	ethylene glycol	2%
Primary fluor	PPO	0,4%
Secondary fluor	POPOP	0,02%
Anti-quencher	naphthalene	6%

With a volume of 1 liter this mixture has a 10% water tolerance.

APPENDIX iii

3% GLUTARALDEHYDE IN 0,025M MILLONIG'S PHOSPHATE BUFFER (pH 7,3)

12ml 25% stock glutaraldehyde solution is added to 88ml 0,025m phosphate buffer. The pH should be 7,3. The fixative may be stored in the cold for 24 hours.

0,025M MILLONIG'S PHOSPHATE BUFFER pH 7,3

A 2,26% $NaH_2PO_4.H_2O$
B 2,52 NaOH

Add 41,5ml of A to 8,5ml B. These should be mixed before use and the pH should be adjusted to 7,3. The buffer is diluted to 200ml to give a 0,025m solution.

1% OSMIUM TETROXIDE IN PHOSPHATE BUFFER (pH 7,3)

A 2% capsule of osmium tetroxide is broken in 2ml 0,025m phosphate buffer to give a 1% solution. Care must be taken in the preparation of osmium tetroxide as this fixative is highly volatile. The osmium tetroxide may be stored at $4^{o}C$ for an indefinite period of time, as long as it remains straw coloured.

SPURR LOW VISCOSITY EPOXY RESIN

A soft modification was used (column C, Spurr, 1969).

ERL	- vinyl cylcohexene dioxide 10g
DER 736	- diglycidylether of polypropylene glycol 7,0g
NSA	- nonenyl succinic anhydride 26,0g
SI	- dimethylamino ethanol DMAE 0,4g

Author Henning Penelope Anne
Name of thesis The Effects Of Some Selected Herbicides And Plant Hormones On The Growth Of Prototheca Wickerhamii. 1974

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