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Mavis Gail Meadows

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PHYSIOLOGICAL CHARACTERIZATION OF PROTOPLASTS OF
ZEA MAYS L. AND HYOSCYAMUS MUTICUS L.

by

Mavis Gail Meadows

Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario, Canada.

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ABSTRACT

Protoplasts isolated from the economically important grasses do not readily divide in culture. This is especially true of Zea mays L. from which only one cell line, B73, has been established from a primary protoplast culture. The cell line is not morphogenetic and has cytogenetic abnormalities. The early physiological events of cell wall regeneration and DNA synthesis in protoplast cultures of Zea mays L. (cultivar Seneca 60), the B73 maize cell line and Hyoscyamus muticus L. were studied to compare the non-regenerating primary protoplast culture of Zea mays with two regenerating protoplast populations, B73 and H. muticus.

Cell wall regeneration and DNA synthesis in the cultured protoplasts were analyzed with fluorescent dyes on a fluorescence activated cell sorter, the FACS II. Cell walls were stained with Calcofluor White which this research showed to be a quantitative stain for cellulose. DNA content was measured by the binding of Hoechst 33258. Hoechst 33258 has been shown to be a quantitative fluorochrome for DNA and a method was developed for staining living plant protoplasts with it for FACS analysis.

The fluorescence activated cell sorter (FACS II) operates by passing stained cells in a flow stream past an argon-krypton laser beam. As the cells pass through the laser beam in a measuring chamber, two measurements are made: 1) the relative size of each cell given by the low angle, forward light scatter; and 2) the amount of fluorescence of each cell. The number of cells of each size and

fluorescence class are stored in a computer. Approximately 2.5×10^4 cells per second can be analyzed, and the data from 10^5 cells were collected.

Survival studies on the three cultures showed protoplast death in the early days of culture: H. muticus had a survival rate of 60 % by day 3 of culture, after which the population stabilized and began to regrow: B73 protoplasts declined to 50 % by day 3, and then stabilized before regrowth began: Se60 protoplasts continued to die throughout the experimental time.

Analysis of DNA showed that all three cultures had protoplasts in G1 and G2 of the cell cycle and that the loss of protoplasts preferentially occurred among G2 protoplasts. All cultures, after day 3 showed an increase of G2 protoplasts indicating that DNA synthesis was occurring in all populations. Se60 protoplasts showed a third peak of DNA content that occurred at less than the G1 peak and, presumably, represented degenerating cells. Hyoscyamus and B73 protoplasts did not show this peak.

Hyoscyamus and B73 protoplasts both showed cell wall fluorescence and both had subpopulations having varying rates of cell wall synthesis. Se60 showed a very small peak of increased synthesis on day 4 of culture but this population was lost by day 6.

Although some portion of the cultivated Se60 protoplasts were capable of undergoing DNA synthesis almost none of the same population were able to regenerate walls in the conditions established for protoplast culture.

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There are many people whom I would like to thank for their help during my studies. First of all, I'd like to acknowledge Dr. D. B. Walden in whose lab I began this work and who, despite my absence from his lab, continued to be supportive of the work and the write-up. I thank him for his conscientious reading of the many drafts of the thesis over 3 years. I would like to acknowledge the agencies that gave me funding during my studies, the National Science and Engineering Research Council of Canada, the Ontario Provincial Government Scholarship committee but especially the Canadian Association of University Women who awarded me the Margaret McWilliams Travelling Scholarship that enabled me to study in the lab of Dr. Ingo Potrykus at the Friedrich Meischer Institute in Basil, Switzerland. Dr. Ingo Potrykus and his colleagues were very helpful during my stay there and I thank them. I would especially like to thank Tim Knaak for the amount of time and effort he gave to running the FACS II for me.

I like to also mention two of the reasons for this thesis write-up taking so long, my two sons, Benjamin and Alexander Gerson and my husband Donald, who always said this thesis was possible.

But most of all, I'd like to thank my parents, Walter and Mavis Meadows, who have always encouraged me and supported me even when they didn't fully understand what I was doing. Their hard work and love over the years is very much appreciated and it is to them that I dedicate this thesis.

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Chapter I: Introduction and Literature Review

One of the most significant advances in plant sciences has been the development of techniques for the isolation and culture of higher plant protoplasts. Protoplasts have been used to study basic biological processes; in particular they have been used extensively as a tool to study plant-virus interactions. As reviewed by Muhlbach (1983), the removal of the plant cell walls has reduced the barrier to high frequency, synchronous infection of host protoplasts and allowed study of the infection and replication processes for both RNA and DNA viruses. Another basic process that has been studied using protoplasts is cell wall regeneration and its relation to the plasmalemma and microtubules of the cytoskeleton (Lloyd et al., 1979; Simmonds et al., 1983; review by Fowke et al., 1983).

Besides providing a tool to investigate some basic biological aspects of plant structure and function, protoplast technology is key to any progress that will be made in genetic manipulation for crop improvement. One method to introduce new genetic information or to generate a new hybrid has been through the fusion of protoplasts from species where sexual incompatibility exists. Most somatic hybrids have been made with solanaceous plants, the best known example being the tomato-potato hybrid of Melchers et al., (1978). Another well documented, well described somatic hybrid is the "Arabidobrassica" of Gleba and Hoffman (1979). There are many more hybrids that have been obtained through protoplast fusion and they are listed and discussed in the recent review by Harms (1983). The advantages and disadvantages

of fusion hybrids have been discussed by Shepard et al., (1983).

In order to improve crop plants, a case can be made for the insertion of single specific genes into these plants now that recombinant DNA technology is becoming available. There has been some skepticism about the value of the transfer of a single gene but as Dixon (1983) has pointed out, modern maize has been developed due to changes in just five genes, and the resistance to the aphids that wiped out the Australian alfalfa crop in 1979 is a single gene trait. The transfer of single genes could be possible by the use of the Ti plasmid of Agrobacterium tumefaciens which can transfer, integrate and express bacterial genes in plant chromosomes. Much research is ongoing on the use of this plasmid to obtain promoter genes that allow expression of foreign DNA in plant cells and yet allow normal plant growth (Manzara and Lurquin, 1983). The Ti plasmid is restricted in its use to dicotyledons but there is some hope that McClintock's transposable elements (Fedoroff, 1984) will prove to be the vectors to transform monocot protoplasts (Dixon, 1983).

The greatest need in protoplast technology in order that genetically-manipulated plants can be achieved is to accomplish the regeneration of plants from protoplasts of the agronomically important plants. Cereals, grasses and legumes have all been unresponsive to protoplast culture. Recently some progress has been made in achieving plant regeneration from protoplasts of both forage and grain legumes and in some Gramineae, (for review, see Dale, 1983); however, to date, there has been no success in inducing protoplasts isolated from corn (Zea mays L.) to become plants. There is an isolated example (Potrykus

et al., 1977) of corn stem protoplasts forming walls and becoming a cell culture but with the loss of morphogenetic potential. There are several examples of callus forming from corn tissues, e.g., from the growing tip (Green et al., 1974); from the mesocotyl (Kunakh et al., 1980; Vuillaume and Deshayes, 1977); from stem sections (Sheridan, 1977); from scutellum (Bartkowiak, 1978); and from endosperm (Motoyoshi, 1971; Oswald et al., 1977). At times the corn callus has given rise to roots and shoots and then to plantlets; e.g., plants have been induced from corn scutellum (Green and Phillips, 1975); from mesocotyl (Harms et al., 1976) and from the growing tip (Mascarenhas et al., 1975). There are also instances where cell cultures have been established from corn callus (Brar et al., 1979; Chourey and Zurawski, 1981; Oswald et al., 1977). There has been no example of corn protoplasts completing the sequence: protoplast to cell to callus to plant, although this sequence has been observed in a large number of other plants, such as Nicotiana (Takebe et al., 1971; Vasil and Vasil, 1974; Gill et al., 1978; Bourgin et al., 1979); carrot (Grambrow et al., 1972), rape (Kärtha et al., 1974), Datura (Furner et al., 1978) and Atropa (Lorz and Potrykus, 1979).

There appear to be at least two parts to the problem of corn protoplast manipulation; one, induction of division to establish a cell suspension, and two, induction of dedifferentiation in the cells so that they become totipotent, i.e., have the ability to become a complete organism. It is the first problem that I have approached. To answer this question, I have studied the physiological behaviour of maize protoplasts in culture with respect to cell wall regeneration and DNA synthesis. Without both of these functions there can be no

protoplast division.

As plant cell walls have been routinely examined by fluorescence staining with Calcofluor White (Nagata and Takebe, 1970; Galbraith, 1981; Galbraith and Shields, 1982), and as Hoechst 33258 has been shown to be a quantitative stain for DNA (Arndt-Jovin and Jovin, 1977; Cesarone et al., 1979; Paul and Myers, 1982) and as there were few reports of the use of flow cytometry with plant cells, (Galbraith and Shields, 1982; Redenbraugh et al., 1982; Slats et al., 1980), I decided to study cell wall regeneration and DNA synthesis in corn protoplasts using the FACS II, Calcofluor White, and Hoechst 33258. At the same time "model systems" were used as controls for these studies. "Model systems" are those plants whose protoplasts can divide under the appropriate culture conditions. To examine early events in the culture of freshly isolated protoplasts, Hyoscyamus muticus was chosen as the model plant. Its mesophyll protoplasts have been shown to regenerate walls and to divide when cultured (Lorz et al., 1979; Wernicke et al., 1979). To study the early events in the culture of maize protoplasts, the B73 cell line of Potrykus et al., (1977) was the model system used. This cell line has been maintained since 1977 when protoplasts isolated from a field grown corn stem were induced to divide and to form a cell culture. As with other cereal cultures, (Gamborg and Eveleigh, 1968; Dudits, 1976), the work could not be repeated and so B73 is the only example of a cell suspension derived from Zea mays protoplasts still in continuous culture. Protoplasts can be readily derived from the cell line and these protoplasts regenerate walls and become cells again. They have not been induced to regenerate into plants.

Using the two model systems, it was possible to study the early physiological events of the dividing cultures with respect to cell wall and DNA synthesis to gain some understanding of the normal progression from protoplast to active cell culture in comparison to the interrupted, unsuccessful process in Zea mays. This is the experimental work reported in this thesis.

From various studies on callus induction in Zea mays (Green and Phillips, 1975; King et al., 1978; Lu et al., 1982), it has become obvious that the genotype of corn is very important for callus formation. The genetic ability to form callus is thought to be related to its ability to respond to the presence of the synthetic auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) in the culture medium (King et al., 1978; Green, 1978a; Green and Phillips, 1975, Harms et al., 1976). Although there is no proven correlation between the ability of protoplasts to divide in culture and the ability of plants to form callus in the presence of 2,4-D, it was felt that it was wiser to do research on protoplasts isolated from a corn cultivar that had a proven record of response to culture. Such a cultivar was Seneca 60 (Se60) which has been used for shoot tip cultures (Raman et al., 1980b) and anther culture (Brettell et al., 1981).

Given that Se60 corn tissue could form callus in response to 2,4-D, it was then necessary to determine the conditions for the isolation of large numbers of protoplasts for the physiological studies (Meadows and Walden, 1978). Before 1960, protoplast isolation was accomplished by the mechanical cutting of cell walls of plasmolyzed plant tissue. Cocking (1972) and Vasil (1976) reviewed work in this field, which began as early as 1892, when Klercker isolated protoplasts from

Stratiodes aloides. The tedious cutting of plant cell walls resulted in very low returns of protoplasts and very few advances were made in the field.

The use of enzymes for protoplast isolation began in 1960 when Cocking showed the release of protoplasts from root tip cells of Lycopersicon esculentum by the action of a cellulase isolated from Myrothecium verrucaria. Shortly thereafter, commercial enzyme preparations became available (Ruesink 1969). Miles Laboratories marketed TAKAMINE 20,000, a cellulase isolated from Aspergillus niger. All Japan Biochemical Company marketed ONOZUKA 1500, and Meiji Seika Kaisha Ltd. marketed MEICELASE, both of these being cellulases of Trichoderma viride. The cellulase preparations, besides containing components that attack cellulose, also contain cellobiases, xylanases, glucanases, pectinases, phospholipases, nucleases, chitinases, peroxidases, and some other harmful components (Cocking, 1972). Schenk and Hildebrandt (1969) purified crude cellulase and found that the purified preparation produced fewer protoplasts than the crude preparation. They suggested that efficient release of protoplasts would result from the use of cellulase in combination with other enzymes. Cellulases release protoplasts from plant cells that have low amounts of arabinose, galactose, and xylose as components of hemicellulose and which have high concentrations of glucose in their walls (Keller, 1970; Cocking, 1972). Some enzymes that are currently available are Driselase, a cellulase and pectinase from a basidiomycete; macerozyme, a pectinase from Rhizopus; Cellulysin (Calbiochem) and hemicellulase (Cocking, 1972; Vasil, 1976). Most enzyme preparations require a further purification step, such as elution over a

Sephadex G-25 or Biogel column to remove phenolics or ribonucleases.

Other conditions that are important in obtaining a high yield of protoplasts are the pH of the enzyme solution (Cocking, 1972; Schenk and Hildebrandt, 1969; Pelcher *et al.*, 1974); the physiological condition of the plant (Pelcher *et al.*, 1974; Shepard and Totten, 1975; Vasil, 1976) and the osmotic pressure of the isolation medium (Cocking, 1972; Ruesink and Thimann, 1964; Ericksson and Jonasson, 1969; Taylor and Hall, 1976). All of these factors were considered as the conditions were determined for isolation of a large number of protoplasts from young corn leaves.

Generally, protoplasts are cultured in media that were initially designed for plant cell and tissue culture. White (1954) compared the compositions of plant cell nutrient media which were designed by early plant physiologists. Since then, there have been a great number of new media recipes published. Two media that are frequently used for protoplast culture are 'MS', designed by Murashige and Skoog (1962) and adapted for tobacco protoplasts by Nagata and Takebe (1971); and the 'B5' medium (Gamborg *et al.*, 1968), initially developed for the culture of soybean cells. Other media have been designed especially for the culture of certain plants or conditions; e.g. 'DPD' medium for the culture of petunia protoplasts (Durand *et al.*, 1973) and later for Hyoscyamus (Potrykus, pers.comm.), and 'K5' medium developed for low density culture of Vicia hajastana cells (Kao and Michaluk, 1975). Schenk and Hildebrandt (1971) defined a medium that supported the growth of maize callus. However, most corn callus culture has been induced on MS medium or variations thereof (Green and Phillips, 1975).

Since there have been no successes in inducing maize protoplasts to divide in culture, I decided to design a culture medium that would allow them to survive at least long enough for analysis on the Fluorescence Activated Cell Sorter (Becton-Dickinson, FACS II). However, all attempts to design a medium for the corn protoplasts were futile as they survived as well in the medium defined by Nitsch and Nitsch (1967) as in any of the novel culture media that I designed. The data obtained from these media testing experiments have been added to the thesis as an appendix.

The final procedures that had to be established before the analysis on the FACS II were the staining techniques for the plant cell nuclei with Hoechst 33258, and for the cell walls with Calcofluor White. Hoechst 33258 is a bisbenzimidazole dye that has been shown to bind quantitatively with DNA (Cesarone et al., 1979; Latt and Stetton, 1976; Arndt-Jovin and Jovin, 1977; Paul and Myer, 1982) via hydrophobic interactions with bases in the major groove in the helix (Comings, 1975). It has often been used to study cell cycle changes in mammalian cells (Cowell and Franks, 1980; Horan and Wheelless, 1977) and for chromosome and nuclear staining in plant cells (Filion et al., 1976). Laloue et al. (1980) determined the conditions for Hoechst staining for fixed cultured cells of five plant species. Galbraith et al., (1981) examined DNA changes in fixed tobacco protoplasts during one week of culture using Hoechst 33258. They observed that all of the protoplasts were in the 2N stage of the cell cycle, but his later work with a flow cytometer proved this observation incorrect, due probably to the small original sample size.

Since one of the attractions for using flow cytometry is that future use may be made of the sorting feature to collect hybrid fusion protoplasts, it was considered essential to establish staining conditions for unfixed, viable protoplasts. Hoechst 33258 is considered to be a non-toxic vital stain and was proven to be so when petunia protoplasts, stained with Hoechst according to the method reported below, continued to grow and divide equally as well as unstained protoplasts (Meadows and Potrykus, unpublished).

Similarly, conditions for staining with Calcofluor were determined for live protoplasts. Calcofluor White is the disodium salt of 4,4'-bis {4-anilino-bis-diethyl amino-S-triazin-2-ylamino}-2,2'-stilbene-disulfonic acid. Because of its fluorescence specificity for configurations of hexopyranosyl polymers (Maeda and Ishida, 1967), it is used in the textile industry as a cotton whitener (Painter et al., 1980). Its use to visualize regenerating walls was first reported for tobacco protoplasts by Nagata and Takebe (1970). It is used also for cell wall staining in microorganisms (Berliner et al., 1978; Darken, 1962) and has become a standard tool in protoplast research (Asamizu et al., 1977; Galbraith, 1981). Calcofluor gives a high background fluorescence and the correct conditions for its use on the FACS II were established as well as an assay to measure cell wall regeneration using Calcofluor and a fluorometer. Galbraith (1981) similarly established a microfluorometric assay to examine and measure regenerating walls in individual protoplasts and cells.

The fluorescence activated cell sorter was developed at Stanford University and is now made by Becton-Dickinson Electronics. Reviews

and machine description have been given by Herzenberg et al., (1976), Horan and Wheelless (1977) and by Herzenberg and Herzenberg (1978).

The FACS II can analyze or sort cells according to physical differences in the individual cells. The general principle of analysis is: cells in suspension, having been stained with a fluorescent dye, are taken, by vacuum pressure, from their test-tube and are forced into a flow stream which is surrounded by a coaxial sheath stream to keep the cells centered in the flow. The sheath and flow stream pressures are adjustable to give the correct flow stream diameter for the cells. The cell concentration can be adjusted to allow single-file flow of the cells in the flow stream. The cells then flow through a nozzle and into the path of a laser beam, which excites the fluorescent-bound tag. The laser is either an argon-ion or an helium-neon laser. Optics placed at 90° to the incident beam collect the fluorescence due to the excitation of the dye. A barrier filter blocks the excitation beam and dichroic mirrors and band-pass filters select the fluorescence for measurement. There are three detectors in the measuring chamber, one to measure the small angle forward light scatter and two to measure the emitted fluorescence. Optical signals are registered as electrical pulses, which are amplified and collected for analysis. Cells that are to be sorted are given an electrical charge and are collected after deflector plates change their direction of flow into a collecting vessel. Cells that have had only an analysis done are discarded after the run.

Two types of data are collected from the FACS analysis: histograms and dot plots. The histograms have the abscissa as units of fluor-

escence or light scatter (size) per cell and the ordinate as the frequency of cells. Dot plots are generated by the measurement of two parameters as a function of a third parameter; e.g., fluorescence and size in the X- and Y directions and the number of cells with those two parameters in the Z direction. Dot plots can be quantitatively replaced by isometric displays of the data.

More recently, flow cytometers have become popular with plant biologists and there are several reports in the literature of flow cytometers being used with plant protoplasts. Slats et al. (1980) have reported that they sorted those protoplasts containing a FITC-tagged alfalfa mosaic virus. Galbraith and Shields (1982) did a time-course study of fixed tobacco protoplasts stained with Hoechst 33258 on a flow cytometer after the protoplasts had been treated with two cell wall inhibitors. They reported that one inhibitor, 2,6-dichlorobenzene (DB) did not affect DNA synthesis but that coumarin did. Unfortunately, they failed to run a control of an untreated sample. Redenbaugh et al. (1982) have reported the flow cytometric detection of two populations of protoplasts which had been stained with two different fluorochromes. They also made preliminary attempts to sort the protoplasts.

Puite and Ten Broeke (1983) have studied DNA histograms of fixed and non-fixed protoplasts of H. gracilis and S. tuberosum after staining with Hoechst 33342, using a FACS IV. Galbraith (1983) has analyzed nuclei isolated from tobacco and stained with Hoechst 33258 on an Epics V flow cytometer and obtained a typical G1 and G2 distribution. Harkins and Galbraith (1984) have reported the

culture of tobacco protoplasts that had been sorted according to their endogenous chlorophyll fluorescence on an Epics V cell sorter. de Laat and Blas (1984) have used a FACS IV to sort ethidium bromide stained chromosomes isolated from burst protoplasts of H. gracilis.

It is apparent that flow cytometry has become a useful tool in plant tissue culture. This technology was used in this research to answer questions on cell cycle kinetics of three different populations of cultured protoplasts. Since the flow cytometer is electronically very sophisticated, the help given by Tim Knaak in running it must be acknowledged. His usual task was to analyze and sort cultured mammalian cells and to run protoplast samples required some adjustments to the sorter; eg. the sheath and flow stream fluids were replaced by protoplast culture medium to accommodate the fragile protoplasts, the nozzle was replaced with one of larger diameter, the flow rate was lowered to 25 % of the normal and the electronic size cut-off gates were set so that small debris was not counted in the analysis. To determine the adjustments necessary to obtain intact protoplasts after an analysis required a number of trial runs and sorts on the machine. When the correct conditions were established, several repeat experiments were performed for each population, each analyzing 10^5 protoplasts. The data presented here are representative of the data obtained for several experiments.

Thesis Organization:

This thesis is not presented in an uniform format because chapters have been published in, or submitted to, journals having diverse editorial standards. References for those chapters that have been published or are to be published have been listed at the end of each chapter. A comprehensive bibliography is presented at the end of the thesis.

Chapter IV, Fluorescence Studies; A. Hoechst 33258 as a Vital Stain for Plant Cell Protoplasts, has been published in Plant Cell Reports 1: 77-79, 1981; chapter V, Characterization of Cells and Protoplasts of the B73 Maize Cell Line, has been published in Plant Science Letters 28: 337-348, 1983; chapter IV, Fluorescence Studies; B. A batch assay using Calcofluor fluorescence to characterize cell wall regeneration in plant protoplasts, has been published in Analytical Biochemistry 141: 38-42, 1984. Chapters VI and VII, Characterization of Protoplasts of Hyoscyamus muticus using Flow Cytometry, and Characterization of Protoplasts of Zea mays using Flow Cytometry, have been submitted for publication.

Chapter II: Isolation and Recovery of Protoplasts
from Se60, a Tissue Culture-Responsive
Genotype of Corn.

Introduction

There are many procedures for the isolation of plant protoplasts from different tissues of various plant species and each procedure must be established or adapted by individual workers for their chosen plant and tissue. In this case the plant species is Zea mays L. but the characteristic that was considered before beginning protoplast isolation was its genotype.

It is recognized (King et al., 1978; Green, 1978a, 1978b; Potrykus, 1980) that the genotype of maize is an important factor in the successful establishment of tissue cultures. Although there has been no correlation between a plant's ability to form callus and its ability to yield dividing protoplasts, it seems appropriate to begin protoplast culture work with a genotype that has a proven ability to respond to being cultured.

Induction of maize callus depends on the presence of the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) in the culture medium, whereas other growth hormones have been shown to have no effect on the initiation of callus (King et al., 1978; Green et al., 1974). There have been several screening programmes to define maize genotypes that can give an "in vitro" response for different tissue sources: scutellum (Green and Phillips, 1975; Bartkowiak, 1978; Lu et al., 1982); stem (Sheridan, 1977); mesocotyl stem and leaf,

(Kunakh et al., 1980). Potrykus (unpublished) established a screening programme to examine callus formation in stem segments of various corn genotypes in response to high levels of 2,4-D. The test medium, P13, (Table 1) was based on "CC" medium (Potrykus et al., 1977) which was used when protoplasts of B73 stem were induced to divide.

Se60, a single cross hybrid sweet corn cultivar that has been used in cell cycle studies (Verma and Lin, 1979), in "in vitro" fertilization studies (Raman et al., 1980a), in shoot tip cultures (Raman et al., 1980b), and in anther culture (Brettell et al., 1981) was tested on the the P13 medium and responded with excellent callus formation. The type of response, the age and origin of the responsive tissue were studied in detail.

Concurrently, methods were established to isolate protoplasts from Se60, first from mature leaves and then from the tissue that proved to be most responsive to the test medium. Once a successful enzyme solution was found, various conditions such as pH, duration of incubation in the enzyme, plant age and pretreatment were varied in order to increase the yield of protoplasts. The final conditions thus established for obtaining high yields of maize protoplasts easily and reproducibly (Meadows and Walden, 1978) and a method for removing cellular debris to give a clean protoplast preparation are reported.

Materials and Methods

Plants: Se60 seedlings were grown in peat pots placed in wooden or plastic flats as described in Table 2. The soil used has been called UWO soil mixture and a description of its composition has been added as appendix 2. The soil was a sandy loam with added

TABLE 1

P13 CULTURE MEDIUM
 MODIFIED FROM CC MEDIUM (POTRYKUS et al., 1977)

<u>Component</u>	<u>mg/l</u>	<u>Component</u>	<u>mg/l</u>
KNO ₃	1212.0	nicotinic acid	6.0
NH ₄ NO ₃	640.0	thiamine-HCl	8.5
CaCl ₂ ·2H ₂ O	588.0	pyridoxine-HCl	1.0
MgSO ₄ ·7H ₂ O	247.0	glycine	2.0
KH ₂ PO ₄	136.0	m-inositol	90.0
FeSO ₄ ·7H ₂ O	27.8	2,4-D	5.0
Na ₂ EDTA	37.3		
H ₃ BO ₃	3.1		
MnSO ₄ ·4H ₂ O	11.15		
ZnSO ₄ ·7H ₂ O	5.76		
KI	0.83		
Na ₂ MoO ₄ ·2H ₂ O	0.24		
CuSO ₄ ·5H ₂ O	0.025		
Co ₂ SO ₄ ·7H ₂ O	0.028		
	Glucose	5.0%	
	cocoanut water (Gibco)	0.4%	
	pH	6.0	
	Agar	0.8%	

TABLE 2

EXPERIMENTAL CONDITIONS FOR TESTING
THE TISSUE CULTURE RESPONSE OF SE60 SEEDLINGS.

EXPERIMENT	SEEDLING AGE	GROWTH CONDITIONS	TISSUE USED
I.	5-day old	moist filter paper in dark, 24° C.	total mesocotyl and stem.
	10-day old	Sandy-loam soil + perlite in 10 cm peat pots; 30°/20° transplanted to 25 cm pots after day 16; watered 2x daily; fertilized weekly.	stem from crown roots to first emerged leaf.
	16-day old		
	21-day old		
	35-day old		
II.	10-day old	grown axenically on 10% P13 salts in graduated cylinders and test tubes.	stem sections as in Exp I. Individual leaves were unrolled and cultured.
III.	Various ages;	UWO soil; 10cm peat pots; then as above.	leaf sheath

trace elements and fertilizer. Germination and growth occurred under standard greenhouse conditions with a 16 hour day length, a daytime temperature of approximately 28° C and a nighttime temperature of approximately 20° C.

Emerged leaves, either whole or macerated, were used in early experiments. Later experiments made use of the leaf sheath.

Surface Sterilization of Tissue: Two methods were used to sterilize the maize tissue before protoplast isolation. One consisted of washing the plant material in a saturated solution of filtered calcium hypochlorite for 15 minutes. The second method used 0.01% mercuric chloride (HgCl_2) plus two or three drops of Tween 80 as a wetting agent, again for 15 minutes. After either treatment, the plant material was washed 5 times in sterile distilled water.

Genotype Testing: Experiment I: Plant Age and Callus Response.

The seedlings were grown as described in Table 2. The stem and mesocotyl of five day-old seedlings and the whole stem segment from crown roots to emerged leaf-blades of 10, 16, 21, and 35-day old seedlings were cut into 1-mm slices under a dissecting microscope and the slices arranged serially on separate drops (Figure 1a) of P13 medium (Table 1) in 9 cm Petri dishes. The Petri dishes were placed in moist boxes in the dark at 28° C and observed after one week for callus growth.

Experiment II: Responsive Tissue: A second experiment was undertaken to establish whether young maize leaves had the capacity to form callus in culture (growth conditions are given in Table 2). The plants had their roots and emerged leaves removed, but not the

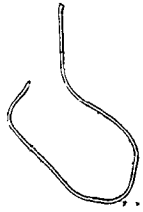
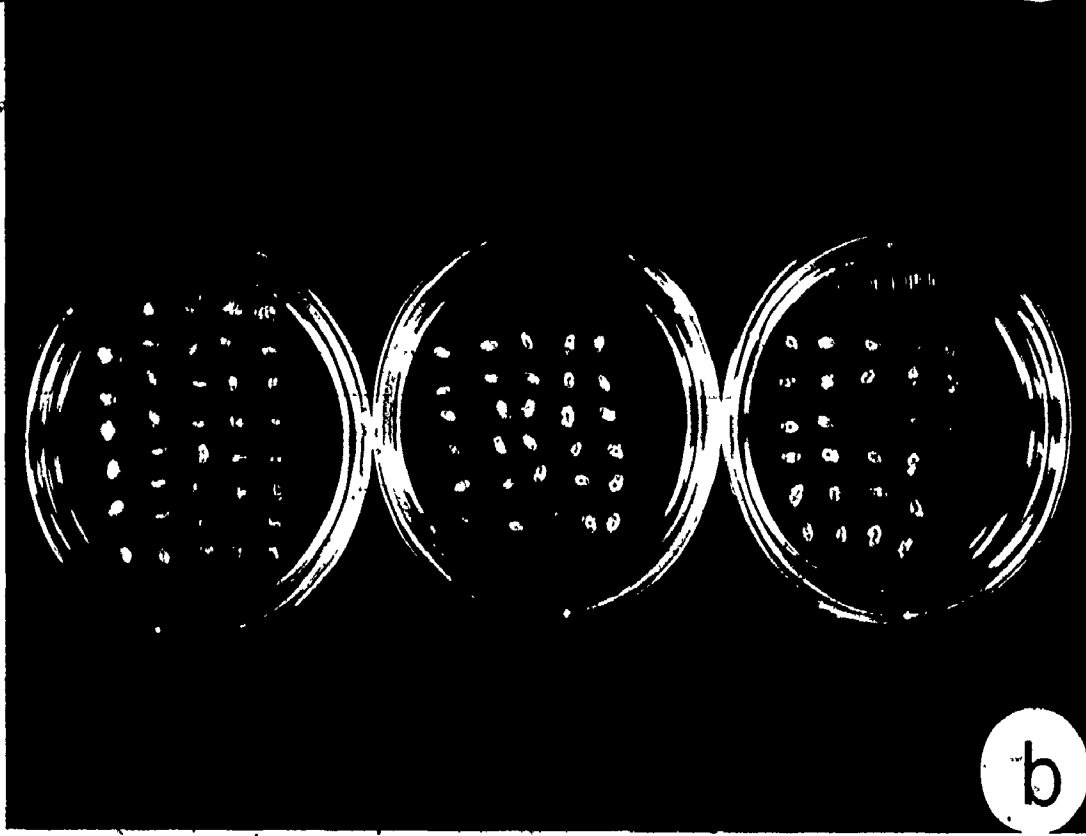
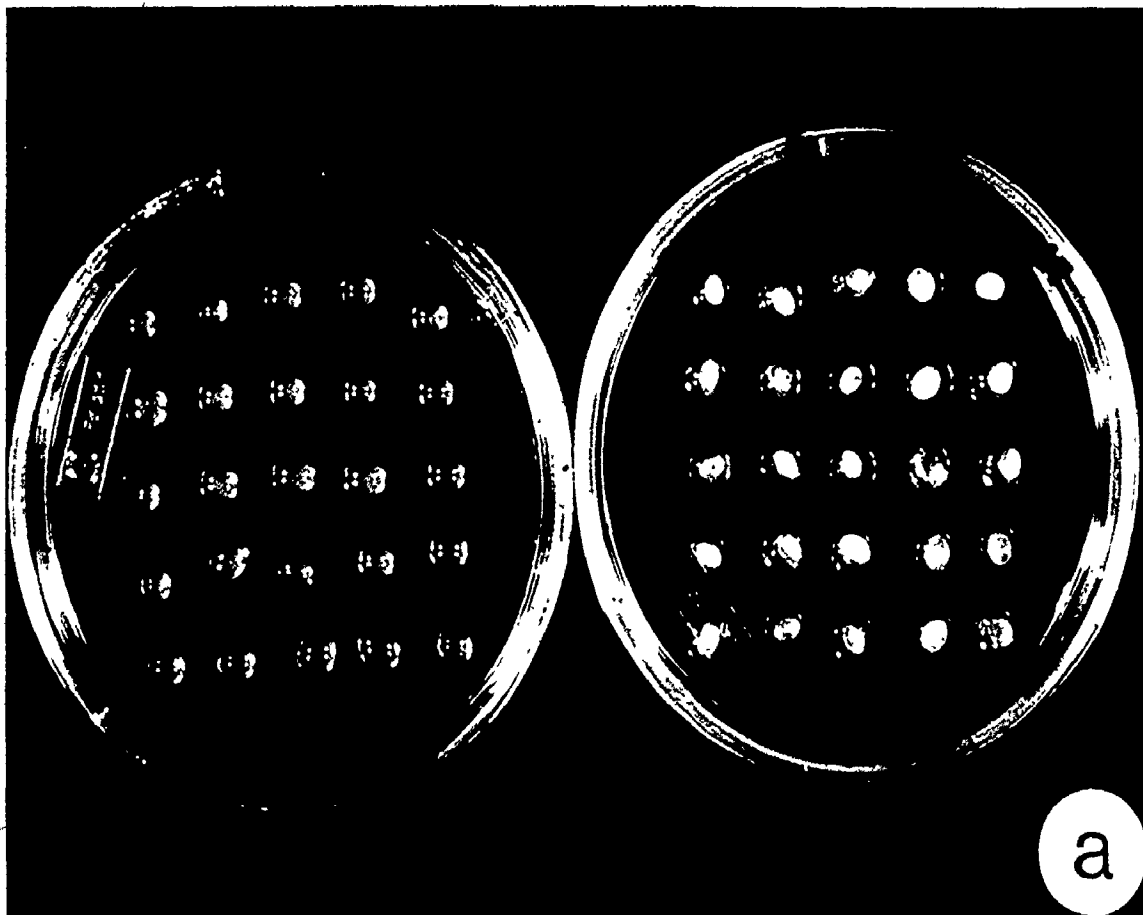


Figure 1: The P13 test system.

a) left: The P13 test medium was arranged in a 5 x 5 drop array;

right: slices of the lower 2.5 cm of maize stem were placed on the drops.

b) serial sections of long stems were placed on drops of P13 in several Petri dishes.



outside leaf sheath or coleoptiles. The plants were cut into thin slices (approx. 3mm) and then the leaf sections were unrolled and cultured individually on the P13 medium following the procedures of Wernicke and Brettell (1980).

Enzymes: The enzymes tested were those generally used for protoplast isolation: Cellulase Onozuka R-10, Macerozyme, (both from Kinki Yakult Mfg. Co., Ltd., Nishinomiya, Japan), Driselase, (Plenum Scientific Research Co., Hackersack, N.J., USA.), Pectinase and Hemicellulase (both from Sigma, St. Louis, USA.) The enzymes were initially used according to recipes given in the literature but were later changed and corrected according to their success in causing protoplast release. The enzymes were used in combination with sugars and salts as osmotic stabilizers, and at varying pH's and temperatures. The combinations of these factors are listed in Table 3. An enzyme solution ESI was defined that released protoplasts from emerged leaf material of the corn seedlings. Further experiments were done to test the effectiveness of the cellulase alone on the isolation of protoplasts both from the emerged leaves and from the tissue-culture responsive leaf sheaths, in comparison to ESI. The concentration of cellulase used were 0.5%, 1%, 2%, 3%, and 4% (w/v) of Onozuka R-10 in 9% mannitol and the salt solution defined by Zapata et al. (1977).

Two other enzyme mixtures were tested for their abilities to release protoplasts from leaf sheafs of corn seedlings. The enzyme mixtures were those most effective for protoplast isolation from wheat and barley (de la Roche, personal communication) and their

TABLE 3

VARIOUS CONDITIONS USED TO ISOLATE PROTOPLASTS FROM SE60.

Macerated Leaves:

Scheme	Cell. (%)	Mag. (%)	Dris. (%)	Pect. (%)	Hemi. (%)	pH	Osm.	Temp. (°C)	Time (Hrs)	Yield
A.	0.5	0.1				5.6	Sucrose 0.3M	25	14	nil
B.	2			0.1		5.5	FVP 2% Sorbitol 0.6M	25	14	nil
C.	1	0.25	0.5			5.9	Sorbitol 0.6M	25	13	nil
D.	0.1	1.5	1			5.9	Sorbitol 0.6M	37	4	some
E.	0.5	0.1	0.7			5.9	Sucrose 10%	37	4	many
F.	0.5	0.1	0.2	0.25	0.25	6.2	Sorbitol 4% Mannitol 6%	37	2	some

Whole Leaves

G.	0.5	0.1	0.2	0.25	0.25	6.2	Same as above	37	14	some
H.	0.5	0.1	0.2	0.25		5.9	Mannitol 13%	37	14	nil
I.	0.5	0.1	0.2	0.25		5.9	Mannitol 13%	25	14	nil
J.	0.5	0.1	0.2	0.25		6.2	Mannitol 13%	25	12	few
									14	few
									16	many
K.	0.5	0.1	0.2	0.25		6.2	Mannitol 13%	37	12	nil
									14	some
									16	many

TABLE 3 continued.

Scheme	Cell. (%)	Mac. (%)	Dris. (%)	Pect. (%)	Hemi. (%)	pH	Osm.	Temp. (°C)	Time (Hrs)	Yield
L.		0.1	2	0.5		5.9	Mannitol 9%	37	4	nil
M.	0.5					5.9	Mannitol 9%	37	4	nil

Abbreviations: Cell = Cellulase Omozuka R-10

Mac = Macerozyme

Dris = Driselase

Pect = Pectinase

Hemi = Hemicellulase

Osm = Osmoticum.

FVP = Poly(vinylpyrrolidone) M.W. 10,000 (Sigma).

compositions are given in Table 4.

Measurement of Yield: The protoplasts were harvested by pouring leaf and enzyme mixture through cheesecloth, following which, the suspension was centrifuged 10 minutes at 300 x g. The protoplasts were resuspended in medium, recentrifuged twice and counted on an haemocytometer. This count was given the value of 100 %.

Effect of Incubation pH: Protoplast yield was measured as a function of pH for four incubation intervals. 0.1M KOH or 0.1M HCl was used to adjust the pH's of the enzyme solutions. Initial experiments did not make use of buffered incubation mixtures so that the enzyme solutions did not remain at a stable pH. The effect of added buffer, 0.5% MES (4-morpholineethane sulfonic acid) on protoplast yield was studied. The immature leaves of 30-day old plants were incubated in 0.5% Cellulase Onozuka R-10, with and without added buffer, Zapata salts, and 9% mannitol. The pH was measured at the beginning of the incubation period and after 2.5 hours at 28° C. Protoplast yield was then measured as a function of pH using the buffered system and 21-day old plants as tissue source.

Pretreatment of Plants: The effect of humidity and darkness on the yield of protoplasts was studied by spraying 12- and 20-day old plants and soaking their soil with water and then placing them in a dark cupboard for 16 hours before enzyme treatment.

Effect of Agitation on Protoplast Yield: Protoplasts were isolated from 25-day old plants under two conditions:

1. the tissue was floated on the enzyme solution and the protoplasts were recovered from the enzyme solution followed by two

TABLE 4

COMPARISON OF PROTOPLAST YIELDS FROM IMMATURE LEAVES
OF CORN USING THREE ENZYME COMBINATIONS

<u>ENZYME MIXTURE</u> * (% w/v)	<u>YIELD FROM IMMATURE LEAVES</u> (# protoplasts/gm. fr. wt.)
A. Driselase 0.7 Macerozyme 1.7 Cellulase 0.5	0.5 x 10 ⁶
B. Cellulase 0.5 Pectinase 1.5 Hemicellulase 1.0	1.6 x 10 ⁶
C. Cellulase 0.5	3.4 x 10 ⁶

* pH 5.7, 0.55 M Mannitol, 0.5 M CaCl₂.

washes; or

2. the leaf tissue was shaken in the enzyme solution at 125 rpm on a New Brunswick Gyrotory-Rotary Shaker.

Effect of Plant Age: To study the effects of plant age on protoplast yield, protoplasts were isolated from plants of different ages, from 10-day old to 35-day old plants, under the optimal conditions that had been thus far established. Plant age was determined from date of planting.

Clean-Up Procedures: During protoplast isolation, cellular debris was generated in the incubation solution. Several methods, as summarized in Table 5, were attempted to clear the protoplast preparation of such debris. The reduction in the amount of cellular debris was judged visually and the yield after each treatment was determined by duplicate haemocytometer counts.

Filtration: The protoplast suspension was filtered through woven filters of decreasing pore size; 250 μ , 100 μ , and 50 μ , and was examined microscopically to ascertain whether the debris had been reduced.

Hormone pretreatment: a) Gibberellic Acid. It was hoped that pretreatment of the plants with hormones would improve the quality of the protoplast preparation. The plants were first sprayed with gibberellic acid (10^{-5} M) which, by causing stretching of the lateral walls in the plant tissues, would make them more amenable to digestion. The plants were returned to the greenhouse for 24 hours and then were placed in the dark at high humidity. Protoplasts were isolated from some plants after 16 hours and from the remaining plants after 40 hours.

TABLE-5
 METHODS USED IN ATTEMPTS TO CLEAR DEBRIS
 FROM PROTOPLAST INCUBATION MIXTURES.

CLEAN-UP PROCEDURE	% RECOVERY OF PROTOPLASTS	REMOVAL OF DEBRIS.
Filtration through 85 μ and 43 μ woven filters.	85	none
GA ₃ (10 ⁻⁵ M) Treatment		
40 hours	59	none
60 hours	89	none
IAA Treatment	22	none
Density and phase separation: 9% Mannitol + Zapata salts over FCS	60	none
Density Separation 0.24 M CaCl ₂ + medium	80	none
Wash with 0.16 M CaCl ₂ + filtration	85	yes.

b) Indole Acetic Acid: It is known that plant tissue exposed to IAA releases endogenous cellulase and swells due to the weakening of the cell wall fibers. It was thought that the pretreatment of the plants with IAA would increase the protoplast yield while decreasing the debris. The plants were sprayed with 10^{-5} M solution of IAA and were used for protoplast production after 16 hours in dark high humidity.

Density and Phase Separation: An attempt was made to separate protoplasts from undesirable debris by a combination of density and phase separation: 16×10^6 protoplasts in 9 % mannitol and Zapata salts were layered on top of 10 ml of fetal calf serum (FCS) containing 9 % mannitol, and allowed to stand for 10 minutes. During that time, large fragments sedimented from the protoplast solution and through the FCS solution. The protoplast suspension was then drawn off the FCS solution using a tipless Pasteur pipette that had been melted into a "J" shape and layered onto a second tube of FCS solution, which was centrifuged at low speed, (100xg), so that small cellular debris would remain in the Zapata salts and mannitol solution while protoplasts would pellet during centrifugation.

Sedimentation in CaCl_2 : The corn protoplasts, resuspended in 9% mannitol, 0.5 % MES and Zapata salts, were mixed with 2 volumes of 0.24 M CaCl_2 according to the method of Potrykus et al. (1977). After centrifugation (300xg) the pelleted protoplasts were resuspended in 0.16 M CaCl_2 and layered on top of 22 % sucrose and recentrifuged. The protoplasts were then collected from the interface between the two phases.

RESULTS

Culture Response Testing: The seedling sections responded to the P13 medium in a number of ways which are illustrated in Fig 2. First, some sections of the plant did not respond. The second type of response was a swelling of the tissues of the slice and the formation of roots from the lower stem sections (Fig 2a). The third type of response was swelling and elongation of the leaf material of the slices (Fig 2b). Watery callus was often formed around the midrib of the leaves. The fourth type of response, (Figs 2 c & d) was the formation of a very loose and watery callus in the outside leaf slices, usually accompanied by elongation of the innermost part of the slices. The callus formation was taken to be indicative of cell division.

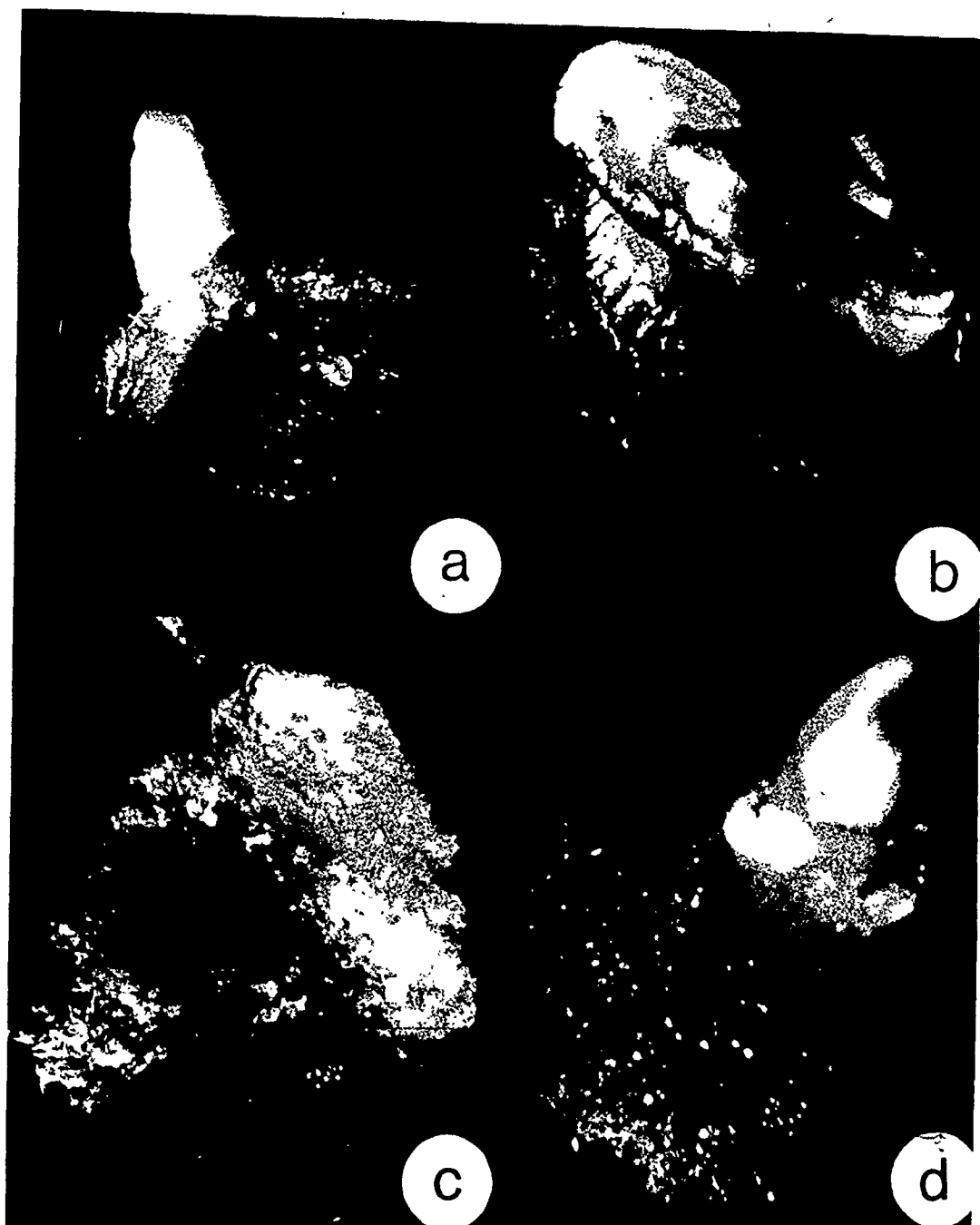
(a) Plant Age and Callus Response: In 5-day old seedlings the maximum response centered around the coleoptilar node. A typical response below the node was one of root formation. Sections near the node gave rise to roots and many root hairs, whereas at the node there was callus formation accompanied by growth of shoots and roots. Occasionally there was callus formation above the node but usually there was only swelling and elongation of the plumule.

Sections from 10 and 16-day old seedlings showed similar responses to being cultured: swelling and elongation of the leaf sheath with some callus formation, usually in the midrib of the outside leaves. The responses also occurred in leaf material located above the apex.

Slices from 21-day old seedlings formed callus in response to

Figure 2: Demonstration of the responses of maize sections to P13 medium.

- (a) Swelling of peripheral tissue.
- (b) Swelling and elongation of leaf sheath.
- (c) & (d) Callus.



being cultured. Only at this age of plant was there a response of the growing tip to culture. Besides the growth of the florets, there was also good response by the leaf material above and below the floret. The lower segments showed pith swelling and leaf expansion. The upper sections showed swelling and callus formation around the midrib of the leaves.

The 35-day old plant slices showed a maximum response of swelling and elongation which occurred along the entire length of the stem.

(b) Responsive Tissue: 10-day-old seedlings, grown in graduated cylinders and test tubes, were sliced and the leaf pieces encircling the stem were unrolled and placed on P13 medium (see Fig 3). The lower six rows of the leaf sections on the Petri dish, counting bottom to top, contained the growing tip. The apex of the growing tip was placed slightly below row 6, at the right of the second Petri dish. The sections are shown after two weeks of culture. The coleoptilar tissue (Fig 3, left) did not respond. The leaf sections which surrounded the shoot tip formed callus (rows 5 through 12). All leaf sections in rows 5 and 6 swelled, elongated and formed callus. The apex tip (placed slightly below row 6) formed a shoot from its callus. In the upper rows, that is, in the more mature leaf sections, callus formation was negligible. The leaf sections located closer to the stem formed more callus than those further from the stem.

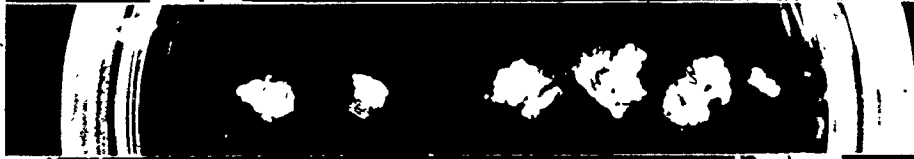
Enzymes: The various combinations of enzymes, osmotic stabilizers, temperatures, pH, and incubation times studied are listed in Table 3. The first series of experiments utilized macerated emerged

Figure 3: Illustrations of the 2-week response of the leaf sections to P13 medium.

Stems of the test tube-grown 10-day old seedlings were sliced and the leaf pieces encircling the stem were unrolled and placed on the test medium; outside leaves on the left of the Petri dish and inside leaves on the right.

The growing tip was contained within the first 6 rows, counting bottom to top. The apex itself was placed slightly under row 6 at the right.

Magnification: 1.2X.



corn leaves. The second series used whole leaves. Initial experiments failed to give protoplasts. The addition of 0.1 M CaCl_2 in experiment "D" resulted in some success. Experiment "E" used a salt solution defined by Zapata et al. (1977) and a substantially larger number of protoplasts was obtained. Experiment "F", in which pectinase and hemicellulase were added to the reaction mixture to reduce the digestion time from 4 to 2 hours, also yielded protoplasts.

The second series of experiments utilized mature leaves that were cut in large fragments and floated on the enzyme solutions.

Experiments "G", "H" and "I", in which osmoticum, temperature and enzyme composition were varied, failed to yield protoplasts. However, experiment "J" in which the pH was raised slightly, gave a small number of protoplasts after 12 and 14 hours incubation and a larger yield after 16 hours. These experimental conditions were repeated in experiment "K" except that the temperature was raised to 37° C. Under these conditions, there was an increase in protoplast yield over the time range of 12 to 16 hours.

In experiments "L" and "M" the importance of cellulase to the digestion mixture was studied using a decreased mannitol concentration and pH 5.9. The replacement of cellulase by pectinase resulted in no yield of protoplasts, as did treatment with cellulase alone under these conditions.

From these experiments, the best enzyme combination for isolating corn mesophyll protoplasts from mature leaf segments was:

0.5 % Cellulase Onozuka R-10,
0.1 % Macerozyme,
0.2 % Driselase,
0.25 % Pectinase,
13 % Mannitol,
Zapata Salts

This enzyme mixture was designated Enzyme Solution 1, (ES1).

Comparison of ES1 and cellulase for different tissues:

It had been shown that Se60 could respond to culturing, but that callus formation was restricted to the leaf sheath material and was not obtained from the more mature leaves. The ability of ES1 to release protoplasts from the leaf sheath of corn plants was tested and found to be negative. At all concentrations tested, however, Cellulase Onozuka R-10 could not cause protoplast release from emerged leaves but was effective in releasing protoplasts from the immature unrolled leaves in the leaf sheaths of 21-day old plants. The location of this tissue is shown in Figure 4. Utilizing this immature leaf tissue from 21-day old plants and 0.5 % Cellulase Onozuka R-10, pH 5.9, protoplast yields in two different extractions were 2×10^5 protoplasts/gram and 4.7×10^5 protoplasts/gram respectively.

A comparison of the activity of ES1 to that of 0.5 % cellulase in Zapata salts and 9 % mannitol at pH 5.9 on emerged and immature leaves of 21-day old plants is presented in Table 6. The enzyme mixture did not release protoplasts from immature leaves, but 0.5 % cellulase did. However, ES1 was required for protoplast production from mature leaves. The yield of protoplasts from immature tissue

Figure 4: Anatomical location of the plant tissue used in protoplast isolation from 21-day old corn plants.

- a) roots, coleoptile, emerged leaves were removed,
- b) the remaining stem was cut in half,
- c) slit open lengthwise and
- d) the young leaf tissue was dissected and put into the enzyme solution.

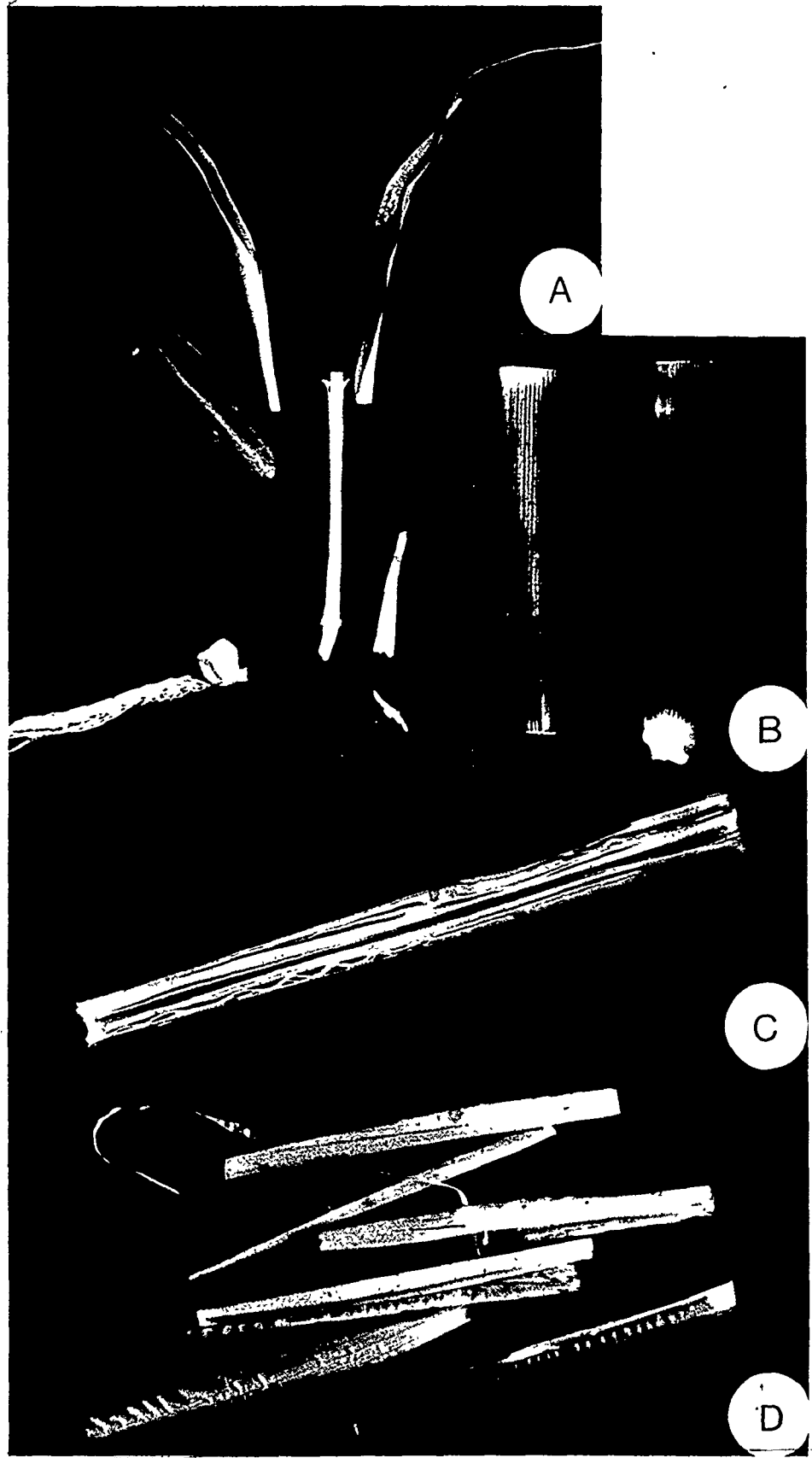


TABLE 6

PROTOPLAST YIELD (NUMBER OF PROTOPLASTS PER GRAM FRESH WEIGHT)
FROM TWO TISSUE SOURCES USING TWO ENZYME MIXTURES.

ENZYMES	LEAF TYPE	
	<u>EMERGED</u>	<u>IMMATURE</u>
ES1	8×10^4	nil
Cellulase (0.5%+ 9% mannitol, Zapata salts)	nil	1.2×10^6

in the presence of 0.5 % cellulase was 15 times that from emerged leaves in the presence of ES1, indicating that immature leaves treated with cellulase alone are a high-yield source of protoplasts.

As is shown in Table 4, neither of the two enzyme mixtures that were effective for protoplast isolation from wheat and barley was as effective as cellulase alone in causing protoplast release from corn leaf sheaths.

Effect of Incubation pH on Protoplast Release: Protoplast yield was measured as a function of pH for four incubation intervals. Figure 5 shows that in 1.5 hours of incubation, protoplast yield was not high at any pH, but increased steadily from pH 7.0 to pH 5.5. For incubation periods between 2.5 and 4 hours, the optimum pH was 6.5. The best conditions for protoplast production were 2.5 hours of incubation at pH 6.5:

The protoplast yields at pH 6.0 and 5.5 for incubation periods from 2 to 5 hours are illustrated in Figure 6. Both curves show a sharp optimum: three hours for pH 6.0 and four hours for pH 5.5. The increased time for optimal protoplast yield (compared with data in Figure 5) may be due to the increased age of the plant material (35 vs 28-day old plants). The pH of incubation mixtures has been reported to drop from pH 6.0 to pH 5.0 during an 18 hour protoplast isolation (Pelcher et al., 1974). This decrease could bring the pH of the solution to below the optimum for protoplast survival or to below that which is optimal for the action of the enzymes. During the decrease in pH, the enzyme's activity would be variable and could be the source of inconsistent yields. The drop in pH was probably the result of cell lysis.

Figure 5: Effect of pH on protoplast yield (number of protoplasts per gram fresh weight) from 28-day old plants during four time intervals.

- 1.5 hours
- 2.5 hours
- 4.0 hours
- 5.0 hours

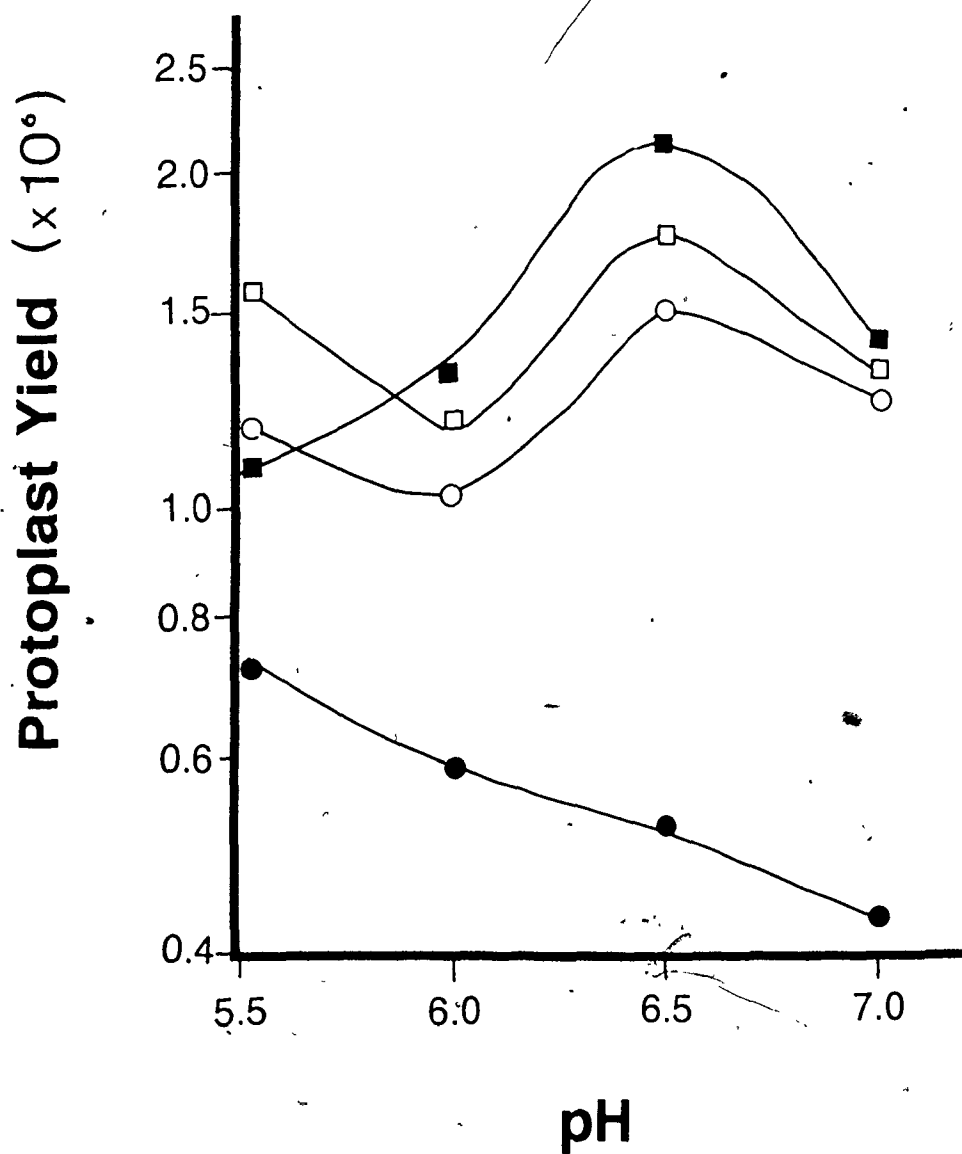
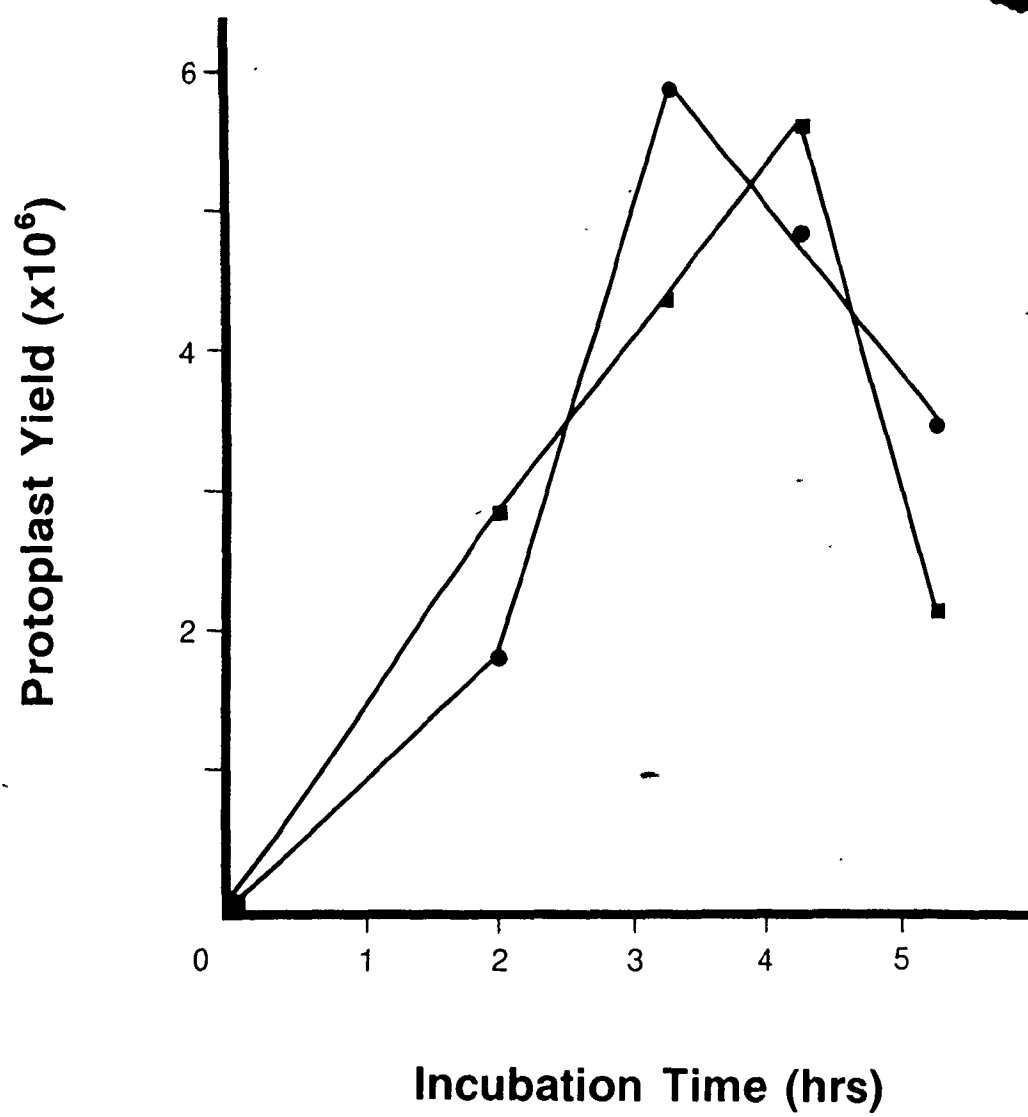


Figure 6: Effect of pH and time on protoplast yield (number of protoplasts per gram fresh weight) for 35-day

- old plants: -

● pH 6.0*

■ pH 5.5



Effect of Added Buffer: The initial pH of the incubation mixture without added buffer was 6.1, which dropped to pH 5.3 after 2.5 hours incubation. A similar incubation mixture, using added MES at 0.5 % (w/v) had a starting pH of 5.5 and a final pH after 2.5 hours of incubation of 5.4.

The optimal conditions for protoplast isolation from 21-day old plants were found to be pH 5.5 and three hours of incubation (see Fig 7) when the enzyme solution was buffered.

Effect of Pretreatment on Protoplast Yield: When the following conditions were used; 0.5 % Cellulase Onozuka R-10, 9 % mannitol, 0.5 % MES, Zapata salts, pH 5.5, 25⁰C, humid, dark treatment of both 12-day old and 20-day old plants (Fig 8) gave approximately three-fold increase in protoplasts over non-treated plants after 2 hours of incubation in the enzyme mixture. This was about twice the final yield obtained from non-treated plants after 4 hours of incubation.

Effect of Agitation on Protoplast Yield: Table 7 shows that more protoplasts can be recovered when leaf material from 25-day old plants was shaken in the enzyme mixture at 125 rpm. Shaking resulted in a 3.6-fold increase in yield.

Effect of Pretreatment and Agitation on Protoplast Yield:

The immature leaves were isolated from 25-day old plants, half of which had been pretreated (as above) and half had not. The extraction medium was 0.5 % Cellulase Onozuka R-10, 9 % mannitol and Zapata salts. Table 7 shows that pretreatment gave a 1.9-fold increase in protoplast yield over those which had no pretreatment, but had been subjected to agitation during enzymatic digestion. Thus, these two

Figure 7: Effect of pH on protoplast yield (number of protoplasts per gram fresh weight) in the presence of 0.5 % MES for 21-day old plants.

■ pH 5.5

● pH 6.5

◆ pH 7.0

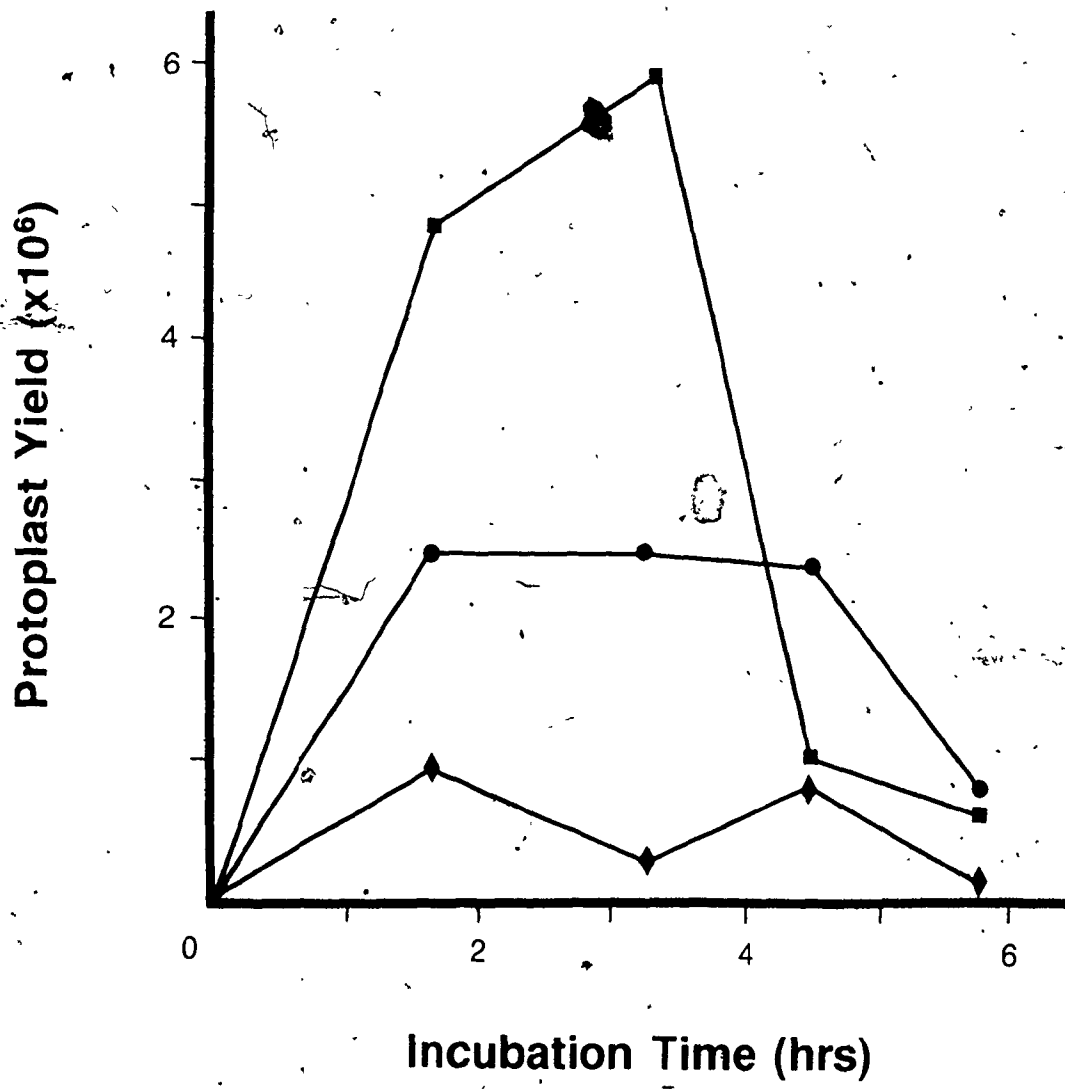


Figure 8: Effect of incubation time, highly humid pretreatment and plant age on protoplast yield (number of protoplasts per gram fresh weight).

- 12 day plants, highly humid
- 12 day plants, not highly humid
- 20 day plants, highly humid
- 20 day plants, not highly humid.

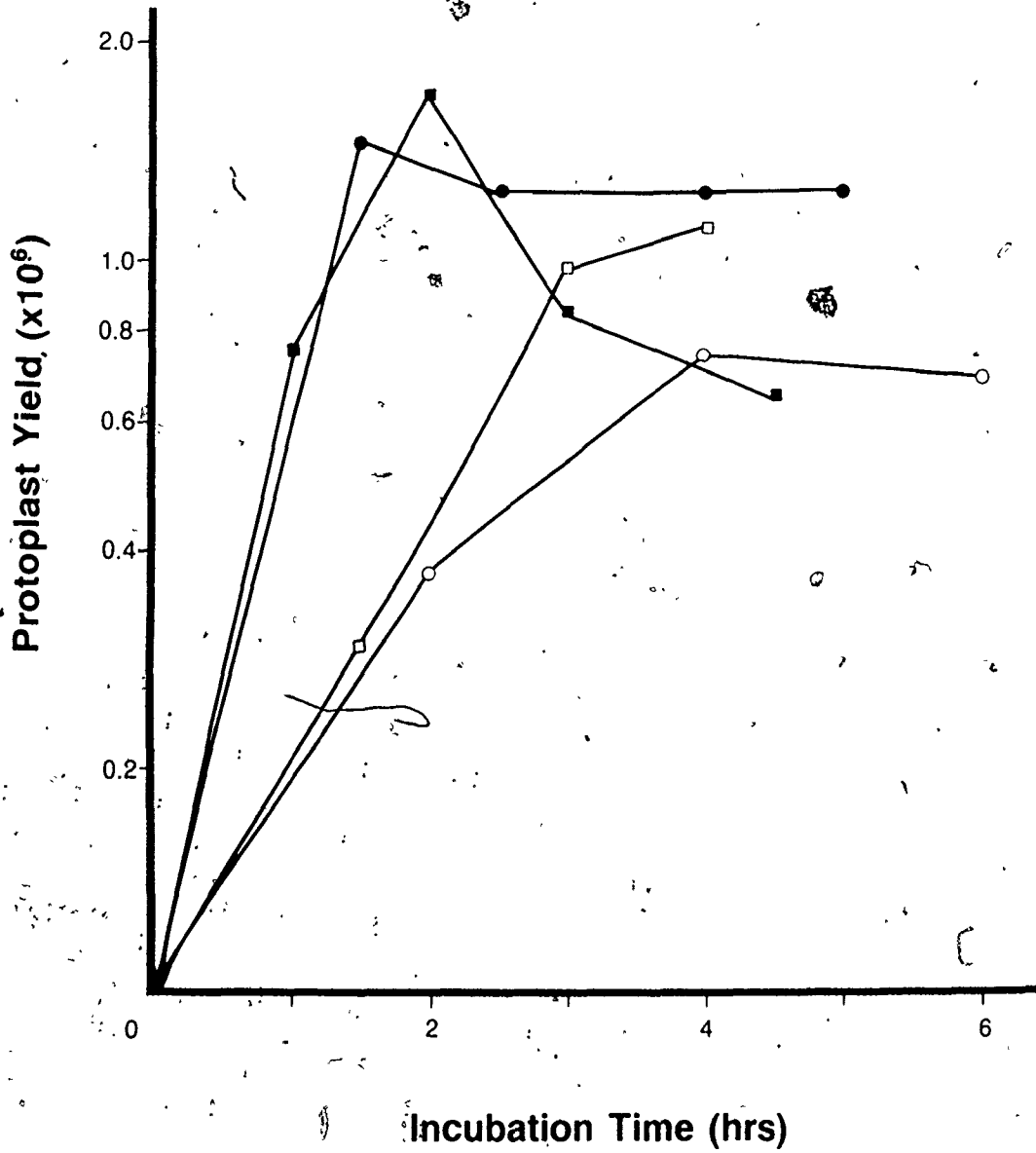


TABLE 7

EFFECTS ON PROTOPLAST YIELD OF SHAKING OF THE INCUBATION MIXTURE AND OF DARK, HIGH HUMIDITY PRETREATMENT OF PLANTS.

CONDITION	YIELD (protoplasts/gm.fr.wt.)
Non-shaking (No pretreatment)	1.8×10^6
Shaking (No pretreatment)	6.6×10^6
Dark, high humidity pretreatment. (with shaking)	1.3×10^7

steps: 1. dark, humid pretreatment of the plants and 2. shaking (125 rpm) during isolation, gave a final 6.9-fold improvement in protoplast yield.

Effect of Plant Age: Figure 9 shows the results of three protoplast isolation experiments using the conditions so far established and plants of various ages. Under these conditions immature leaves of 21-day old plants gave the greatest yield of protoplasts, which was 1.6×10^7 protoplasts/gram fresh weight. This represents a 100-fold increase in protoplast yield as a result of optimizing isolation conditions.

Clean-Up Procedures: Filtration: The general method (Gamborg and Wetter, 1975) of filtering the protoplast suspension through several woven filters of decreasing pore size, proved to be unsuccessful in removing the debris.

Hormone Pretreatment: a) Gibberellic Acid: As is shown in Table 5, GA_3 pretreatment 40 hours before protoplast isolation gave 58.8 % of the yield obtained from untreated plants. Isolation of protoplasts from GA_3 -treated plants 64 hours after treatment gave 89.1 % of the yield obtained from the untreated plants. There was no obvious reduction in the debris following either procedure.

b) Indole Acetic Acid: The effect of IAA pretreatment on protoplast yield is shown in Table 5. It is possible that the IAA-induced cellulase activity plus the exogenous treatment with Cellulase Onozuka R-10 caused extensive cell damage as only 22.2 % as many protoplasts were recovered from the IAA-treated plants as from the untreated plants.

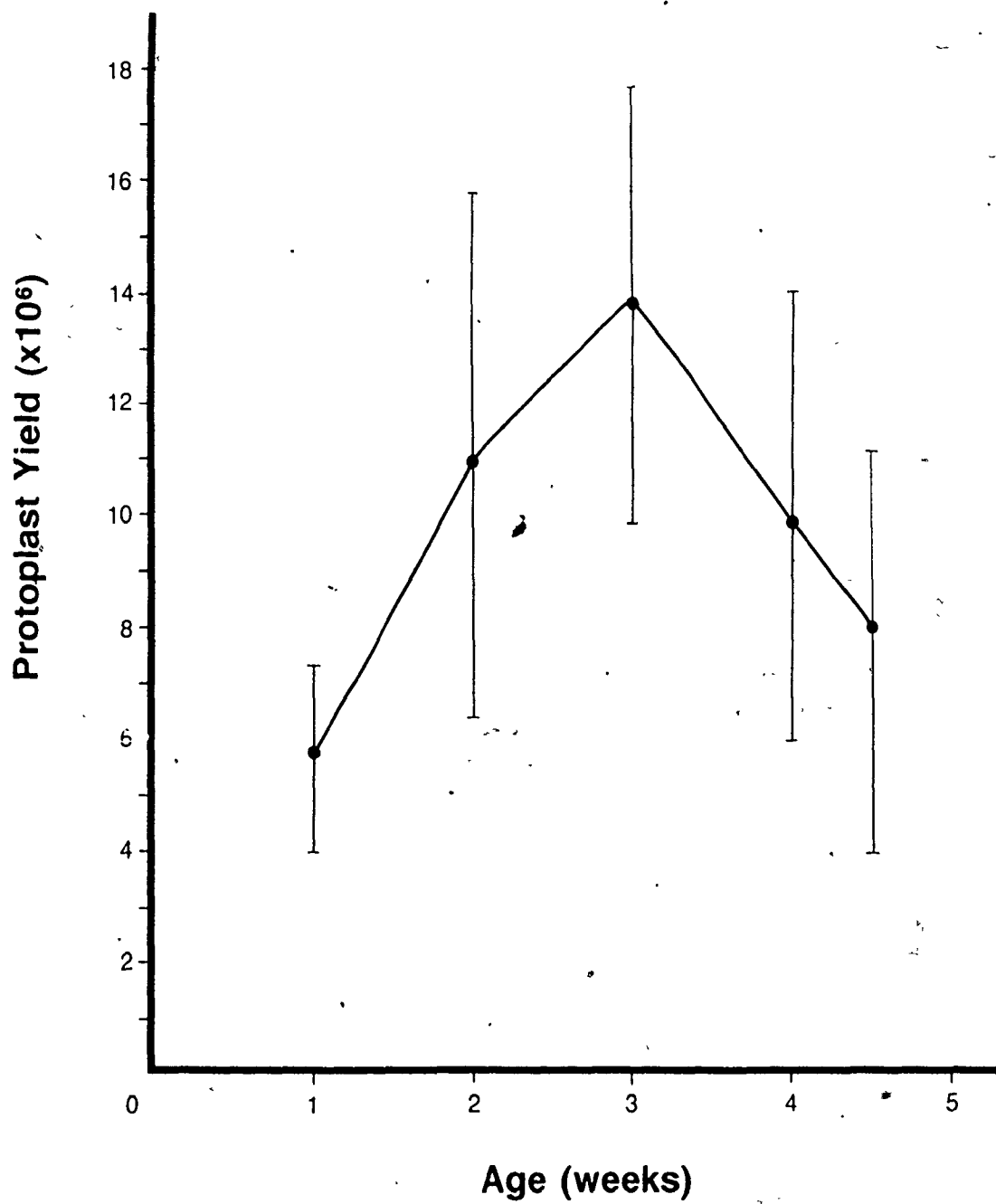
Figure 9: Protoplast yield (number of protoplasts per gram fresh weight) as a function of plant age.

Isolation conditions: 0.5 % Cellulase Onozuka

R-10, 9 % mannitol, 0.5 % MES, pH 5.5, shaking,

2.5 hours.

Bar = \pm one standard error.



Density and Phase Separation: This method failed to give clean protoplasts. During the procedure, 40.% of the protoplasts had been lost.

Sedimentation in CaCl_2 : The method used by Potrykus to obtain a clean preparation of B73 protoplasts failed to separate protoplasts from debris in the case of Se60 leaf sheath protoplasts. However, the Se60 protoplasts were found to be stable in 0.16 M CaCl_2 and were resuspended in this solution during harvest. It was observed that after about 10 minutes, debris particles clumped whereas the protoplasts did not. After filtration through a 50 μ stainless steel sieve the protoplasts preparation was cleared of most of the cellular debris. There was approximately 85 % recovery of the protoplasts using this procedure.

Conclusions

Seneca 60, a single cross hybrid, showed that it could respond to the P13 medium by callus formation, by elongation and swelling of tissues and by root formation. These three responses occurred in different regions of the stem and leaf sheath and they varied with plant age. Callus formation was most pronounced in 21-day old seedlings of Se60, although it occurred in all of the seedlings except those 35-days old. Callus also formed in leaf material located above the growing tip of the plant. Further analysis of callus growth in leaf tissue entailed the dissecting of leaf sections from the slices of axenically grown seedlings. There was callus formation in leaf material not associated with the apex, as well as

in those leaf slices which would have contained leaf bases of the growing tip:

The conditions for obtaining high yields of viable protoplasts from the responsive leaf sheath material of 21-day old greenhouse grown plants were established. The young leaves were incubated in a solution of 0.5 % Cellulase Onozuka R-10 in 9 % mannitol, Zapata salts and 0.5 % MES at pH 5.5 for 2.5 to 3 hours.

The protoplasts are harvested by centrifugation and resuspended in 0.16 M CaCl_2 plus 0.5 % MES, pH 5.5. After 10 minutes, the solution is filtered through a 50 μ stainless steel sieve and the protoplasts are washed twice in 0.16 M CaCl_2 + 0.5 % MES and then resuspended in culture medium. The final yield of debris-free protoplasts is from 1×10^6 to 1×10^7 protoplasts per gram fresh weight of plant tissue, which compares favourably with other reports in the literature (Giles, 1974; Aldrich et al., 1977).

The protoplasts, once isolated were studied for survival under many physical and cultural conditions and several new media were designed for their maintenance. However, none of these media proved better than that designed by Nitsch and Nitsch (1967) and modified by Potrykus et al., (1979) (see Chapter III), for the survival of the corn protoplasts. Thereafter, NN67 medium was used for the culture of the Se60 protoplasts. The results of the media testing experiments are reported in appendix 1.

Chapter III: Model Systems: Hyoscyamus muticus L. and the B73
maize cell line.

Introduction

This chapter describes the conditions that were already well-established and routinely used with Hyoscyamus muticus protoplasts and the B73 maize cell line protoplasts (Potrykus et al., 1977) in the plant tissue culture laboratories of the Friedrich Miescher Institute. These are the methods and media that I used to obtain the data in this chapter and for other studies presented in this thesis.

(a) HYOSCYAMUS MUTICUS

Introduction:

Mesophyll protoplasts isolated from Hyoscyamus muticus L. (Egyptian henbane) have been induced to divide in culture to form cells and callus and to regenerate plants via shoot formation and somatic embryogenesis (Lorz et al., 1979; Wernicke et al., 1979). Since the techniques for this process are established and reproducible, H. muticus is a good model system in which to examine processes taking place in the regeneration of protoplasts to cells and to study the factors affecting the differentiation of cells to whole plants. Information and techniques learned from the manipulation of a responsive plant could lead to the establishment of similarly effective techniques for less responsive plants such as the cereals.

Materials and Methods

H. muticus plants were grown in a greenhouse from seeds in 6 to 9 inch diameter pots for 8 to 10 weeks at 27° C day/18° C night. They were kept under low light for a 12 hour photoperiod. An 8-week-old H. muticus plant grown in a 9 inch pot is shown in Figure 1.

Protoplast Isolation: The series of steps leading to protoplasts from fresh material is illustrated in Figure 2. The third to the sixth leaves, counting from the apex, were surface sterilized in 0.01 % HgCl₂ + a few drops of Tween 80 as wetting agent for 15 minutes. They were then rinsed five times with sterile distilled water. The midrib of a leaf (Fig 2a) was removed as shown in Figure 2b, and the two halves of the leaf were stacked (Fig 2c) and cut diagonally into thin (1-2 mm) strips (Fig 2d). Cutting of the leaves vertically or horizontally resulted in lower yields of protoplasts. The leaf slices were placed in an osmotically balanced enzyme solution (Fig 2e) consisting of 1 % Cellulase Onozuka R10 (Kinki Yakult, Nishinomiya, Japan), 1 % Pectinol Fest (Rohm, Darmstadt), and 0.2 % hemicellulase (Rhizopus, Sigma H2125), 0.3 M mannitol, 0.04 M CaCl₂ and 0.5 % MES, at pH 5.7. The leaf slices were vacuum infiltrated with the enzyme solution (Fig 2f) and then transferred to fresh enzyme solution in a 9 cm Petri dish (Fig 2g) and allowed to stand for 2 to 3 hours at 30° C. Protoplast release caused the enzyme solution to become visibly green and turbid (Fig 2h). Protoplasts being released from tissue clumps and leaf veins can be seen at higher magnification in Figures 2i and 2j. The protoplasts became

Figure 1: *Hyoscyamus muticus* L.

Left: The greenhouse grown plant is 8 weeks old and 25 cm. tall.

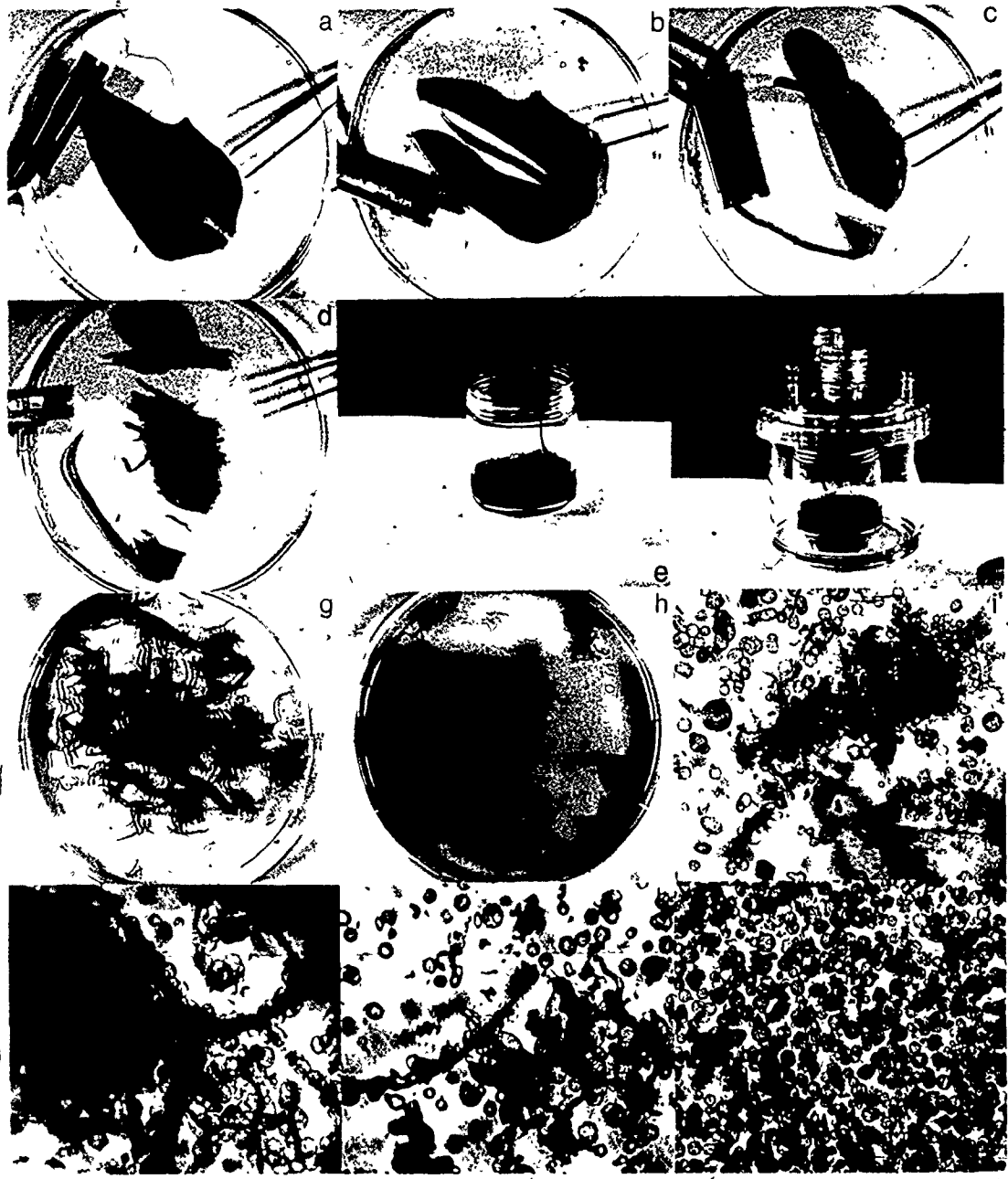
Right: A close-up photograph of the leaves used for the isolation of protoplasts. The plants were 8 to 9 weeks old when used; the third to sixth leaves, counting from the apex were used. Typically, these were leaves 13-16 counting from the cotyledons.



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Figure 2: Procedures for the isolation of mesophyll protoplasts from H. muticus.

- (a) A leaf from H. muticus was placed in a few drops of CaCl_2 in a 9 cm Petri dish.
- (b) The midvein of the leaf was removed.
- (c) The two halves of the leaf were stacked together and
- (d) sliced diagonally in 1 to 2 mm. strips.
- (e) The strips were transferred to a 5 cm screw top vessel containing the enzyme mixture and
- (f) vacuum-infiltrated with the enzyme mixture and then
- (g) returned to a 9 cm Petri dish containing fresh enzyme.
- (h) After 2 to 3 hours at 30°C , there was protoplast release as shown by the turbid background in the Petri dish.
- (i), (j), (k) Protoplasts are shown in clumps connected to the leaf veins (i) X 100 ; (j) X 150 ; (k) X 150 .
- (l) Final protoplast suspension X 100 .



more dispersed as digestion continued (Fig 2k) and finally a protoplast suspension resulted (Fig 2l).

The protoplasts were separated from large debris and tissue by filtration through 250 μm , 100 μm and 50 μm stainless steel sieves. The protoplast suspension was diluted 1:2 with 0.16 M CaCl_2 + MES and centrifuged at 300 g for 5 minutes. This resulted in two fractions of protoplasts: a floating layer and a pellet. Both were handled separately during the clean-up procedures. The floating protoplasts usually did not have an excessive amount of debris associated with them. They were pelleted by centrifugation in 0.16 M CaCl_2 + MES, rewashed in the same medium, and then resuspended in medium. The pelleted protoplast fraction was resuspended in 2 ml of 0.16 M CaCl_2 + MES and layered over a 22 % sucrose solution. After slow centrifugation (300 x g) for 10 minutes, protoplasts were harvested from the interface, washed twice in 0.16 M CaCl_2 + MES and then recombined with the floating fraction of protoplasts in culture medium.

Protoplast Culture: The density of the protoplasts was adjusted to $2-4 \times 10^5$ /ml in DPD medium (Durand et al., 1973) containing 0.3 M mannitol, 2 % sucrose, 4 mg/l pCPA (parachlorophenoxyacetic acid) and 1 mg/l kinetin, pH 5.8. They were cultured in thin layers (2-4 mm) of DPD medium in 9 cm plastic Petri dishes, overnight at 12° C and for 2-3 weeks at 26° C, in the dark.

Callus Culture: Small samples of the 2-3 week old suspension were cultured in multi-dishes (Costar, U.S.A.) in three different media: DPD (Durand et al., 1973), NT (Nagata and Takebe, 1971) or NN67 (Nitsch and Nitsch, 1967). The compositions of the three

media are given in Table 1. Each medium was made up in 0.15 M mannitol and 1 % sucrose. Each multi-dish contained one medium with serial dilutions (by 1/4) of the phytohormones to determine the optimal concentration for rapid callus proliferation. Two-way gradients were made as follows: pCPA was serially diluted right to left from 4 mg/l to 0.06 mg/l; kinetin was diluted serially top to bottom from 4 mg/l to 0.004 mg/l. The multi-dishes were sealed and placed in a 26° C growth chamber in the light at 5000 lux for a 16 hour photoperiod.

Plant Regeneration: After 14 days, actively growing, green calli were transferred to agar (0.8%) containing the same growth medium from which they had been selected, but without mannitol. Once these calli established shoots, they were transferred to medium of the same composition, but containing 0.5 % sucrose and 0.1 mg/l NAA for the induction of roots. From this stage these calli could be induced to regenerate plants through hydroponic culture followed by transplantation into soil.

Results

The stages of H muticus protoplast development in culture are shown in Figures 3 and 4. Initially, the protoplasts appeared spherical with chloroplasts evenly distributed throughout the cytoplasm. After 3-5 days in culture, the protoplasts underwent shape changes and the chloroplasts clustered around the nucleus (Fig 3a). After 7-10 days of culture, the two cell stage shown in Figure 3b was common. Thereafter, 4 and 8 cell clusters became frequent (Fig 3c and d). Eventually small calli formed (Fig 3e-h). The results of transferring small calli and cell clumps to 3 different media having

TABLE 1

DEFINED MEDIA USED IN THE CULTURE OF PROTOPLASTS, CELLS AND CALLI
 OF HYOSCYAMUS muticus L. AND THE B73 MAIZE CELL LINE.

MEDIA				
Macro-elements (mg/l)	DPD	NT	NN67	CC
NH ₄ NO ₃	270	825	-	640
Ca(NO ₃) ₂ ·4H ₂ O	-	-	500	-
KNO ₃	1480	950	125	1212
CaCl ₂ ·2H ₂ O	570	435	-	588
KH ₂ PO ₄	800	680	125	136
MgSO ₄ ·7H ₂ O	340	125	125	247
Micro-elements (µg/l)				
H ₃ BO ₃	200	620	10000	3100
MnSO ₄ ·4H ₂ O	500	1690	25000	11150
ZnSO ₄ ·7H ₂ O	150	860	-	5760
KI	25	83	830	-
Na ₂ MoO ₄ ·7H ₂ O	150	25	250	240
CuSO ₄ ·5H ₂ O	1.5	2.5	25	25
CoCl ₂ ·6H ₂ O	1.0	3.0	25	28
Vitamins (µg/l)				
soluble starch	-	+	-	50000
i-inositol	1000	10	100000	90000
folic acid	40	-	500	-
thiamine HCl	400	100	500	8500
nicotinic acid	400	-	500	6000
glycine	140	-	2000	2000
pyridoxin HCl	70	-	500	1000
biotin	4	-	50	-
Iron (mg/l)				
FeSO ₄ ·7H ₂ O	27.8	27.8	27.8	27.8
Na ₂ ·EDTA	37.3	37.3	37.3	37.3

Figure 3: Cells and calli derived from cultured protoplasts of H. muticus.

(a) A cultured "protoplast" after 3 to 5 days showing a changed shape, presumably because of cell wall resynthesis.

Magnification: 1.5×10^3 .

(b) After 7 to 10 days of culture, some cells had divided and appeared as doublets.

Magnification: 1.5×10^3 .

(c) to (f) Clusters of four, eight and more cells appeared after 10 to 14 days.

Magnification: (c) and (d): x 1000

(e) and (f): x 500.

(g) and (h) Calli with many cells appeared after 2 to 3 weeks. Magnification: x 100.

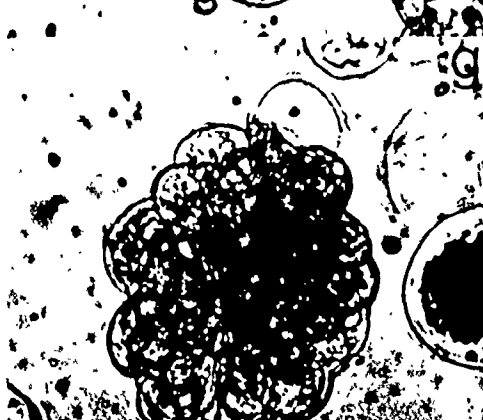
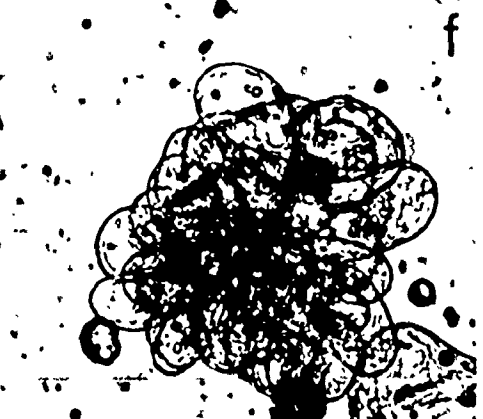
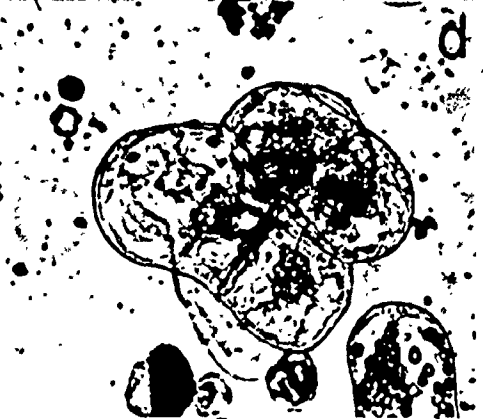
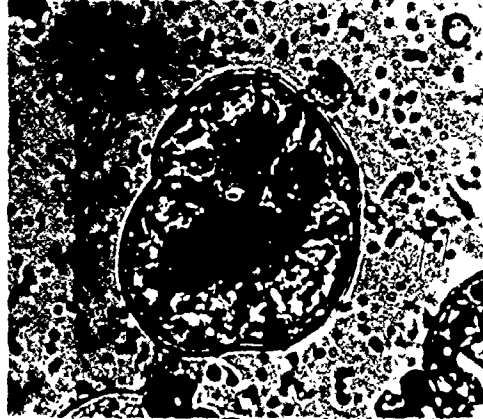
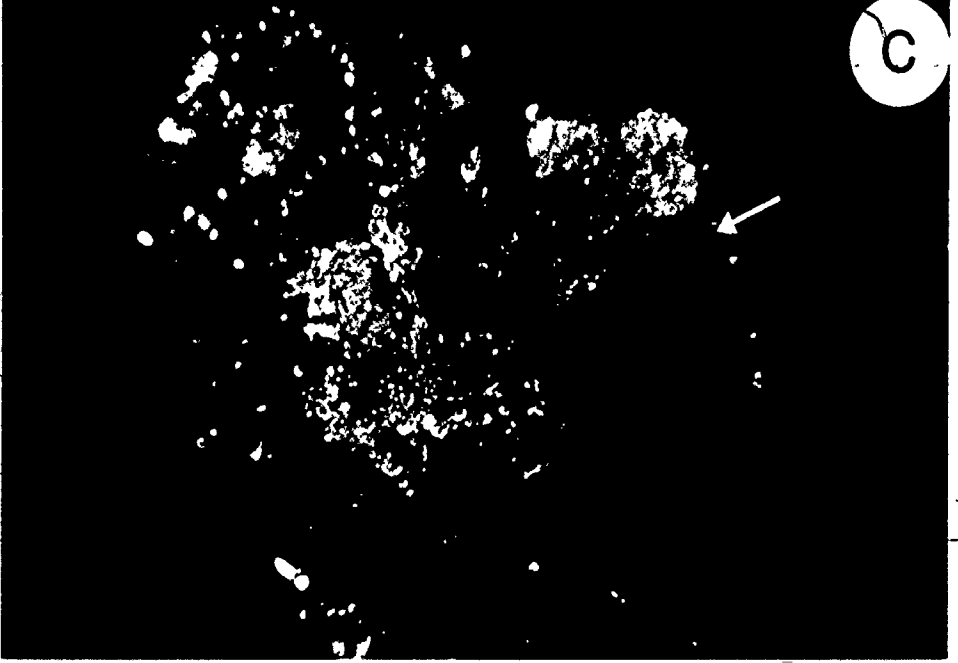
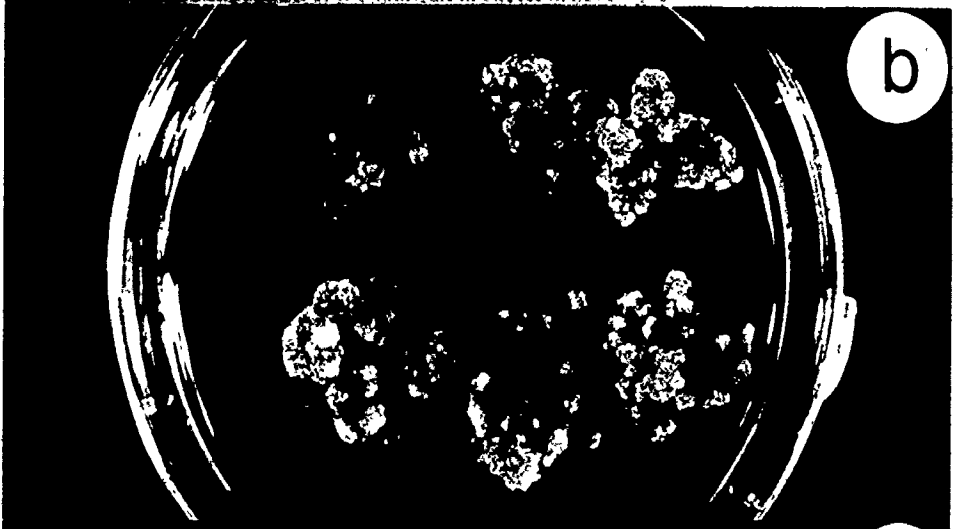
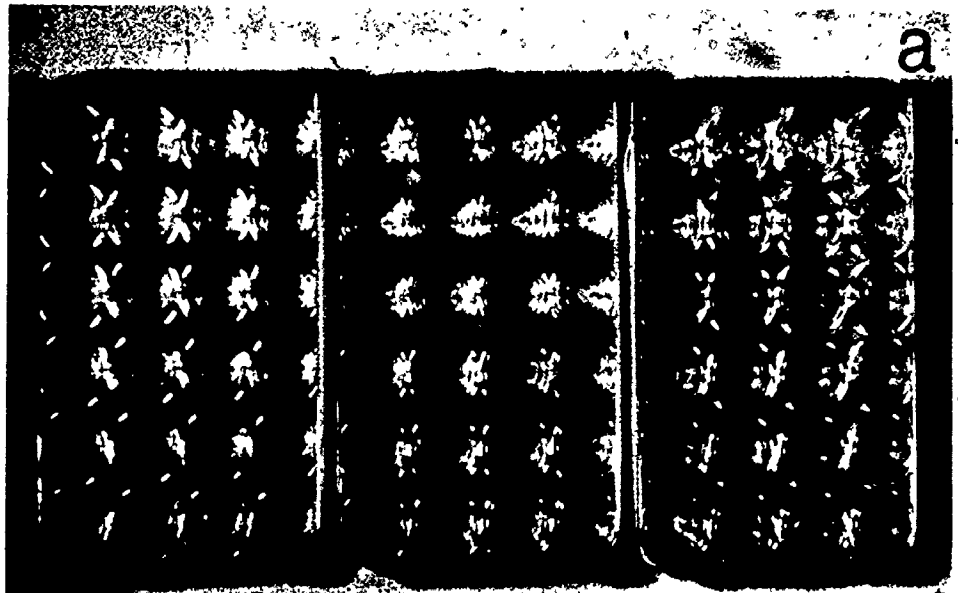


Figure 4: Callus proliferation and organ regeneration in H. muticus.

- (a) Multi-well dishes used for the screening (described in text) of those phytohormones inducing rapid callus proliferation.
- (b) Calli after 14 days after transfer from the multi-well to 6 cm Petri dishes, formed small leaves as shown in the upper left callus. Magnification: x 1.5.
- (c) A 20 X magnification of the leaf shown in Figure 5(b).



various phytohormone concentrations are shown in Figure 4a. Most rapid callus growth was observed in NT medium with 0.25 mg/l pCPA and 0.06 mg/l kinetin (Fig 4a, center).

When the calli from the wells were placed onto agar (as described above) there was rapid proliferation and the development of young leaves (upper left callus in Fig 4b and 4c).

(b) The B73 Maize Cell Line.

Introduction:

The B73 maize cell line was established by Potrykus and co-workers (1979). It was the first and only cell line to be established from freshly isolated corn protoplasts. The source tissue was the stem of a 10 week-old field grown inbred, B73. The protoplasts divided in culture and can still be maintained either as suspension culture or as callus. The cells of this line can be enzymatically returned to protoplasts but cannot be induced to regenerate plants. As with other non-morphogenetic cell lines, the B73 cell line has cytogenetic abnormalities (see chapter V). The B73 cell line was used as a model system in order to study the growth and maintenance characteristics of a cereal.

Materials and Methods:

Culture Maintenance: The B73 cells were cultured in CC medium (Table 1). During maintenance, the cells were diluted 1:10 with medium every 5 or 6 days. During experiments, they were diluted 1:4 every 2 days or 1:2 every day. It was important to dilute the cells the night before protoplast isolation.

Callus Culture: The B73 cell line can be maintained as callus by plating the large cell clumps onto CC-agar (0.8%). The calli that grow are friable and loose and can be easily resuspended in liquid medium to reinitiate suspension cultures.

Protoplast Isolation: Cells from suspension culture were centrifuged at 200 x g for 5 minutes and the pellet was resuspended

in the following enzyme mixture: 1 % Cellulase Onozuka R-10, 0.5 % Pectinol Fest, 0.5 % hemicellulase, 0.3 M mannitol, 0.04 M CaCl_2 and 0.5 % MES, pH 5.7. They were placed on a rotary shaker at 75 rpm for 2 hours at 26°C , in the dark. To harvest the protoplasts, the suspension was passed through a 50 μm sieve, diluted 1:1 with 0.16 M CaCl_2 + 0.5 % MES and centrifuged at 300 g for 10 minutes. The protoplasts were washed twice in the CaCl_2 -MES medium and cultured in NN67 medium (Table 1) plus 0.17 M mannitol, 2 % sucrose and 2 mg/l 2,4-D. They were kept in the dark at 12°C overnight, then transferred to 26°C for 10-14 days. Over this period the protoplasts reformed walls and divided. The cells were washed and resuspended in CC medium for further growth as shake cultures (100 rpm) at 26°C in the dark.

Results:

Cell Cultures: The growth characteristics of the B73 maize cell line are discussed in chapter V. The cells grow in clusters, are often elongated and are relatively free of plastids. Some cells are shown, after staining with Hoechst 33258 according to the method of Laloue et al., (1980) (but with no background clearance), in Figure 5. The cells are similar to root cortical cells in appearance. Unlike cells that maintain their ability to regenerate plants, the B73 cells are not rounded and full of plastids.

Callus: The calli that grew from cell clumps, which were transferred from liquid culture to agar, are non-morphogenetic in appearance, i.e., they are not tight and compact calli, able to give rise to embryoids but are loose, watery and friable (Fig 6a). Aging calli often turn brown or yellow (Fig 6b).

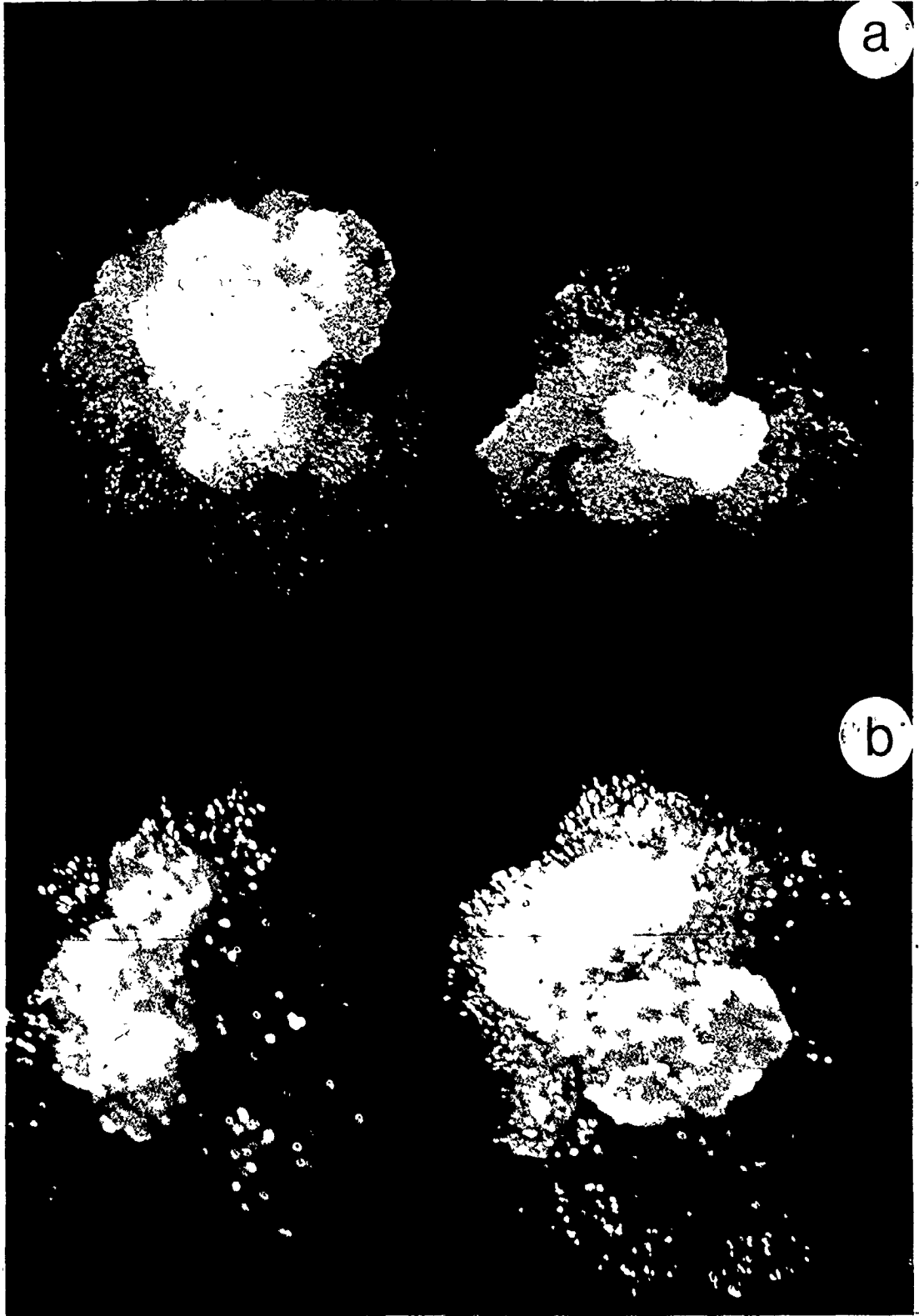
Figure 5: B73 maize cell line cells stained with Hoechst 33258 according to the method of Laloue et al., (1980) without background stain clearance. Magnification x 1000. (Photographed on a Leitz Orthoplan Fluorescence microscope, light source, a 200 w Osram HBO high pressure mercury lamp used with excitation barrier filter UV-UGI and dichroic filter assembly Ploempak A; film, Ilford HP-4).



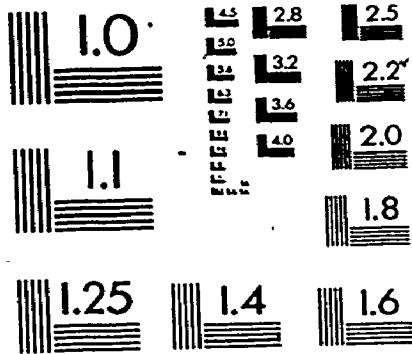
Figure 6: Callus culture of the B73 maize cell line.

- (a) Calli were very friable and loose.
- (b) Aging calli accumulated phenolics and turned brown.

Magnification: x 10.



2



Protoplasts: The B73 cells and their resulting protoplasts are shown in Figure 7. The protoplasts in Figure 7b were produced from the cells in Figure 7a. Figures 7c and 7d are higher magnification photos of the same cells and protoplasts. The average size of newly isolated protoplasts was 20 μm . The stages of B73 protoplast development in culture are shown in Figure 8. Spherical, newly isolated protoplasts can be seen in Figure 8a. There was often spontaneous fusion of protoplasts during isolation. After 7-9 days in culture some protoplasts had changed shape and divided (Fig 8b). The elongation and division were accompanied by cell clumping due to cell wall regeneration (Fig 8c).

Summary

Hyoscyamus muticus has been shown to be an ideal "model plant" (Lorz et al., 1979) in that it can be easily manipulated in culture. Primary protoplast cultures have been established and cultured under various conditions giving cell suspensions and calli from which plants can be regenerated. Such a system is useful for the study of the physiological events that occur in the early culture of the protoplasts and which lead to the complex events of embryogenesis and regeneration.

Although the B73 maize cell line has lost its ability to regenerate plants and thus, is not useful for the study of embryogenesis, it is still a useful system for the culture needs and manipulative techniques necessary for the handling of cereals in culture. The protoplast-to-cell line sequence of events can be studied and compared to that of non-cereal tissue culture and non-responsive cereal tissue cultures.

Figure 7: Cells and protoplasts of the B73 maize cell line.

- (a) Stationary phase cell clusters of the B73 cell line.
X 200.
- (b) Protoplasts derived from the cell culture shown
in Figure 7(a). X 500.
- (c) A higher magnification of Figure 7(a). X 500
- (d) A higher magnification of Figure 7(b). X 800.

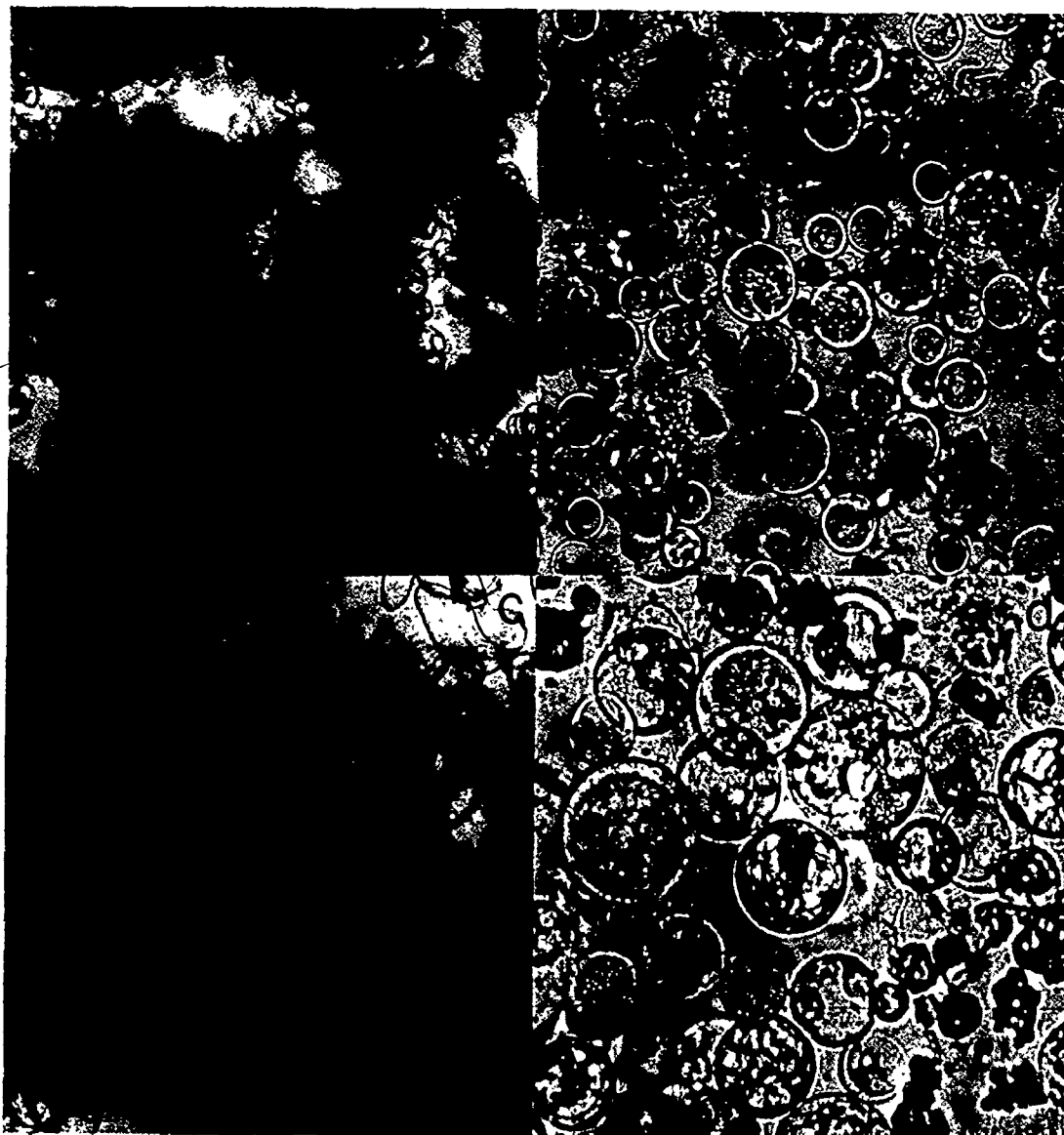


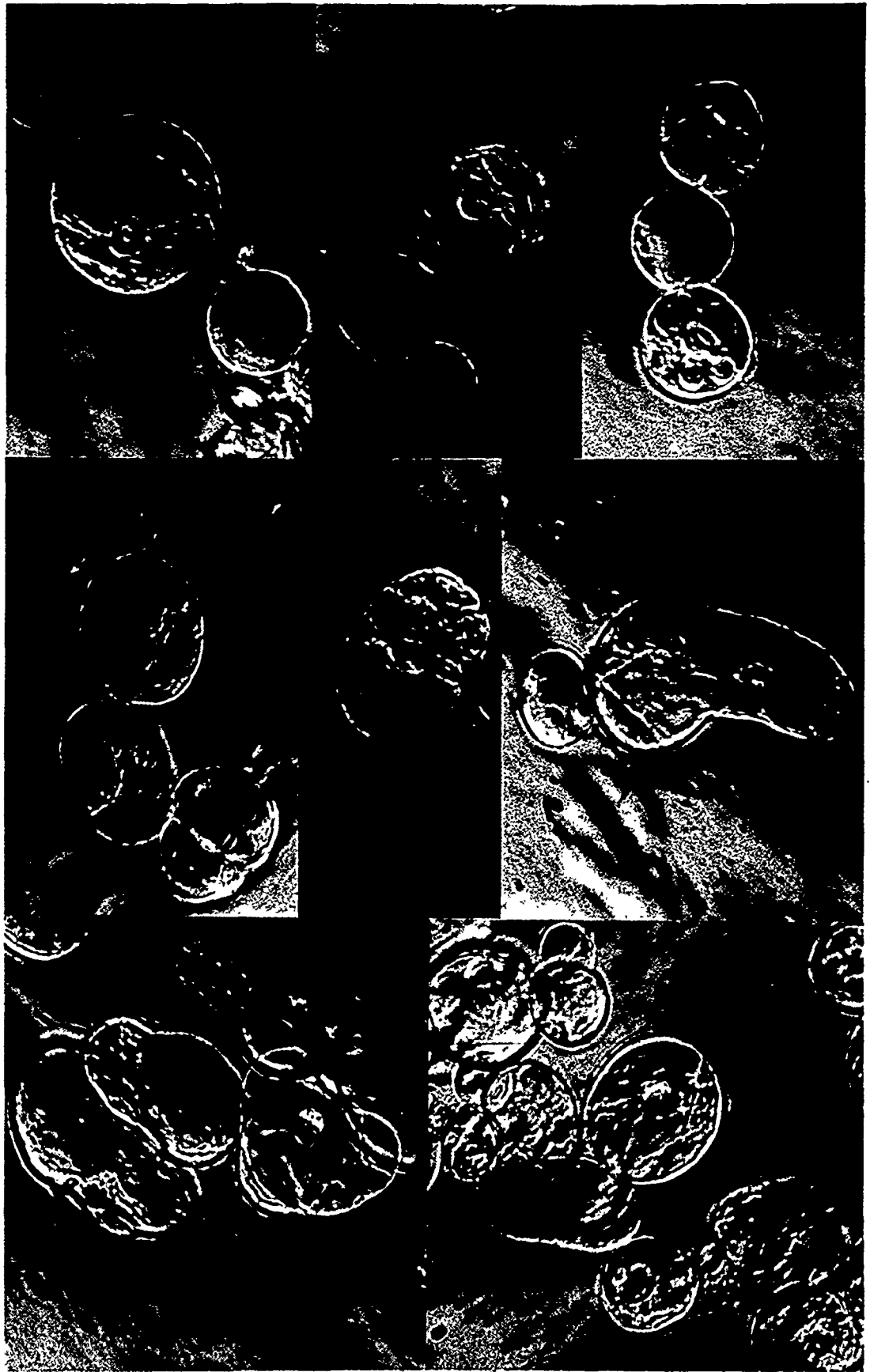
Figure 8: Protoplasts of the B73 maize cell line.

(a) Newly isolated protoplasts.

(b) Protoplasts after 7 to 10 days of culture.

(c) Protoplasts and cells after 10 days of culture.

Magnification: $\times 10^3$.



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Chapter IV: Fluorescence techniques.

- A. A method for staining plant cell protoplasts with Hoechst 33258. (Plant Cell Reports 1: 77-79).

Introduction:

It would be of great interest for plant scientists to be able to use flow cytometric techniques with plant cells and protoplasts. These techniques would be useful to analyze cell cycle events in cultured cells and also to sort cycling from non-cycling cells. More importantly, flow cytometry may provide a general method for separating hybrid fusion products from parental fusion products and non-fused protoplasts. Flow cytometry requires the use of fluorescent dyes which bind to the cells to be analyzed. A prerequisite for sorting and further culturing of the cells is that the fluorescent dye be a vital and non-toxic stain.

The bis-benzimidazole dyes, Hoechst 33258 and Hoechst 33342, are vital, non-toxic in cultured mammalian cells and are considered to be quantitatively specific for DNA (Arndt-Jovin and Jovin 1977; Cesarone et al., 1979). Previous uses of Hoechst 33258 in plant cells for nuclear and chromosome studies (Filion et al., 1976; Laloue et al., 1980; Galbraith et al., 1981) have used material fixed prior to staining. We describe a procedure for the vital staining with the dye, Hoechst 33258, of nuclei of protoplasts derived from cultured plant cells.

Materials and Methods

Protoplast Isolation: The protoplasts were derived from the maize cell line, inbred B73, established by Potrykus *et al.*, (1977). The cells were routinely maintained in suspension culture as described by Potrykus *et al.*, (1979). To isolate protoplasts from the cells, a stationary phase culture was diluted 1/4 with fresh medium and cultured overnight. The cells were then harvested by gentle centrifugation and resuspended in an iso-osmotic solution containing 1 % Cellulase Onozuka R-10 (Kinki Yakult, Nishinomiya, Japan), 0.5 % Pectinol Fest (Rohm, Darmstadt, FRG) and 0.5 % Hemicellulase (Rhizopus, Sigma H2125, St. Louis, USA) in 0.3 M mannitol and 0.04 M CaCl_2 at pH 5.8. After incubating with gentle shaking for 2 to 3 h at 24° C in the dark, protoplasts were separated from remaining cell clumps and large debris by successive passages through 250 μm , 100 μm and 50 μm stainless steel sieves. They were then harvested, washed three times in 0.16 M CaCl_2 + 0.5 % MES (2-N-morpholinoethane sulfonic acid), pH 5.8, and cultured in NN67 medium (Nitsch and Nitsch, 1967) + 0.17 M mannitol, 2 % (w/v) sucrose and 2 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid).

DNA Staining: The Hoechst 33258 (H33258) was a gift from Dr. H. Loewe, Hoechst AG, Frankfurt, FRG. It was stored as a 1 mg/ml stock solution in water and stored at 4° C in the dark. H33258 was found to precipitate from solution at 4° C when dissolved in phosphate-containing buffers.

To establish the relationship between pH, concentration of the

dye, time in stain and other constant aspects of the staining procedure, the manipulations were carried out with the protoplasts resuspended in 0.16 M CaCl_2 . The pH was adjusted with KOH.

Microscopy: The microscopic examination of the protoplasts was done on an Orthoplan microscope equipped with an Orthomat automated camera (Leitz). The light source was a 200 W HBO high pressure mercury lamp used with excitation barrier filter UV-UG1 and dichroic filter assembly Ploempak A.

Toxicity of H33258 to Protoplasts: Hoechst 33258 is known to be non-toxic to mammalian cells but has not been tested on plant cells. Petunia hybrida protoplasts were stained with H33258 according to the procedure established below, for 1 to 20 min. The survival and plating efficiency of stained cultures were identical to those of control cultures.

Results and Discussion

Effect of pH: The relationship between the pH of the medium and H33258 uptake is shown in Fig. 1. The dye is optimally taken up at pH 7.5; however, even at this pH, no more than 80% of the protoplasts stained. A typical preparation of protoplasts is shown in Fig 2 where some protoplasts have stained and others have not.

Use of Surfactants: To increase protoplast uptake of H33258, two non-ionic detergents were added to the protoplast solutions at varying concentrations in the presence of the dye at pH 7.5. The results are in Fig 3. Tween 80 inhibited the uptake of the dye at all concentrations whereas Triton X-100 increased nuclear staining to

Figure 1: The effect of pH on the staining of the plant protoplast nuclei by H33258. Even at the optimum pH, the nuclei of only 80 % of the protoplasts stained.

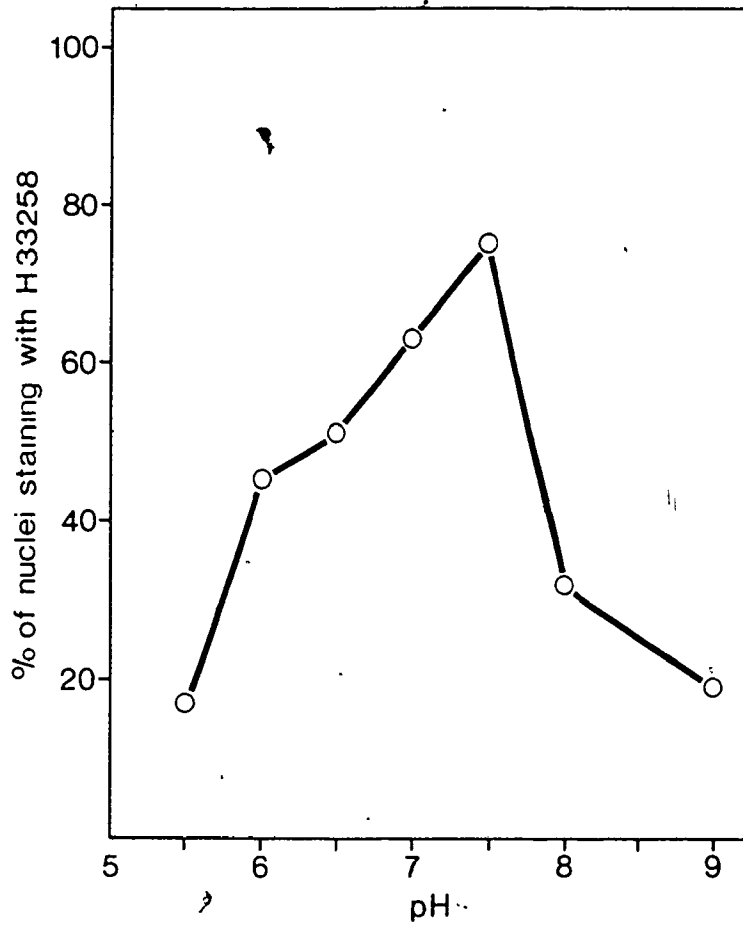


Figure 2: Illustration that not all plant protoplasts stain with H33258 in the absence of detergent.

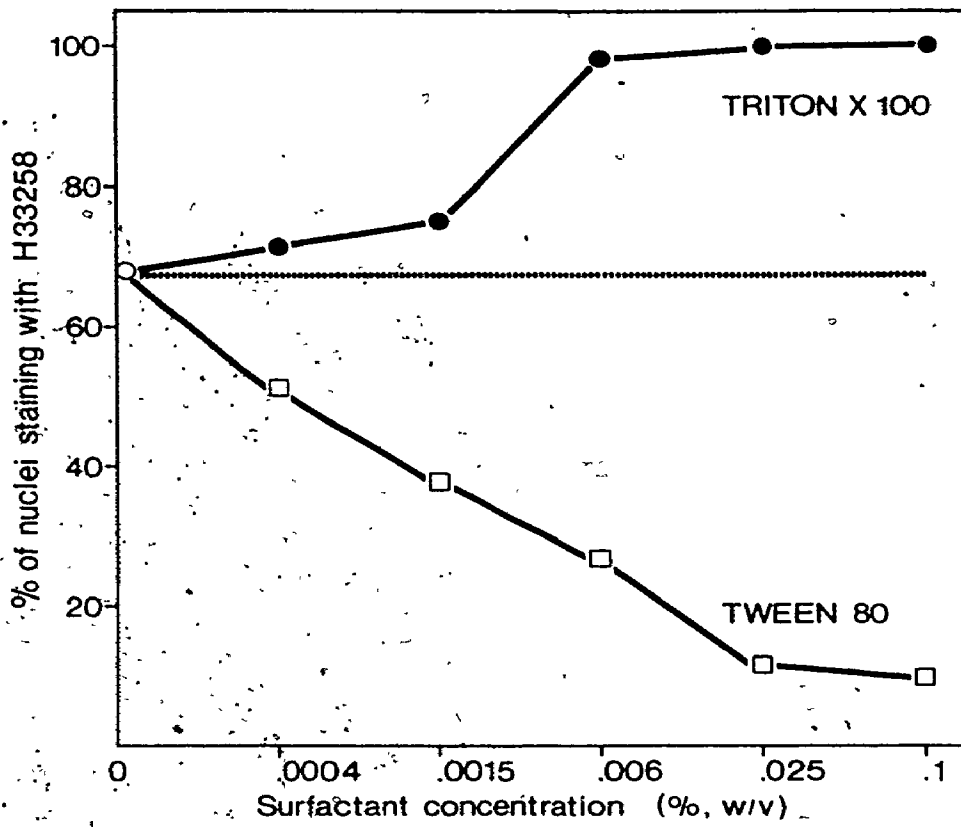
(a) Transmitted-light photomicrographs of B73 protoplasts

(b) Fluorescence photomicrographs of the same protoplasts shown in panel "a". The protoplasts labelled "1" have not taken up the stain. Arrows indicate nuclei.



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Figure 3: The effect of two non-ionic detergents on H33258 uptake into the protoplast nuclei. Tween 80 prevented uptake at all concentrations whereas Triton X-100 enhanced uptake.



100 % at concentrations from 0.006 % and higher. Taylor and Milthorpe (1980) have recently reported similar results for Triton X-100 with mammalian cells stained with Hoechst 33342.

H33258 Concentration: The effect of H33258 concentration on the percentage of protoplast nuclei staining with and without Triton X-100 treatment is shown in Fig 4. The measurements were made after the protoplasts were in the stain for 1 h. In separate experiments, it was found that good staining was obtained in 15 min with an H33258 concentration of 20 µg/ml, and a Triton X-100 concentration of 0.006%.

Background Staining: We found that the membranes of the protoplasts fluoresced slightly after treatment with H33258. This background staining was eliminated if the protoplasts were treated with DNAase (50 µg/ml final concentration) for 20 min at 37° C before exposure to the stain.

Conclusions

In anticipation of using flow cytometric techniques with plant protoplasts, we have defined conditions for the staining of plant protoplast nuclei with H33258, a vital fluorescent stain for DNA.

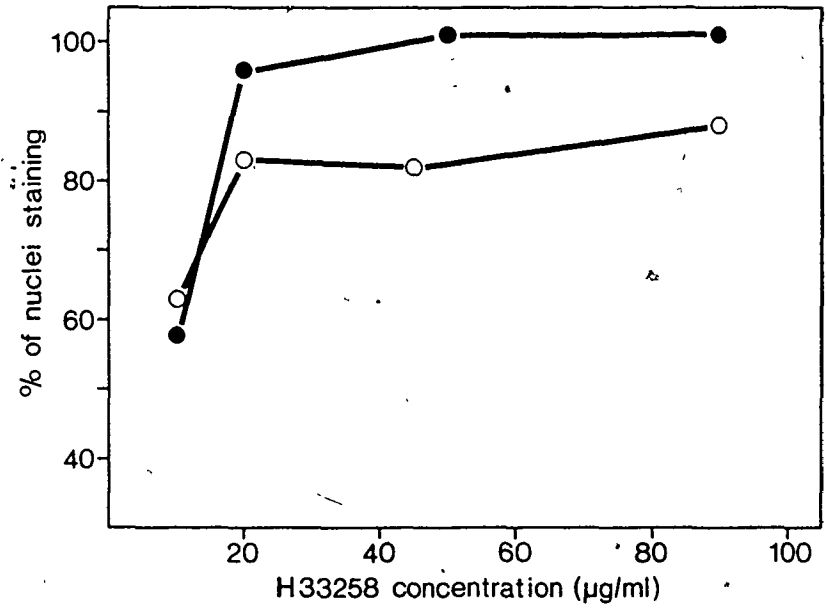
The following protocol summarizes these conditions:

- (1) Protoplasts in medium are treated with DNAase (50 µg/ml final concentration) for 20 min at 37° C.
- (2) After washing, the protoplasts are resuspended in 0.16 M CaCl₂, pH 7.5, in the presence of 0.006 % Triton X-100.
- (3) H33258 is added to a final concentration of 20 µg/ml.
- (4) After 15 min, the protoplasts are washed and resuspended in medium for sorting or for microscopic examination.

Figure 4: The effect of H33258 concentration on the percentage of nuclei staining at pH 7.5

●—● with 0.006 % Triton X-100

0--0 without added surfactant.



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B. A batch assay using Calcofluor fluorescence to characterize cell wall regeneration in plant protoplasts. (Anal. Biochem. 141: 38-42, 1984).

Introduction

A pronounced and important feature of the early culture of plant protoplasts is the regeneration of their cell walls. The new walls must be deposited in order for cytokinesis to occur. Cellulose biosynthesis can be measured by a nitration method used by Spencer and Maclachlan (1) for pea epicotyl or by the anthrone method of Viles and Silverman (2). Both methods involve extraction and drying steps and are thus time consuming. Incorporation of radioactive precursors into cellulose has also been a measure of wall regeneration in protoplasts (3,4) but the relative incorporation of labelled precursors into cellulose and other metabolites is variable (5). Peberdy and Buckley (6) used Tinopal, a fluorescent optical brightener, to quantitate the cell wall regeneration in protoplasts of Aspergillus nidulans.

Calcofluor White is also an optical brightener used in the textile industry because of its high affinity for cellulose (7). It has been used to visualize cell walls in bacteria, yeast, and higher fungi (8) and in Cosmarium (9). Since Nagata and Takebe (10) first used Calcofluor to observe regenerating walls in tobacco protoplasts, it has been used extensively for that purpose. Calcofluor has also been used in conjunction with a flow cytometer to observe cell wall regeneration in B73 maize protoplasts (11). Recently, Galbraith (5) measured Calcofluor fluorescence due to cellulose deposition on

individual tobacco protoplasts and correlated it with the weight of extracted cellulose as determined by the anthrone method (2). This paper describes a quantitative batch assay for cell wall regeneration in protoplasts isolated from Hyoscyamus muticus. It is based on the specific binding of Calcofluor to cellulose, and is an extension of earlier flow cytometry work (11). Isolated mesophyll protoplasts of H. muticus were studied because they have been shown to regenerate walls and to divide in culture (12,13).

Materials and Methods

Protoplast preparation: Mesophyll protoplasts were isolated from the 3rd and 4th leaves of 8-week old Hyoscyamus muticus plants. After surface sterilization with HgCl_2 and washing 5 times with sterile distilled water, thin leaf slices were incubated for 3 hours at 30°C in 1 % Cellulase Onozuka R-10 (Kinki Yagult, Nishinora, Japan), 0.5 % Pectinol Fest (Rohm and Haas, Darmstadt, FRG), 0.5 % hemicellulase (Rhizopus, Sigma, St. Louis), 0.3 M mannitol and 0.04 M CaCl_2 at pH 5.7. After passage through 250, 100 and 50 μm stainless steel sieves, which removed the large cellular debris and clumps, the protoplast suspension was mixed with 0.5 volumes of 0.16 M CaCl_2 plus 0.5 % MES (Sigma, St. Louis) at pH 5.7, and centrifuged at 300 x g for 10 minutes giving a pellet and a fraction of floating protoplasts. The floating protoplasts were removed, then pelleted by centrifugation in 0.16 M CaCl_2 , combined with the first pellet, and washed in 0.16 M CaCl_2 . The protoplast density was adjusted to approximately $10^5/\text{ml}$ (haemocytometer counts) in the medium defined by Durand, Potrykus and Donn (14) containing 0.3 M mannitol, 2 % sucrose, 4 mg/l para-chlorophenoxyacetic acid (pCPA) and 1 mg/l

kinetin. The protoplasts were incubated overnight at 12° C and then transferred to 26° C, always in the dark. At daily intervals, aliquots of the protoplast suspension were collected by centrifugation at 300 x g for 10 min, washed in 0.16 M CaCl₂, counted on a haemocytometer and assayed for cell wall.

Staining: Calcofluor White is a fluorescent dye which stains the cellulosic components of plant cell walls (7,8,9,10). It has an excitation wavelength of 350 nm and, when bound to cellulose, an emission maximum of 450 nm (7).

Initial experiments showed that a high concentration of Calcofluor gave a high background fluorescence. When the concentration of Calcofluor was high, addition of the protoplasts or cells to the Calcofluor gave quenching of this background. It was necessary, therefore, to determine the concentration of Calcofluor which gave low background fluorescence but increasing fluorescence in the presence of cells. This concentration was determined to be 0.5 µg/ml. The Calcofluor was kept as a 0.1 mg/ml stock solution in distilled water.

All fluorescence measurements were made on a Kontron FM23 spectrofluorometer equipped with a magnetic stirrer beneath the cuvette. Before each run, the baseline was set with 0.16 M CaCl₂. The fluorescence of unstained newly isolated protoplasts was less than 0.4 % of the fluorescence of stained protoplasts at the concentration of Calcofluor used in the assay, and less than 4 % for unstained 6-day old protoplasts.

Assay procedure: To determine background fluorescence, 10 μ l of the 0.1 mg/ml Calcofluor solution was added to 2.0 ml of 0.16 M CaCl_2 , and the fluorescence emission spectrum was scanned between 400 and 500 nm at an excitation wavelength of 350 nm. Known volumes of the protoplast suspension (eg. 200 μ l) were then added, and the emission spectrum was repeated after each addition. In this way, a series of spectra were generated which, after volume correction, correlated with the number of cells in the cuvette. The effect of this procedure was to titrate all free Calcofluor with cellulose in the cell walls. The end point was reached when further addition of cellulose (cells) caused no further increase in fluorescence. Thus, the cellulose/Calcofluor ratio was relatively constant for each determination. The assay was performed for protoplasts at various stages of wall regeneration. Control assays were performed with Merck microcrystalline cellulose (Fig 1.).

Results and Discussion

H. muticus protoplasts, whose average diameter is 20 μ m immediately after isolation, resynthesize cell walls and divide in culture. (12, 13). The first shape change from spherical to spheroid due to cell wall regeneration occurs around day 3 or 4. Some clumping due to cell wall formation can be seen by day 6 or 7 and divisions by day 8 to 10.

The series of emission spectra obtained for regenerating Hyoscyamus muticus protoplasts for days 0 and 3 of culture are shown in Fig 2a and Fig 2b. The spectrum labelled '1' is the background fluorescence

Figure 1: Standard Curve using microcrystalline cellulose and Calcofluor.

There was a linear relationship between the fluorescence (450 nm) due to Calcofluor and the amount of added cellulose.

The graph represents two experiments each having duplicate samples taken.

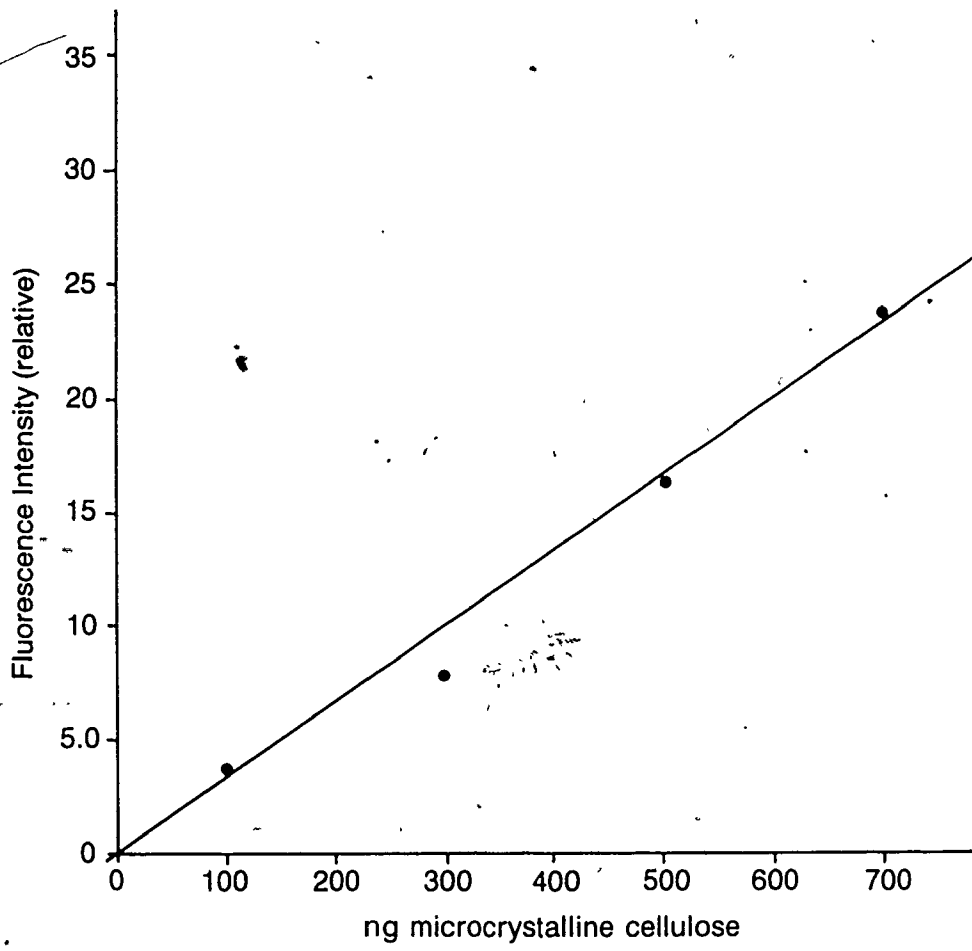
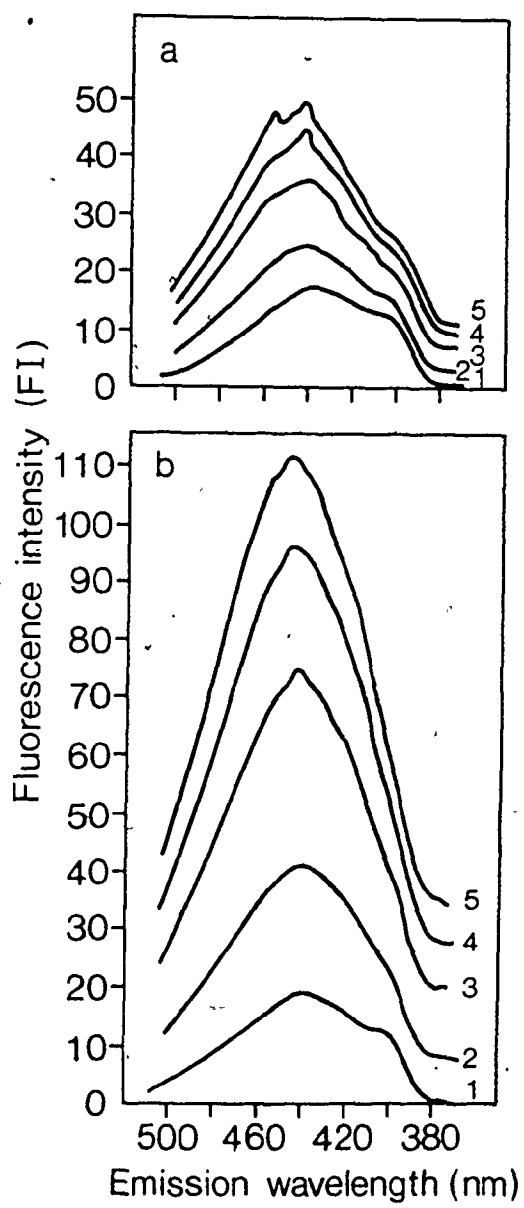


Figure 2: Fluorescence emission spectra of Calcofluor and of Calcofluor plus protoplasts. Line 1 is Calcofluor alone, lines 2,3,4,5, are Calcofluor plus increasing numbers of protoplasts. a) day 0 of culture;
b) day 3 of culture.

Fluorescence is in arbitrary units.



of the Calcofluor solution, and the subsequent spectra are due to the sequential addition of 200 μ l aliquots of protoplast suspension.

The peak fluorescence, corrected for background, was plotted as fluorescence intensity against the number of protoplasts. Fig 3a gives these results for cells which had been cultured for 0 through 7 days. As the protoplasts regenerated more wall material, the cellulosic constituents of the wall absorbed more of the Calcofluor at lower cell densities. The rate of change of fluorescence with respect to the number of cells in the cuvette, or the initial slopes of the lines on Fig 3a, is a measure of the average wall content of the cells; i.e., the amount of fluorescence per cell is related to the amount of cellulose per cell. Fig 3b gives the relation between the initial slope and the number of days in culture for Hyoscyamus protoplasts. The increasing slope reflects the increase in the quantity of cell wall per cell with increasing time in culture.

After this methodology was established, these measurements were repeated for five independent repetitions of the regeneration of cell walls by freshly prepared Hyoscyamus protoplasts. The results of these studies are summarized in Fig 4. Since Calcofluor is highly specific for cellulose, there appears to be incomplete wall removal in some preparations on day 0. This was previously observed by flow cytometry (11). Unstained protoplasts gave very little background fluorescence. There was an increase in the cell wall content per cell until day 6 of culture, when the cell wall content reached a plateau. This occurred about the same time as the Hyoscyamus cells clumped and when there were some very early divisions.

Figure 3: Example of a set of cell wall determinations for Hyoscyamus protoplasts to establish the assay conditions. Each point is the average of two determinations.

(a) Fluorescence Intensity as a function of protoplast or cell number. F.I. is the peak fluorescence due to adsorption of Calcofluor to the protoplast or cells.

● day 0;

■ day 3;

▲ day 5;

◆ day 8.

(b) Time course of cell wall resynthesis in the Hyoscyamus protoplasts. Measurement of the initial slopes in the graph (part a) gave the relative amount of cell wall synthesis for a given number of cells at each time.

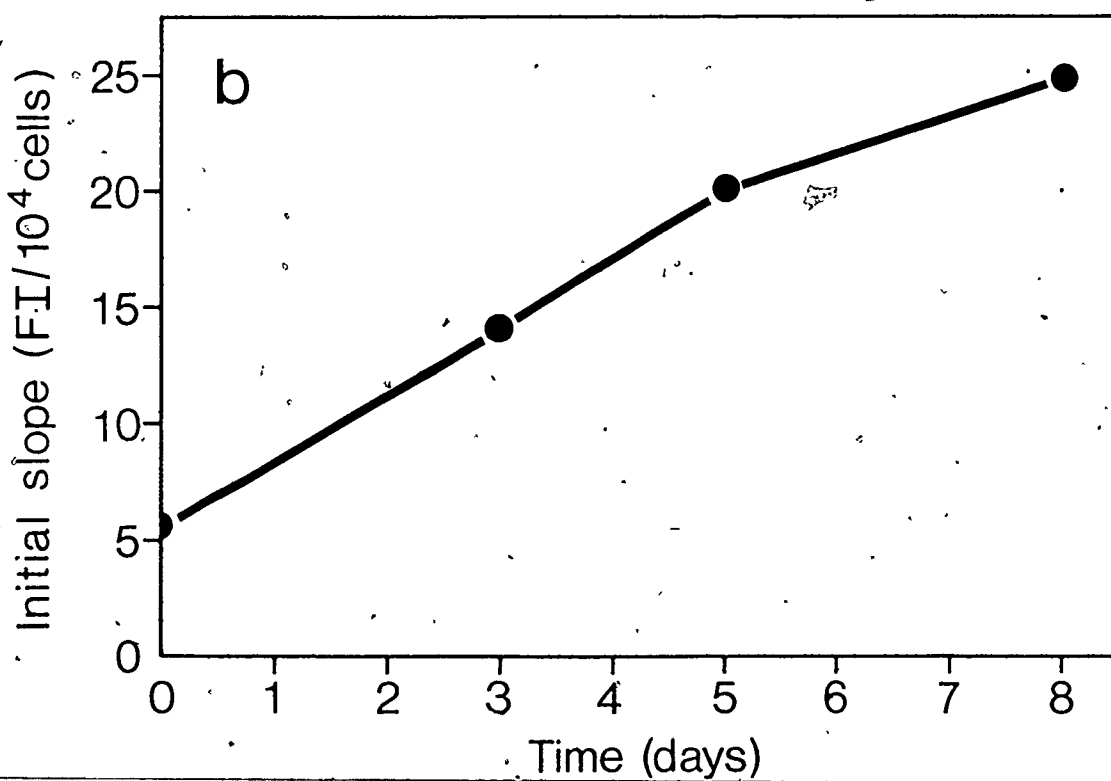
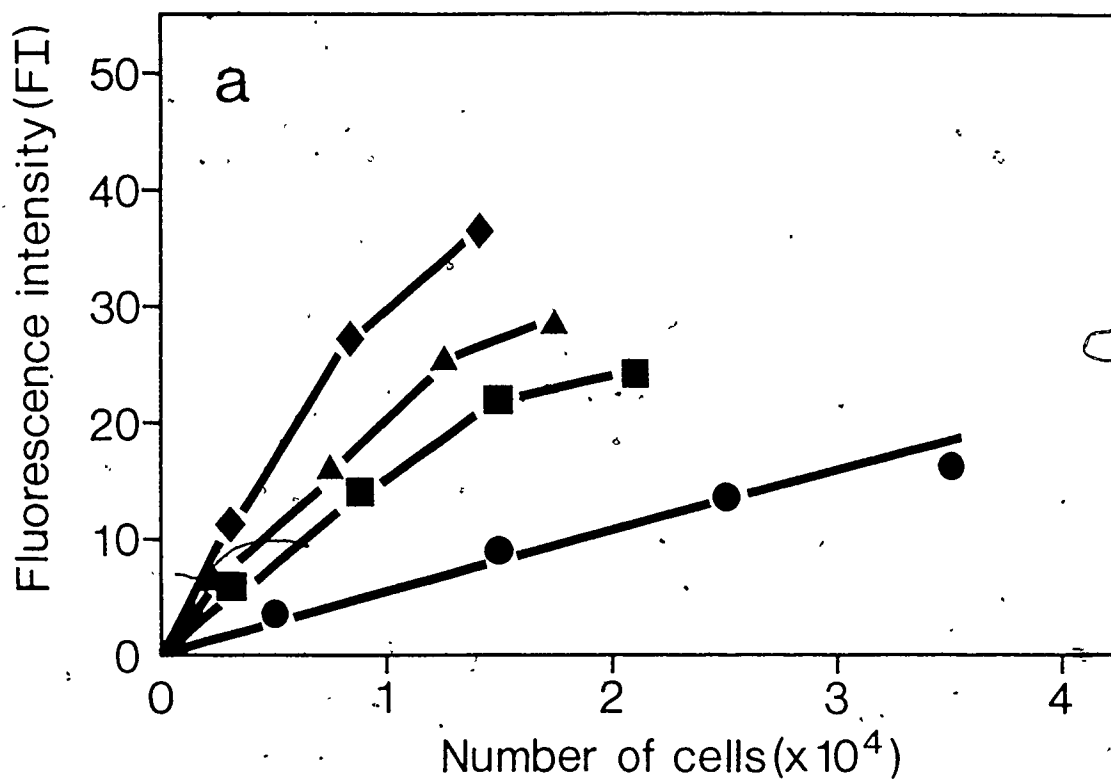
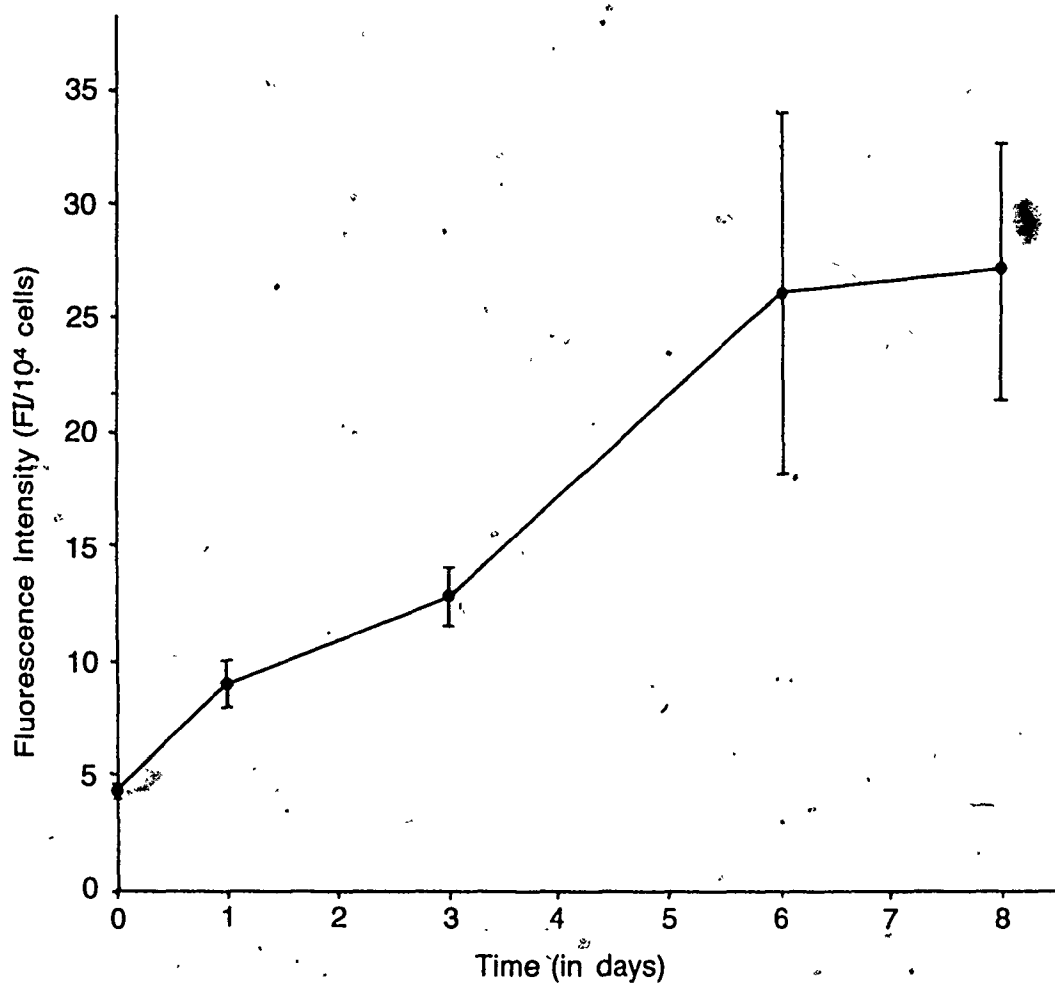


Figure 4: Average rate of cell wall synthesis in *Hyoscyamus muticus*.

The data are averages of 5 different experiments.



Discussion

The assay that has been developed here can measure cell wall resynthesis in plant protoplasts using the fluorescent dye Calcofluor in a scanning fluorometer. The fluorescence measurements can be correlated to micro-crystalline cellulose equivalents as shown in Table 1.

Galbraith (5) has also established an assay for tobacco protoplast wall regeneration by measuring the fluorescence of individual protoplasts and correlating these values to the actual amount of cellulose measured as alkali-insoluble polysaccharide.

The two methods can be compared; eg. for the cellulose content of "mature" protoplasts, in his case, 5-day old tobacco protoplasts; and in this case 6-day old Hyoscyamus protoplasts. From his data, a 5-day old protoplast gave a fluorescence of 45 mVBk, which when multiplied by the factor $1\text{mV} = 22 \text{ }\mu\text{g}$ of cellulose, gave a cellulose content of 99 μg per protoplast. Considering 6 day-old Hyoscyamus protoplasts, from Table 1, 10^4 protoplasts had a cellulose equivalence of 785 ng (78.5 μg per protoplast). Considering that the two protoplasts are quite different in initial average size (Hyoscyamus, 20 μm ; tobacco, 33.6 μm) the two methods are in good agreement with each other.

Both methods have the advantage of being fast and convenient compared to extraction methods (1,2). In this assay, a given level of fluorescence is correlated with a particular quantity of micro-crystalline cellulose. As yet, this estimate has not been compared

TABLE 1

Fluorescence Intensity (in arbitrary units) and cellulose equivalence due to the binding of Calcofluor White to regenerating cell walls of H. muticus protoplasts during 8 days of culture.

Time in Culture (days)	FI/10 ⁴ Cells (+ SEM)	ng Cellulose/10 ⁴ Cells
0	4.2+0.49	135
1	9.0+ 1.0	280
3	12.8+ 1.3	385
6	26.0+ 8.01	785
8	26.8+ 5.5	805

to quantities of extracted cellulose. Neither assay indicates what fraction of the protoplasts are undergoing cell wall synthesis and neither measures the non-cellulosic components of the cell wall. The assay designed here is a batch assay and as such is performed more quickly and conveniently to determine and to compare rates of cell wall synthesis in cultured protoplasts.

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Chapter V: Characterization of cells and protoplasts
of the B73 maize cell line. (Plant Sci. Lett.
28: 337-348, 1982/3).

Introduction

To date, only one cell line, the B73 line of Potrykus et al. (12) has been established from a primary culture of protoplasts from plant tissues of Zea mays L. The cells grow well in suspension culture, can be maintained on agar medium and can be enzymatically returned to protoplasts, which in turn, regenerate walls and become proliferating cells again. The cells of this line cannot be induced to regenerate plants and, as is known in other non-morphogenetic cell lines, they have ploidy abnormalities.

The B73 cell line is useful for the characterization of a maize cell culture and for comparison with morphogenetic cell cultures. We have, firstly, characterized the growth of the B73 cells in suspension culture. Secondly, we have examined the DNA synthesis and cell wall regeneration of B73 protoplasts during the first 5 days of culture. For the second study, we have used two fluorescent dyes, Hoechst 33258, which binds quantitatively to DNA (1, 2, 15), and Calcofluor White, which stains plant cell walls (10). To study protoplasts stained with these dyes, we have used a fluorescence-activated cell sorter, the FACS II (Becton-Dickinson Electronics Lab.) which allows the collection of data on a large number (10^5) of individual protoplasts.

Materials and Methods

B73 Cell Line: The cells are routinely grown as suspension cultures in CC medium as defined by Potrykus *et al.* (13) and are diluted with medium either every 5 to 6 days by 1:10 during maintenance, or every 2 days by 1:4 or every day by 1:2 during experiments. An alternate method of maintaining the line is to plate the cell clusters on CC-agar (0.8%) medium (13). The calli which develop are friable and can be easily resuspended in liquid medium to re-establish cell suspension cultures.

Chromosome Staining: The chromosomes of the B73 line have been difficult to stain; thus, several methods were attempted to obtain a chromosome count and the mitotic index of the cell suspension. The dyes used were: Feulgen, Toluidine Blue O, Acridine Orange and Hoechst 33258.

Feulgen: Staining of the cells was done according to the procedure established for corn root tips (3). The time required for hydrolysis of the cells varied with every preparation (from 1 to 8 min). Since a series of test samples had to be set up for each batch of cells, this method proved to be tedious.

Toluidine Blue O: After the cells were fixed overnight in 3:1 (v/v) ethanol: acetic acid, they were centrifuged at 300 g for 10 min and washed in borate buffer, pH 8.4, stained for 2 min in 0.05 % toluidine blue O, rewashed and mounted in water.

Acridine Orange: The cells were fixed in 3:1 (v/v) ethanol: acetic acid, and then, according to the method of Darzynkiewicz (5), 0.2 ml of the suspension was washed in 40% ethanol, mixed with an

equal volume of KCl/HCl solution for 1 minute and then with 2 ml of acridine orange (0.8 µg/ml) made up in citric acid buffer, pH 2.6. The high background staining by acridine orange made the visualization of chromosomes difficult.

Hoechst 33258: The method is described below.

Determination of Chromosome Number: Colchicine, at a final concentration of 10^{-4} M was added to a 2 ml aliquot of the cell suspension for 2 h, after which the cells were washed in 0.16 M CaCl_2 , pH 7.5, stained with Hoechst 33258, and resuspended in 0.16 M CaCl_2 . To soften the walls for squashes, the cells were treated with 0.5 % Cellulase Onozuka R-10 in CC-medium for 20 to 30 min at 30°C , before being returned to 0.16 M CaCl_2 for mounting and observation. They were examined with a Leitz Orthoplan fluorescence microscope equipped with an Orthomat automated camera. Its light source was a 200w, Osram HBO high pressure mercury lamp used with excitation barrier filter UV-U1 and dichroic filter assembly Ploemopak A.

Packed Volume Determinations: Stationary phase cultures were diluted 1:4 with fresh medium, and 2 ml of the suspension was centrifuged at 300 g for 10 min in graduated conical centrifuge tubes. The packed volume, expressed as a percentage of the initial total sample volume, was determined in duplicate every 24 h until the culture reached stationary phase.

Mitotic Index Determination: Aliquots of cells were washed twice in 0.16 M CaCl_2 , pH 7.5. They were stained with Hoechst 33258 and duplicate samples of at least 500 cells were scanned. The mitotic index was the percentage of all cells at any stage of

mitosis. The determination of the mitotic index throughout the growth curve was repeated three times.

Protoplast Isolation: Protoplasts were isolated from B73 cells that had reached stationary phase, were diluted 1:4, and allowed to grow overnight. The cells were then treated with 1 % Cellulase Onozuka R-10, 0.5 % Pectinol Fest and 0.5 % hemicellulase in 0.3 M mannitol and 0.04 M CaCl_2 at pH 5.8. After 2 to 3 h of shaking (125 rpm, 24° C, in the dark), the protoplasts were separated from the remaining cell clumps and large debris by successive passages through 250 μm , 100 μm and 50 μm stainless steel sieves. The protoplasts were then harvested according to the method described by Potrykus et al. (12). The resulting protoplasts were cultured in NN67 (11) medium containing 0.17 M mannitol, 2 % sucrose and 2 mg/l 2,4-D, in 6 cm Petri dishes, overnight at 12° C, and thereafter at 24° C, and always in the dark. By 7 days they had reformed walls and could be harvested and returned to shaking culture in CC medium.

Survival of the Protoplasts: Since haemocytometer counts of the protoplasts had proven (by Evans Blue exclusion) to be a good indication of the number of viable protoplasts, this method was used to determine the survival of the protoplasts in culture. Initial densities of the protoplasts were adjusted to approximately 10^5 per ml. The protoplasts were counted every 24 h until clumping occurred, and further measurement became difficult. This experiment was repeated five times and all samples were counted in duplicate.

Hoechst 33258 is a bisbenzimidazole dye that binds quantitatively to DNA (1, 2, 9, 15). The dye was a gift from Dr. H. Loewe,

Hoechst, AG, BRD. At an excitation wavelength of 350 nm, Hoechst 33258 fluoresces at 465 nm when conjugated to DNA. The B73 protoplasts were stained with H33258 as previously described (9): the protoplasts were first treated with DNAase I (Sigma) at 50 µg/ml final concentration for 20 min at 37° C, to remove DNA absorbed from the culture medium and adhering to the outer surface of the cells, then washed and resuspended in 0.16 M CaCl₂, pH 7.5 in the presence of 0.006 % Triton X-100. Hoechst 33258 was added at a final concentration of 20 µg/ml and the mixture was allowed to stand for 15 min. The protoplasts were centrifuged at 500 g for 10 min, then washed and resuspended in culture medium for FACS analysis.

Calcofluor White: Calcofluor White ST preferentially stains the cellulosic components of plant cell walls. It has an excitation wavelength of 350 nm, and an emission wavelength maximum around 450 nm when bound to cellulose. Commercial samples have some background fluorescence. At high concentrations of Calcofluor, the background fluorescence is quenched by the cells. The optimal concentration of Calcofluor giving little background while still allowing fluorescence detection of cell walls was determined with a Kontron FM-23 spectrofluorometer. It was found that a final Calcofluor concentration of 0.5 µg/ml in a cell or protoplast suspension of 10⁵/ml was optimal. At this fixed concentration of Calcofluor, the fluorescence increased linearly with the number of cells or protoplasts added, the slope depending on their cell wall content. This concentration of Calcofluor also gave linearly increasing fluorescence when calibrated with Whatman CM52 microgranular carboxymethyl cellulose particles. The Calcofluor-treated protoplasts were analyzed on the FACS.

The FACS: Flow cytometers can make measurements of each individual of a large population of cells. The analysis of the cells is based on the quantity of fluorescent probe bound to a particular cellular component. As the stained cells pass single file through a krypton-argon laser beam, two measurements are made on each: 1) an estimate of cell size given by the low angle forward light scattering and 2) a fluorescence emission intensity given by the amount of bound dye. For the protoplasts, two changes in the operation of the FACS were made. To accommodate the osmotically fragile protoplasts, the sheath flow solution was replaced by NN67 medium as described above, so that during flow and analysis the protoplasts remained stable, and the FACS was refitted with a 100 μm nozzle to allow passage of the protoplasts. Immediately before analysis, to prevent blockage of the flow lines, the protoplast suspension was filtered through a 100 μm sieve to remove any large clumps that may have formed.

The FACS II is linked to a computer system which is programmed to produce histograms and scatter or contour plots of the data. The FACS II used in these experiments is owned and operated by the Institute for Immunology (Hoffmann-La Roche, Basel). A log amplifier was designed for the FACS by Dr. H. Koller, Hoffmann-La Roche, Basel.

Results

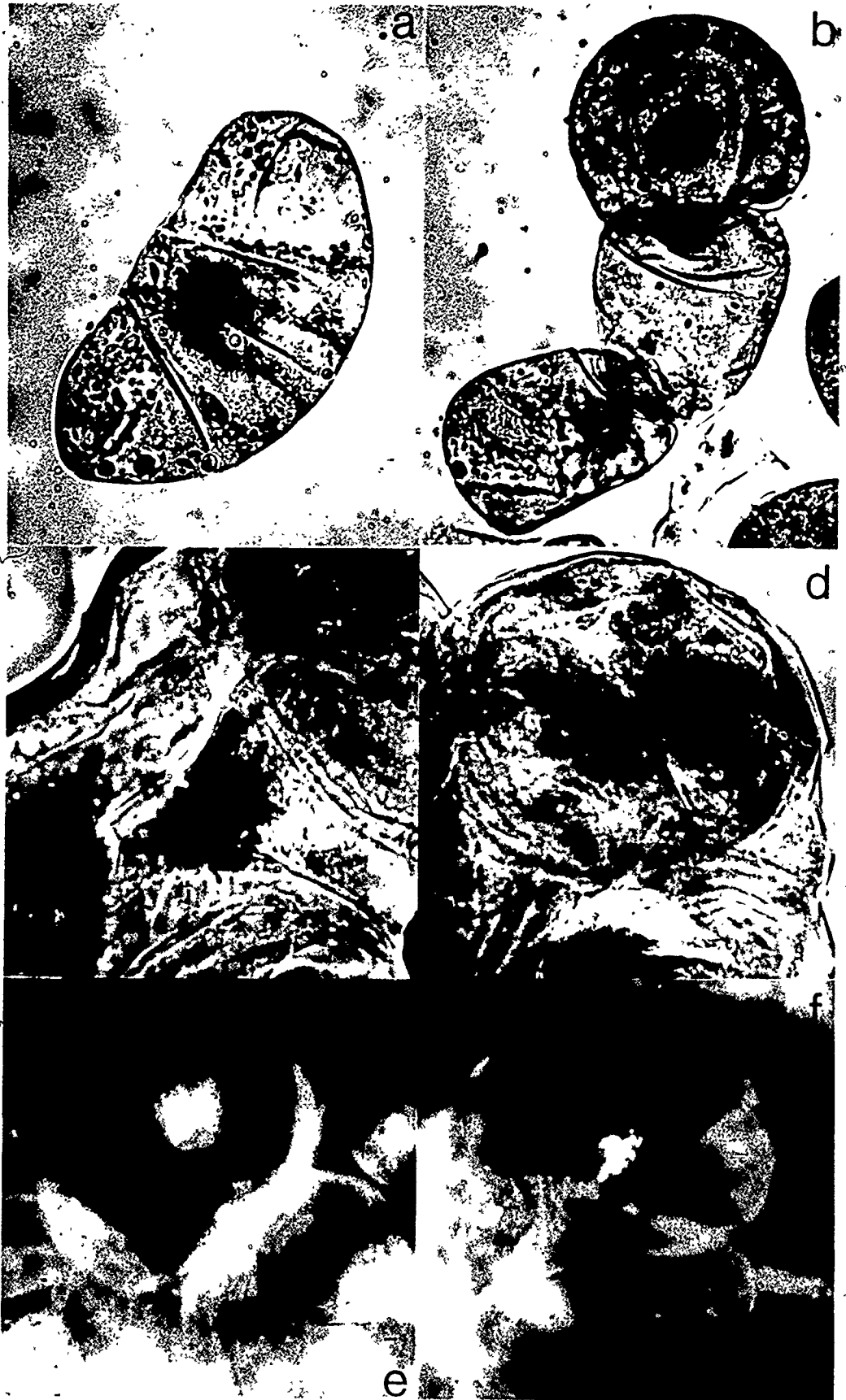
Chromosome Staining and Distribution: B73 cells, as stained by several nuclear dyes, are shown in Fig 1. The photographs were taken before clearing the background with ethanolic BaOH. Chromosomes of H33258 stained cells (Fig 1f) are most distinctly visible. The

Figure 1: Nuclear Staining of B73 Cells.

la, b, c, d, Toluidine Blue O

le Acridine Orange staining gives a yellow nucleus,
orange nucleolus and green walls. Background
staining was very difficult to remove.

lf . Hoechst 33258 staining: gave blue white chromosomes
Background fluorescence due to the walls could
be removed by ethanolic BaOH wash.



distribution of the chromosomes of B73 is shown in Fig 2. Approximately 50 % of the cells have the expected diploid number of 20 chromosomes for Zea mays. The distribution also shows that the line is partially aneuploid. A small fraction of the cells have 30 chromosomes.

Growth Curve and Mitotic Index: The growth curve (packed cell volume) for the B73 cell line, diluted 1:4 and allowed to regrow for 6 days is shown in Fig 3. There is a linear increase in growth from the time of dilution until day 4, after which stationary phase is reached. The mitotic index is also given in Fig 3. The number of cells in mitosis increases from the time of dilution until day 2, after which the number of dividing cells decreases as the culture reaches stationary phase. By day 4, there is a very small percentage of cells in division.

Survival of Protoplasts Isolated from B73 Cells: The survival of the B73 protoplasts during the first 6 days of culture is shown in Fig 4. There is appreciable net protoplast death during the first 2 days of culture, after which the population stabilizes. About 50 % of the protoplasts remain viable after the 3rd day of culture, 45 % remain after 6 days. Presumably these survivors become the cells which perpetuate the culture.

FACS Analysis of B73 Protoplasts: After staining with either H33258 or with Calcofluor White, protoplasts were screened for DNA and for cell wall content using the FACS. Distributions of DNA content in the protoplast populations, as shown by Hoechst fluorescence, are presented in Fig 5. Figs 5a to 5d are histograms of the DNA contained in 10^5 protoplasts on days 0 to 5. Figs 5e to 5h are

Figure 2: The Distribution of the Number of Chromosomes in B73.

About 50 % of the cells have 20 chromosomes, the diploid number for Zea mays L. A small number of cells are triploid.

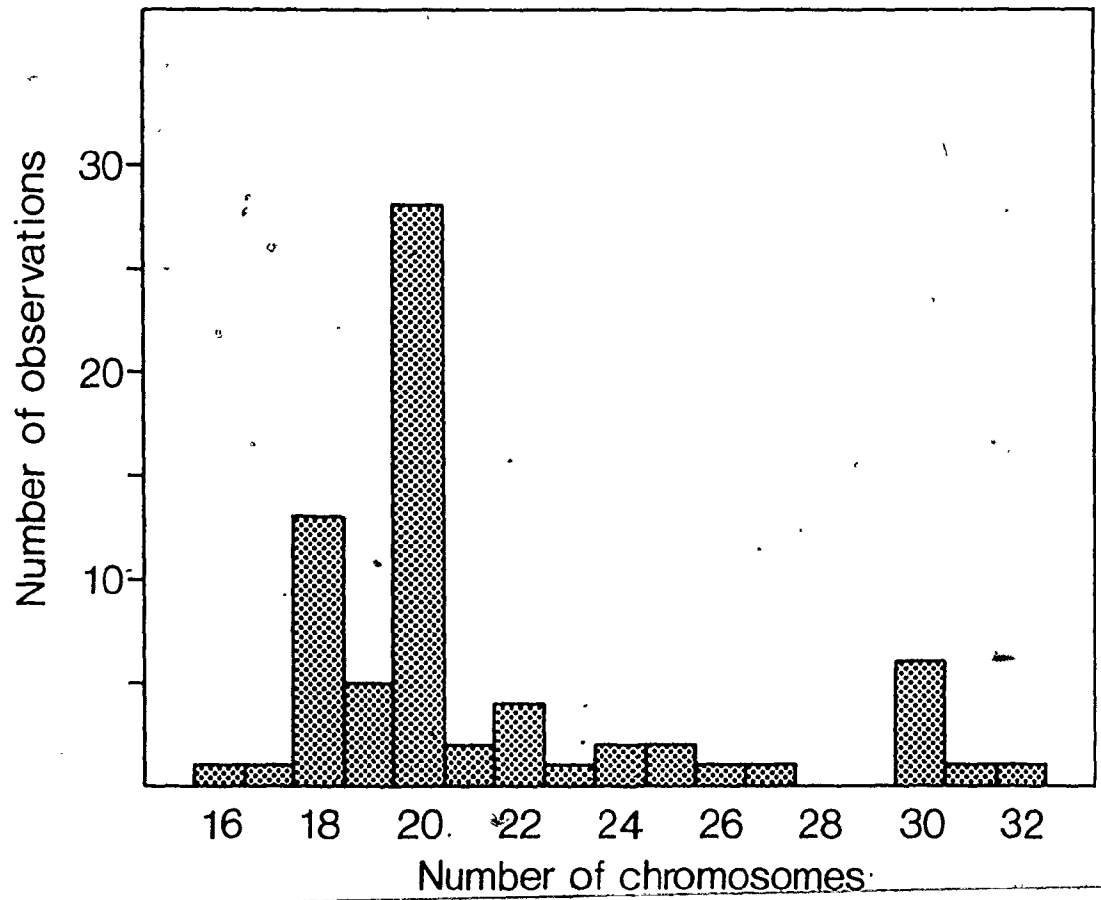


Figure 3: Growth Curve and Mitotic Index of B73 Suspension Cultures.

Growth was measured as the percentage packed volume of cells. Mitotic Index is expressed as the percentage of cells in any stage of mitosis. Duplicate samples of 500 cells were scanned every 24 h in 3 repeated experiments. Indicated range is \pm one standard error of the mean.

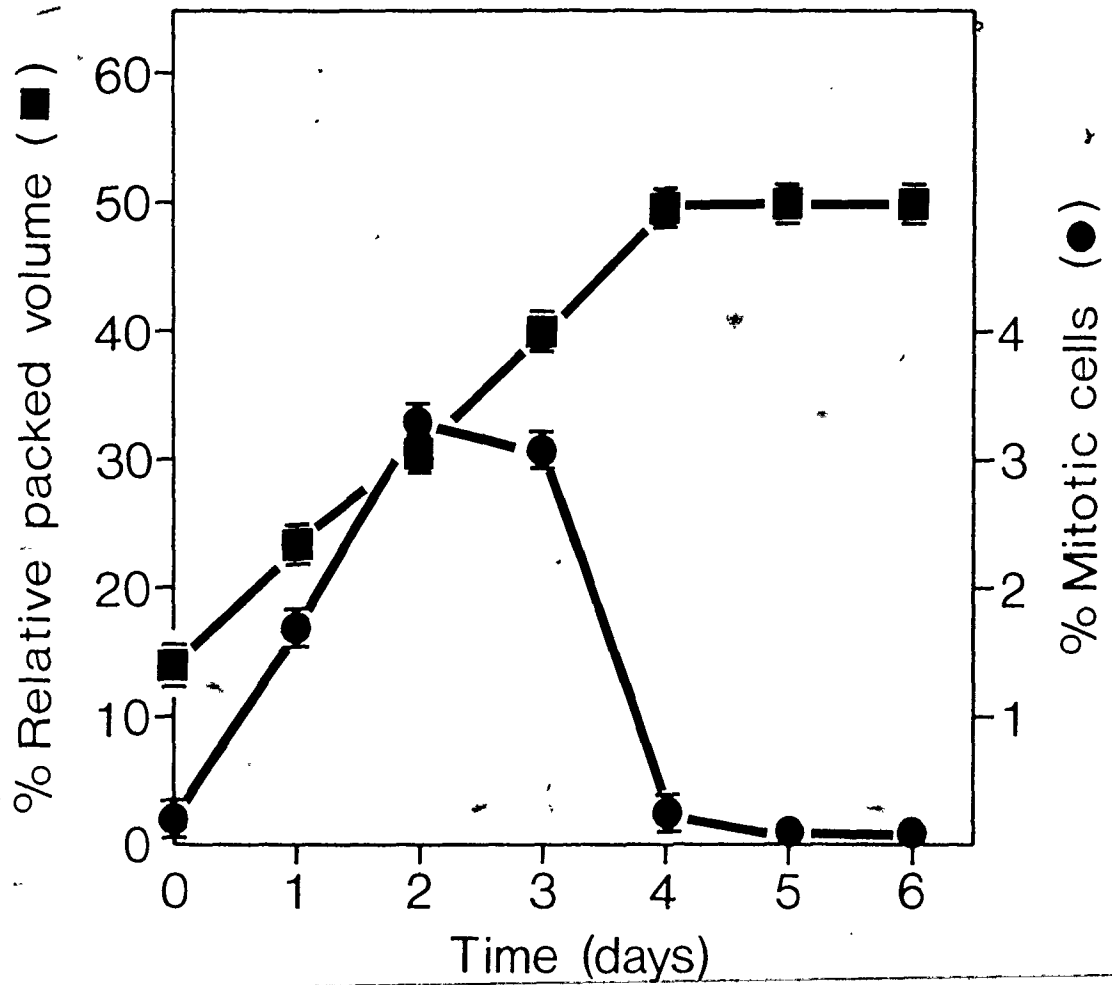


Figure 4: Survival of B73 Protoplasts in Culture.

Haemocytometer counts, in duplicate, every 24 h for 3 experiments showed that 50% of B73 protoplasts survived to become cells. Indicated range is \pm one standard error of the mean.

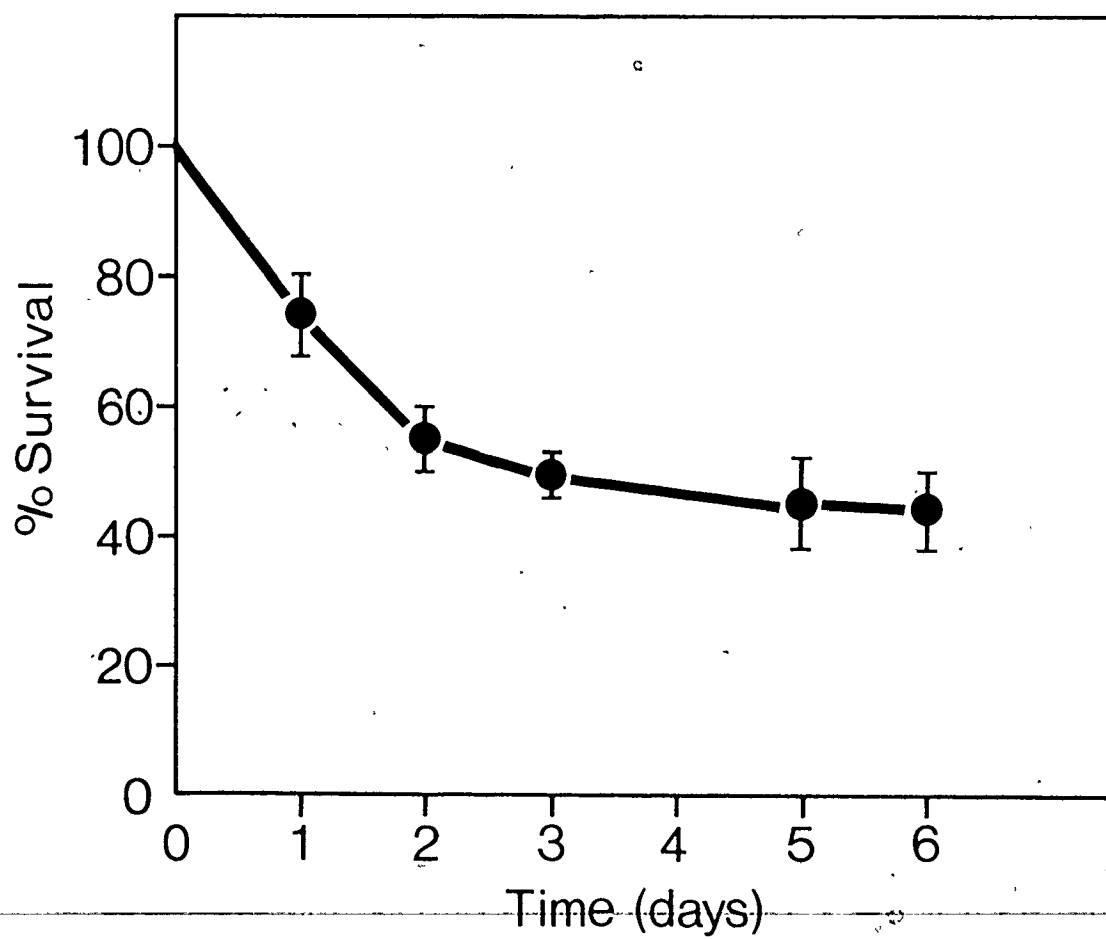
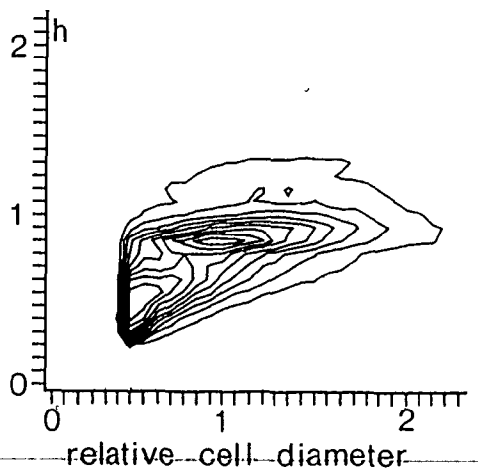
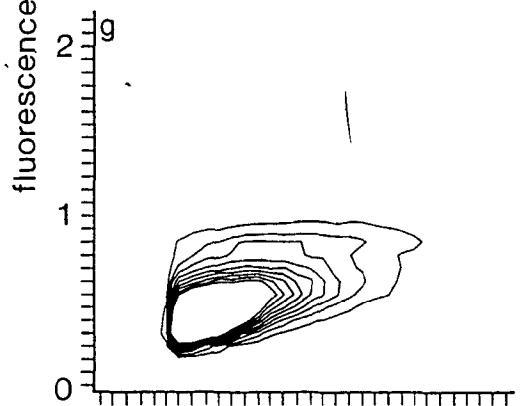
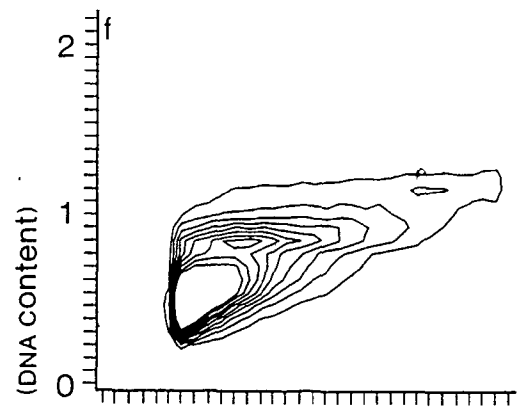
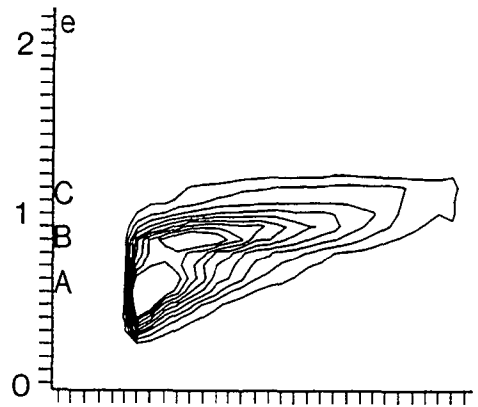
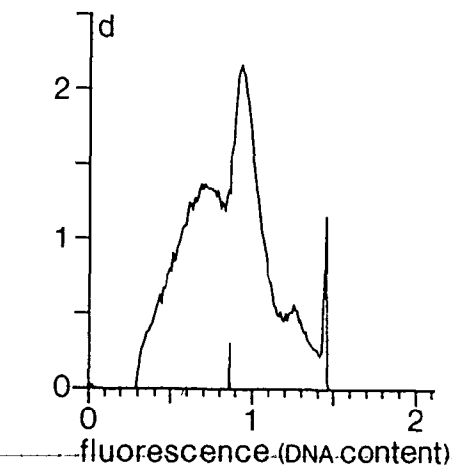
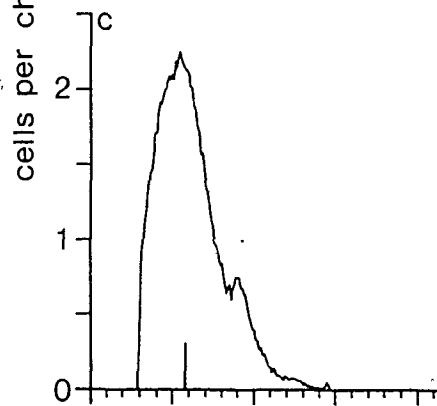
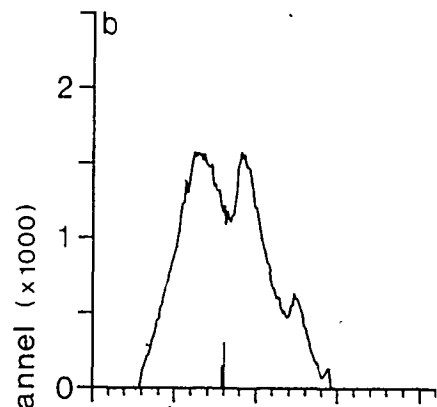
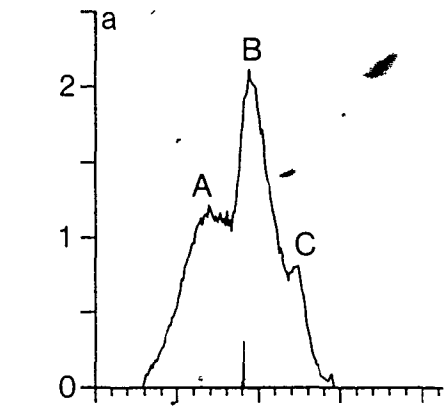


Figure 5: Fluorescence-Activated Cell Sorter (FACS) Analyses
of Hoechst 33258 Staining of Protoplasts of Cultured
Zea mays Cells.

Figs 5a to 5d are the histograms of number of protoplasts as a function of DNA content for days 0, 1, 3 and 5 of culture. The abscissa, on a log scale, is the fluorescence intensity of bound Hoechst 33258 and the ordinate is the number of protoplasts per channel. Throughout culture there is a progressive loss of the "B" peak representing those protoplasts in G2. By day 5, new DNA synthesis has occurred and a new G2 population is present.

Figs 5e to 5h are scatter plots of the intensity of staining as a function of cell size for days 0, 1, 3 and 5 of culture. The abscissa is the relative size of the protoplasts. The ordinate is the fluorescence due to Hoechst. The 3rd dimension is the number of protoplasts for a given size and DNA content. There is a loss of large protoplasts up until day 3 but by day 5 an increase in larger protoplasts is coincident with the reappearance of a new G2 population.



the corresponding contour plots of the number of protoplasts (indicated by the contour lines) as a function of size and DNA content. As shown, the protoplasts, after one night's growth from stationary phase, had their DNA distributed in 3 peaks. The analysis of the 3 peaks is difficult since there was spontaneous fusion among some newly isolated protoplasts, so that a small percentage of the protoplasts were multinucleated.

Immediately after isolation, as is shown in Table 1, most of the protoplasts were uninucleated, but about 6 % were multinucleated. The percentage of multinucleated protoplasts decreased over the course of the experiment. Some of the multinucleate cells would have been present in peak C, Fig 5a. It is generally accepted (4, 6, 7, 8) that the DNA profiles obtained from cytofluorometry correspond to cell cycle analyses obtained by other methods. We have assumed this to be so also for plant protoplasts and have interpreted the data on the assumption that the lowest fluorescence peak of the histograms corresponds to G1 of the cell cycle of the B73 cells. Precise identification of the peaks obtained by these methods with intracellular DNA content is a continuing problem in flow cytometry.

Non-morphogenetic cells are often aneuploid and this is certainly true of B73 cells. The number of chromosomes ranges from 18 to 32 with approximately half the cells being the expected $2n$ for Zea mays, and a small proportion being $3n$. This broadens the peaks of the DNA distribution. Peak A then corresponds to the G1 state of those cells whose diploid number of chromosomes is near 20, and peak B corresponds to the G2 of that sub-population. Peak B also contains

TABLE 1

Distributions of nuclei per cell after various days of incubation since protoplasting.

DAY	Number of Nuclei per Cell				
	1	2	3	4	4+
	<u>% of Protoplasts</u>				
0	94.1	5.0	0.4	0.5	0.0
1	94.4	5.0	0.2	0.4	0.0
4	96.0	2.9	0.2	0.6	0.3
6	95.0	3.4	0.5	1.1	0.0

Percentages of uninucleate and multinucleate protoplasts on days 0 to 6 following protoplast isolation. Each value is the average of 3 separate experiments in which 600 protoplasts were counted for each time point.

two other classes of protoplasts: those with a diploid number greater than 20 and those which are binucleated. The G2 for these two classes would then be Peak C, which is broadened by multinucleated, spontaneously-fused protoplasts. The relative contribution of binucleate cells to the apparent G2 peak would be approximately 5 %, and the total contribution of cells with 3 or more nuclei to the DNA distribution would be approximately 1 % (Table 1).

From Fig 5, it can be seen that in days 1 to 3, there was a relative increase in the number of protoplasts in G1, so that by day 3 most of the cells were in peak A. Since there was no cytokinesis during that time, it would seem that there was a loss of protoplasts in G2. Only 50 % of the isolated protoplasts survive until day 3 and become the cells which perpetuate the culture (Fig 4). It would seem that there was a selective death of protoplasts in G2. Those protoplasts remaining in G1 by day 3 begin to resynthesize DNA so that by day 5 of culture, some protoplasts are in G2 again. The large G1 peak on day 5 suggests that a proportion of the protoplast population was not cycling rapidly. The contour plots show the same trend, i.e., loss of those protoplasts in G2 which are also the larger protoplasts (Fig 5g).

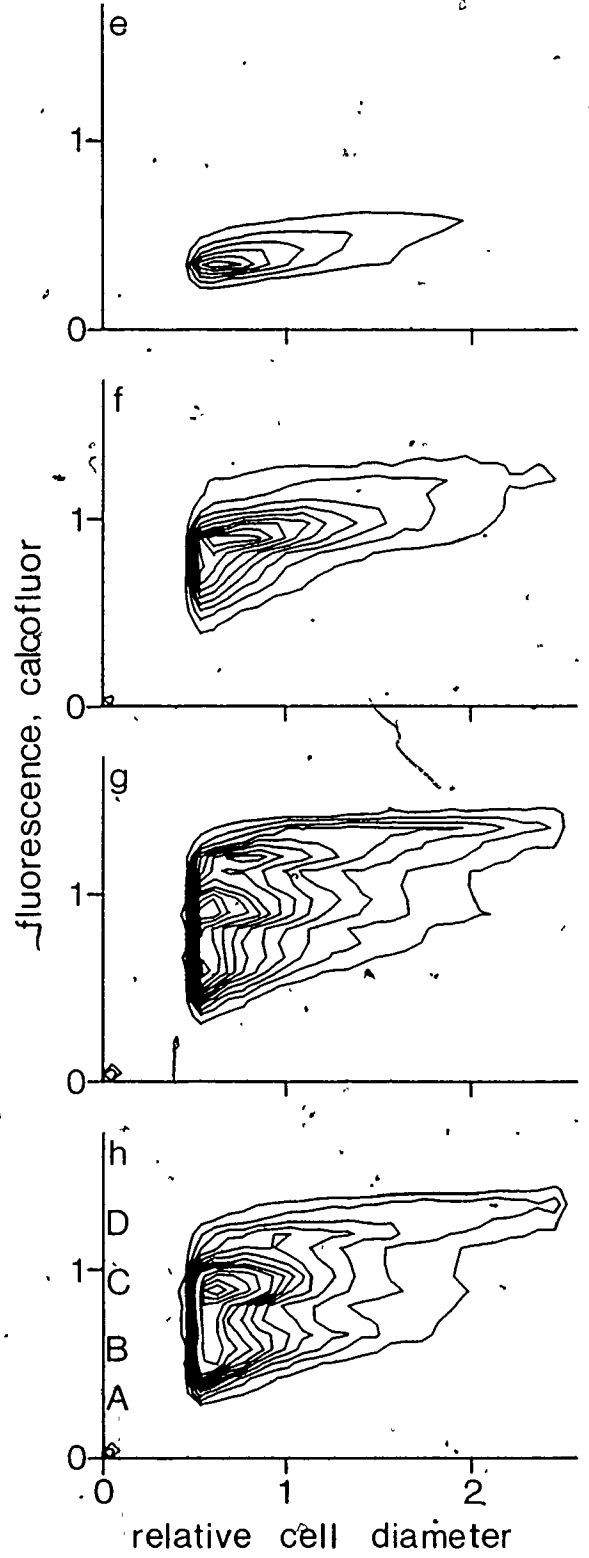
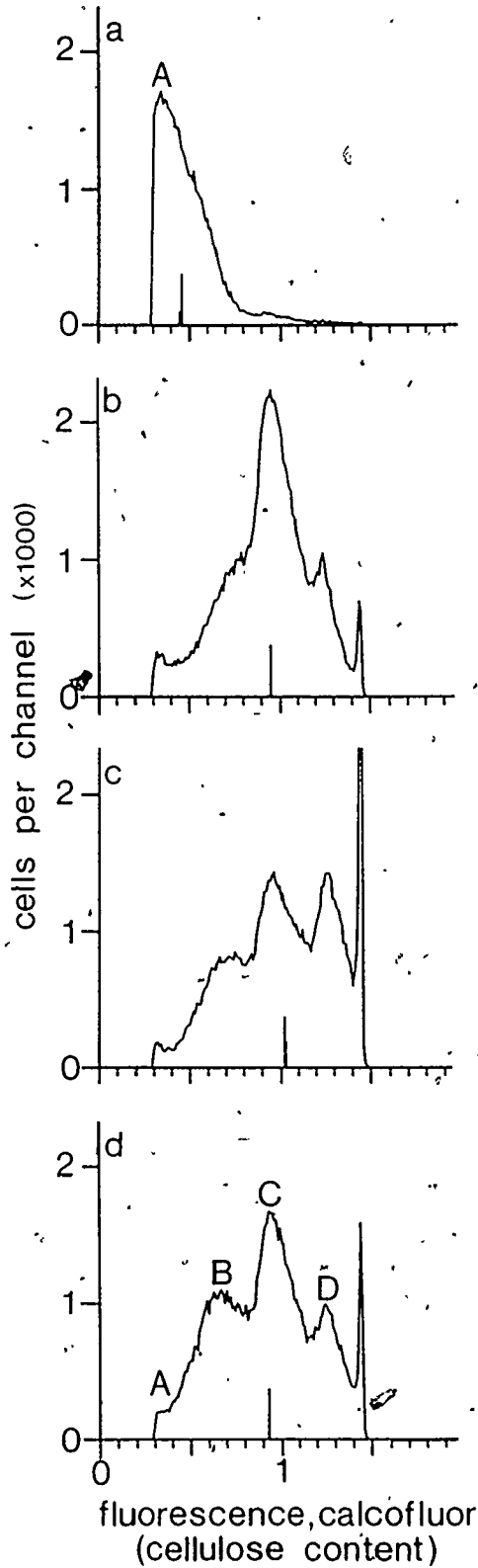
The corresponding graphs for cell wall resynthesis are given in Fig 6. Initially, the newly isolated protoplasts showed fluorescence in the presence of Calcofluor (Fig 6a) and this fluorescence was present in all sizes of protoplasts (Fig 6e). This population of protoplasts could be those which did not have their walls completely removed during isolation. Although peak A, Fig 6a, decreased on

Figure 6: Fluorescence-Activated Cell Sorter (FACS) Analyses

of Cell Wall Regeneration as shown by Calcofluor
staining of Protoplasts of Cultured Zea mays cells.

Figs 6a to 6d are the histograms of number of protoplasts as a function of cell wall content for days 0, 1, 3 and 5 after isolation. The abscissa, on a log scale, is the fluorescence intensity of bound Calcofluor White. The ordinate is the number of protoplasts per channel. There is a small population that does not completely lose its wall (peak A) during isolation. With time in culture, there is the emergence of several populations of protoplasts having different rates of cell wall regeneration.

Figs 6e to 6h are scatter plots of the cell wall regeneration as a function of size for days 0, 1, 3 and 5. The abscissa is the relative size of the protoplasts. The ordinate is the fluorescence due to Calcofluor. The 3rd dimension is the number of protoplasts per size class and cell wall content. All sizes of protoplasts resynthesize cell wall but the smaller protoplasts appear to be most active in the process.



subsequent days, it was always present, indicating that there was a subpopulation of protoplasts not resynthesizing walls. The progressive increase in cell wall fluorescence is given in Figs 6b through to 6d. By the first day after isolation, there were 3 subpopulations of protoplasts having varying amounts of cell wall, indicating that there were subfractions of protoplasts having different rates of cell wall synthesis. By the third day of culture (Fig 6c), the population with high fluorescence (peak D) had increased at the expense of those in the medium fluorescence peak (peak C). There was also an increase in the population with low fluorescence (peak B) probably due to resynthesis of walls by those protoplasts which had not their walls completely removed during isolation. Although cell wall resynthesis was detectable in all sizes of protoplasts (Figs 6e to 6h) it would seem that most of the resynthesis of wall material occurred in the smaller protoplasts.

Conclusions

The B73 cell line, established by Potrykus et al. (12), has been the only maize cell line to be obtained from a primary protoplast culture. It has been maintained either as a suspension culture in liquid medium or as callus on agar medium.

Stationary phase cells, when diluted 1:4 and allowed to grow, reach stationary phase again within 4 days. The mitotic index of the culture is highest on day 2 after dilution and by day 4 there is very little mitotic activity. Protoplasts are easily isolated from the cells and are able to resynthesize walls and divide. During the

first 3 days after isolation, there is a 50 % loss of the protoplasts from the culture.

Two synthetic processes, DNA synthesis and cell wall synthesis, were followed using fluorescent dyes and the FACS II during the early days of culture of the protoplasts. The DNA analysis showed that there were 3 peaks of DNA content. The lowest two have been assumed to represent the G1 and G2 + M of the B73 cell cycle. The third subpopulation was those protoplasts which had greater than G2 + M content, assumed to have arisen by spontaneous fusion during protoplast isolation.

The aneuploid nature of the protoplasts made the DNA distributions complicated but it was obvious that there was a selective loss of those protoplasts with higher DNA content during the first 3 days of culture. This population reappeared by day 5 owing to the reinitiation of DNA synthesis in the G1 protoplasts.

Concurrent with the resurgence of the G2 population of protoplasts was the occurrence of populations of protoplasts resynthesizing walls at various rates.

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Chapter VI: Characterization of protoplasts of Hyoscyamus
muticus L. using flow cytometry.

Introduction

Automated flow cytometers are becoming increasingly important for the analysis of the components of large populations of cells. They allow fast, multiparameter screening and measurement of cellular constituents, yielding data that are statistically precise. The analysis of cells with automated cytometers is based on the quantity of a fluorescent probe which is bound to a particular cellular component. Up to 10^5 cells per second can be examined with a cytometer.

Two fluorescent dyes can be used to follow the early events in the culture of newly isolated plant protoplasts: Hoechst 33258, a bisbenzimidazole dye that binds quantitatively to DNA (Arndt-Jovin and Jovin, 1977; Cesarone et al., 1979; Taylor and Milthorpe, 1980) can be used to follow the cell cycle, and Calcofluor White, a fluorescent cellulosic substantive, can be used to follow cell wall regeneration (Nagata and Takebe, 1971).

Automated flow cytometers have been used to characterize DNA synthesis and cell wall regeneration in the early culture of the B73 maize cell line (Meadows, 1982/83) and DNA synthesis in fixed tobacco protoplasts treated with cell wall inhibitors (Galbraith and Shields, 1982). The FACS IV (Fluorescence Activated Cell Sorter, an automated flow cytometer produced by Becton-Dickinson), has been used to select and sort protoplasts containing FITC-tagged alfalfa mosaic viruses (Slats et al., 1980) and chromosomes of H. gracilis (de Laat and

Blas, 1984). Redenbaugh et al. (1982) have also presented preliminary data on the use of the FACS IV for detecting doubly stained populations of protoplasts as a possible means for selecting hybrid fusion products and Puite and Ten Broeke (1983) have studied Hoechst 33342 staining of DNA in fixed and non-fixed protoplasts on a FACS IV. Harkin and Galbraith (1983) have established the conditions for sorting protoplasts of tobacco on an Epics V cell sorter that allow the protoplasts to be collected and cultured after the sort.

This paper reports the use of the FACS II to characterize the early events in a primary protoplast culture of Hyoscyamus muticus L. Hoechst 33258 was used to determine relative DNA content and Calcofluor White was used to determine relative cell wall content at various times after initiation of culture.

Materials and Methods

Plant material: Hyoscyamus muticus ($2n = 28$), or Egyptian henbane, is an herbaceous dicot whose mesophyll protoplasts reproducibly divide and grow in vitro (Lorz et al., 1979; Wernicke et al., 1979). Under appropriate culture conditions, its protoplasts can be induced to regenerate into whole plants.

Protoplast Isolation: The third and fourth leaves (counted from the apex) of 6- to 8-week old plants were surface sterilized in 0.01 % (v/v) $HgCl_2$ with 0.01 % (v/v) Tween 80 added as a wetting agent, for 15 min. They were then washed five times with sterile distilled water. Thin slices of the leaf blades were vacuum-infiltrated with and incubated in the following medium for 2 to 3 hrs at $30^{\circ}C$: 1 % Cellulase Onozuka R-10 (Kinki Yakult, Nishinomiya, Japan), 1 % Pectinol

Fest (Rohm, Darmstadt, FRG), 0.2 % Hemicellulase (Rhizopus, Sigma H2125, St. Louis, U.S.A.), 0.3 M mannitol and 0.04 M CaCl_2 at pH 5.7.

The protoplasts were harvested by the methods described by Potrykus et al. (1979). They were collected by successive passages through 250, 100 and 50 μm stainless steel sieves, then mixed with an equal volume of 0.16 M CaCl_2 + 0.5 % 2-N-morpholinethane sulfonic acid, (MES, Sigma) at pH 5.7, and centrifuged. The protoplasts were washed twice more in the CaCl_2 solution and finally resuspended in DPD (Durand et al., 1973) culture medium plus 2 % sucrose, 0.3 M mannitol, 4 mg/liter of parachlorophenoxyacetic acid (pCPA) and 1 mg/liter kinetin.

The protoplast concentration was adjusted to approximately 10^5 protoplasts/ml. The protoplasts were incubated at 12°C overnight in the dark in 6 cm Petri dishes and then transferred to a 24°C dark incubator for the duration of the experiment.

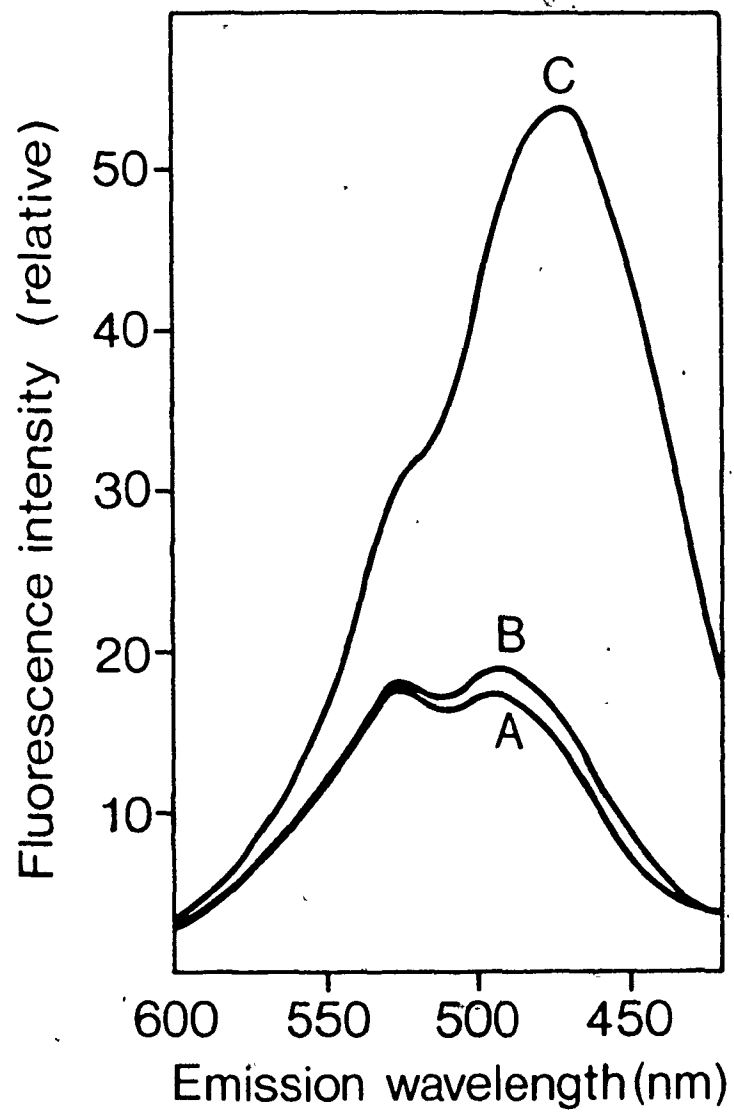
Survival of the protoplasts: The viability of the protoplasts approached 100 % (as checked by microscopic examination and the exclusion of Evans' blue) at all times after the initiation of the cultures. Therefore, survival was determined only by haemocytometer counts of protoplasts in each sample.

Hoechst 33258 staining. The Hoechst 33258 dye (H33258) was kindly provided by Dr. H. Loewe of Hoechst AG, BRD.

At an excitation wavelength of 350 nm, H33258 has a fluorescence emission maximum at around 520 nm in distilled water and an emission of around 465 nm when conjugated to DNA. The fluorescence spectra of Hoechst dye alone or in the presence of either RNA or DNA are shown

Figure 1: Emission spectra of Hoechst 33258 when excited at 350 nm;

、 A, Hoechst alone; B, Hoechst + RNA; C, Hoechst + DNA.



in Figure 1. Hoechst 33258 is a DNA specific fluorescent dye, and shows a large increase in fluorescence in the presence of DNA, but shows no increase of fluorescence in the presence of RNA. In the FACS II, the cells were excited with a laser line at 351 nm and emission was observed at 450 ± 5 nm, using a band-pass interference filter (Balzers, Lichtenstein).

The Hyoscyamus protoplasts were stained with H33258 using the method previously established for the B73 maize cell line (Meadows and Potrykus, 1981). The protoplasts were then washed and resuspended in DPD medium for FACS analysis, or mounted onto a slide for microscopic examination.

Calcofluor staining: Calcofluor White has an excitation wavelength of 350 nm and an emission maximum around 450 nm. It has been shown to be a quantitative stain for cell wall (Galbraith, 1981; Meadows, 1984). The protoplasts were washed in 0.16 M CaCl_2 and resuspended in 2 ml of culture medium to which 10 μl of a 0.1 mg/ml stock solution of Calcofluor was added. The protoplasts were either analyzed on the FACS, with an excitation wavelength of 351 nm and an emission wavelength of 450 ± 5 nm, or mounted for microscopic examination.

Microscopy: Protoplasts were examined with a Leitz Orthoplan fluorescence microscope equipped with an Orthomat automated camera. Its light source was a 200w, Osram HBO high pressure mercury lamp used with excitation barrier filter UV-UG1 and dichroic filter assembly Ploempak A (excitation maximum wavelength 350 nm and emission maximum 450 nm).

The Fluorescence Activated Cell Sorter. The FACS II used for these experiments (Becton-Dickinson Electronics Lab.) is owned and operated by the Basel Institute for Immunology (F. Hoffmann-La Roche, Ltd., Basel).

Flow cytometers have been used extensively with animal cells (Crissman and Tobey, 1974; Krishan, 1975; Morselt et al., 1979) and microbes (Hutter and Eipel, 1979), but only recently with plant protoplasts (Meadows, 1982/83; Galbraith and Shields, 1982; Slats et al., 1980; Redenbaugh et al., 1982; Puite and Ten Broeke, 1983; de Laat and Blas, 1984; Harkins and Galbraith, 1984). Some adjustments were made on the machine to accommodate the large, fragile protoplasts (Meadows, 1982). To do this, the sheath flow solution was replaced by culture medium (DPD), and the FACS was fitted with a 100 μm nozzle. The flow rate was reduced to about 25% of normal. To eliminate interference from small fragments and cellular debris which are detected close to the origin, the electronic size cut-off was set at slightly below the size of the smallest protoplasts (at approx. 13 to 15 μm). The gates on the input amplifier were set to allow maximum detection of the protoplasts as they passed singly through the krypton-argon laser, where two measurements were made: the low angle forward light scatter which gave an estimate of cell size, and the fluorescence due to bound dye. The FACS II was interfaced to an Apple II+ computer which was itself interfaced to an IBM 370 computer. The histograms and contour plots were produced on a Tektronics 4662 plotter using software designed for this purpose.

Results

The protoplasts isolated from H. muticus regenerate walls and divide in culture (Lorz et al., 1979). The stages of this process are shown in Figure 2. By approximately day 5, the protoplasts have undergone shape changes due to cell wall regeneration. The nucleus appears centered and surrounded by chloroplasts. By about 10 days, small colonies have formed due to the continued division of the cells and the clumping of cell clusters.

Survival: The survival of Hyoscyamus protoplasts during the first 8 days of culture is shown in Figure 3. Only during the first 3 days was there appreciable net protoplast death in culture. Approximately 60 % of the starting number of protoplasts remained viable and showed cell wall regeneration, plastid rearrangement and sustained division.

Hoechst staining: The staining of the Hyoscyamus protoplast nuclei by H33258 is illustrated in Figure 4. Although most protoplasts had only one nucleus, a small fraction (1-4 %) was multinucleated, probably due to spontaneous fusion during isolation (Table 1).

Calcofluor White staining: The fluorescent staining of the cell walls of the protoplasts after 6 days is shown in Figure 5. The Hyoscyamus protoplasts regenerated continuous walls, which were detected by blue fluorescence, and they changed shape and formed some cell clusters by the 6th day of culture.

DNA content as measured by the FACS: Plots of the DNA content of Hyoscyamus, as shown by H33258 staining, are given in Figure 6. The data are presented in two forms. Plots of the number of cells of

Figure 2: The stages of development of protoplasts from Hyoscyamus muticus in culture.

- (a) newly isolated mesophyll protoplasts (x 1000);
- (b) protoplast-derived cells, 5 to 7 days in culture (x 1000).
- (c) protoplast-derived cells and cell-colonies after 10 to 14 days in culture. (x 850).

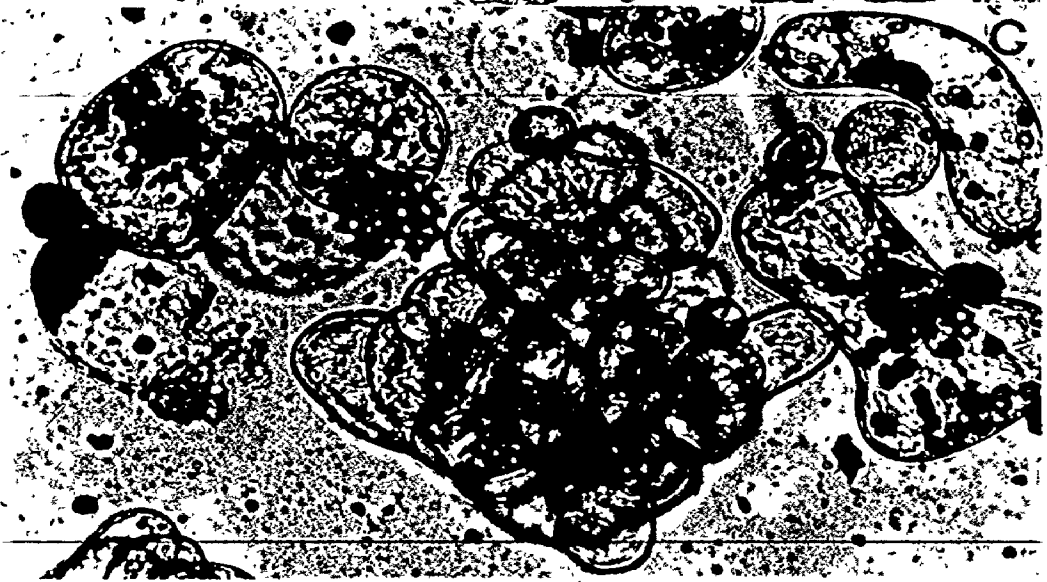
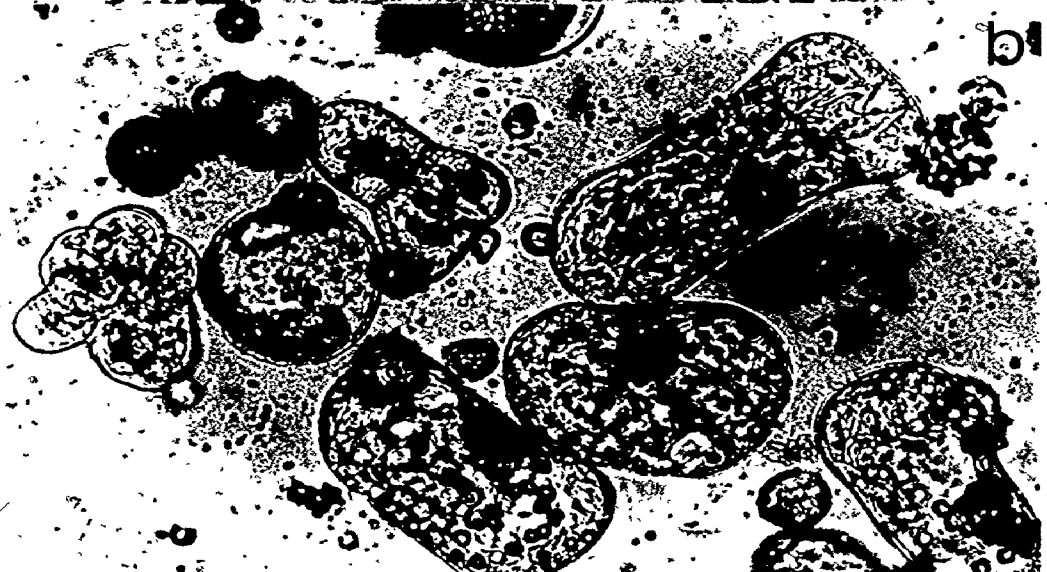
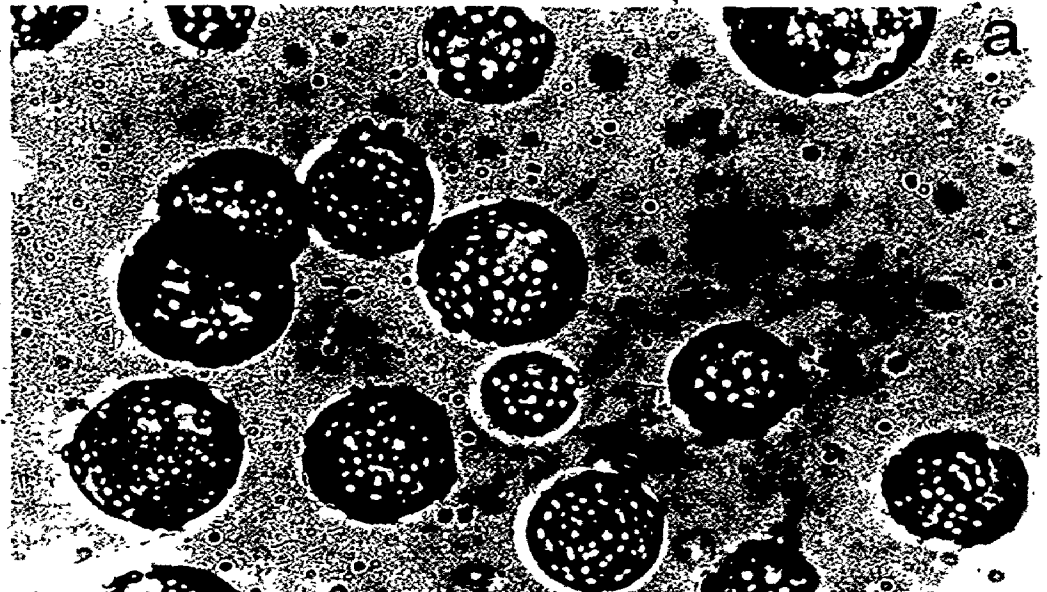
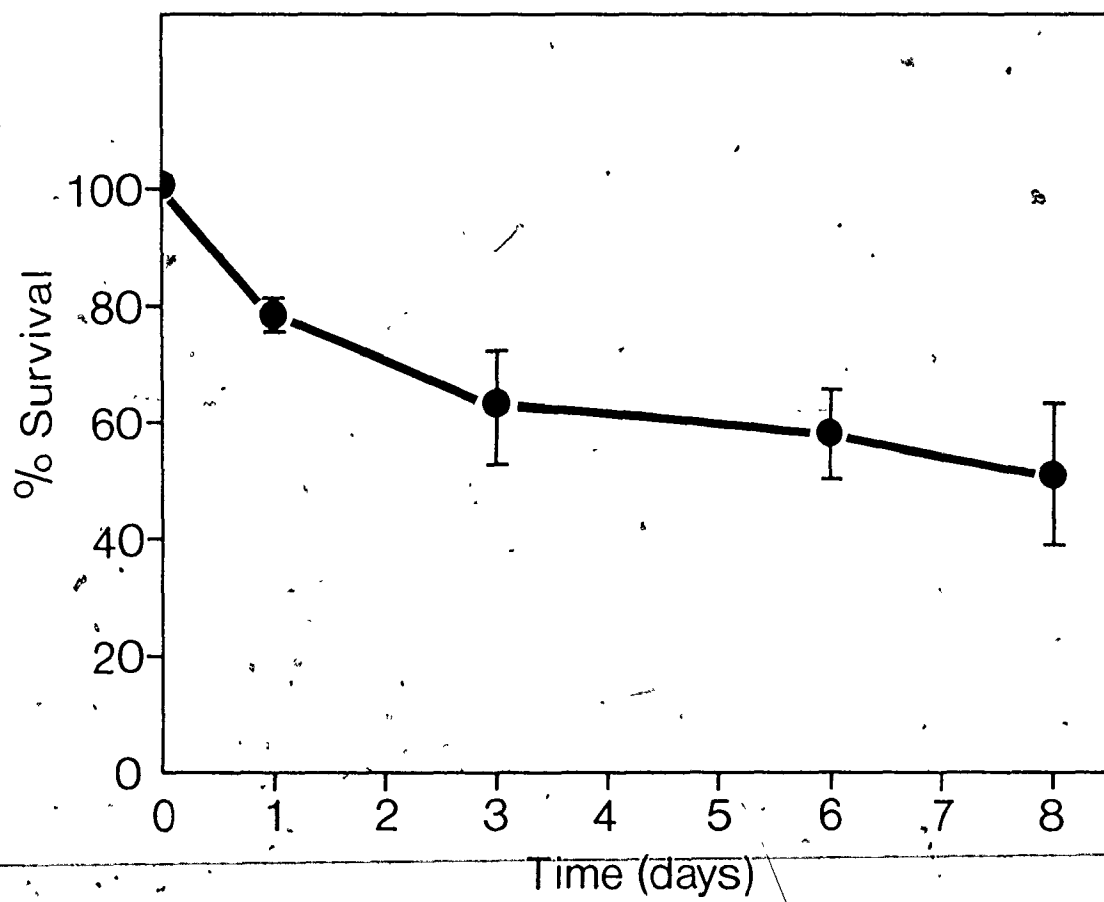


Figure 3: Survival of Hyoscyamus protoplasts for 8 days in culture. The graph is an average of five experiments of double counts of cells for each time point.

Bar = S.E.M.



8

Figure 4: Examples of Hoechst 33258 staining of Hyoscyamus nuclei
in protoplasts and protoplast-derived cells. (x 1000).

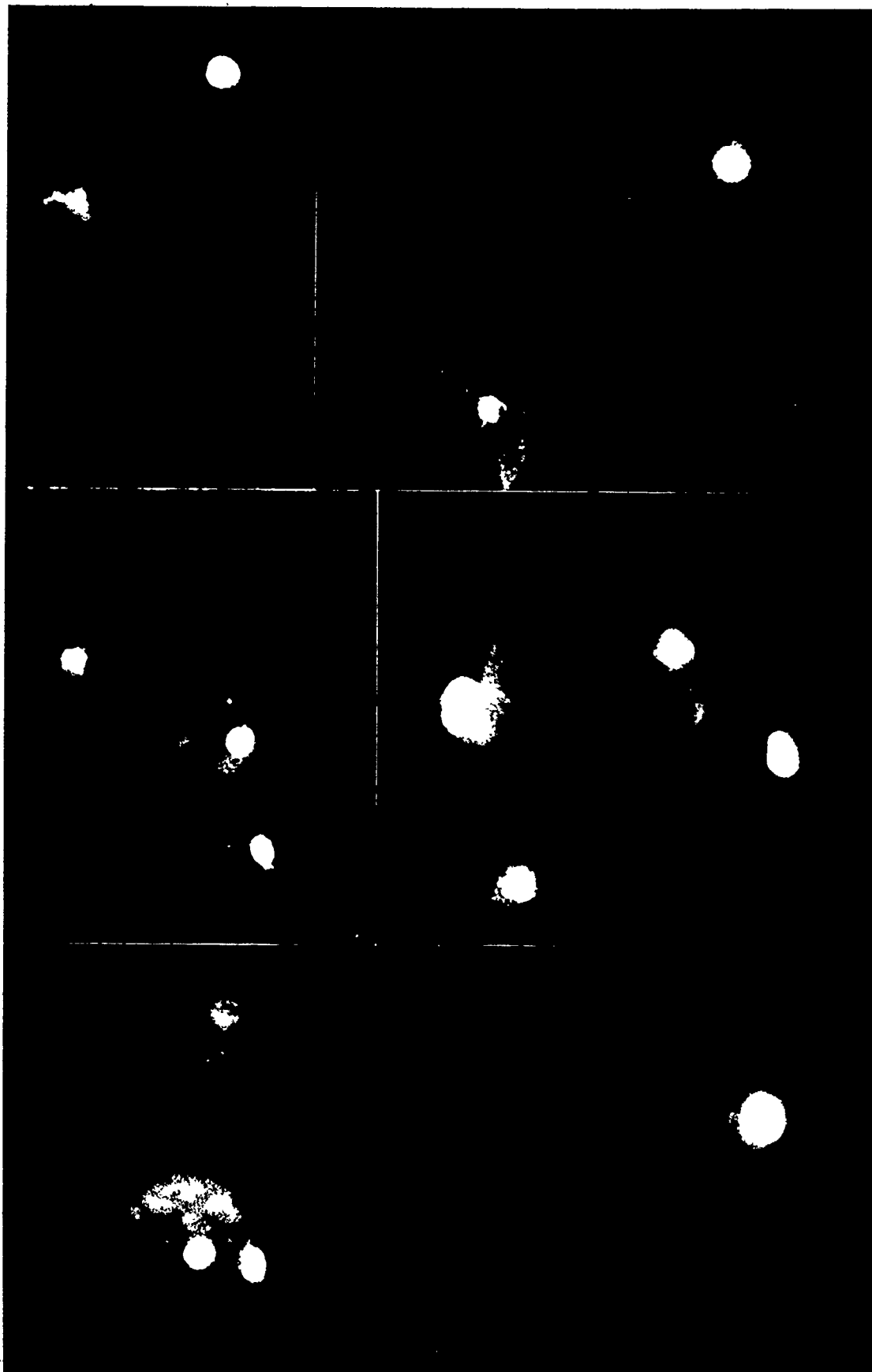


TABLE 1.
 THE PERCENTAGE OF UNINUCLEATE AND MULTINUCLEATE PROTOPLASTS
 PER DAY DURING 8 DAYS OF CULTURE.

(Each value is the average obtained for 3 separate experiments in which 600 protoplasts were scored on each day).

NUMBER OF NUCLEI PER CELL				
DAYS	1	2	3	4
% of cells.				
0	99.14	0.912	0.08	-
2	97.3	2.45	0.25	-
3	100.0	-	-	-
4	99.06	0.94	-	-
6	96.36	3.26	-	0.38
8	97.16	2.84	-	-

Figure 5: Calcofluor staining of cell walls of Hyoscyamus
muticus after 14 days in culture (x 1000).

Upper frame, transmitted light; lower frame, the same
cell cluster stained with Calcofluor.

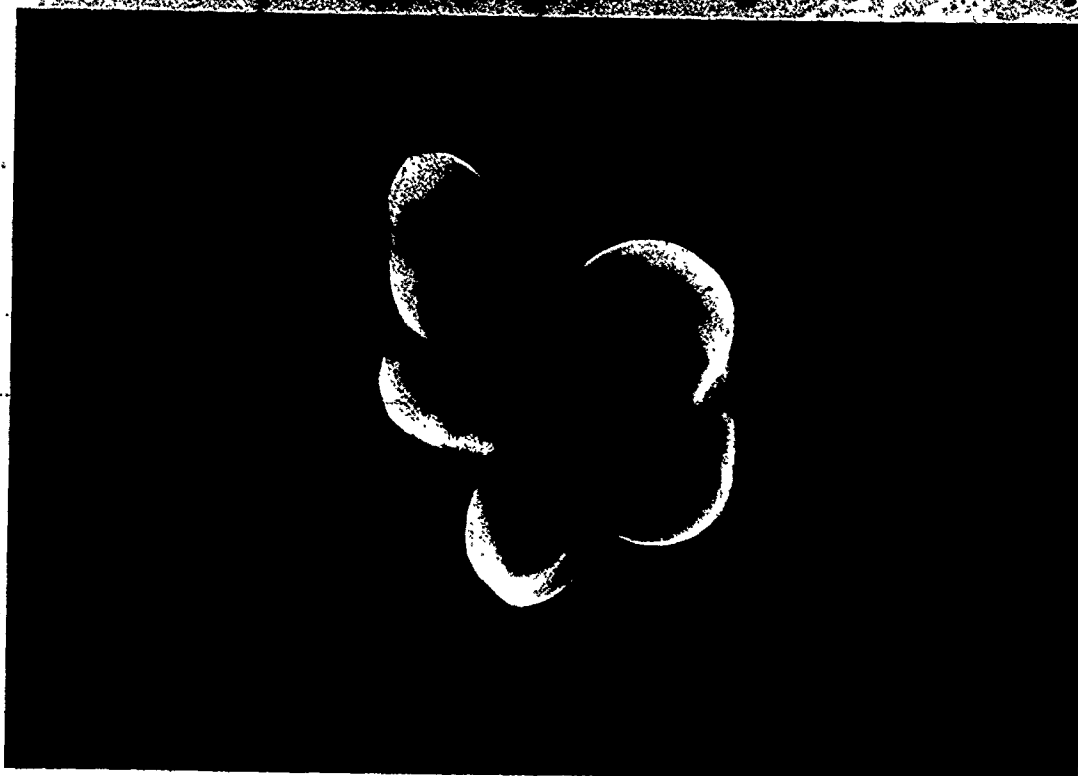
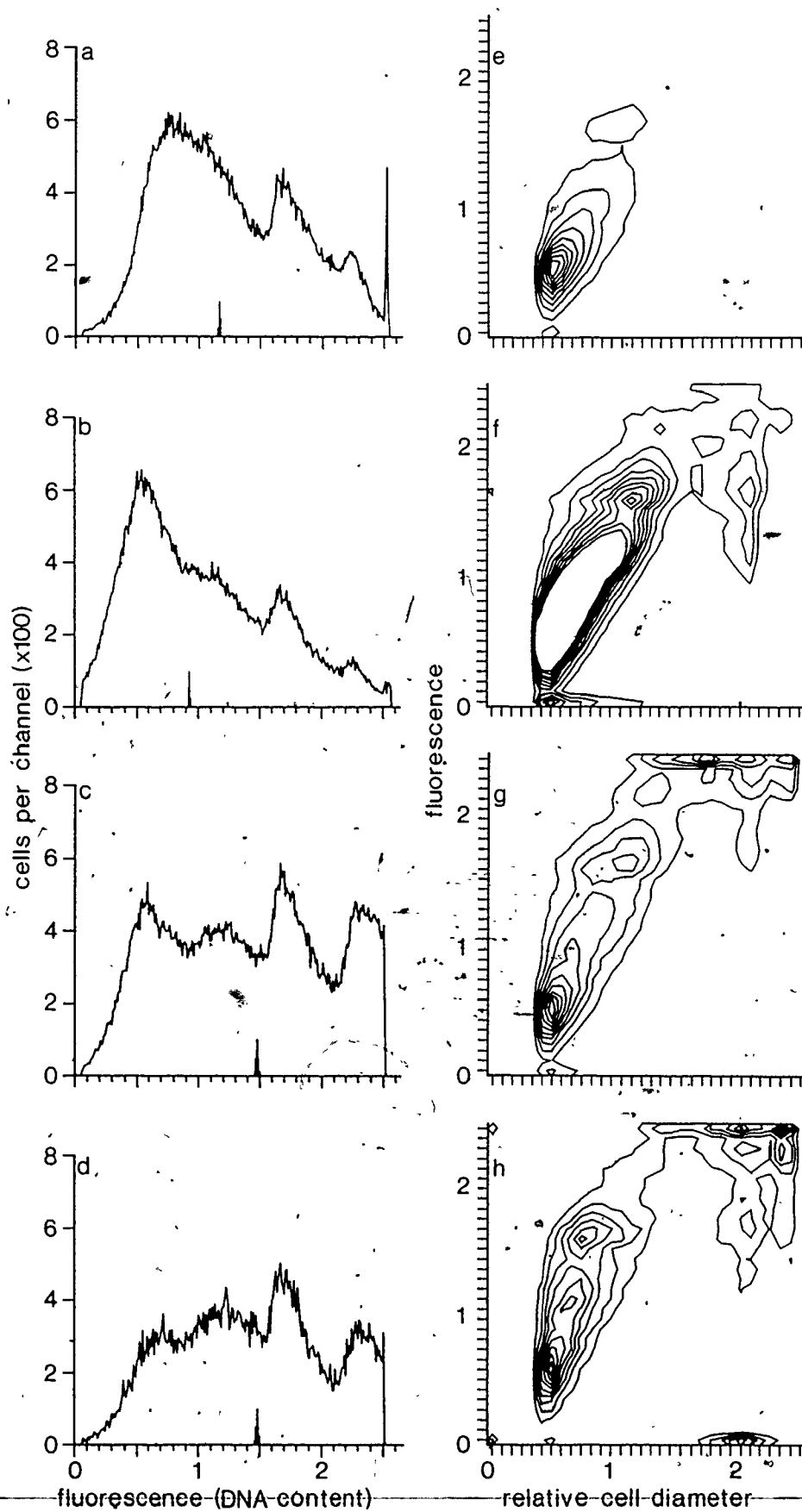


Figure 6: Histograms of DNA distribution of Hyoscyamus protoplasts as determined by the FACS II, for days 0, 1, 3 and 5 of culture. In the histograms, Figs 6a-d, the abscissa is the logarithm of the fluorescence intensity due to the amount of Hoechst 33258 bound to the DNA of the individual protoplasts and the ordinate is the number of protoplasts per channel. The tick marks indicate the median fluorescence and is bracketed by 1% and 5% confidence limits. The contour plots, Figs. 6e-h, record the relative size of the protoplasts as the abscissa and fluorescence due to Hoechst 33258 as the ordinate. The number of protoplasts of a given size and DNA content is plotted as the third dimension as contour lines spaced at intervals of 10% of the maximum.



a given fluorescence intensity (DNA content) are referred to as histograms. Plots of the number of cells (contour) of a given size (abscissa) and a given DNA content (ordinate) are referred to as contour plots.

Initially, the distribution of DNA in Hyoscyamus mesophyll protoplasts had 3 peaks (Fig 6a). The first, lowest fluorescence peak is presumed to correspond to the G1 state of the cell cycle and included most protoplasts. The valley between the first and second peaks presumably corresponded to S-phase protoplasts. A substantial number of the newly isolated protoplasts appear to be in G2 (peak 2). A smaller fraction had a DNA content apparently greater than G2 as shown by the small peak of highest fluorescence intensity. This was considered to result from multinucleated protoplasts formed by spontaneous fusion during isolation or from small clumps (see Table 1) and from a small portion of polyploid nuclei. About 99 % of the protoplasts were uninucleate immediately after isolation and this did not change. The sharp peak at the end of the distribution is from the electronic accumulation of oversized particles.

The contour plot of the number of protoplasts as a function of size and fluorescence for the newly isolated protoplasts is given in Figure 6e. It shows that all 3 fluorescent subpopulations were of small diameter.

By the second day of culture, the relative proportion of protoplasts in G1 had increased and the relative number in G2 had decreased (Fig 6b). From survival data (Fig 3), there had been 20 % protoplast death by day 2, and it appeared that those protoplasts in

G2 had been selectively lost. The G2 protoplasts increased considerably in size (Fig 6f). By day 3 (Figs 6c and g), there was a relative increase in the number of protoplasts in S and G2 at the expense of those that had been in G1, indicating that the protoplasts were proceeding through the cell cycle. The protoplasts in S-phase were visible as a distinct subpopulation. The population of protoplasts with very high fluorescence increased. This could have resulted from new DNA synthesis in the multinucleated protoplasts or from new DNA synthesis in those protoplasts that had been in G2 but which could not undergo cytokinesis, or to the formation of clumps. These latter two explanations are favoured. The protoplasts which had been in G2 at isolation were presumed not to have undergone cytokinesis until they had resynthesized walls, and thus could not return nuclei to the G1 compartment of the cell cycle. However, these protoplasts could, presumably, have initiated a new round of DNA synthesis, resulting in protoplasts having greater than 4N DNA. In Figure 6g, such protoplasts would be the larger subpopulation in the very high fluorescence class, on the predominant trend between size and fluorescence. The population of very large protoplasts outside the trend was presumed to be aggregated protoplasts.

By day 5, the relative number of protoplasts in the very high fluorescence peak had decreased slightly which may have been due to the removal of the clumps by filtration before analysis. The contour plots for day 5 (Fig 6h) show an increase in the very large cells of medium fluorescence. There is also a small population of essentially non-fluorescent, very large cells. It is presumed that these large cells are actually cell aggregates, and that the non-fluorescent

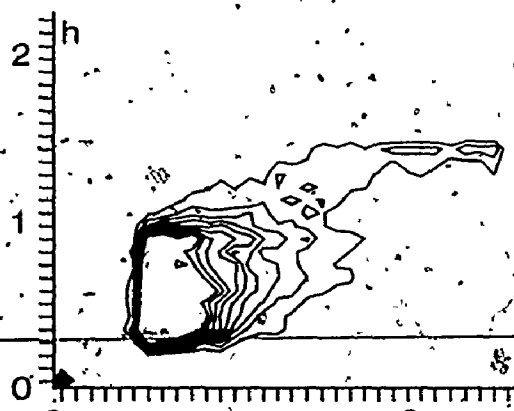
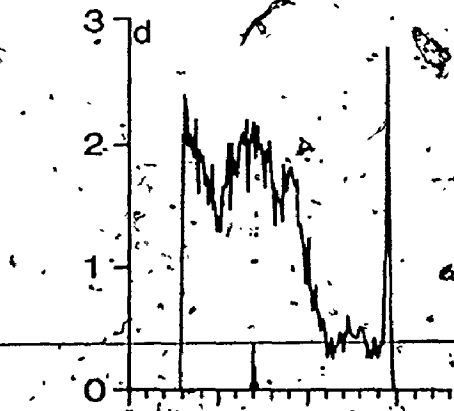
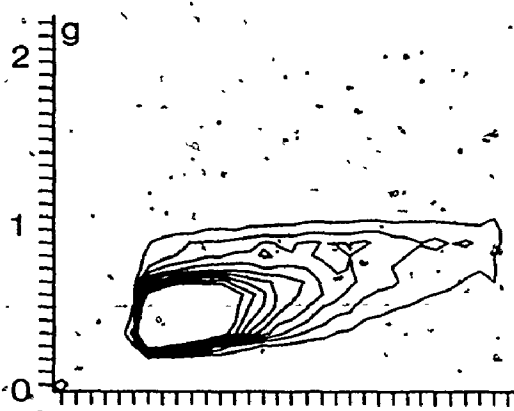
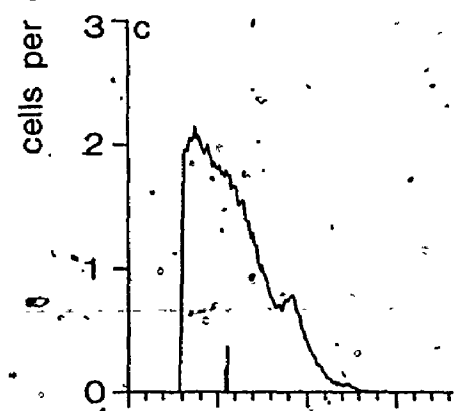
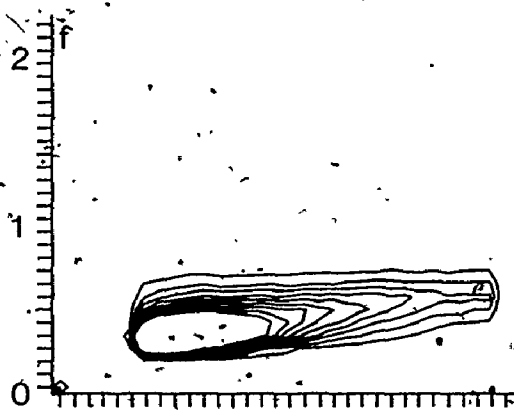
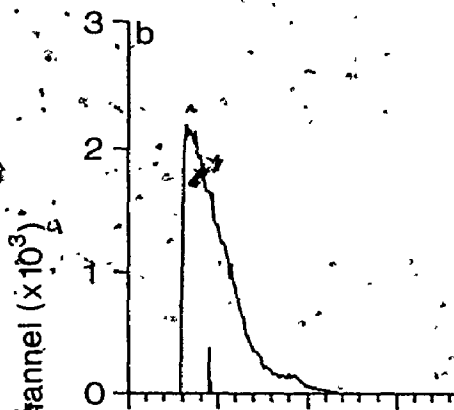
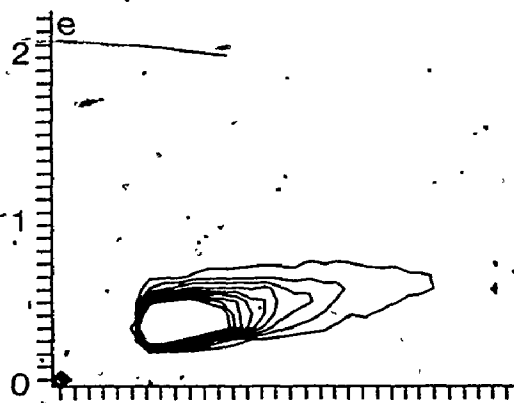
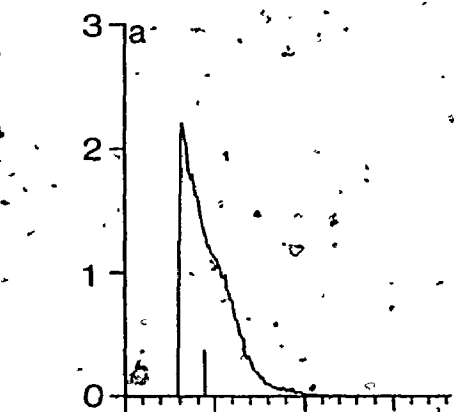
cells were wall fragments from degraded, dead protoplasts.

Cell Wall Regeneration as Shown by Calcofluor Fluorescence: The fluorescence due to the binding of Calcofluor to cell walls of Hyoscyamus protoplasts is shown in Figure 7. Initially (Fig. 7a) there was a background fluorescence peak, due probably to the incomplete removal of walls from some protoplasts. For the remainder of the experimental time (1, 3 and 5 days), there was a continual increase in the amount of cell wall regenerated by the protoplasts. The presence of the initial background peak indicated that there was always a population of protoplasts possessing some cell wall. The appearance (Fig 7b) and increase (Fig 7c) of a medium fluorescence shoulder and finally of several distinct peaks on day 5 (Fig 7d) indicated subpopulations of protoplasts which had varying rates of cell wall synthesis. The contour plots showed that the initial background fluorescence occurred among all sizes of protoplasts (Fig 7e) as did the increased fluorescence due to cell wall regeneration on days 1 and 3 (Fig 7f,g). By day 5, the small and medium sized protoplasts were the most populous and showed further increased wall synthesis. There was also a population of very large, brightly fluorescent protoplasts which could have been small clumps.

Summary

The FACS II has been used to study DNA and cell wall synthesis in newly isolated protoplasts of Hyoscyamus muticus during their early days of culture. The primary advantage of the method is that it allows analysis of large numbers of viable protoplasts for both DNA and cellulose content. Protoplasts from H. muticus which can

Figure 7: Histograms of cell wall fluorescence due to Calcofluor White for days 0,1,3, and 5 of culture of the protoplasts. In the histograms, Figs 7a-d, the abscissa is the logarithm of the fluorescence intensity due to the bound Calcofluor and the ordinate is the number of protoplasts per channel. The tick marks indicate the median fluorescence and are bracketed by 1% and 5% confidence limits. The contour plots, Figs 7e-h, have the relative size of the protoplasts as the abscissa and the fluorescence due to Calcofluor as the ordinate. The number of protoplasts of a given size and cellulose content is plotted in the third dimension at intervals of 10% of the maximum.



fluorescence, calcofluor

relative cell diameter

regenerate cell walls and divide (Lorz et al., 1979) are a good model system to study the early events in the culture of plant tissues.

The DNA distribution patterns for the Hyoscyamus showed that there were subpopulations of mesophyll protoplasts whose nuclei were in the G1, S and G2 compartments of the cell cycle. Similar results were obtained for fixed tobacco protoplasts by Galbraith et al. (1981).

Survival studies on the cultures of Hyoscyamus showed that there was considerable cell death during the first three days of culture.

The FACS analysis of the protoplasts during that time showed that there was an initial selective loss of those protoplasts that were in G2. However, with time, there was a resurgence of the G2 subpopulation and an increase in the number of cells with greater than G2 DNA content. Galbraith and Shields (1982) examined fixed tobacco protoplasts that had been cultured in the presence of 6-dichlorobezonitrile (DB) on an Epics V flow cytometer. DB is a cell wall inhibitor that apparently does not inhibit DNA synthesis. Despite the lack of data for untreated protoplasts, it appeared that with time, the G1 subpopulation decreased and the G2 and greater than G2 subpopulations increased. Similar patterns of DNA distributions were observed in the FACS analysis of B73 protoplasts (Meadows, 1982). Thus, in all systems which have been studied, there is a general progression into G2 and some polyploidy in the early days of culture of the protoplasts.

Cell wall synthesis occurred simultaneously with DNA synthesis in the cultured protoplasts of H. muticus, and was studied on the FACS. There was an ever-increasing amount of cell wall fluorescence

distributed among subpopulations with slightly different rates of synthesis. This pattern was similar to regenerating maize B73 protoplasts (Meadows, 1982).

The use of flow cytometry with protoplasts is relatively new. In this study, live protoplasts were used to examine nuclear changes during the early culture of H.muticus. The data obtained were in good agreement with that published for fixed tobacco (Galbraith and Shields, 1982) and for unfixed maize B73 protoplasts (Meadows, 1982). The cell wall regeneration data were similar to that observed for cultured maize protoplasts.

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Chapter VII: Characterization of protoplasts of Zea mays L.
using flow cytometry.

Introduction

The use of the FACS II to examine DNA and cell wall synthesis in protoplasts isolated from cultured corn cells and from leaves of Hyoscyamus muticus has been reported (Meadows, 1982; Meadows, 1984). Both plant systems could be considered as "responsive" to culturing as they are capable of synthesizing DNA and cell walls, and undergoing division in the appropriate culture conditions. In this study, primary protoplasts of a "non-responsive" plant have been examined for DNA and cell wall synthesis with the FACS II. The fluorescent dyes were Hoechst 33258 for DNA and Calcofluor White for cell walls. The plant material was from a single cross hybrid sweet corn, Se60. The results obtained allow comparison with the earlier findings on protoplast cultures of the B73 maize cell line and primary protoplast cultures of Hyoscyamus muticus.

Materials and Methods

Plant material: Protoplasts isolated from any organ of Zea mays L. ($2n = 20$), with one exception (Potrykus et al., 1979), do not divide in culture. The cultivar of corn used in the experiments reported below was Seneca 60, (Se60), a single cross hybrid sweet corn. Tissues from its immature leaf sheath or from its immature nodes and internodes respond to culture on high auxin medium by callus formation. However, protoplasts isolated from these tissues do not divide in culture.

Protoplast Isolation: The stalks of 21-day old plants were surface sterilized in HgCl_2 and were cut open lengthwise. The leaf sheaths were removed, separated and floated on a solution containing 0.5 % Cellulase Onozuka R-10, 9 % mannitol and the salts described by Zapata et al. (1977). They were shaken gently on a reciprocal shaker for 3 hours at 24°C in the dark.

Isolated protoplasts were harvested and collected by the method described by Potrykus et al. (1977). There was a large amount of cellular debris after the final wash that could be reduced by resuspending the protoplasts in 0.16 M CaCl_2 and allowing it to stand for 10 min. The debris aggregated under these conditions and was retained on a 50 μm filter. After centrifugation, the protoplasts were resuspended in NN67 (Nitsch and Nitsch, 1967) plus 2 % sucrose, 0.17 M mannitol and 2 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D). The concentration was adjusted to 10^5 protoplasts/ml. before incubation which was at 12°C overnight and then at 24°C always in the dark.

Survival of the Protoplasts: The survival of the corn protoplasts was determined by duplicate haemocytometer counts of samples taken each day of the experiment.

The methods for fluorescent staining with Hoechst 33258 and Calcofluor White, the adjustments to the FACS II and the microscopy were as described earlier (Meadows, 1982; Meadows, 1984; Meadows and Potrykus, 1981).

Results

The protoplasts from Zea showed little shape change after one week in culture. There was some aggregation, but no cell division.

Survival: Zea protoplasts continuously died in culture (Fig 1). By day 6 of culture, less than 10 % of the starting number of protoplasts was still viable.

Hoechst Staining: The staining of the corn protoplast nuclei by H33258 is illustrated in Figure 2. Most protoplasts had only one nucleus; less than 1 % were multinucleated due to spontaneous fusion during isolation.

Calcofluor White Staining: The fluorescence of the cell walls of the corn protoplasts after 6 days is shown in Figure 3. There appeared to be no continuous wall formation; rather, wall formation appeared to be in patches.

DNA Content as Measured by the FACS: The data for the DNA content of the corn protoplasts are shown in Figure 4. The number of newly isolated protoplasts in the presumptive G1 and G2 classes was approximately equal. Verma and Lin (1979), using the same variety of corn, Se60, and ^3H -thymidine autoradiography, have shown that for root tip cells, the number of cells in G1 and G2 classes are equal. Van't Hoff (1974) has also shown that the percentage of corn primary root cells arrested in G1 and G2 after sucrose starvation are equal. Recently, Harris *et al.* (1984), studied the changes in the nuclear DNA of corn leaves and found that the DNA was almost equally distributed between G1 and G2 in young leaves. Thus, it appears that the data obtained with the FACS are in good agreement with the results of these earlier studies. A large number of protoplasts were in S phase, corresponding to its large proportion of the cell cycle time (Verma and Lin, 1979). This broadened the G1 and G2 peaks (Fig 4).

Figure 1: Survival of Se60 protoplasts for 6 days in culture.

The graph is an average of five experiments with
double counts for each time point.

Bar = S.E.M.

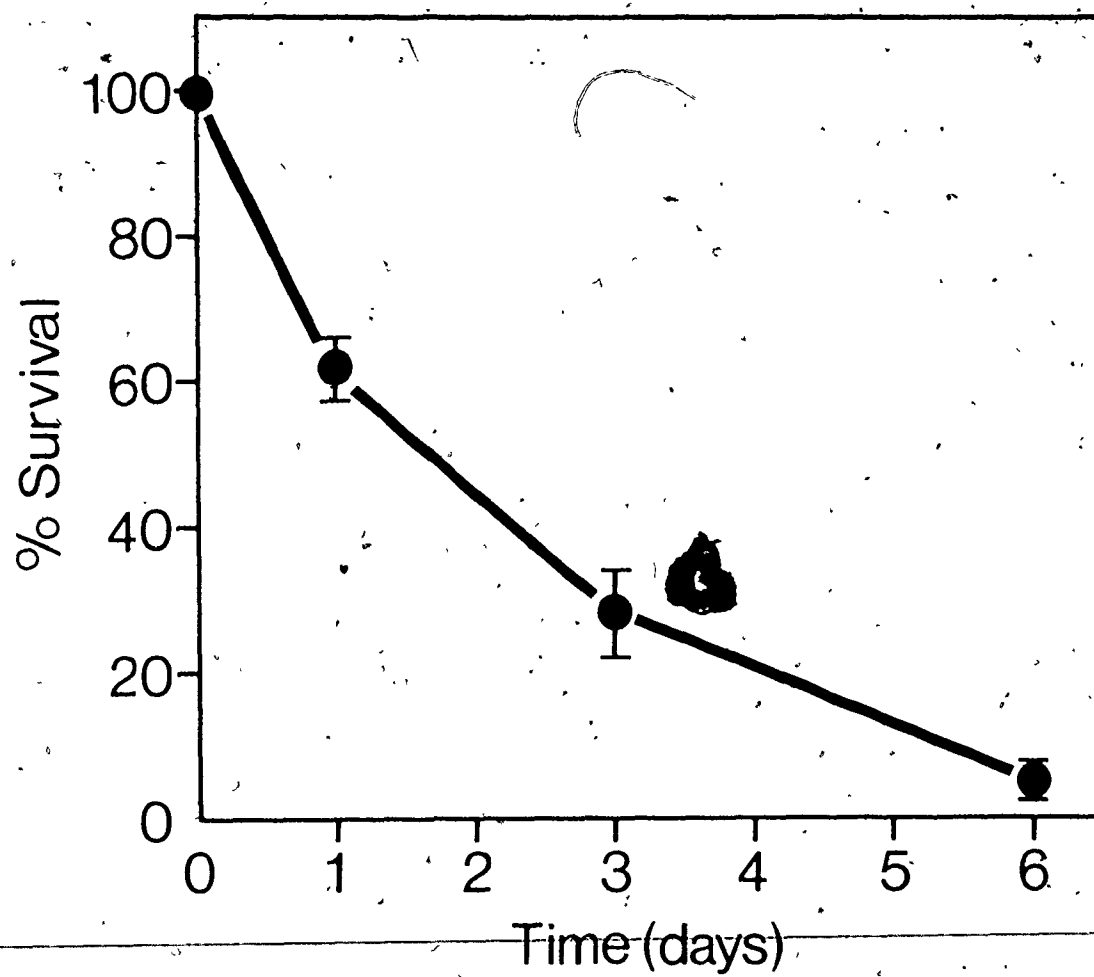
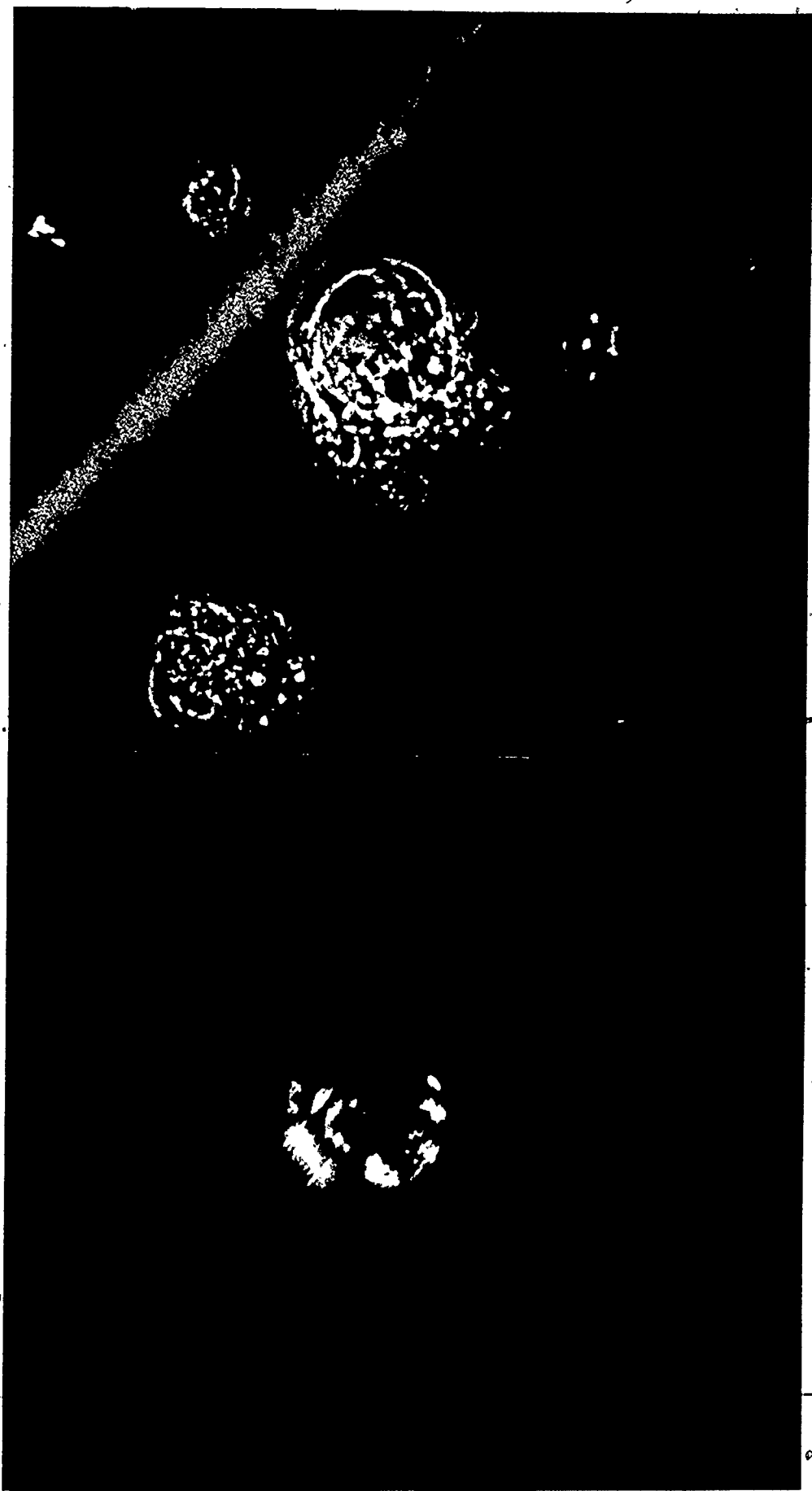


Figure 2; Hoechst 33258 staining of Se60 protoplast nuclei.

(x 1000).



Figure 3: Calcofluor staining of cell walls of a Zea mays protoplast after 6 days of culture. Upper frame, transmitted light; lower frame, same protoplast stained with Calcofluor. (x 2000).



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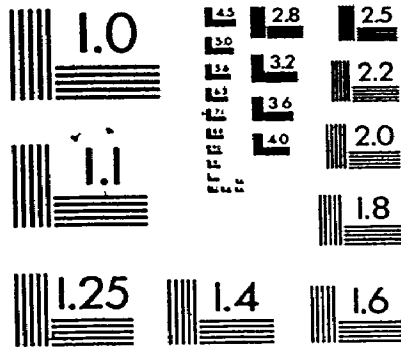
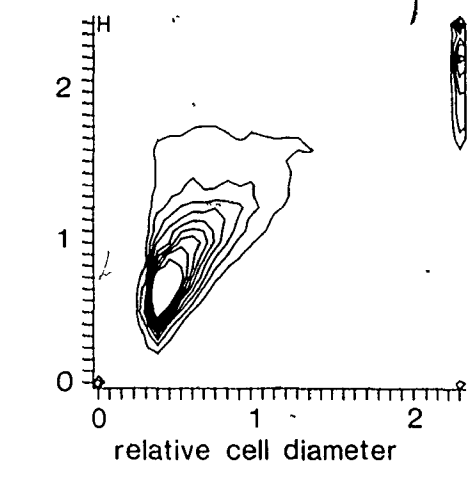
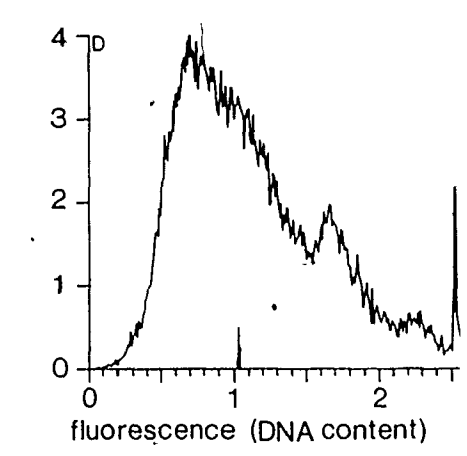
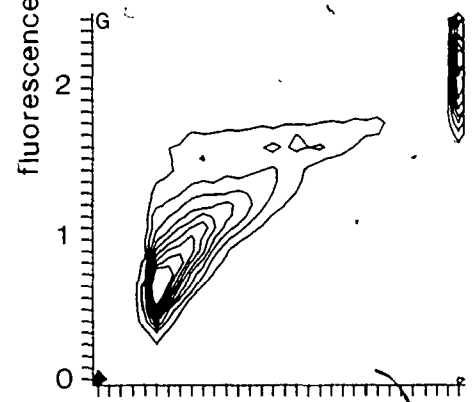
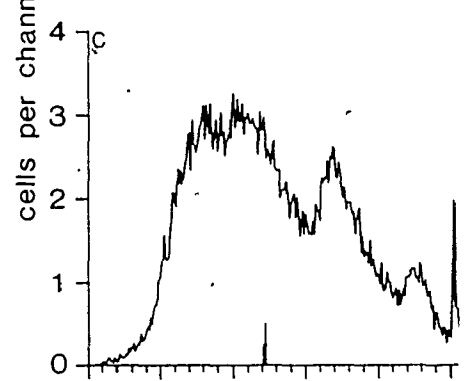
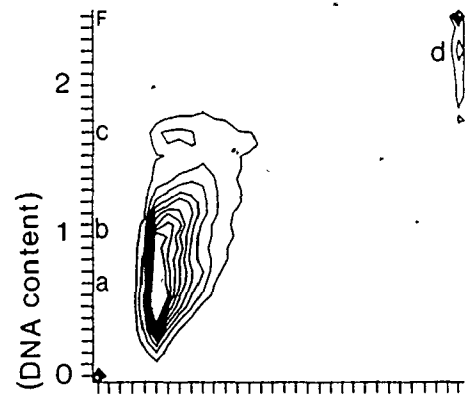
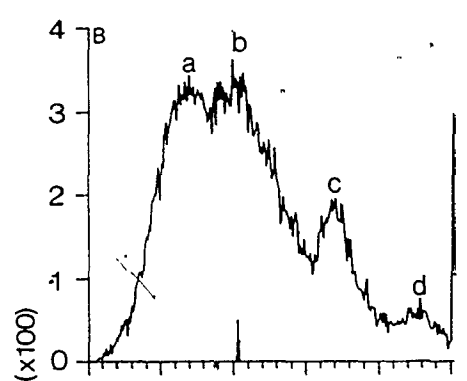
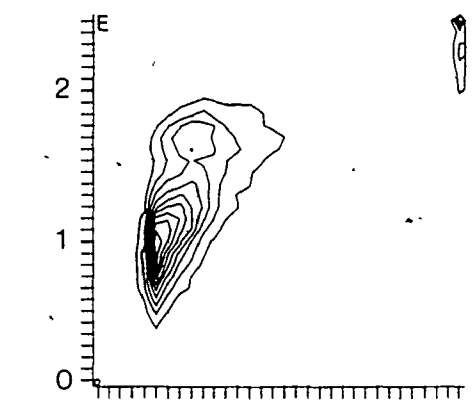
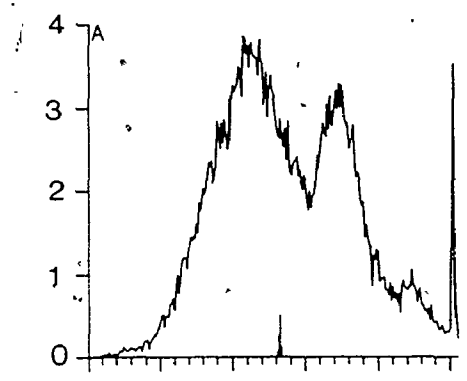


Figure 4: Facs histograms of DNA distribution of Zea mays protoplasts.

The abscissa in figures 4a-d is the logarithm of fluorescence due to bound H33258 for days 0,2,4 and 6 respectively and the ordinate is the number of protoplasts per channel. Figures 4e to h are the contour plots wherein the relative size of the protoplasts is recorded on the abscissa; the H33258 fluorescence intensity is recorded on the ordinate and the third dimension is number of protoplasts per size and fluorescence for days 0,2,4 and 6 respectively. The contour lines are drawn at 10% of the maximum.



The protoplasts at all stages of the cell cycle were of relatively uniform size (Fig 4e).

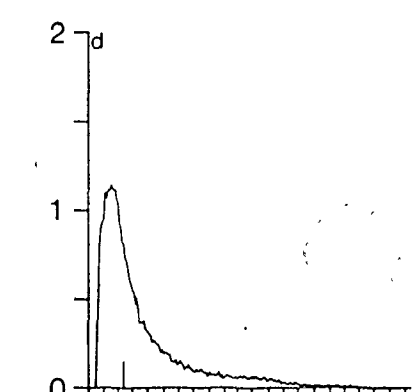
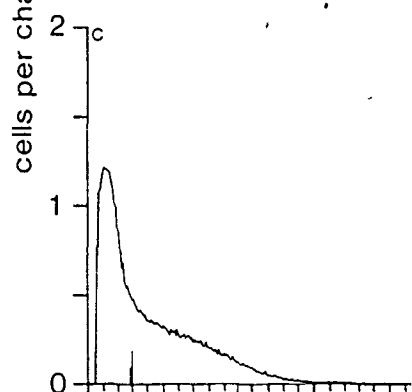
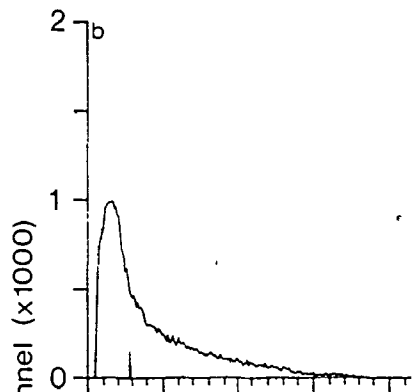
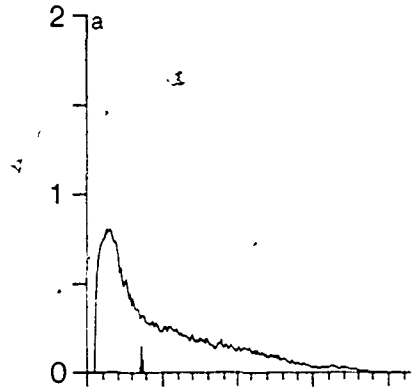
By the 2nd day of culture a new population appeared (Fig 4b, peak a) which had less DNA than the presumptive G1 population (Fig 4b, peak b). At the same time, there was a significant relative decrease in the number of protoplasts in G2 (Fig 4b, peak c). There was a small population (Fig 4b, peak d) which appeared to result from clumping.

As shown by survival studies, the corn protoplasts died rapidly (Fig 1). The cell death appeared to occur mostly among G2 protoplasts. The appearance of the "a" population may have been due to DNA degeneration in the dying population. At day 2, there was no significant increase in the number of protoplasts with greater than G2 DNA content (Fig 4b, peak d). The median DNA content had decreased from 1.3 to 1.0 fluorescence units. However, on the fourth day of culture, DNA content and cell size had increased (Fig 4C,G). The proportion of protoplasts in G2 had increased (peak c) and there were relatively fewer protoplasts in G2 and G1 but more in peak "a". Some of those protoplasts which had enlarged by day 4 appeared to be lost by day 6 (Fig 4G,H).

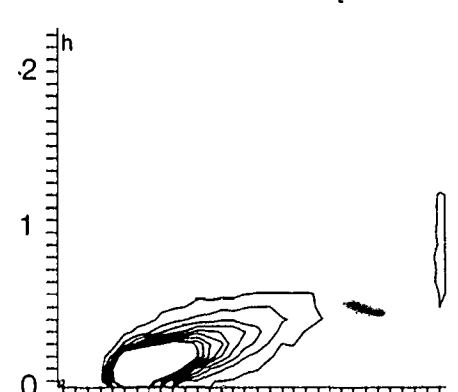
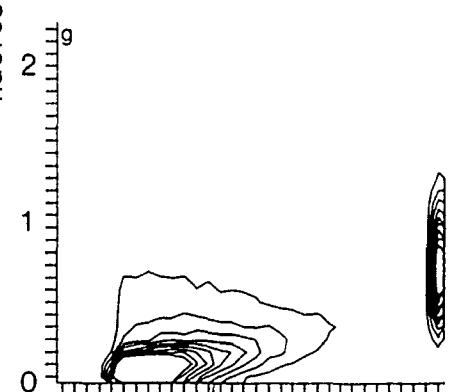
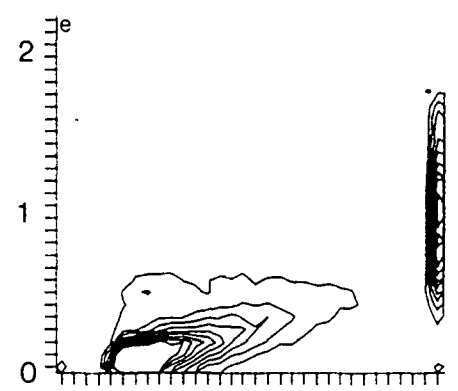
Cell Wall Regeneration as Shown by Calcofluor Fluorescence:

Freshly isolated Zea protoplasts had some fluorescence with Calcofluor presumably due to the presence of protoplasts bearing wall fragments (Fig 5a). However there was little increase in fluorescence above initial levels for the duration of the experiment. At day 4, there appeared to be a small population of protoplasts that

Figure 5: Histograms of cell wall fluorescence due to Calcofluor for days 0,2,4 and 6. The axes are as described in figure 4, except that the fluorescence is due to Calcofluor.



fluorescence, calcofluor
(cellulose content)



relative cell diameter


had increased their cellulose content, as shown by the median fluorescence shoulder. This increased fluorescence may have been due to cell wall synthesis by smaller protoplasts (Fig 5g). However, by day 6 of the experiment, this population of protoplasts had decreased in proportion to the whole, and the fluorescence was again confined to the peak corresponding to the background fluorescence. Throughout the experiment, there was a small proportion of large fluorescent particles (Fig 5e through h) which are presumed to be clumps of cells and debris. This population could contribute to the "tail" of the fluorescence histograms.

Discussion:

FACS II studies of DNA and cell wall synthesis in newly isolated protoplasts of Zea mays L. can be compared to similar studies done on protoplasts isolated from a cultured cell line, B73, (Meadows, 1982) and from Hyoscyamus muticus (Meadows, 1984) The DNA distribution patterns for Hyoscyamus, B73 and Zea appeared to be similar in some respects. There was an initial loss of protoplasts in G2, followed by cell enlargement, a resurgence of G2 protoplasts, and an increase in the number of cells with greater than G2 DNA content. The major difference was the appearance of a new peak in the Zea DNA distribution pattern which had a fluorescence lower than that assumed to be due to G1 nuclei. The peak may represent degenerating nuclei of senescing protoplasts. Harris et al., (1984), studying changes microspectrophotometrically of Feulgen stained nuclear DNA of corn leaves, observed a 17.4% shift from G1 to less than G1 content in nuclei of senescing leaves compared to young

leaves. Yataganes and Clarkson (1974) have also observed such a peak in drug-killed mammalian cells. This less than G1 peak became predominate in the Zea protoplast cultures whereas no such peak occurred following culture of either the Hyoscyamus protoplasts or the B73 protoplasts. Hyoscyamus and B73 protoplasts synthesized DNA and the G2 peak became predominate in readiness for cytokinesis:

The cell wall resynthesis patterns for the various types of protoplasts were quite different. Whereas Hyoscyamus and B73 protoplasts showed an ever-increasing amount of wall fluorescence distributed among subpopulations having different rates of synthesis, (Meadows, 1982; Meadows, 1984), the Zea protoplasts showed only a small increase in cell wall fluorescence on day 4 of culture, when a very small population of protoplasts appeared to be making walls. The appearance of this population was concurrent with the increase in the number of protoplasts in G2. However, by day 6 of culture this subpopulation was lost due to continued death of the culture. One could speculate that indeed there is a fraction of the isolated Zea protoplasts capable of division and cell wall synthesis but that culture conditions did not permit their continued growth.



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Chapter VIII: Discussion.

Protoplast technology has become fundamental to basic morphological and physiological studies in many plants and is an absolute requirement if genetically transformed plants are to be achieved in the future. Although there have been many reports of plant regeneration from protoplasts no such success has been reported for protoplasts isolated from Zea mays although there is a great deal known both biochemically and genetically about this plant. There has been some recent success in inducing protoplasts derived from cell cultures of Z. mays var Black Mexican Sweet (Chourey and Zurawski, 1981) to divide in culture but as yet plants cannot be regenerated from these cultures.

One of the requirements for successful genetic engineering is that the isolation and cultivation of the protoplasts be repeatable and applicable to all varieties of the plant. There is a need to examine the protoplasts in culture to define what processes are occurring physiologically. Despite the fact that the regeneration of a plant from a protoplast usually excites the scientific community, the data reported usually are of a descriptive nature, stating the final conditions of the success and not of the "in vitro" occurrences that had lead to it.

This study was undertaken to examine the progression of protoplast to cell culture in three systems. Two of them, protoplasts from H. muticus and the B73 maize cell line were used as "model" systems since they are repeatedly capable of regeneration of wall and sustained divisions in culture (Lorz et al, 1979; Potrykus et al, 1979). H. muticus can be induced to regenerate plants in the correct

conditions (Iorz et al., 1979). The third system studied was Se60, a cultivar of corn, used to evaluate the unsuccessful process of protoplast to cell culture in comparison to the other two. DNA and cell wall synthesis were studied in all three protoplast cultures. To do this, use was made of the FACS II, a fluorescence activated cell sorter and two fluorescent stains: Hoeschst 33258, which binds quantitatively to DNA (Cesarone et al., 1979; Arndt-Jovin and Jovin, 1977) and Calcofluor White, which has been used to visualize cell wall (Darken, 1962; Nagata and Takebe, 1970; Berliner et al., 1978; Galbraith, 1981) and which this research showed to be quantitative for cell wall.

It has become accepted that the genotype of the corn is very important for callus production (Green and Phillips, 1975; King et al., 1978; Lu et al., 1982) and there have been many screening programmes developed to choose those genotypes of corn capable of responding to culture: scutellum, (Green and Phillips, 1975; Bartkowiak, 1978; Lu et al., 1978), stem (Sheridan, 1977), mesocotyl and leaf (Kunakh et al., 1980).

Se 60 was shown to be able to respond to the genotype testing medium P13, developed by Potrykus (personal communication) by forming callus from leave sheath tissue. This tissue was subsequently shown to be ideal for protoplast isolation.

The methods were developed to isolate a large quantity of protoplasts routinely. The conditions defined for primary corn protoplast isolation were: leaf sheaths from 21-day old plants, greenhouse grown approximately on a 30⁰/20⁰, 16/8 hour cycle were pretreated by placing them in a dark, highly humid cupboard overnight before protoplast

preparation; the enzyme preparation used to isolate the protoplasts was a solution of 0.5 % Cellulase Onozuka R-10, 0.5 % MES, 9 % mannitol, the salt solution defined by Zapata et al., (1977), pH 5.5, 2.5 to 3 hours at 25°. Yields obtained were always greater than 10⁶ per gram fresh weight.

The conditions for the repeatable isolation and culture of protoplasts from H. muticus and from the B73 maize line were already established (Potrykus, pers. comm; Potrykus et al., 1979). The culture conditions that were adopted in the culture of the newly isolated Se60 protoplasts were the same as those used in the culture of the B73 maize cell protoplasts (Potrykus et al., 1979). This medium based on that of Nitsch and Nitsch (1967) was shown to be as good as any of the several that were designed after testing the effects of many components individually and in combinations on the survival of the protoplasts. The tedious and time-consuming testing of culture media components, based on Se60 kernal extract and stem exudate analyses, did not result in induction of division in the protoplasts. Since media testing experiments tend to be qualitative, the effect of an added component was quantitated by measuring protoplast survival in the presence of that component as compared to survival in 9 % mannitol.

A medium, based on the component testing results, plus two other media were tested and compared to the defined medium of Nitsch and Nitsch (1967). NN67 medium supported protoplast survival marginally better than the others. Se60 protoplasts were not induced to divide and after 8 days of culture in any of the media, 10 % was the maximum

survival obtained. Data collected from the unsuccessful media testing are included as Appendix I of this thesis.

Hoechst 33258 is a bisbenzimidazole dye that binds quantitatively to DNA (Cesarone *et al.*, 1979; Latt and Stetton, 1976; Arndt-Jovin and Jovin, 1977; Paul and Myer, 1983). Besides being used routinely for animal cells, it has been used for staining plant cell nuclei, most often in fixed material (Filion *et al.* 1976; Laloue *et al.*, 1980; Galbraith *et al.*, 1981). In anticipation of using the "sort" option on the FACS II, conditions were established for staining the nuclei of unfixed plant protoplasts. It was found that Hoechst 33258 was not taken up in to the nuclei of all of the living protoplasts. Treatment of the protoplasts with a low concentration of Triton X-100 allowed 100 % of the protoplast nuclei to be stained. The optimal concentration for the staining of the protoplast nuclei was found to be 20 $\mu\text{g/ml}$ at pH 7.0, after treatment with DNAase at a final concentration of 50 $\mu\text{g/ml}$ at 37 $^{\circ}$. This method has been adopted for visualizing nuclei of protoplasts in DNA microinjection experiments (Miki, pers. comm.). Petunia protoplasts, treated with Hoechst 33258 according to this protocol, have been observed to have the same plating efficiency as non-stained protoplasts (Meadows and Potrykus, unpublished.)

Calcofluor White is a dye that has been used in the textile industry as a cotton whitener because of its affinity for hexopyranosyl polymers (Maeda and Ishida, 1967). It was first reported as a specific stain for cell wall in cultured protoplasts by Nagata and Takebe (1970) using tobacco protoplasts. Since then, it has become routine stain in most protoplast research laboratories. A titration assay

developed in this research showed that its fluorescence was linear with microcrystalline cellulose concentration. Cell wall regeneration in H. muticus protoplasts and cells was studied microfluorometrically and the amount of cellulose deposited per protoplast could be calculated. The amount observed by this method was in good agreement with the amount calculated for tobacco protoplasts by Galbraith (1981) who measured the amount of cellulose by the anthrone method (Viles and Silverman, 1969) and related it to the amount of calcofluor fluorescence.

The appropriate conditions for using the FACS II with the large fragile plant protoplasts were found to be: replacement of the sheath and flow stream fluids with protoplast culture medium; adjustment of the flow rate to 25 % of normal; and adjustment of the electronic size cut-off to allow analysis of protoplasts of the correct size. When the methods for culturing, staining and analyzing the protoplasts of H. muticus, B73, and Se60 on the FACS II were completed, they were each analyzed for survival in culture, DNA and cell wall content for approximately the first week of culture.

Se60 protoplasts had very poor survival in culture, as already stated. It was also shown that both H. muticus and B73 showed death in culture; only 50 % of the B73 protoplasts and 60 % of the Hyoscyamus protoplasts survived beyond the first three days of culture.

All three cultures initially had G1 and G2 populations of protoplasts. This finding agrees with other cell cycle analyses of plant cells: Galbraith and Shields (1982) showed that fixed protoplasts of tobacco, stained with ethidium bromide and analyzed on an Epics V flow cytometer had nuclei in the G1 and in the G2 compartments of the

cell cycle; Galbraith (1983) showed that nuclei isolated from tobacco plants and analyzed on the Epics V were also equally distributed in the G1 and G2; Verma and Lin (1979), using ^3H -labelled thymidine uptake in root cells of Se60, showed that the nuclei were distributed equally between G1 and G2. FACS data showed that protoplast death appeared to occur preferentially among the G2 population.

By day 3 of culture, when the B73 and the Hyoscyamus protoplasts had stabilized, (there was continuous death in the Se60 protoplasts), there was a resurgence of the G2 protoplasts, showing that all three cultures underwent DNA synthesis. The difference was that a DNA peak having less than the G1 DNA content appeared in the Se60 protoplasts, and was presumed to be the result of the degenerating DNA in the senescing and dying population. Harris et al., (1984) have also shown such a low peak of DNA content in senescing leaves of corn, as have Yataganes and Clarkson (1974) in drug-killed mammalian cells..

Both B73 and H. muticus began to resynthesize their walls immediately and showed subpopulations of protoplasts having different rates of cell wall resynthesis. Se60 protoplasts showed almost no cell wall resynthesis. Briefly, there was a small population having increased wall content on day 4 of culture but it was lost by day 6.

From the FACS data, the two most important pieces of information gained are: (1) that there is a small portion of the corn protoplasts that do begin DNA synthesis in culture but (2) they do not regenerate cell walls. It appears that the culture conditions were not sufficient to maintain those protoplasts which were undergoing a round of DNA synthesis and that the whole question of maize protoplast culture

returns to the definition of the medium and the physical conditions of their culture. I think that there are two avenues to be followed to achieve routine protoplast to cell culture techniques in corn. First, most obviously, a new approach is needed to study the cultural requirements of the protoplasts and the second is to study the physiological differences between those plants that are recalcitrant to culture and those that are easily cultured. Corn is a monocotyledon, and has a C4 metabolism. Most plants that have proven to be not easy to culture have been monocots but not all are C4. Those plants that culture easily are dicots and are generally of C3 metabolism.

When protoplasts of any plant are cultured, it is observed that slowly the chloroplasts draw to the centre of the protoplasts and appear to surround the nucleus and eventually they disappear. The chloroplast is the source of the reducing power and nitrogen metabolism enzymes in the cell and is therefore necessary to most cellular metabolism. With the loss of the chloroplasts, it could be speculated that there is also the loss of the differences between C3 and C4 plants and that all protoplasts should be equal and open to manipulation in culture; yet, those dicot protoplasts that divide in culture are later able to show regeneration of the chloroplasts. Monocots, along with loss of their walls, lose their chloroplasts and don't regenerate either.

It is the regeneration of the rigid cell wall that appears to be the prerequisite for protoplast division in culture. Schilde-Rentschler (1977) has shown that tobacco protoplasts, cultured in the presence of cellulase, did not regenerate walls nor go through nuclear division.

Kinnersley et al. (1978) measured the potential changes on the surface of cultured protoplasts. They showed that the dividing tobacco protoplasts changed potential from negative to positive as wall was deposited. Corn and oat protoplasts failed to change their initial negative charge. Meyer et al. (1975) showed that tobacco protoplasts in a sugarless osmoticum could form a discontinuous, non-rigid wall and could undergo two or three rounds of nuclear division. They postulated that the synthesis of wall polysaccharide and its secretion to the outside of the membrane did take place but that the assembly of the polysaccharide did not occur in the salt osmoticum. Herth and Meyer (1978) showed that the tobacco protoplasts divided by budding and cleavage as in yeast in salt osmoticum. Their later work (Meyer and Herth, 1978), substantiated by Galbraith and Shields (1982), showed that nuclear division was not dependent upon cell wall formation. The data presented in this thesis show that there is a fraction of the maize protoplasts which were able to synthesize DNA in the absence of cell wall formation.

The question that remains to be addressed is whether the corn protoplasts fail to manufacture cell wall polysaccharide, fail to secrete it or fail to deposit it on the plasmalemma. The cytoskeletal elements, composed partially of microtubules, influence the rate and direction of deposition of cell wall microfibrils on the plasmalemma (MacLachlan and Febve, 1982) and it is perhaps this area that should be investigated in the non-regenerative cultures of protoplasts.

Lloyd et al. have shown that plant microtubules occur in cortical hoops at right angles to the long axis of the cell. The microtubules are cross-linked to each other and to the membrane. Tubulin has

also been observed in the plant cell membrane and is postulated to facilitate the association between the membrane and the cytoskeleton (Gunning and Hardham, 1982).

Lloyd and Barlow (1982) have shown that the microtubule hoops in cultured Sycamore cells have no skeletal function and do not maintain cell shape in the absence of cell wall. Cellulose microfibrils are synthesized and oriented under the influence of the microtubule hoops though to give the cell its shape. Obviously, this may be true of those protoplasts that do regenerate walls in culture but not of those that do not, as in Z. mays.

There are several questions that could be investigated in non-dividing protoplasts. Is the fact that cell wall is not made in maize protoplast culture a function of microtubule (MT) assembly? MT assembly is dependent upon the condensation of tubulin in the cell and on its polymerization (Gunning and Hardham, 1982). The condensation is concentration dependent and the polymerization is very sensitive to calcium concentration. Calcium induces disassembly of the MT arrays.

The DNA profiles from the FACS II also showed that there was a degeneration of DNA in the corn protoplast population, very similar to that observed microspectrofluorometrically in senescing corn leaves (Harris et al., 1984). The underlying causes of senescence in plants are not known but one of them may be the inability of the plant to utilize nutrients (Thimann, 1980). Molisch (1938) stated that even annual plants are capable of living forever if enough nitrogen were supplied to them. It would appear that the problem of corn culture is still one of nutrition and culture, i.e. provision of the correct

combinations of sugars, salts and vitamins in the correct physical environment, photoperiod, pH, osmotic pressure, or it could be one of inherent differences between monocots and dicots that allow, on the average dicots to be cultured and monocots, not. Descriptions in the literature of early protoplast culture, especially of tobacco and other regenerating systems, say that with the removal of the wall, cell organelles migrate to the centre of the cell and surround the nucleus. This description is very similar to that in tissues that are mounting a wound response to injury. Such wounded cells, usually storage tissue, become dedifferentiated, lose their function and regain their mitotic activity (Barckhausen, 1978). Thus, it would appear that those protoplasts that do divide in culture are mounting a wound response. To extrapolate this idea, wounded plant cells show increased respiration, degradation of carbohydrate to supply energy as ATP in order to produce phenylpropanoids for the production of lignin and suberin, activation and biogenesis of mitochondria, and stimulation of the pentose phosphate pathway for the production of nucleic acids (Uritani and Asahi, 1980). Ethylene is also released from the surface of the wound. Most data on this response is reported for dicots and for storage tissue in particular, but very little information can be found for monocots. Is it possible that the dicots show these responses to being cultured, secrete suberin to stabilize the membrane and monocots do not? These biochemical changes have yet to be compared in the two cultured systems. At this time in protoplast culture research the fundamental questions of metabolic and regulatory events should be addressed in order to understand the

differences among various plants and their protoplasts. I think that the question of protoplast culture, especially the physical parameters, ought to be addressed and new, different and perhaps drastic measures be tried, such as culturing on filters and not in immersion cultures, in different atmospheric conditions, eg. in the presence of ethylene. Perhaps the isolation and culture of protoplasts could be done in the presence of tubulin-stabilizing agents such as glycerol or taxol, a product obtained from the western yew. Taxol stabilizes MT's and lowers the critical concentration for tubulin assembly (Gunning and Hardham, 1982). Culture conditions should also use less calcium.

The FACS II analyses of three populations of protoplasts for DNA and cell wall content during the early days of culture have shown that DNA was synthesized by the protoplasts. Also shown was that those protoplasts that are known to be "responsive" to being cultured could and did make new cell walls. The "unresponsive" plant, Zea mays L., which is known to be difficult to culture, did not show cell wall regeneration as early nor to the same extent as the other cultures. This inability to make wall may be a reason that the protoplasts of the crop plants do not respond to being cultured. The reason for the lack of cell wall regenerating capacity in Z. mays may be inherent or it may be due to incorrect culture conditions.

This thesis demonstrates that the adaptation of histological techniques to the cell sorter, and the adaptation of cell sorter techniques to plant protoplasts can bring sophistication of FACS technology to plant cell physiology. Study of critical physiological functions, such as DNA and cellulose synthesis, in large numbers of

individual cells may lead to a greater understanding of not only the variability of plant cell cultures but also the failure of important crop plants to survive in protoplast culture.

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Appendix I: Definition and testing of culture media.

Introduction

Although there are hundreds of media defined for growing plants hydroponically or in tissue culture, none has been sufficient to induce cereal protoplasts to routinely regenerate walls and to divide in culture. Potrykus' (1977) establishment of the B73 maize cell line from protoplasts is the only example of a primary corn culture and he found the correct conditions at that time in his "CC" medium (Table 1). Since then though, NN67 medium (Nitsch and Nitsch, 1967) plus additional sugars and hormones has been the culture medium for the protoplasts of B73 and the "CC" medium has been the maintenance medium for the cells derived from the protoplasts (Potrykus *et al.*, 1979).

Once the techniques for the routine isolation of large numbers of Se60 protoplasts were determined, it was necessary to define a culture medium to maintain the protoplasts for as long a time as possible. The effects of single additions of macro- and micro-nutrients, of sugars, of nitrogen sources and hormones, as well as the effects of pH and osmolality were studied using the survival of the protoplasts as the measure of effect. The stability of the protoplasts in 9 % mannitol was the standard to which the survival of the protoplasts in the presence of the added element was compared.

Finally, when the best conditions were defined, a new medium was made and the survival of the protoplasts in it was compared to their survival in NN67 as used by Potrykus *et al.*, (1979) and in two other culture media.

TABLE 1

COMPOSITION (mg/l) OF TWO MEDIA USED WITH THE B73 MAIZE CELL LINE.

	CC-MEDIUM	NN67-MEDIUM.
NH ₄ NO ₃	640	
CaCl ₂ ·2H ₂ O	588	
Ca(NO ₃) ₂ ·4H ₂ O		500
KNO ₃	1212	125
MgSO ₄ ·7H ₂ O	247	125
KH ₂ PO ₄	136	125
MnSO ₄ ·4H ₂ O	11.15	25
H ₃ BO ₃	3.1	10
ZnSO ₄ ·4H ₂ O		10
ZnSO ₄ ·7H ₂ O	5.76	
NaMoO ₄ ·2H ₂ O	0.24	0.25
KI	0.83	
CuSO ₄ ·5H ₂ O	0.025	0.025
CoCl ₂ ·6H ₂ O	0.028	0.025
Na ₂ EDTA	37.3	0.032
FeSO ₄ ·7H ₂ O	27.8	0.028
glycine	2	2
myo-inositol	90	100
nicotinic acid	6	5
pyridoxin HCL	1	0.5
thiamine HCl	8.5	0.5
folic acid		0.5
biotin		0.05
cocoanut water	100 ml/l.	
sucrose	20 gm/l.	
mannitol	36.4 gm/l.	
2,4-D	2 mg/l.	

Materials and Methods

Survival of Protoplasts: In order to quantitate and to compare the effects of various additives to the culture medium, survival rates of the protoplasts were determined. Se60 protoplasts were cultured in a salt medium defined by Zapata et al., (1977), plus 9 % mannitol at pH 5.5. Aliquots were taken from the culture; one was counted on a haemocytometer, one was mixed with Evan's Blue at a final concentration of 0.1 % and then counted on a haemocytometer. Those protoplasts excluding the dye were counted as viable. The count obtained immediately after isolation and clean-up was given the value of 100 % survival. (Usually, between 10^5 and 10^6 protoplasts/ml).

pH: The survival of the protoplasts was measured as a function of the pH of the culture medium (Zapata salts plus 9 % mannitol). The pH was adjusted with 0.1 M KOH or with 0.1 M HCl to pH values within the range pH 4.0 to pH 6.0. The protoplasts were incubated in Petri dishes in the dark at 25° C for 16 hours, at which time the survival was determined.

Osmolality: Schenk and Hildebrandt (1969) have stated that protoplast viability is adversely affected by high osmolality of a culture medium. However, the osmotic conditions of the medium must be high enough to prevent protoplast rupture. Equal volume aliquots of Se60 protoplasts, immediately after isolation, were centrifuged and were resuspended in mannitol solutions ranging in osmolality from 50 mOsm to 750 mOsm. as determined by a freezing point depression osmometer. One aliquot of the protoplasts was suspended in dH_2O . After 16 hours, the protoplasts were counted in each sample.

Elemental Analysis: An extract was obtained from 10 to 21-day

kernels. After a 2 minute spin in a Waring blender, this breeze was strained through double layers of cheesecloth to produce the final extract. Stem exudate was obtained by cutting off the top leaves of greenhouse grown, 15 to 21-day old plants and removing the liquid with a pasteur pipette as it formed in drops at the surface of the cut.

Samples of both extract and exudate were analyzed by Barringer Research Ltd., Rexdale, Ontario, for their elemental constituents. The cations were determined by Plasma Arc Emission and the anions by ion chromatography. The elemental analyses are given in Table 2.

Component Testing: Once results were obtained for the mineral constituents of corn, their individual effect on protoplast survival was studied using Costar 24-well plates. The control for each test was 9 % mannitol at pH 6.0. Each well of the multi-well dish contained a final volume of 1 ml. The Costar dish was used so that there were 4 columns and 6 rows. Each of 5 rows was a dilution series for a different component and the 6th row was the control containing mannitol only. All dilutions were 1/10 into 9 % mannitol, pH 6.0 and 550 mOsm. Each well initially had 0.9 ml of the mannitol added except those in the 4th column, where there was 0.8 ml of the mannitol solution added. The component to be tested was kept, as a 10-times concentrated stock solution, of which 100 μ l was added to the well in column 1 and diluted through to column 4. Finally, 100 μ l of the protoplast suspension was added to each well. After 24 hours, the survival of the protoplasts in each well was determined by duplicate haemocytometer counts and graphed as a function of the concentration of the added chemical.

TABLE 2

ELEMENTAL ANALYSIS OF KERNAL EXTRACT AND STEM EXUDATE
OF ZEA mays L. (SENECA 60).

<u>ELEMENT</u>	<u>KERNAL EXTRACT</u>	<u>STEM EXUDATE</u>
CATIONS (ppm)		
Al	1.9	1.9
B	0.86	1.32
Ba	0.08	0.14
Ca	1.41	191.0
Cu	3.01	0.6
Fe	1.94	4.79
K	3750.0	839.0
Mg	303.0	96.7
Mn	1.49	0.99
Na	0.0	0.0
P	714.0	83.0
Pb	0.1	0.38
Si	9.6	38.7
Zn	5.4	1.6
ANIONS (ppm)		
Cl ⁻	700.0	590.0
PO ₄ ³⁻	2519.0	401.0
NO ₃ ⁻	N.D.	N.D.
Br ⁻	707.0	N.D.

N.D. = Not Detectable.

Mineral Elements: The initial concentrations of the components tested were: 0.1 M each of KH_2PO_4 ; MgSO_4 ; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$. The fifth component tested was a serial dilution of the micro-elements of the medium defined by Nitsch and Nitsch (1967) (see Table 1).

Sugars: Five sugars were tested in the multi-well dishes: D-galactose (purified grade, Sigma), sucrose, myo-inositol, D-glucose, and D-sorbitol (all Sigma grade). The sugars (0.55 M) were placed in the wells of the first column and were then diluted serially by tenths into 9 % mannitol, all at pH 6.0.

Nitrogen source: Various sources of nitrogen were tested in the multiwell dishes. KNO_3 , glutamine, urea, $(\text{NH}_4)_2\text{SO}_4$ and NH_4NO_3 were tested for their effect on the protoplast survival. Each had an initial concentration of 0.1 M in 9 % mannitol and each was diluted by tenths. The control was 9 % mannitol. The optimal concentration within the range tested of each nitrogen source was used to study the effect of two nitrogen supplies together on the survival of the protoplasts.

Other Additions: The following were also tested for their effect on protoplast viability; each was diluted serially by tenths into 9 % mannitol, pH 6.0, from the given initial concentrations:

Lipids in the form of corn oil (Mazola) were added at an initial concentration of 1 % (v/v).

Tween 80 (Sigma) had an initial concentration of 0.1 % (v/v).

Corn Syrup (Beehive) - 1 % (v/v).

Malate (Sigma) - 0.01 % (w/v).

Corn Starch (Maizena) - 0.1 % (w/v).

Hormones: Qualitative determination of the value of added hormones was made. The protoplasts were incubated for 5 days in the presence of hormones at concentrations of 20, 2, 0.2, 0.02 mg/l. Each day, the cultures were examined microscopically and a qualitative judgement was made as to the viability and health of the protoplasts. 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), para-chlorophenoxyacetic acid (pCPA), kinetin, zeatin, 6-benzyl-aminopurine (6BAP) and N-6- δ -2-isopentenyl adenine (2iP) were the hormones studied.

Other: The composition (Table 3) of commercial mineral water (CMW) appears to contain most of the ions found in the stem exudate of the corn plant. The degassed mineral water was used as the mineral basis of two test culture media.

Final Culture Media Tested: Based on the observations from the survival studies of Se60 protoplasts in the presence of various salts, sugars, and hormones, three culture media were constructed in which protoplasts were cultured for eight days. NN67 medium, as used by Potrykus *et al.*, (1979) (Table 1) was the control for these experiments. The compositions of the three test media are given in Table 4.

RESULTS

Protoplast Survival: The survival rate data obtained by haemocytometer counts with and without Evan's Blue dye are shown in Figure 1. The total number of protoplasts present was a good indication of the number of viable protoplasts as shown by the exclusion of the Evan's Blue dye. Haemocytometer counts alone were used as the index of protoplast survival in culture as it was more convenient than staining before counting.

TABLE 3

MINERAL COMPOSITION (mg/kg) OF A COMMERCIAL MINERAL WATER.

Ammonium	0.01	Lithium	0.7
Sodium	5.2	Potassium	1.8
Magnesium	44.1	Calcium	348.8
Strontium	2.0	Manganese	0.2
Copper	2.0	Zinc	0.054
Iron	0.037	Aluminum	0.009
Fluorine	0.55	Chlorine	5.1
Bromine	0.0006	Iodine	0.001
Sulfate	864.0	Nitrate	2.2
Carbonate	88.4	Silicate	6.0
Molybdenum	0.003	Boric Acid	0.91
Carbonic Acid	179.8	Phosphoric Acid	0.5
Arsenic	0.057	Lead	0.015

Total = 1462 mg/kg

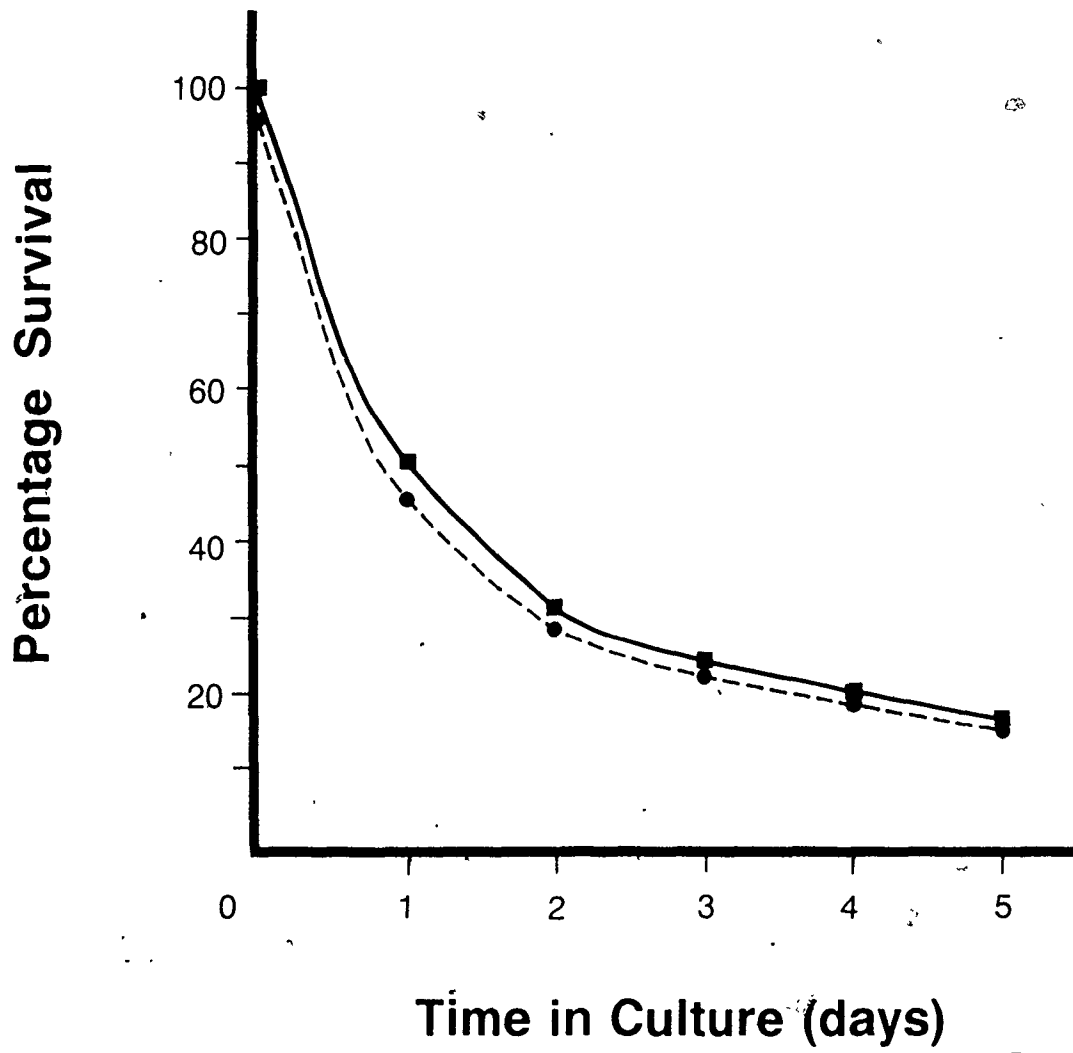
TABLE 4

COMPOSITION OF FOUR TEST MEDIA.

Medium 1:	Commercial mineral water plus NN67 vitamins 1.5 % sucrose 2.5 % mannitol 0.01 M Malate 0.001 M Urea 0.02 M Glutamine 1 mg/l GA ₃ 2 mg/l 2,4-D 1 mg/l hydroxyproline 1 mg/l adenine
Medium 2:	Medium 1 plus 0.1 % (v/v) corn oil 0.001 % (v/v) Tween 80.
Medium 3:	10 ⁻³ M KH ₂ PO ₄ 10 ⁻⁴ M MgSO ₄ 10 ⁻³ CaCl ₂ ·2H ₂ O 10 ⁻³ Na ₂ SiO ₃ ·9H ₂ O 10 ⁻³ dilution NN67 micro-elements NN67 vitamins 1/2 dilution "CC" Iron 10 ⁻⁴ M Malate 0.001 % (v/v) Tween 80 0.01 % (v/v) corn oil 0.001 % (w/v) corn starch 2.0 % sucrose 3.0 % mannitol 2 mg/l 2,4-D.
Medium 4:	NN67 plus 0.17 M mannitol 2 % sucrose 2 mg/l 2,4-D 10 % coconut water.

Figure 1: Comparison of the measurements of protoplast survival rates.

Se60 protoplasts were counted on a haemocytometer without the addition of Evan's Blue dye (■) and counts were compared to those obtained after Evan's Blue treatment (●). Each curve is the average of two experiments.



pH: The survival of the protoplasts after 16 hours as a function of pH is shown in Figure 2. There were two peaks of increased survival, one at pH 4.0 and another at pH 6.0.

Osmolality: The survival of the Se60 protoplasts as a result of osmolality is shown in Figure 3. The lowest osmolality at which maximum survival occurred was 500 mOsmolal. Osmolalities above 700 resulted in protoplast loss.

Elemental Analysis: The elemental compositions of liquid endosperm and stem exudate of Se60 as determined by Plasma Arc Emission and ion chromatography are shown in Table 2. The concentrations of potassium, magnesium, phosphorus and phosphates were high in both tissues. The stem exudate had a high concentration of silicate and calcium.

Mineral Elements: The survival of the Se60 protoplasts in the presence of decreasing concentrations of the various macro- and micro-nutrients is shown in Figure 4. KHPO_4 (Fig 4a) at any concentration tested failed to improve the survival above that in mannitol. MgSO_4 (Fig 4b) at all concentrations tested, improved the survival of the protoplasts above control. The maximum survival occurred at 10^{-5} M. CaCl_2 (Fig 4c) improved the survival of the protoplasts above background at one concentration only, 10^{-4} M. Both silicate (Fig 4d) and the NN67 micro-elements (Fig 4e) improved protoplast survival above that in mannitol at all concentrations.

SUGARS: The effect of 0.55 M concentrations of five sugars and the dilution series of each on the survival of the protoplasts is shown in Figure 5. None of the sugars at 0.55 M improved the survival

Figure 2: Survival of Se60 protoplasts as a function of pH.

The protoplast survival was measured as a function of pH after 16 hours incubation at 25^o C. The plots are an average of two experiments.

SURVIVAL OF PROTOPLASTS VS. pH

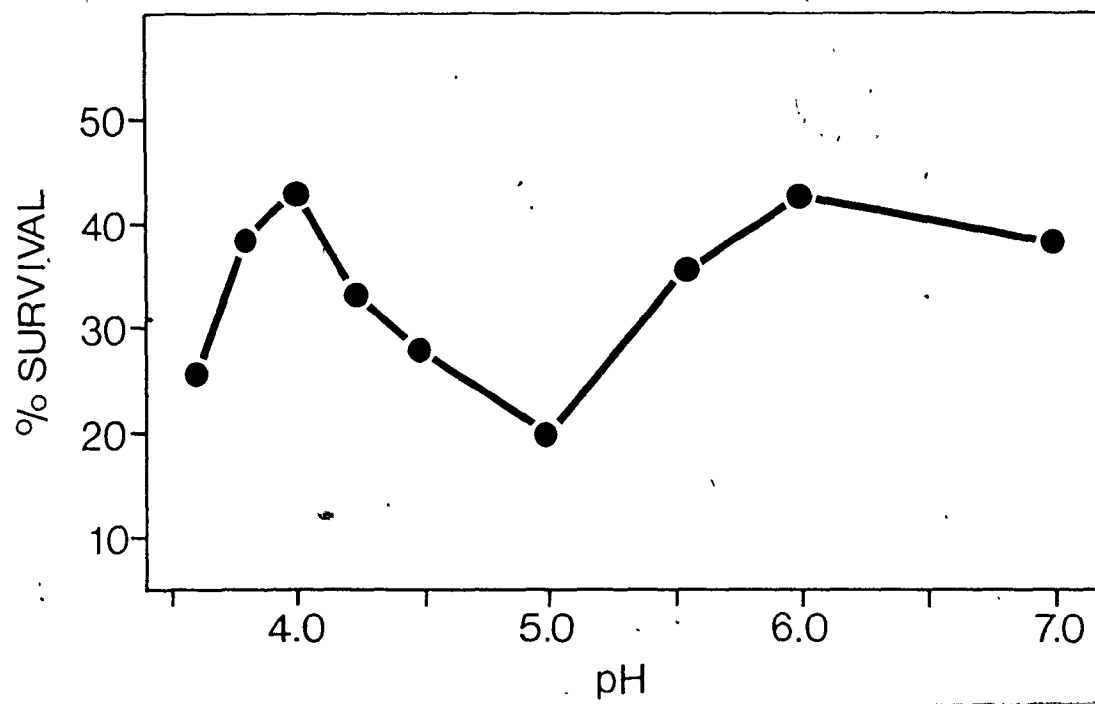


Figure 3: Survival of Se60 protoplasts as a function of osmolality. Measurements of media osmolality were made on a freezing point osmometer. All experimental counts were done in duplicate and each experiment was repeated 3 times. Bar = S.E.M.

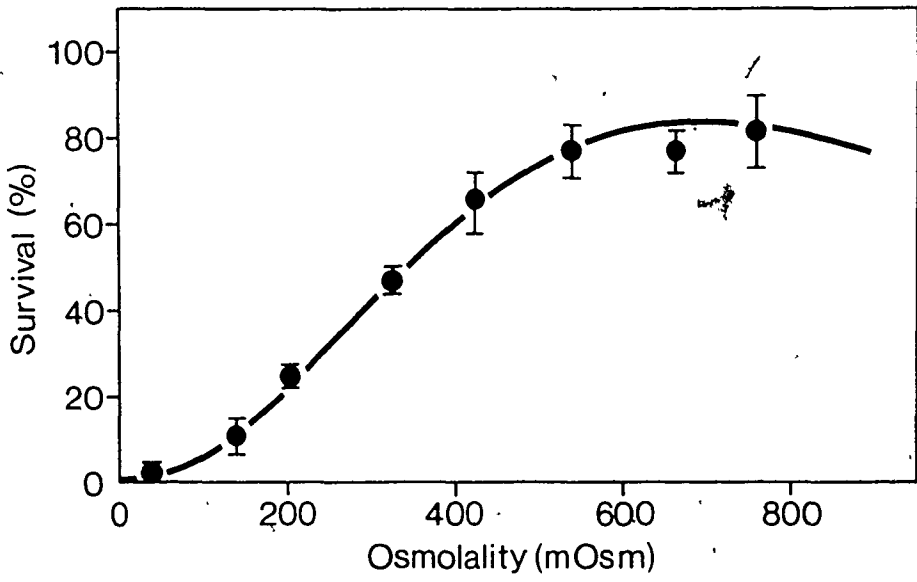
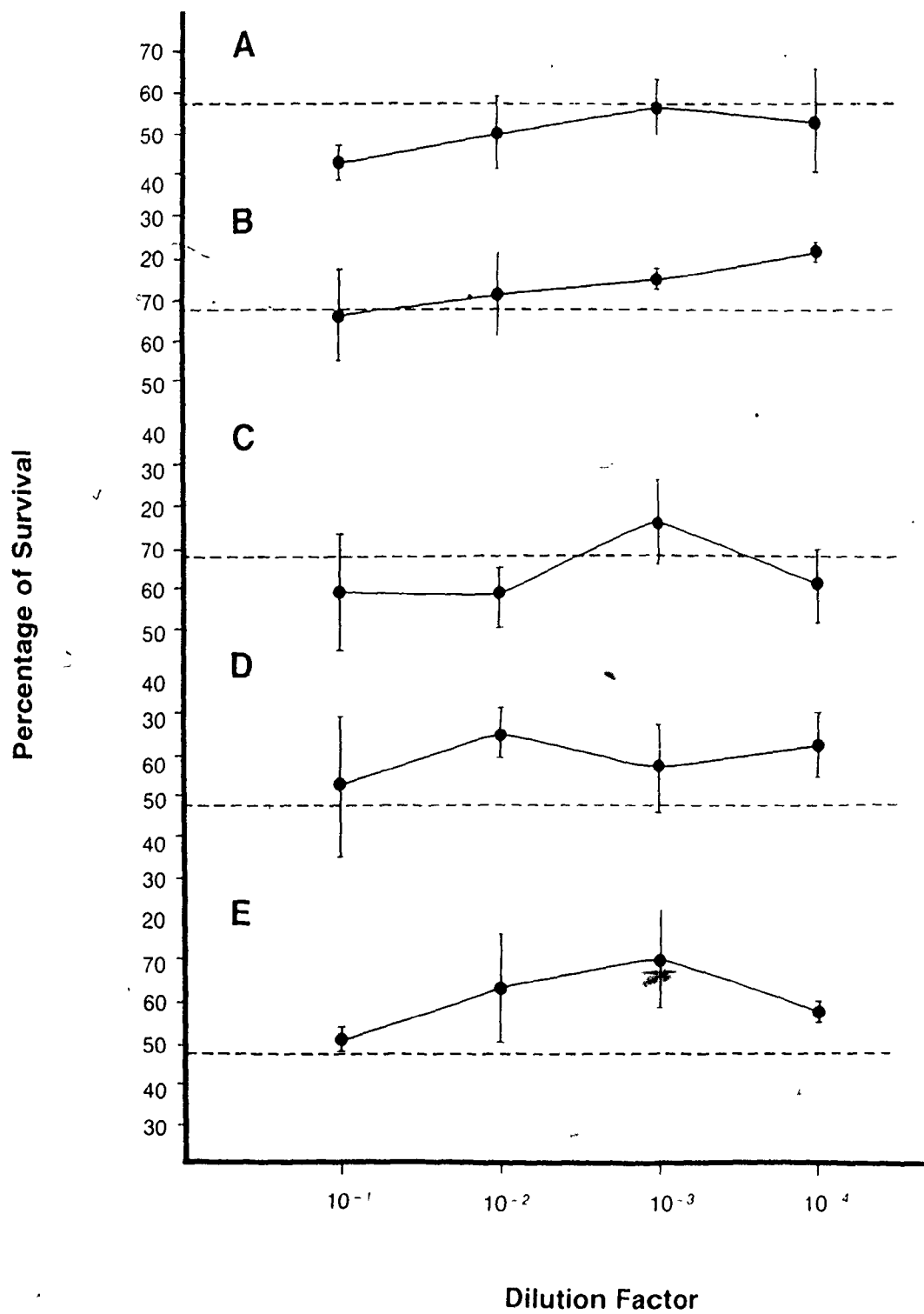


Figure 4: Effect of individual elements on the survival of Se60 protoplasts.

Macro- and micro- elements were diluted into 9 % mannitol and their effects at decreasing concentrations on the survival of the protoplasts were studied. The dashed line represents protoplast survival in 9 % mannitol.

Bar = S.E.M. Stock solutions were 0.1 M each.

- A. KH_2PO_4
- B. MgSO_4
- C. $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
- D. $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$
- E. NN67 micro-nutrients.



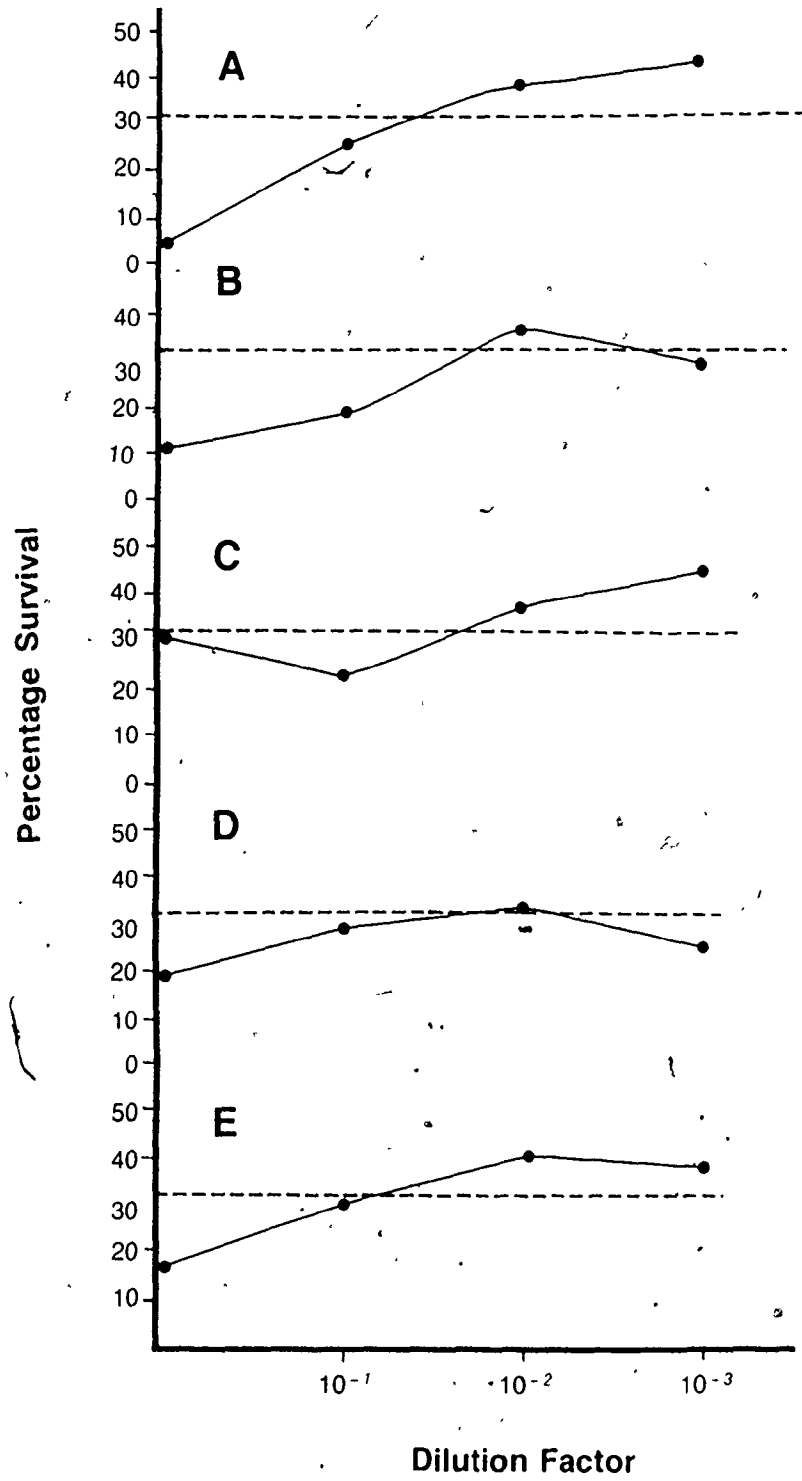
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Figure 5 : Effect of added sugars on the survival of protoplasts in culture.

Various sugars, alone (0.55 M) and diluted into mannitol were studied for their effect on protoplast survival.

Each sample was taken in duplicate and each experiment was repeated. The dashed line represents protoplast survival in 9 % mannitol.

- A. D-galactose.
- B. Sucrose.
- C. Myo-inositol.
- D. D-glucose.
- E. D-sorbitol.



above that in mannitol. Sucrose (Fig 5B) and glucose (Fig 5D) did not increase protoplast viability at any concentration tested. The other three sugars, galactose (Fig 5A), inositol (Fig 5C), and sorbitol (Fig 5E) allowed slightly increased survival at lower concentrations.

Nitrogen Source: The effects of the various nitrogen salts on protoplast survival are shown in Figure 6. In all cases protoplast survival was improved slightly above their survival in 9 % mannitol, only in the lowest concentrations of the nitrogen sources tested. However, when the lowest concentration of the nitrogen sources were tested in combination with each other, most combinations improved the viability of the protoplasts above control. The results are presented in Table 5. The presence of 10^{-6} M each of urea and glutamine had the greatest effect in increasing the survival of the protoplasts. Only urea and $(\text{NH}_4)_2\text{SO}_4$ in combination gave survival rates lower than control.

Other Additions: Several compounds were tested for their effect on the survival of the Se60 protoplasts. The results are shown in Figure 7. Corn syrup (Fig 7A) at a concentration of 0.01 % (v/v) showed increased survival above control, as did 10^{-5} M malate (Fig 7B). Increased viability of the protoplasts was also seen with corn oil at a concentration of 0.1 % (v/v) and with Tween 80, at 10^{-3} and 10^{-4} dilutions. Addition of corn starch at concentrations of 0.1, 0.01, and 0.001 % increased the survival of the protoplasts.

Hormones: Qualitative estimations of the effect of added hormones on protoplast survival over a week in culture determined that only

Figure 6: Effect of nitrogen source on the survival of the protoplasts in culture.

Various sources of nitrogen (0.1 M each) were serially diluted in mannitol and tested for their effect on the survival of the protoplasts. Each sample was counted twice and each experiment was repeated three times.

The dashed line represents protoplast survival in

9 % mannitol. Bar = S.E.M.

A. KNO_3

B. Glutamine

C. Urea

D. $(NH_4)_2SO_4$

E. NH_4NO_3

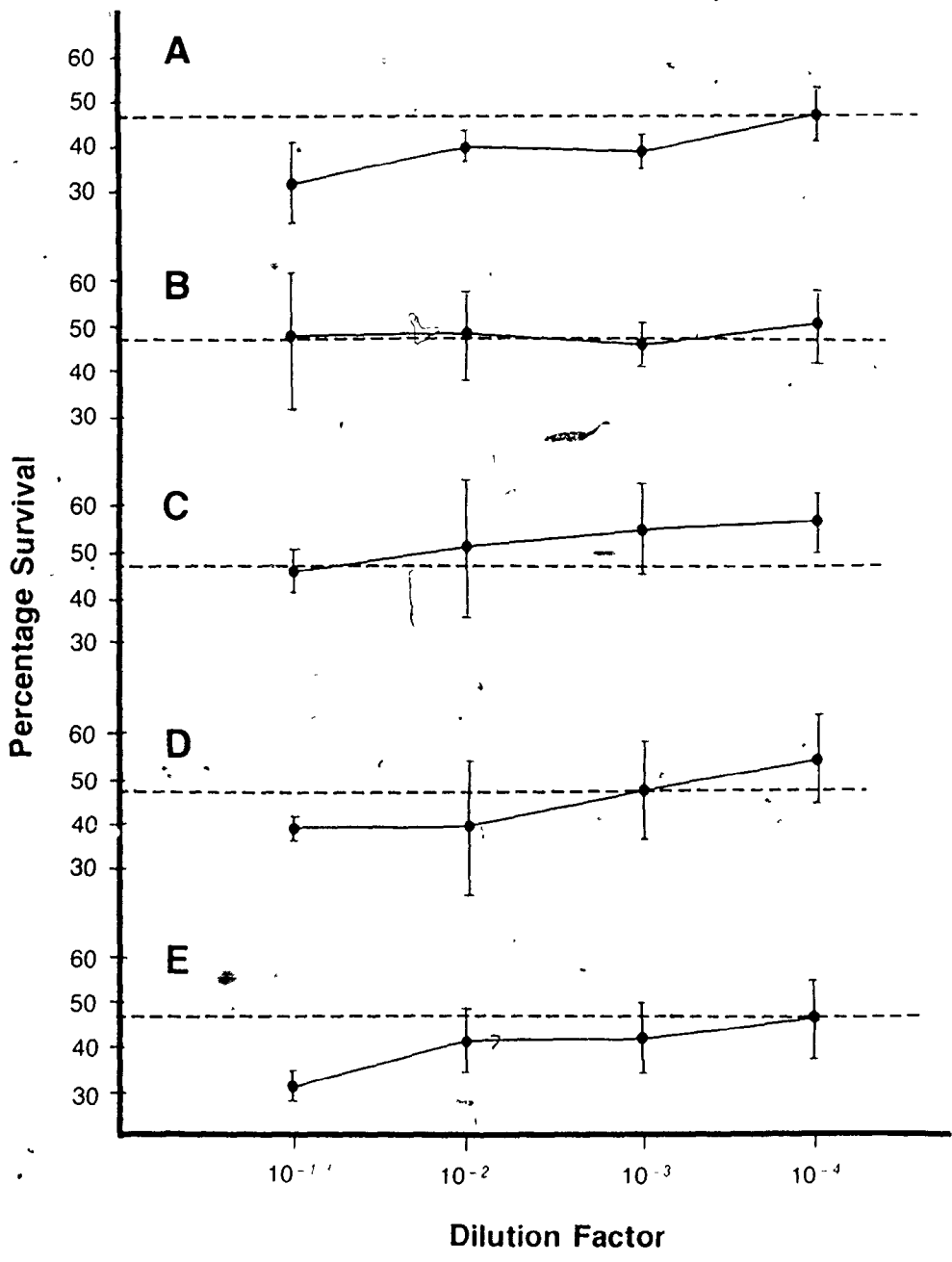


TABLE 5
 COMPARISON OF THE EFFECT OF NITROGEN COMBINATION ON THE
 SURVIVAL OF SE60 PROTOPLASTS AFTER 1 DAY.

Nitrogen Source Combination	% Survival
urea + glutamine	75.2
glutamine + $(\text{NH}_4)_2\text{SO}_4$	73.6
NH_4NO_3 + KNO_3	70.5
NH_4NO_3 + $(\text{NH}_4)_2\text{SO}_4$	68.2
glutamine + KNO_3	67.4
urea + KNO_3	67.4
KNO_3 + $(\text{NH}_4)_2\text{SO}_4$	61.3
glutamine + NH_4NO_3	59.6
urea + NH_4NO_3	58.2
mannitol 9% (control)	57.5
urea + $(\text{NH}_4)_2\text{SO}_4$	51.5

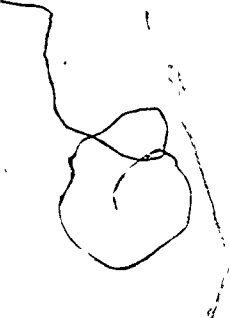
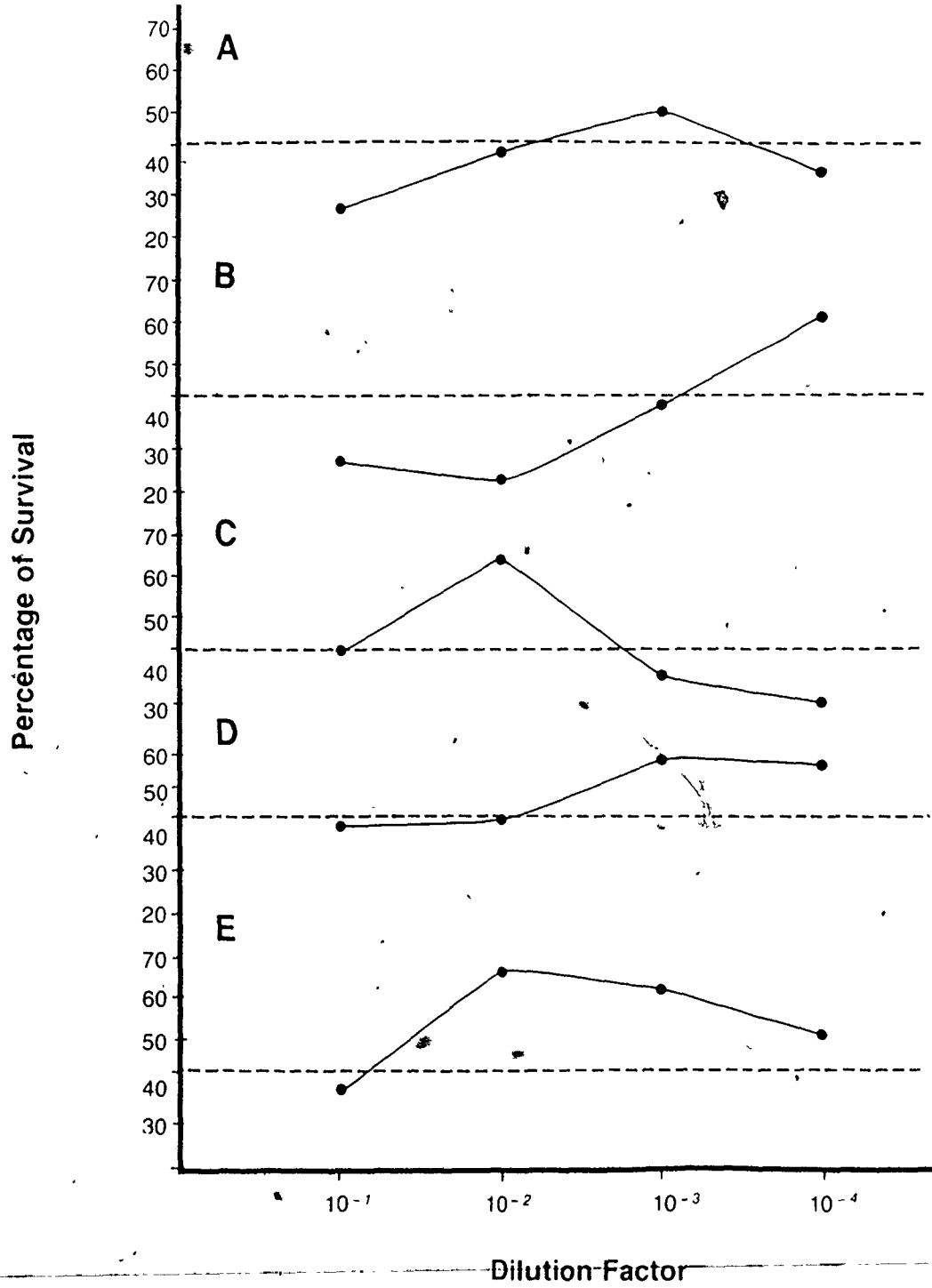


Figure 7: Effect of other additives on the survival of protoplasts in culture.

A variety of compounds were diluted in 9 % mannitol and tested for their effects on protoplast survival. All samples were counted twice and the experiments were repeated. The dashed line represents protoplast survival in 9 % mannitol. Stock solution concentrations are given.

- A. Corn Syrup - 10 % (v/v)
- B. Malate - 0.1 % (w/v)
- C. Corn Oil - 10 % (v/v)
- D. Tween 80 - 1 % (v/v).
- E. Corn Starch - 1 % (w/v).



2,4-D at 20 mg/l and 2 mg/l had any effect on protoplast survival. All other hormones at all concentrations tested did not improve the survival nor the quality of the protoplasts as compared to 9 % mannitol.

Culture Media Testing: As the commercial mineral water (CMW) contained the necessary elements for a tissue culture medium, it formed the basis for two test media whose recipes are given in Table 4. The third medium to be tested was composed by using the results obtained from the testing of the individual compounds. It was called medium 3 and its recipe is given in Table 4. Finally, NN67 as used by Potrykus et al., (1979) for the B73 protoplasts was used as the control medium. Its composition is presented in Table 1.

Figure 8 shows the survival of the protoplasts in the three media described in Table 4 and in NN67 during 8 days of culture. The protoplasts did not survive well in any of the media. Although the survival rates were similar in all of the media, it was slightly better in NN67, despite all of the testing of individual components.

Conclusions

Numerous chemical and physical elements were tested for their ability to improve the survival of Se60 protoplasts in culture. Those that did increase the viability of the protoplasts to above that in 9 % mannitol, pH 6.0, were used in combination to define a new medium. In addition, a commercial mineral water, because it contained many of the same ions found in corn stem exudate, as determined by elemental analysis, was used as a base for two other culture media. The media were adjusted to pH 6.0 and 550 mOsm, the

Figure 8: Survival of Se60 protoplasts in four different media.

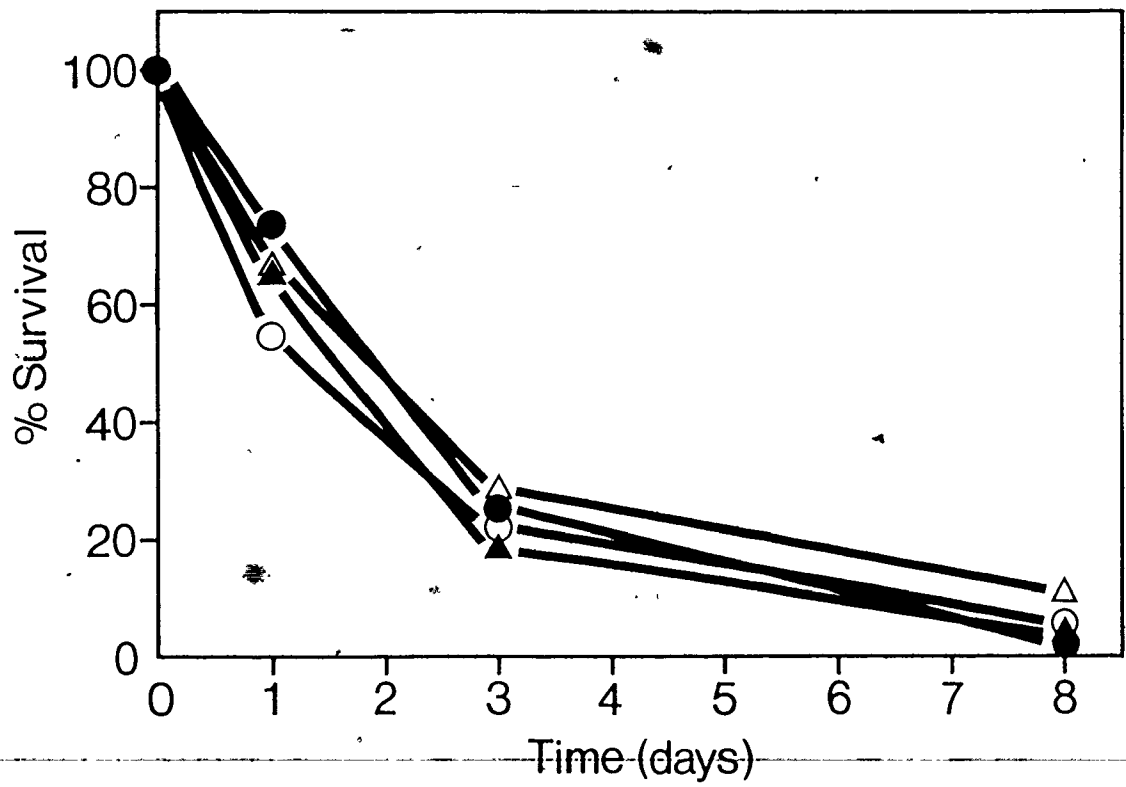
The protoplasts were cultured in three experimental and in NN67 media. All samples were taken in duplicate and the experiments were replicated.

NN67 △

Medium 1 ▲

Medium 2 ●

Medium 3 ○



conditions determined to be most suitable for survival. The only hormone found to be effective at enhancing protoplast survival was 2,4-D at a concentration of 2mg/l and this was added to the culture media. The positive effect of 2,4-D on corn protoplast survival has been observed by other workers (King et al., 1977; Green et al., 1974). Finally, the Se60 protoplasts were cultured in the NN67-based medium (Table 1) that has proven successful for the culture of the B73 protoplasts (Potrykus et al., 1979). This medium proved to support protoplast survival slightly better than any of the many other conditions tested. There was no prolonged survival of the protoplasts in any of the four media. From this information, the NN67 medium plus 2 % sucrose, 0.17 M mannitol, 2 mg/l 2,4-D and 10 % coconut milk, at pH 5.8, was elected as the medium for further Se60 protoplast culture experiments.

Appendix 2: Composition of UWO soil mixes used in the growth of corn.

MATERIAL	APPROX. VOLUME
Before 1978:	
sand	3 cu. ft.
muck	3 cu. ft.
loam	12 cu. ft.
peatmoss	4.5 cu. ft.
perlite	1.35 cu. ft.
fertilizer (5-20-20)	1500 c.c.
Fritted Trace Elements	50 c.c.
Iron 20 %	
zinc 8 %	
manganese 8 %	
copper 4 %	
boron 1.6 %	
molybdenum 0.05 %	
After 1978:	
loam	0.5 cu. m.
sand	0.17 cu. m.
peatmoss	0.13 cu. m.
Osmocote (14-14-14)	1500 c.c.
Fritted Trace Elements	50 c.c.
Muriate of Potash (0-0-62)	200 c.c.
Magnesium sulphate (Mg 10%)	220 c.c.
Superphosphate (0-20-0)	1500 c.c.

END

1 3 1 1 8 5

FIN