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SOME FACTORS AFFECTING THE ISOLATION, VIABILITY, CULTURE AND PROPERTIES OF TOBACCO (*NICOTIANA TABACUM VAR XANTHI N.C.*) MESOPHYLL PROTOPLASTS

CLIVE RAYMOND MOULDING

THESIS SUBMITTED FOR THE DEGREE OF MASTER OF PHILOSOPHY

SEPTEMBER 1989

UNIVERSITY OF SOUTHAMPTON

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ABSTRACT

FACULTY OF SCIENCE

BIOLOGY

Master of Philosophy

SOME FACTORS AFFECTING THE ISOLATION, VIABILITY, CULTURE AND PROPERTIES OF TOBACCO (NICOTIANA TABACUM VAR XANTHI N.C.) MESOPHYLL PROTOPLASTS

by Clive Raymond Moulding

Tobacco mesophyll protoplasts were isolated using commercial and purified enzymes at various times during the year. It was found that the time of year, rather than enzyme purity, affected protoplast viability. Attempts were made to correlate this effect with daylength, change in daylength and sunshine hours.

Populations of protoplasts were separated on continuous and discontinuous Percoll gradients, the latter succeeded in separating a population of protoplasts more likely to divide.

Media, and their components, used in culture of protoplasts are affected by events which occur during autoclaving, which alter their composition and render defined media, undefined. Thiamine hydrochloride is one such component affected. The type of agar used to solidify media for protoplast culture also affected the ability of protoplasts to regenerate cell walls and divide.

During the early stages of culture, a suitable osmoticum is used to maintain protoplast stability and prevent bursting. The strength of this osmoticum can be reduced immediately on isolation and still further in a short period of time, with protoplasts retaining the ability to regenerate cell walls and divide with a relatively high plating efficiency approaching 60%.

ACKNOWLEDGEMENTS

I express my appreciation and gratitude to my supervisor Dr. Peter Evans for his encouragement, helpful advice, valuable guidance, suggestions and constructive criticism throughout my period of study as a part-time research student. Above all I thank him for his never failing good humour.

On technical matters, I thank Mrs R. Bell and Mrs. V. Wilson for their help and advice and in particular their assistance during those times when I could not be present.

Part of my research period was made possible by financial assistance from Hampshire County Council and by some remission from teaching duties granted by my principal Dr. John Potter and my head of department Dr. Gareth Rees, to whom I am especially grateful for giving me the occasional gentle reminder.

ABBREVIATIONS

NAA.	α-Naphthalene acetic acid
IAA.	Indole-3-acetic acid
BAP.	6-Benzylaminopurine
CPW.	Cereal Protoplast Washing Salts (Evans, Keates and Cocking, 1972)
CPW. 11M	Cereal protoplast washing salts to which has been added 11% (w/v) mannitol
CPW. 20S	Cereal protoplast washing salts to which has been added 20% (w/v) sucrose
LSI.	Leaf shape index
MSP.	Murashige and Skoog Protoplast culture medium (Murashige and Skoog, 1962)
MSP. 9M	Murashige and Skoog medium to which has been added 9% (w/v) mannitol
x g.	Gravity

Chapter 1

INTRODUCTION

The isolation, culture and manipulation of plant protoplasts has considerable potential as a technique for studying the biology of plant cells and as sources of material for hybridisation and genome manipulation.

The term "protoplast" has been defined as "that part of the plant cell which lies within the cell wall and can be plasmolysed, and which can be isolated by removing the cell wall by mechanical or enzymatic procedures. The protoplast is, therefore, only a naked cell - bounded by the plasma membrane - which is potentially capable of cell wall regeneration, growth and division". (Vasil and Vasil, 1980).

The absence of the cell wall makes the protoplast suitable for a variety of experimental manipulations that are not possible with intact cells. (Galun, 1981). However, one basic prerequisite for the use of protoplasts in such studies is the ability to isolate them readily in large numbers and with a high viability and to be able to culture them *in vitro* to form cell colonies and whole plants.

All of the early attempts to isolate plant protoplasts relied entirely on mechanical methods and was limited to plant tissues containing large vacuolated cells. (Whatley, 1956). Cells in such tissues were induced to plasmolyse by immersion in a hypertonic solution, causing retraction of the plasma membrane away from the cell wall. Subsequent sectioning of plasmolysed tissues into thin strips released protoplasts when sections were placed in gradually decreasing osmotic potentials. This was caused by swelling and extrusion of the protoplast from the confines of the cut cell wall. (Klercker, 1892). Such methods of protoplast isolation are only suitable for a limited variety of higher plant tissues, such as storage tissues. Another serious limitation of this technique is the very small number of intact, undamaged protoplasts obtained. Nonetheless, many of the pioneering plant protoplast studies were carried out on such mechanically isolated protoplasts. (Chambers and Hofler, 1931; Tornava, 1939; Whatley, 1956.)

The disadvantages inherent in mechanical methods were largely overcome by the development of an enzymatic procedure that used a crude cellulase fraction from the fungus Myrothecium verrucaria to isolate protoplasts from tomato roots, (Cocking, 1960).

Similar enzyme preparations were later used to isolate protoplasts from a variety of tissues and species. (Eriksson, 1985). With this development it became possible to obtain comparatively large populations of active and viable protoplasts. Additionally, it was possible to avoid the deleterious effects of excessive plasmolysis and to obtain protoplasts from relatively non-vacuolated meristematic cells, which do not plasmolyse easily. (Gamborg et al, 1975).

Protoplast preparations, obtained by mechanical or by enzymatic means, almost always contain considerable amounts of cellular debris in the form of cuticle, vascular elements, cell walls, tissue fragments, membranes, nuclei, plastids and other cell organelles, all of which must be removed rapidly and adequately. The simplest method of removal involves filtration through various filters followed by washing or by centrifugation at low speeds. Two-phase density systems have proven useful in some cases for purification, (Kanai and Edwards, 1973; Larkin, 1976). The cleanest preparations, particularly from cell cultures, are obtained by floating the protoplasts on sucrose solutions, (Evans et al, 1972; Vasil and Vasil, 1980). A discontinuous density gradient system for separation of protoplasts based on their buoyant densities has also been described, (Harms and Potrykus, 1978; Hughes et al, 1978; Fitzsimons and Weyers, 1983).

During the 1970's a number of potent, but crude enzyme preparations became available commercially, and with a fair degree of quality control. These include cellulase from Trichoderma viride, " Driselase" from a basidiomycete rich in cellulase and a pectinase "Macerase" from Rhizopus and various other pectinases and hemicellulases. Many toxic substances and impurities were present in these preparations, including nucleases, lipases, peroxidases, proteolytic enzymes and some phenolics. Partial purification through Sephadex G-25 or Biogel P6 is sometimes recommended, (Schenk and Hildebrandt, 1969).

However, most workers routinely use commercial enzyme preparations without any purification to obtain high yields of viable protoplasts that are capable of normal growth and development. Indeed, very highly purified and crystalline enzymes are not only prohibitively expensive, but are also relatively useless for protoplast isolation. Such enzymes as these are unable to breakdown the chemically and structurally complex plant cell wall, which contains hemicellulose, pectin, and protein in addition to cellulose.

Complex enzyme mixtures, cleaned of impurities and toxic components are, therefore, most efficient for the complete breakdown of cell walls and release of protoplasts.

Two principal methods have been used for the enzymatic isolation of protoplasts. In the sequential method, plasmolysed material is cut into small pieces and incubated in "Macerozyme", a pectinase, (Takebe et al,
1968). This causes maceration of the tissues and release of whole cells. The enzyme mixture and debris is removed and cells are then suspended in a cellulase solution to digest the cell walls and release protoplasts.

The second, and more commonly used method, involves a mixture of pectinase and cellulase which macerates the plant tissue by attacking the middle lamella, but also releases protoplasts by digesting cell walls, (Power and Cocking, 1969). During and after liberation, the protoplast must be protected against osmotic swelling and bursting. This is easily accomplished by the inclusion of osmotic stabilisers such as mannitol, sorbitol, glucose or sucrose, in the enzyme mixture, or with a combination of ionic and non-ionic osmotica. Reducing the osmotic potential to as small a value as possible appears to aid the subsequent culture of the protoplasts, (Vasil and Vasil, 1980).

The determination of the viability of protoplasts is an important aspect of protoplast culture. The best test for viability will always be regeneration of the cell wall and induction of cell divisions. Alternatives include examination for cytoplasmic streaming, vital staining with Evans blue or fluorescein diacetate, (Widholm, 1972; Evans and Cocking, 1975; Larkin, 1976).

The yield, as well as the viability of protoplasts is closely related to the conditions under which the source material has been grown and maintained. Plants grown under field conditions or in the greenhouse provide a most inconsistent source material. Light intensity, day/night cycle and inorganic nutrition appear to be the most critical factors affecting yield and viability. In only a few instances have the various parameters controlling yield been studied methodically, (Watts et al, 1974; Shepard and Totten, 1975 and 1977; Cassells and Cocker, 1982).

The techniques for the culture of protoplasts are adapted, with only minor modifications, from well established procedures developed for the culture of intact single cells or cell suspensions. In suspension and drop cultures, protoplasts are suspended in a liquid medium at a density of 5×10^4 per ml. and cultured either in drops on the base or lid of a Petri dish, or in medical flats. The technique for drop culture developed by Kao et al (1971) has also been used successfully and extensively. Protoplast suspensions are placed in 50 μl drops in plastic Petri dishes, sealed with parafilm and incubated. Regular monitoring is possible with an inverted microscope.

In plating procedures, protoplasts are suspended in liquid media and mixed gently, but rapidly with an equal volume of media prepared in agar (1.6%) at 45°C. Small volumes are used to give a thin poured layer containing a final plating density of 2.5×10^4 per ml.. The Petri dishes are sealed with parafilm, (Nagata and Takebe, 1971). Examination of such cultures is not as easy as drop cultures, but has the advantage that protoplasts remain in a fixed position, and the development of individual protoplasts can be followed.

Freshly isolated protoplasts cultured in suitable nutrient media rapidly regenerate cell walls. The newly synthesised wall can be observed at the light microscope level by Calcafluor staining, (Nagata and Takebe, 1970). Cell wall regeneration precedes cell division in cultured protoplasts and is considered to be a prerequisite for cytokinesis, (Vasil, 1976).

Mitosis and cytokinesis are normal and occur after 2-7 days in culture. Protoplasts isolated from differientiated cells, such as mesophyll cells of leaves, which do not divide in nature, take longer

to undergo the first division than those isolated from cells dividing rapidly in culture, (Vasil and Vasil, 1974). Multicellular clumps or colonies are formed within 1-3 weeks and these can be either further subcultured as callus tissues or used to obtain suspension cultures.

Although single, isolated protoplasts can be cultured in complex nutrient media under certain conditions, maintenance of a minimum cell density is generally necessary for inducing cell wall regeneration and cell division in most cases, (Kao and Michayluk, 1975). Experience has shown that a population density of 10^3 per ml. to 10^5 per ml. gives the best results. Exogenously supplied plant growth substances do not appear to directly influence cell wall formation but are required for cell division, (Vasil and Vasil, 1980; Muller et al, 1983).

Plant regeneration from protoplasts is a prerequisite for the utilisation of protoplast technology in somatic hybridisation and genetic manipulation. In order that this technology may be used to improve crops, produce new hybrids with increased vigour or modification of existing species, especially legumes and cereals, it will be necessary to ensure that plant regeneration from protoplasts becomes a routine, easily achievable process with a high degree of reproducibility.

Many of the published procedures involve lengthy and numerous manipulations in the production of a cleaned protoplast preparation, followed by further manipulations during the culture period in order to produce successful plant regeneration. Many of the procedures used, are used simply because they are known to work, although they may involve complex manipulations and require numerous transfers of protoplasts from isolation enzyme mixtures, through cleaning operations, possible fractionating procedures and finally into culture media, where still further operations may take place. Any methodology which reduces the complexity and number of these operations is to be encouraged.

To this end, this study investigated the possibilities of achieving these aims by devising new techniques, or modification and improvement of existing techniques.

Chapter 2

MATERIALS AND METHODS

CHEMICALS AND GLASSWARE

Analar grade chemicals and water were used where possible with the exception of mannitol,which was reagent grade, (BDH Chemicals Ltd., England). All solutions were autoclaved at 121°C for 15 minutes. The enzyme mixture was filter sterilised (see later). To ensure chemical cleanliness, all culture vessel glassware was recoated with a silica mono-layer between each use. This was achieved by placing the glassware in a solution of 0.8% (w/v) sodium metasilicate, 0.08% Calgon, (Albright & Wilson Ltd., England) in tap water and bringing it to boiling point. The glassware then remained in the solution whilst it cooled. This was then removed and the glassware steeped overnight or longer in tap water, followed by an overnight bath in 0.1M hydrochloric acid. The glassware was then rinsed in tap water, double rinsed in distilled water and dried in a hot air oven. The dried glassware was then placed in autoclavable nylon bags, (Portex, Hythe, Kent) and autoclaved and again air dried in the bags after removal from the autoclave.

Volumetric glassware was washed using Teepol detergent, followed by two rinses in tap water and two rinses in distilled water and air dried in a hot air oven. Petri dishes used in some experiments were supplied by Falcon Plastics, (California, USA) via a UK supplier, (Becton Dickinson, Wembley). Screw cap 15 ml. culture tubes were supplied by Corning Glass, (New York) via a UK supplier, (Jennings and Co., Nottingham).

Aseptic techniques were employed throughout the isolation and subsequent procedures where necessary in a "Laminar Flow Clean Air Cabinet", (Bassaire Ltd., Southampton).

PLANT GROWTH CONDITIONS

Seeds of Nicotiana tabacum var xanthii NC were planted in Levington Universal Compost, (Fisons Ltd., UK) in 5" plastic pots. The seeds were lightly scattered over the surface of the compost, well watered and germinated at 24°C.

Approximately two weeks after sowing, the seedlings were pricked out into 3 " pots again containing Levington Universal Compost. Three seedlings were transferred to each pot. The seedlings were maintained at 24°C for a further two weeks. After this, individual plants were transferred to 3" pots containing John Innes No 2 Compost. A certain amount of selection took place at this stage so that only healthy and relatively uniform plants were potted on. The root ball of plants was not stripped of the Levington compost when potting on to avoid shock which might have arisen from a compost change. The newly potted plants were maintained at an average temperature of 24°C.

After a further two weeks growth, plants were potted on into 4" pots containing John Innes No 2 compost, to which had been added hoof and horn fertiliser. Plants were then maintained within a temperature range of 20-30°C with a 16 hour day length by warm white fluorescent tubes. Lighting was adjusted to take account of changes in day length. Plants were used as sources of leaf material about 7-8 weeks after sowing. At certain times of year, this occurred a little later as plants were less actively growing. In general, plants were used one week after final potting.

LEAF PEELING

Protoplasts were isolated from greenhouse grown plants using the following procedure, adapted from Power et al., (1976). Young, but fully expanded leaves were cut from the plant and surface sterilised for 30 minutes in 7.5% Domestos solution, (Lever Bros., London) contained in a sterile casserole dish. The sterilised leaves were then washed four times in approximately 500 ml. of sterile distilled water. Continuing to work aseptically, the lower epidermis of the leaves was removed by peeling. With practice, this was readily achieved by using a pair of fine pointed forceps and the midrib or areas containing large veins as starting points for peeling. The peeled areas were cut from the leaf and transferred, peeled surface downwards, onto the surface of 30 ml. of plasmolysing solution, either CPW 11M or CPW 13M contained in a sterile 14 cm. glass petri dish (Table 2.1). Peeling and cutting continued until the whole surface of the solution was covered. After at least 60 minutes in contact with the plasmolysing solution, the CPW was removed using a sterile 60 ml. Brunswick syringe and replaced with 30

TABLE 2.1

COMPOSITION OF X10 STRENGTH CPW SALTS

Constituent	Quantity (mg l^{-1})
KH_2PO_4	272.0
KNO_3	1010.0
$CaCl_2.2H_2O$	14800.0
$MgSO_4.7H_2O$	2460.0
KI	1.6
$CuSO_4.5H_2O$	0.25
Analar water	to volume

PREPARATION OF WORKING STRENGTH CPW SOLUTIONS

(1 litre)

x10 strength CPW salts 100ml

		Mannitol
	CPW 5M	50g
	CPW 7M	70g
	CPW 9M	90g
	CPW 11M	110g
	CPW 13M	130g
Sucrose	CPW 20S	200g

Analar water to volume

pH adjusted to 5.8 using 0.1M HCl or 0.1M KOH

Autoclave and store in dark

TABLE 2.2

COMPOSITION OF PROTOPLAST ISOLATING ENZYME SOLUTION

prepared as 100 ml lots

Component	Quantity(mg l^{-1})
" Meicelase" [a](cellulase)	50,000.0
" Macerozyme"[b](pectinase)	5,000.0
Mannitol	90,000.0*
Tetracycline[c]	10.0
Ampicillin[d]	400.0
Gentamycin[e]	10.0

Made up to volume with CPW salts solution.
pH adjusted to 5.8, filter sterilised and stored at $-4^{o}C$.
* Mannitol content may be varied to give desired isolation level

a Meiji Seika Kaisha Ltd., Japan
b Kinki Yakalt Manufacturing Co. Ltd., Japan
c Sigma Chemical Co. Ltd., England
d "Penbritin" Boots Pure Drug Co., England
e Flow Laboratories Ltd., Scotland

ml. of isolation enzyme solution, (Table 2.2). The dish was then placed in the dark and incubated at 27°C overnight.

FILTER STERILISATION OF ISOLATION ENZYME MIXTURE

The isolation enzyme mixture was prepared using the components contained in Table 2.2. The enzymes were dissolved in CPW working strength salts solution and antibiotics and the appropriate mannitol content added to the mixture. The pH of the solution was adjusted to 5.8 using 0.1M HCl or 0.1M KOH.

Filter sterilisation was achieved for small volumes using a Swinnex filter system, (Millepore, UK) or Sartorius filter system, (Sartorius, GMBH, W.Germany), for larger volumes. Filtration using a 50 mm, 0.2μm pore size and acid purified Keiselguhr as pre-filter was achieved in about 30 minutes using compressed air from a cylinder to provide a small positive pressure. The sterile enzyme mixture was transferred to sterile medical flats in 30 ml. aliquots for storage at -4°C.

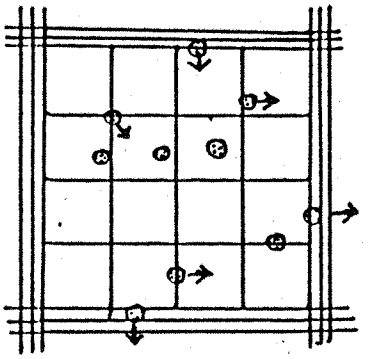
A degree of quality control was exercised at this stage by placing a small quantity of "sterile" enzyme mixture in nutrient broth and incubating at 37°C for four hours. If the incubated broth remained clear, the enzyme mixture was placed in storage, if it did not, the mixture was subjected to a further filter sterilisation before being placed in storage. Each 30 ml. of enzyme was only subjected to one freeze-thaw cycle before use.

PROTOPLAST ISOLATION

After incubation in enzyme solution, the protoplasts were obtained in the following way. Usually, 16 hours was sufficient to ensure that protoplasts would be readily liberated, however, the yield could be increased by gently agitating the dish and teasing with sterile forceps. This helped release protoplasts not liberated by enzyme action alone. Larger undigested pieces of leaf were removed and the protoplast suspension transferred to sterile 15 ml. screw top tubes using a sterile 60 ml. plastic syringe. It was important to carry out this stage gently, to ensure that shearing forces were not exerted on the protoplasts causing them to burst.

FIGURE 2.1

Counting convention used in the determination of protoplast number



Area within triple rulings = 1.0 mm^2
Depth = 0.2 mm
Volume = 0.2 mm^3

The tubes were transferred to a bench centrifuge and spun at 100 x g, for 15 minutes. The supernatant was discarded. The pellet, containing protoplasts, cells, starch grains and debris was resuspended in CPW 20S and centrifuged at 100 x g for 10 - 15 minutes.

Protoplasts were recovered from the surface of the supernatant with a sterile Pasteur pipette and resuspended in CPW 9M to remove the sucrose. A further centrifugation at 100 x g for 10 minutes yielded a protoplast pellet. The supernatant was discarded and replaced with a known volume of culture medium.

Protoplast number was obtained using a haemocytometer with modified Fuchs and Rosenthal rulings, (Weber and Sons, England). The suspension was then adjusted to a final culture density of 5 x 10^4 per ml. by dilution.

Protoplast viability was determined by the fluorescein diacetate method, (Evans and Cocking, 1975), using a Vickers microscope with a 50w HB50/W.3 mercury vapour lamp, excitation filter BG 12, and suppression filter K510.

COUNTING OF PROTOPLASTS

Using a sterile Pasteur pipette a small sample was removed and placed on a clean haemocytometer slide. The sample was then counted using x100 magnification. A total of 80 squares was counted for each sample, (5 x 16). The convention shown in Fig. 2.1 was adopted for those protoplasts which fell on the rulings.

The mean number of protoplasts was obtained from the counts and, knowing the volume, the mean number of protoplasts per ml. calculated. Since this number of protoplasts was contained in a known volume,the factor by which this must be diluted to obtain the final culture density could be determined.

PLATING EFFICIENCY

Plating efficiency was calculated by counting protoplasts at various periods of time following plating into liquid or agar culture media. Plating efficiencies were always calculated on counts of at

least 500 protoplasts and using the formula below:-

$$\text{Plating Efficiency} = \frac{\text{Total no. of protoplasts dividing}}{\text{(No. dividing)+(No. not dividing)}} \times 100\%$$

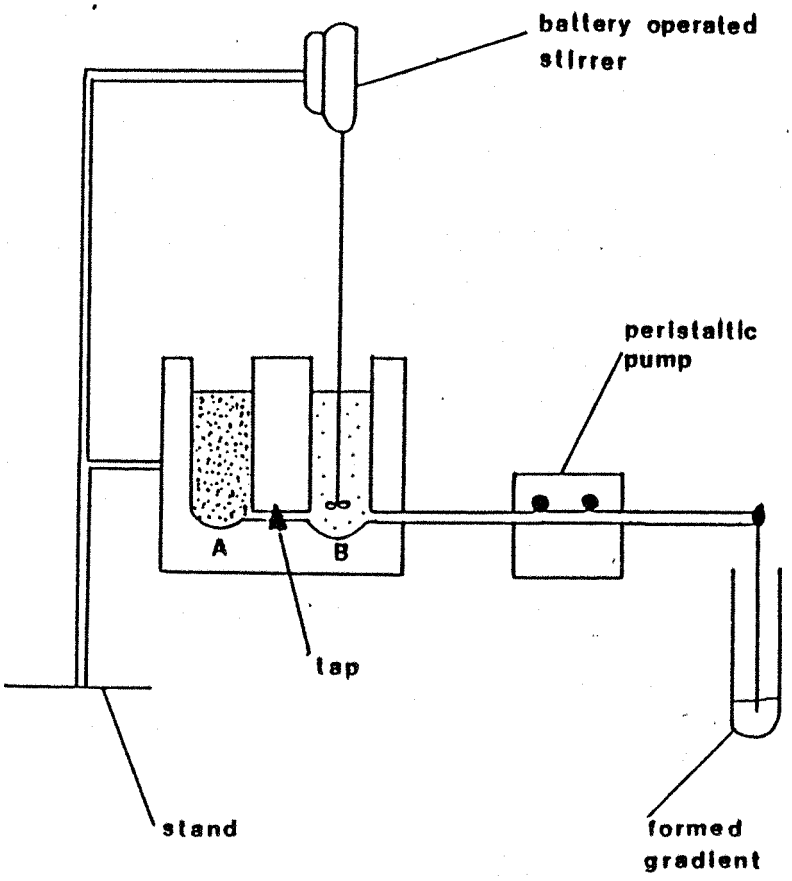
PURIFICATION OF "MEICELASE"

The purification of "Meicelase" was carried out on a Bio-Gel P6 column. The dry gel was added gradually to water in a beaker. The amount of gel required to pack a column of known volume may be estimated by using the "packed bed volume". The gel hydrated at 8 ml/g with a water regain factor of 3.7. It was advisable to use twice as much water as was the expected packed bed volume.

The gel was allowed to hydrate at room temperature for four hours. After hydration was complete, half the supernatant was decanted off and de-aeration achieved by aspirating at reduced pressure. A funnel was fixed to the top of the column and water poured in to nearly fill the column. The stirred gel slurry was then carefully poured into the column. When 2-5 cm of the column bed had formed, the exit from the column was opened and a gentle flow of water allowed to flow from it. A further quantity of stirred gel slurry was poured into the column, allowed to settle, and water again removed from the base of the column. This process was repeated until the column was packed.

The void volume of the column was determined using Dextran Blue. Samples were loaded onto the column after the water level had been drained down to the upper level of the gel bed. The sample was then carefully drained into the gel column. This was followed with an additional water wash to ensure all the sample went into the column. The drained water volume was replaced with an equal volume of fresh water and the column run.

The effluent from the column was monitored using a Uvicord type 4701A set at 280 nm to identify the protein containing fractions from an initial loading of 100 ml containing 10g of "Meicelase". Fractions were collected in 10 ml aliquots and those containing the majority of protein bulked together. Collection continued until a volume equal to the void volume had been collected. Using the figures of Patnaik et al (1981) working strength enzyme mixture was then prepared. This required the addition of the following materials:-



A - dense component

B - light component

FIGURE 2.2

Apparatus used to produce continuous gradient

0.5g "Macerozyme", 13.0g mannitol (for a 13M isolation level) or 11.0g mannitol (for an 11M isolation level), and 10 ml of x 10 strength CPW salts. To this mixture was added 1.0 mg Tetracycline, 40.0 mg Ampicillin, 1.0 mg Gentamycin and the whole mixture adjusted to a pH of 5.8 using 1M NaOH and diluted to 100ml with analar distilled water. This gave a final solution containing 5% "Meicelase" and 0.5% "Macerozyme" mixture in CPW 13M or CPW 11M. The mixture was then filter sterilised and stored at -4°C.

PREPARATION OF CONTINUOUS GRADIENT

The continuous gradient was formed using a gradient forming machine as shown in Fig. 2.2. Before use, the apparatus was run using distilled water and then the tube to the pump was clamped. Approximately 2 ml of low density component (8% mannitol) was placed in chamber B of the apparatus, with the tap open. The mannitol flowed through the interconnecting channel into chamber A. The tap was then closed. The chamber A was then emptied using a Pasteur pipette and rinsed with distilled water. The mannitol lost from chamber B was replaced and the chamber almost filled with additional mannitol. Chamber A was filled to a similar level with the heavy component Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) plus 8% mannitol.

The stirrer was started by making a connection to the battery and the tap between the chambers opened. The clamp on the tube to the peristaltic pump (LKB Bromma 12000, Varioperspex) was removed and the pump set to operate at 5 ml per minute. As the component in chamber B became depleted, the heavy component from chamber A flowed into chamber B and the two components mixed by stirring. The mixture was delivered to the bottom of a screw capped tube via a small catheter. By this means, an increasingly dense mixture was introduced into the tube. The gradient so formed was continuous with the lightest mixture at the top of the tube and the heaviest at the bottom. The apparatus was throughly rinsed in distilled water after use.

Protoplasts for fractionation were loaded on the gradient in 8% mannitol and spun at 100 x g for half an hour. Following centrifugation samples were removed from the gradient using a calibrated 1 ml Pasteur pipette.

PREPARATION OF DISCONTINUOUS GRADIENT

Two stock solutions were prepared, the first was an 8% (w/v) mannitol solution in analar distilled water. Although it would have been desirable to use CPW salts as a stabilising agent for the protoplasts this was not possible, as they caused the Percoll to precipitate on autoclaving. The second solution was Percoll to which was added 8%(w/v) mannitol. The gradient consisted of eight discontinuous bands, with the heaviest band containing the greatest proportion of Percoll at the bottom of the tube. The various mixes were prepared in 10 ml. volumes and placed in screw top 1 ounce universals and autoclaved. (See Table 2.3).

Following the autoclaving and cooling, the gradient was prepared in the following way. Using aseptic technique, and starting with the heaviest band, 1 ml. volumes were placed in 15 ml. screw top tubes using sterile Pasteur pipettes.

The first band was easily achieved. For subsequent lighter bands it was necessary to ensure that they were placed on the preceeding band with the minimum of mixing. This was easily achieved with practice and the use of a fine drawn out Pasteur pipette.This enabled the next band to be placed directly on top of the preceeding band by allowing it to run on to the top surface of the lower band under the slightest of pressure exerted by the rubber teat on the pipette. If desired, it was possible to indicate the interface of the bands by a mark placed on the outside of the tube.

After addition of the final lightest band the protoplasts for fractionating were placed on the gradient. These were also in 8% mannitol. The gradient was then placed in a centrifuge and spun at 100 x g for half an hour.

Following centrifugation the bands of protoplasts were clearly visible and could be recovered as separate sub-populations using sterile Pasteur pipettes.

Each sub-population was washed twice in CPW salts to remove the Percoll and then resuspended in MSP solution of appropriate mannitol content. By counting protoplast numbers before and after it was found

TABLE 2.3

PREPARATION OF DISCONTINUOUS GRADIENT

Band No	Volume of Mannitol(ml)	Volume of Mannitol/Percoll(ml)
1	10.0	0
2	9.5	0.5
3	9.0	1.0
4	8.5	1.5
5	8.0	2.0
6	7.5	2.5
7	7.0	3.0
8	6.5	3.5

that 85-90% efficiency of recovery could be achieved for a 1 ml loading containing 10^5 to 10^6 protoplasts.

DENSITY MEASUREMENTS

An organic solvent density column was devised to determine the density of each successive 1 ml volume from the continuous gradient and each of the bands in the discontinuous gradient. Four different mixtures of kerosene, (Esso Blue - density 0.777 g ml^{-1}) and carbon tetrachloride (density 1.595 g ml^{-1}), were layered into a 500 ml measuring cylinder.

The following mixtures of kerosene:carbon tetrachloride were used, 75:25 (lightest), 70:30, 65:35, 60:40 (heaviest), in 100 ml volumes. The lightest mixture was added first using a filter funnel and a long glass tube sufficient to reach to the bottom of the cylinder. This mixture was followed by the mixtures of increasing density, each introduced in turn at the bottom of the cylinder. When the column was complete the tube was removed with as little disturbance as possible. The column was allowed to stand for 24 hours so that a linear gradient would form by diffusion between layers. The gradient remained usable for up to 14 days if left undisturbed in a fume cupboard.

COLUMN CALIBRATION

The column was calibrated using stock standard density solutions of sucrose in distilled water. Using a fine drawn out Pasteur pipette, two or three drops of the standard sucrose solution were dropped onto the surface of the gradient from about 1 cm above it. The drops sunk and eventually came to rest in their density equilibrium phase. This position was recorded by using the graduations on the side of the cylinder. This procedure was repeated for the remaining sucrose standards and the results used to plot a graph of graduation marks against density. The calibration curve could then be used to measure the density of the various fractions from the different gradients.

MEDIA PREPARATION

Gibberellins Stock solutions of gibberellic acid, GA_3 were prepared at 10mg in a 100 ml of distilled water. The solution was transferred to a medical flat and stored in a refrigerator. As GA_3 is heat labile it was filter sterilised and added to the medium after autoclaving.

Auxins The auxins were;

1-naphthalene acetic acid, NAA
2,4-dichlorophenoxy acetic acid, 2,4-D
3-indole butyric acid, IBA
3-indole acetic acid, IAA (Sigma Chemical Co. Ltd.).

Solutions of the above compounds were made by dissolving 20mg in 1ml of ethanol in a small beaker.The solution was taken up by a Pasteur pipette and transferred to 75ml of distilled water in a 100ml volumetric flask. The beaker was rinsed out with a further 1ml of ethanol into the volumetric flask and the solution then made up to 100ml. NAA and 2,4D were stored at 4°C for two weeks, IAA and IBA for one week only.

Cytokinins The cytokinins used were;

6 Benzylaminopurine, 6BAP
Kinetin, N -(γ -isopentenyl) adenine, 2 iP
Zeatin

Solutions of the above compounds were made by weighing out the required amount of compound to the nearest mg, and putting into a dry 100 ml volumetric flask. To this were added 2ml of 0.1M HCl to dissolve the cytokinin, which was assisted by a brief period of steaming. The solution was then made up to 100 ml with distilled water and transferred to a medical flat and stored in a fridge. As cytokinins are relatively stable in solution the stock solution was kept for one month.

MS medium. MS medium was supplied as dry packs, (Flow Laboratories, Irvine Scotland). (Table 2.4). An appropriate sized flask for the dry pack being used was three-quarters filled with distilled water and swirled. Using a dry filter funnel the powder from the pack was added to the flask whilst the water was still swirling.

TABLE 2.4

COMPOSITION OF PROTOPLAST CULTURE MEDIUM
(Murashige & Skoog,1962)

Component	Quantity(mgl^{-1})
$CaCl_2.2H_2O$	440.000
KNO_3	1900.000
$MgSO_4.7H_2O$	370.000
KH_2PO_4	170.000
$MnSO_4.4H_2O$	22.300
H_3BO_3	6.200
$ZnSO_4.7H_2O$	8.600
$NaMoO_4.2H_2O$	0.250
KI	0.830
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
$FeNa_2EDTA$	36.700
NH_4NO_3	1650.000
i-inositol	100.000
nicotinic acid	0.500
thiamine HCl	0.100
glycine	2.000
pyridoxine HCl	0.500

These materials were provided as one litre dry packs purchased from Flow Laboratories, Irvine Scotland.
The packs contained no sucrose and no hormones.

A small amount of water was used to remove the last traces of powder from the pack. The appropriate amount of sucrose was weighed out and also added to the flask using a dry funnel. The flask was shaken until all powder and sucrose had dissolved. Using a pipette the required amounts of growth regulators were added to the flask from the stock solutions. Any other supplements required were added at this stage. (Table 2.5).

The mixture was then made up to volume and after shaking well dispensed to a large beaker and the pH adjusted to 5.8 using a pH meter and 1M HCl or 1M NaOH. The culture medium was then dispensed into appropriate volumes for autoclaving.

If agar medium was required, sufficient agar powder was weighed to produce a double strength solution. The agar powder was added to swirling liquid and this was continued to give good dispersal of the agar powder. The flask was capped with foil and placed in a steamer and heated until the agar dissolved. The mixture was then either autoclaved and dispensed into appropriate sterile disposable containers in a laminar flow cabinet, or dispensed to non-sterile autoclavable containers and then autoclaved.

CULTURE OF PROTOPLASTS

Following isolation, protoplasts were fragile due mainly to the delicate nature of the plasma membrane. It was therefore necessary to take care to avoid rupturing the membrane when transferring protoplasts from vessel to vessel especially if Pasteur pipettes were used which had a tendency to produce shearing forces if filled or emptied too quickly.

For liquid culture, small volumes of "concentrated" protoplasts were transferred to culture media to give the desired culture density, (5×10^4 per ml.), either in screw capped tubes, or medical flats. As protoplasts readily sink in culture media, only small volumes of culture media were used. In the screw capped tubes about 3 -4 ml. appeared to be the ideal volume to ensure adequate aeration. In the medical flats the culture volume was increased to about 10 - 12 ml. with the bottle stored horizontally in the culture room. Both methods of culture afforded easy access to remove samples for examination on cavity slides using a photomicroscope.

TABLE 2.5

PREPARATION OF MURASHIGE AND SKOOG MEDIUM

To prepare working strength MS medium the following materials were added in addition to one litre dry pack.

Component	Quantity(mg l^{-1})	
sucrose	30,000.0	
NAA	2.0	
6-BAP	0.5	
mannitol	30,000.0	MSP 3M
	50,000.0	MSP 5M
	70,000.0	MSP 7M
	90,000.0	MSP 9M
	110,000.0	MSP 11M
	0.0	MSP 0M

Analar water to one litre volume adjusted to pH 5.8 and autoclaved.

Protoplasts were also cultured in agar. For this method, sterile medium containing agar at twice the final concentration required was held in a molten state at about 50°C. Protoplasts in liquid culture medium were also obtained at twice the final plating density required, 2.5×10^5 per ml. Using sterile pipettes 2 ml. of protoplasts in liquid medium were transferred to sterile plastic Petri dishes, to this was added and equal volume of molten agar. The dishes were rotated to thoroughly mix the two and then left to set for thirty minutes. The Petri dishes were cultured in an inverted position in a larger petri dish containing a sterile filter paper moistened with sterile water. Protoplasts could be observed under the microscope through the Petri dish, without having to open the dishes for sampling, which reduced the risk of contamination.

CULTURE CONDITIONS

Protoplasts were usually cultured at 24 - 25°C in approximately 40% relative humidity and in darkness. This regime was changed for some experiments and some protoplasts were given a period of culture in light with an illumination of $4 - 6 \times 10^2$ lux provided by daylight fluorescent tubes. The cultures were not agitated for either set of culture conditions and were only disturbed when removing samples for examination or for the addition or removal of culture medium. Aeration was achieved by surface diffusion alone.

STATISTICAL ANALYSES

Results from some experiments were subjected to statistical analysis. These tests have been explained at the appropriate points in the text, and include analysis of variance, Duncan range test, one sample X^2 test, regression analysis and the Durbin-Watson test for auto-correlation.

Chapter 3

ISOLATION AND CULTURE OF MESOPHYLL PROTOPLASTS AND THE USE OF PURIFIED ENZYMES

3.1 Introduction

Patnaik et al, (1981) suggested that plating efficiency of leaf mesophyll protoplasts of Petunia parodii in microdrop cultures could be increased significantly by using purified enzymes for protoplast isolation and that division of single protoplasts in such microdrops was only possible when purified enzymes were used.

Whilst this may be true, factors other than enzyme purification also affect the viability of protoplasts. These include, for mesophyll protoplasts, seasonal factors, plant age, culture media, (Frearson et al, 1973), and even light conditions under which the protoplasts are cultured. However, this is by no means the whole story.

Increased plating efficiency is particularly important when attempting to culture single heterokaryons, (fusion products from a somatic hybridisation), since the chance of survival and division is increased when the two parental protoplasts have a high plating efficiency under the same cultural conditions. A period of pre-culture for 1-3 days is carried out, sometimes in a special nutritionally enriched medium, but the use of purified enzyme eliminates the need for this period of pre-culture.

In addition, Patnaik et al examined the effects on plating efficiency of type of medium, specifically MSP 9M, and KM 8P, (Kao and Michayluk, 1975). For suspension cultures, the increased plating efficiency of purified enzyme as compared to commercial enzyme preparations was some 5-6 times more on MSP 9M and 3 times more on KM 8P. For microdrop cultures, prepared using commercial preparations, no division occurred at all, irrespective of medium, but a plating efficiency of 25% was obtained when purified enzyme was used. Other reports had shown the benefits of purification of cell wall degrading enzymes in increasing stability of protoplasts from callus cultures, (Schenk and Hildebrandt, 1969), to improve the viability of protoplasts isolated from carrot suspension cultures, (Slabos et al,

1980) and to elicit division in leaf protoplasts of Medicago sativa L., (Santos et al, 1980).

The following series of experiments were designed to ascertain whether the purification of enzymes used to isolate tobacco mesophyll protoplasts improved subsequent plating efficiency in liquid MSP 9M liquid culture. As increased plating efficiency is particularly important when attempting to culture single heterokaryons, culture in agar rather than liquid offers particular advantages, as it allows the progress of individual heterokaryons to be followed. The effects on plating efficiency of various types of agar was also investigated. All protoplast culture depends on the ability of a protoplast to regenerate a cell wall and undergo cytokinesis (Vasil, 1976). The development of a procedure which could identify those protoplasts most likely to undergo this process would improve the likelihood of achieving a culture with a high plating efficiency. The use of a discontinuous gradient to identify and separate possible sub-populations within a given population of protoplasts was also examined.

Comparison of plating efficiences of isolated protoplasts prepared using purified and commercial enzyme preparations and cultured on various agars following discontinuous gradient treatment.

3.2 Variations in materials and methods

Protoplasts were obtained from Nicotiana tabacum plants in the usual way, except that one batch was isolated using a commercial preparation and one batch was isolated using purified enzyme obtained by the method described in the materials and methods section. The donor material was obtained from greenhouse plants and divided randomly between the two isolation dishes.

Protoplasts were cleaned and counted in the usual way and adjusted to give a final density of 5×10^4 per ml. Viability was determined using fluorescein diacetate. Both preparations were subjected to treatment on a discontinuous gradient prepared as described in materials and methods. Following centrifugation, the separated protoplasts were carefully removed and washed in MSP 9M and pelleted by gentle centrifugation. Counts were made and suitable

dilutions made so that all bands from both preparations contained 5 x 10^4 per ml.

Plating media had been previously prepared. This was MSP 9M containing 1.6% agar of various brands, Sigma type IV, (Sigma Poole UK), Bacteriological and Oxoid No 3,(Oxoid UK) and New Zealand,(Davis Gelatin Division UK). Several replicates of each band, from both preparations and for each agar type were prepared and incubated in the light and examined at weekly intervals for 5 weeks.

Non-gradient treated protoplasts from the same isolation were placed in culture to act as controls. A second set of controls, containing gradient and non-gradient treated protoplasts were set up in liquid MSP 9M culture medium.

3.3 Results and Discussion

Fig. 3.1 and 3.2 show the appearance of protoplasts after isolation and cleaning using the methods described in chapter 2 (Materials and Methods). The protoplasts shown in Fig. 3.1 were isolated using commercial enzyme preparations, whilst those in 3.2 were isolated using enzymes which had been purified. The protoplasts from this particular isolation were characterised by a wider range of sizes than those obtained from the isolation with commercial enzyme and a noticeable absence of chloroplasts within the protoplasts.

When placed in liquid MSP 9M culture both sets of protoplasts regenerated a cell wall and the first division occurred after four days in culture.(Fig. 3.3) After two weeks in culture, colonies of callus cells had formed in both cultures.(Fig. 3.4) At the end of three weeks in culture large colonies of cells had been produced and were of a sufficient size to allow them to be removed from the liquid culture vessels for subsequent procedures if so desired.(Fig. 3.5) The extent of protoplast division and colony formation was assessed in experiments by measuring plating efficiency. Fig. 3.6 shows low, medium and high plating efficiencies.

The results from this series of experiments (Table 3.1) showed that there was nothing to be gained by purification of the enzymes used to isolate protoplasts with respect to subsequent plating efficiency. Indeed, after a somewhat lengthy and tedious procedure

FIGURE 3.1

(a) Uncleaned protoplast preparation. Preparation obtained from enzymatic isolation stage of protocol showing whole protoplasts surrounded by collapsed, dead protoplasts and cellular debris. (x100).

(b) Collapsed, dead protoplast. Collapsed, dead protoplasts removed by centrifugation in 20% sucrose together with cellular debris.(x100).

(c-d) Cleaned protoplast preparation. Whole protoplasts obtained from surface of sucrose after centrifugation. Protoplasts characterised by noticeable absence of chloroplasts. (x100).

(e-f) Cleaned protoplast preparation. Protoplasts obtained as in (c-d). Protoplasts characterised by prominent chloroplasts and are of mesophyll origin. Note presence of polar protoplast (assymmetric distribution of chloroplasts in (e). (x100).

Protoplasts isolated in 30 ml. of a mixture containing 5% (w/v) Meicelase (cellulase) and 0.5% (w/v) Macerozyme (pectinase) and mannitol in CPW salts as osmoticum.

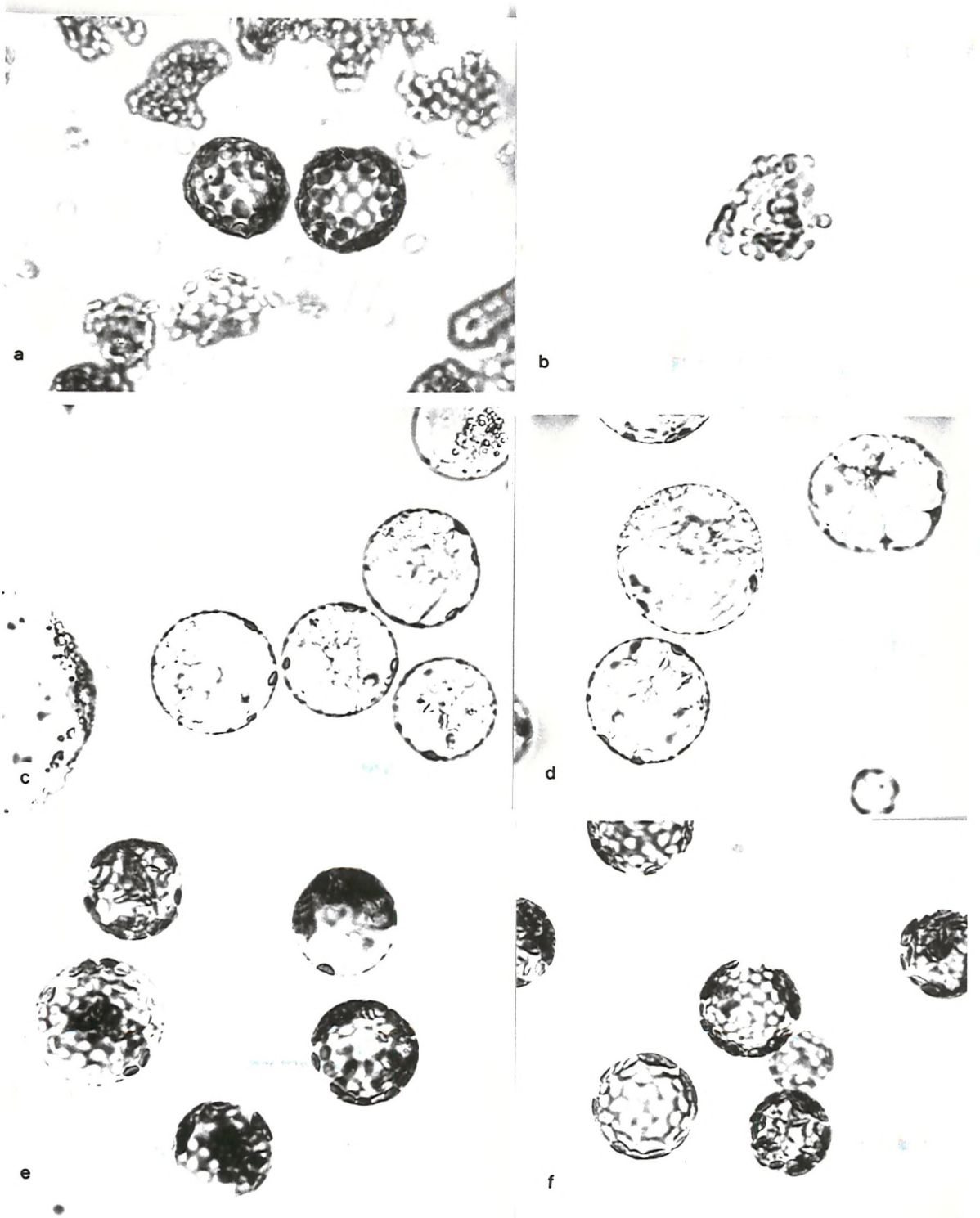

a
b
c
d
e
f

FIGURE 3.2

(a-c) Cleaned protoplast preparation from isolation using purified enzyme. Protoplasts obtained, characterised by presence of those with few chloroplasts and a wider range of sizes. (x100)

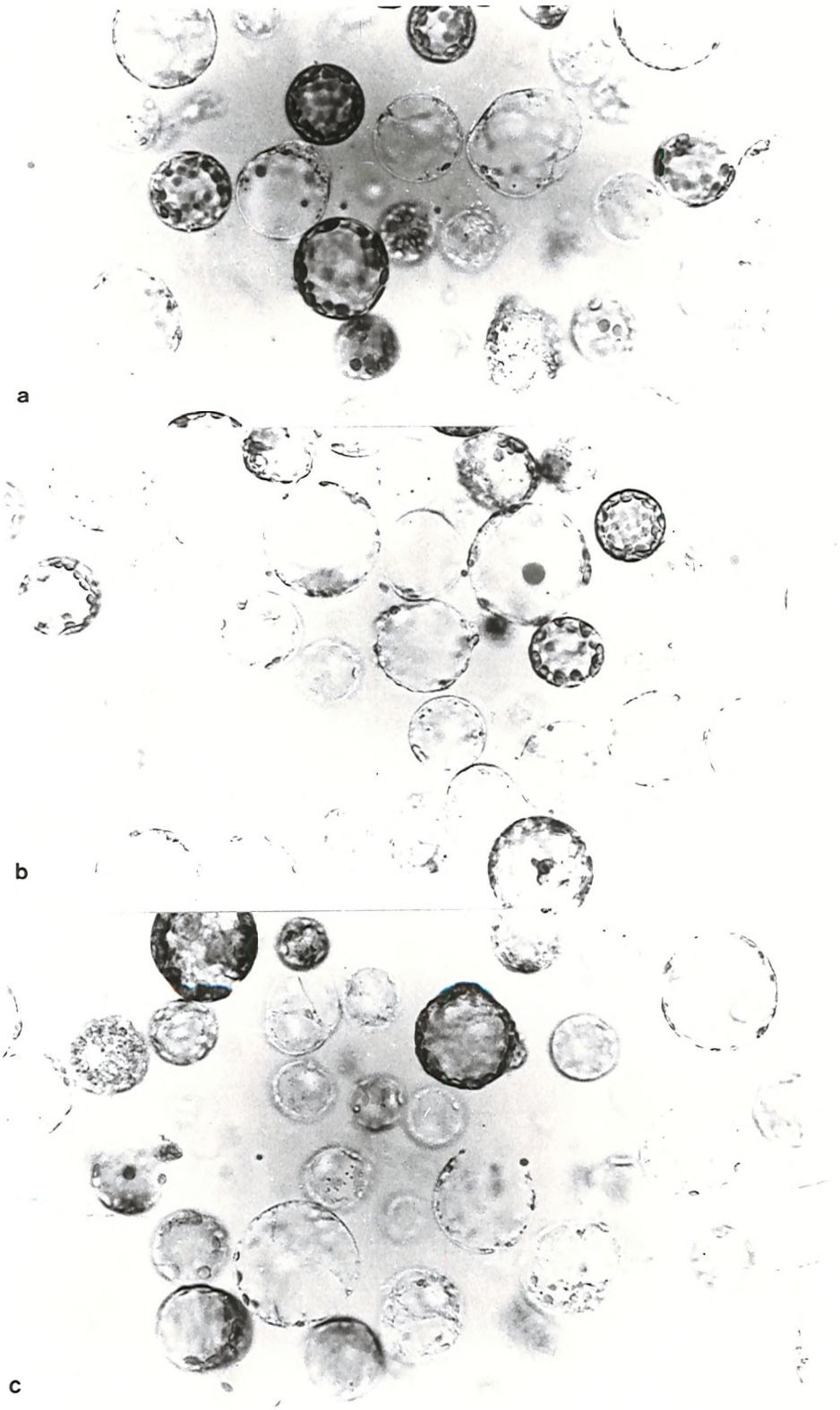
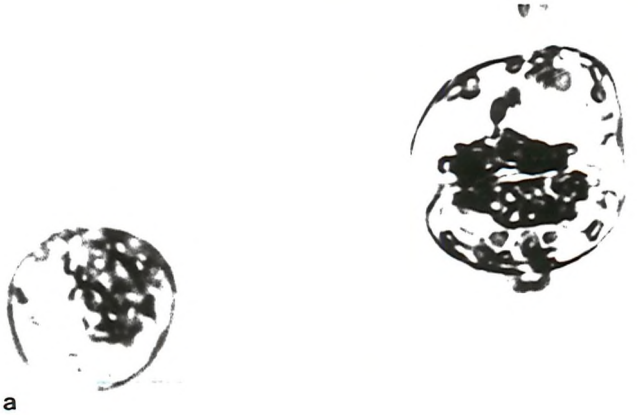

a
b
c

FIGURE 3.3

(a) First protoplast division. Undivided and first division in protoplast after four days in culture in MSP 9M. (x100).

(b) First protoplast division. Protoplast which has undergone a first division and produced spontaneous budding on one of the daughter cells. (x100).

(c) First protoplast division. Late first division stage, with two well formed daughter cells. (x720).



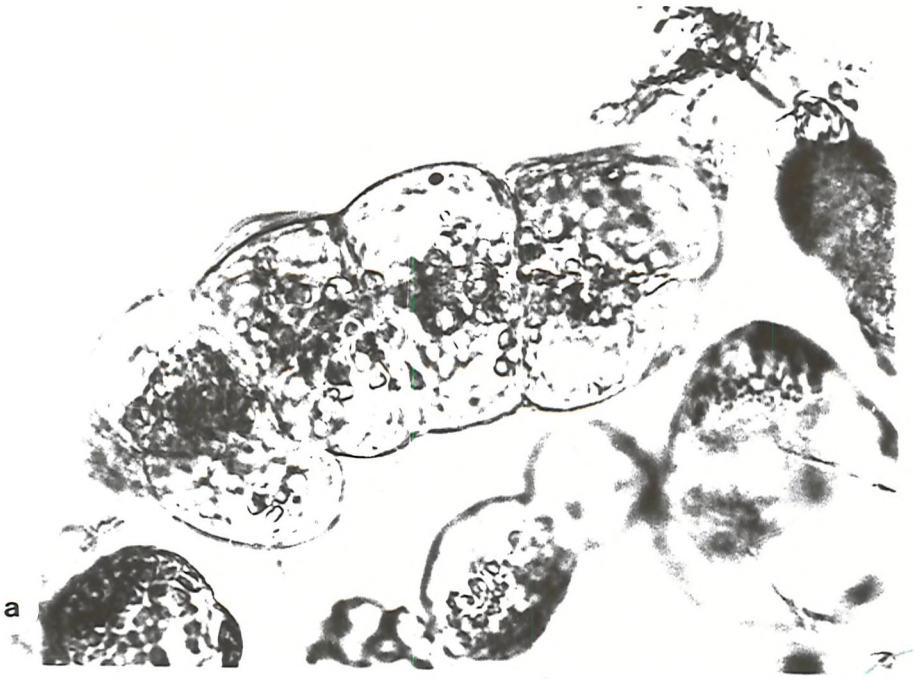
a

b

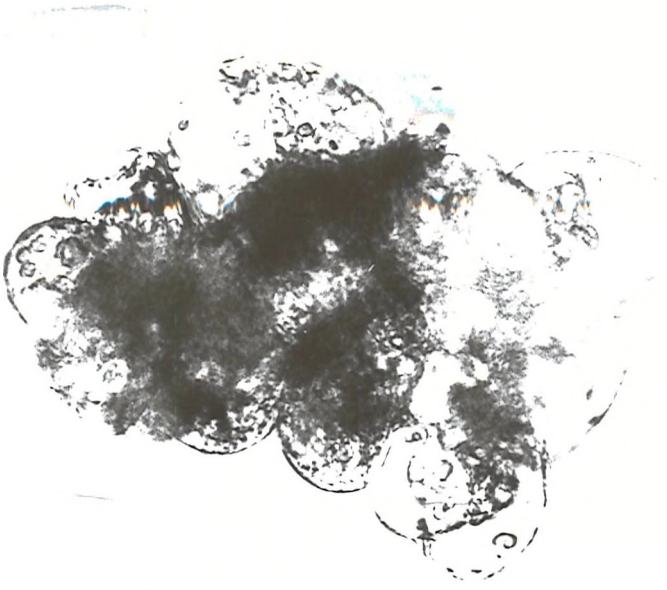
c

FIGURE 3.4

(a-b) Colonies of callus cells derived from protoplast divisions after two weeks in culture in MSP 9M. (x100).



a



b

FIGURE 3.5

(a-c) Colonies of callus cells derived from cell divisions after three weeks in culture in MSP 9M. (a,b;x40 c;x100).

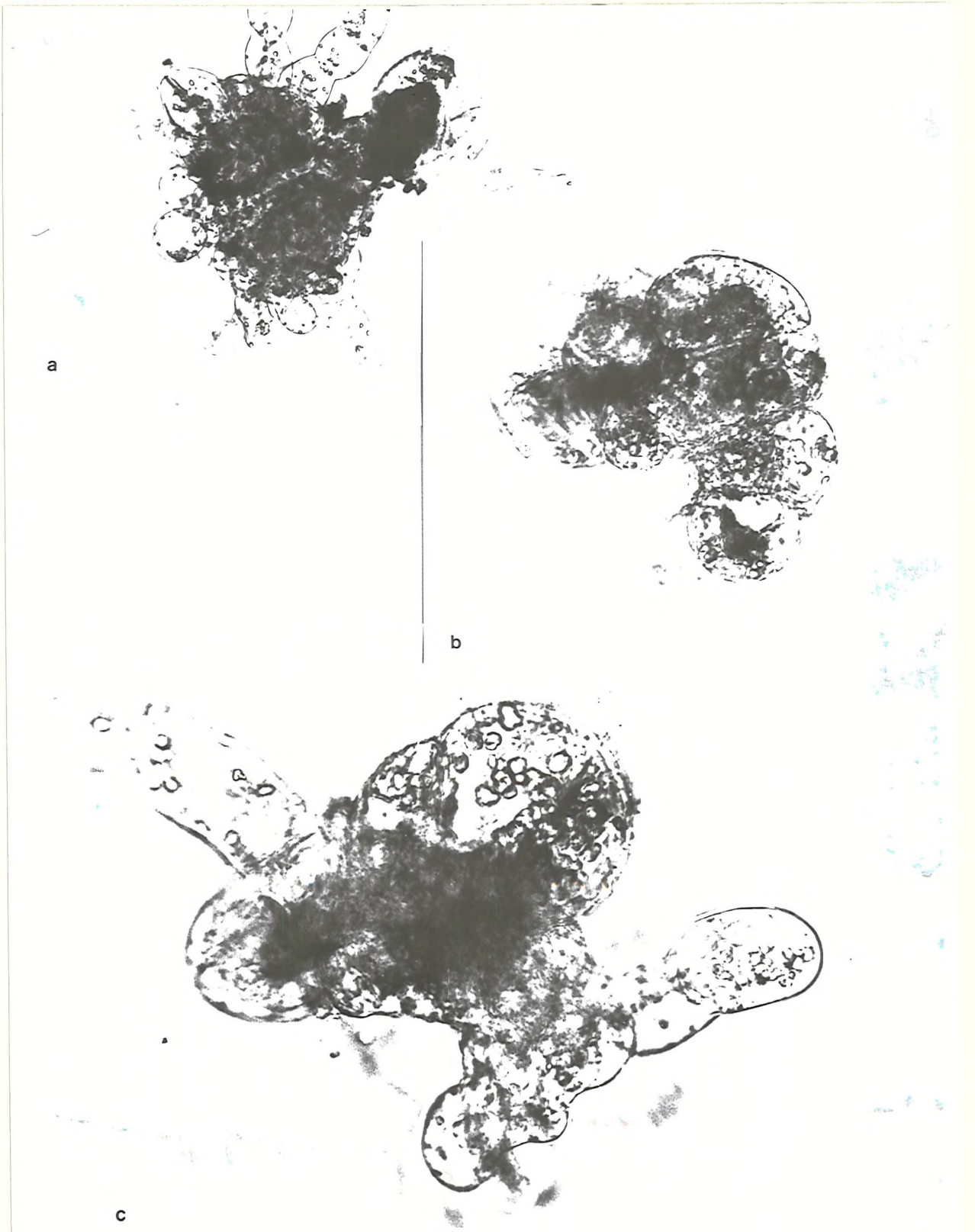
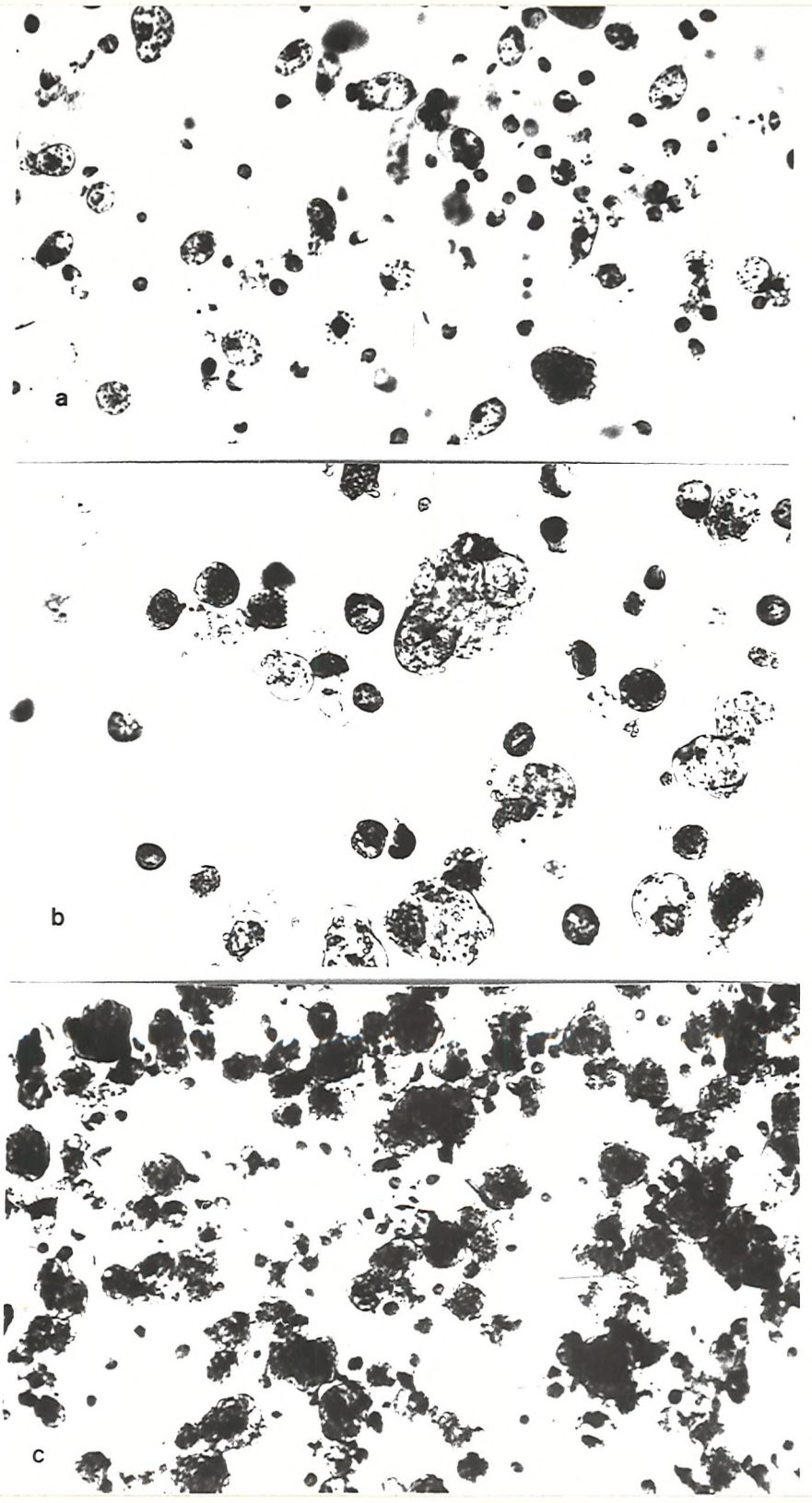

a
b
c

FIGURE 3.6

(a–c) Plating efficiencies. Figure shows a range of plating efficiencies, from low (a) where few protoplasts have commenced divisions, through (b) where some divisions have occurred to high (c) where most protoplasts have commenced dividing. Culture density 2.5 x 10^4 per ml and after one week in culture in MSP 9M. (x40).


a
b
c

the plating efficiencies were reduced if purified enzymes were used, 82% for commercial enzymes compared with 76% for purified enzymes. The viability of protoplasts isolated using the two different enzyme preparations were very similar at 93.6% and 93.9% respectively. In this instance, any impurities present in the crude enzyme preparation did not seem to have an immediate effect on protoplast viability although on subsequent culture the plating efficiency of protoplasts isolated using purified enzyme was less than that for commercial preparation, suggesting than even if toxic materials were present in the commercial preparation they had no effect in these experiments. It appeared that the only observable effect of purified enzymes on protoplasts of tobacco were those of greater variability in size and absence of chloroplasts.

Protoplasts isolated using both enzyme preparations were then subjected to centrifugation on a discontinuous gradient. This procedure produced two distinct bands on the gradient, both of similar viability for each type of enzyme preparation. This showed that gradient treatment had no immediate effect on protoplast viability. With the commercial preparation, a third band, lower down on the gradient, was also present but not so for the purified preparation. The pellet present at the base of the gradient is shown as band IV in Table 3.1, which was also investigated for the presence of viable protoplasts.

On subsequent culture in liquid, both bands I and II from both preparations divided to produce colonies, although band II from both had the greater plating efficiency. Band I gave plating efficiencies of less than 10% for both.

Culture in agar also produced similar results, but although the plating efficiency of band II was again the highest, it was affected by the brand of agar used to produce the culture medium and was less for purified enzyme than for commercial enzyme.

The results from the cultures set up using protoplasts isolated with commercial enzyme were subjected to an analysis of variance (Table 3.2) and tested for statistical significance in an Anova table.(Table 3.3). The same procedure was repeated for the culture obtained using protoplasts isolated with purified enzymes.(Table 3.4 and 3.5)

TABLE 3.1

Effect on plating efficiency of agar type for protoplasts isolated using commercial and purified enzymes and separated by discontinuous gradient.

	Commercial Gradient Bands				Purified			
	I	II	III	IV	I	II	III	IV
Agar								
Sigma	0.31	9.20	0.15	-	0	1.10	0	0
Bact.	2.78	26.98	1.8	-	2.62	7.97	0	0
Oxoid	15.66	79.97	0.56	-	0.25	6.94	0	0
N.Zeal.	14.27	50.81	0	-	0.29	22.95	0	0
Viability of whole population	93.6				93.9			
Viability of bands I & II	94.3 and 94.2				94.5 and 93.8			
Plating efficiency of whole population	82				76			
Plating efficiency of bands I & II in liquid culture	8.1 and 63.4				7.8 and 56			

TABLE 3.2 Commercial Enzyme

Analysis of Variance

Bands

	I	II	III	IV	Total
Sigma	0.31	9.2	0.15	0	9.66
Bact	2.78	26.98	1.8	0	31.56
Oxoid	15.66	79.57	0.56	0	95.79
NZ	14.27	50.81	0	0	65.08
Total	33.02	166.56	2.51	0	202.09

$$S = (0.31^2 + 9.2^2 + 0.15^2 + \ldots\ldots 0^2) - 202.09^2/16$$
$$= 10185.87 - 2552.52$$
$$= 7633.35$$

$$S_B = 1/4(33.02^2 + 166.56^2 + 2.51^2 + 0^2) - 2552.52$$
$$= 4657.19$$

$$S_T = 1/4(9.66^2 + 31.56^2 + 95.79^2 + 65.08^2) - 2552.52$$
$$= 1073.60$$

$$S_E = S - S_B - S_T$$
$$= 1302.56$$

TABLE 3.3

Anova Table

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Bands	3	4657.19	1552.40	10.7 **
Agars	3	1673.60	557.87	3.86
Error	9	1302.56	144.73	
Total	15	7633.35	-	

DUNCAN RANGE TEST

$$= \sqrt{\text{error mean sq}/(\text{bands}-1)}$$

$$= \sqrt{144.73/3}$$

$$= 6.946$$

Therefore, the least significant range was

6.946 x 3.41 = 23.68

I	II	III	IV
8.255	41.64	0.628	0

Arranged in rank order,

IV	III	I	II
0	0.62	8.25	41.64

TABLE 3.4 Purified Enzyme

Analysis of Variance

	Bands				
	I	II	III	IV	Total
Sigma	0	1.10	0	0	1.10
Bact	2.62	7.97	0	0	10.59
Oxoid	0.25	6.94	0	0	7.19
NZ	0.29	22.95	0	0	23.24
Total	3.16	38.96	0	0	42.12

$$S = (0^2 + 1.10^2 + 0^2 + ...) - 42.12^2/16$$
$$= 646.61 - 110.88$$
$$= 535.73$$

$$S_B = 1/4(3.16^2 + 38.96^2) - 110.8$$
$$= 271.09$$

$$S_T = 1/4(1.10^2 + 10.59^2 + 7.19^2 + 23.24^2 - 110.88$$
$$= 68.06$$

$$S_E = S - S_B - S_T$$
$$= 196.56$$

TABLE 3.5

Anova Table

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F
Bands	3	271.09	90.36	4.14 *
Agars	3	68.09	22.70	1.04
Error	9	196.56	21.84	
Total	15	535.73	-	

DUNCAN RANGE TEST

$$= \sqrt{\text{error mean square}/(\text{bands}-1)}$$
$$= \sqrt{21.84/3}$$
$$= 2.698$$

Therefore, least significant range was

2.698 x 3.41 = 9.20

I	II	III	IV
3.16	38.96	0	0
0.79	9.74	0	0

Two null hypotheses were proposed, firstly that agar has no effect on the plating efficiency of protoplasts in culture, and secondly, there was no difference in the behaviour of protoplasts from different bands of the gradient.

In the first analysis of variance, (commercial enzyme) the result for agars was not significant and the null hypothesis was retained. However, the results for the bands was significant at the 1% level and a Duncan Range Test revealed that there was a significant grouping of bands with regard to subsequent behaviour of the isolated protoplasts in culture.

The Duncan Range Test identifies those treatments which lie within the same experimental group and for which there is no significant difference in the effects produced. If there is less than the least significant difference between the means of two treatments then they lie within the same experimental group.

Bands I, III and IV were not significantly different, but the behaviour of band II was significantly different to that of the other bands.

When the analysis was repeated using the results from purified enzyme this significance was retained,but only at the 5% level, and although there was no division of the protoplasts from bands III and IV, they were not significantly different from protoplasts from band I, but all three bands were significantly different from II.

The retention of the null hypothesis for agars meant that although different bands produced different plating efficiencies on different agars, no one agar was better or worse at promoting or inhibiting protoplast division. This held whether commercial or purified enzyme was used in the isolation, although overall plating efficiencies were lower if purified enzyme was used.

The null hypotheses for the bands obtained from the gradient were rejected in favour of an alternate hypothesis which stated that there was a difference in plating efficiency obtained in culture. The plating efficiencies obtained were significantly different at the 1% level for commercial enzyme preparations and at the 5% level for purified enzyme. Thus separation of protoplasts on a gradient prior

to culture is of more benefit to obtaining high plating efficiencies than purification of isolating enzymes or brand of agar used in the culture medium.

Chapter 4

USE OF GRADIENTS IN PROTOPLAST TECHNOLOGY

4.1 Introduction

The use of density gradient centrifugation has played a major role in the advances made in cellular and molecular biology. Various media have been used such as sucrose, $CsCl_2$ and Ficoll, which take advantage of certain properties including density range, ease of preparation and low cost. Media are also chosen which do not alter the particles to be separated, and most workers choose a familiar and inexpensive medium, such as sucrose, that provides a useful density range for separation.

The most common problems encountered are those of osmotic pressure changes and toxicity. Low molecular weight materials, (sucrose, $CsCl_2$, potassium tartrate and sodium bromide), are useful but create high osmotic pressure, an advantage for protoplast work in some instances. The use of high molecular weight substances such as Ficoll, (MW 400,000), Percoll and Dextran T40, (MW 40,000) allows adjustment of osmotic pressure to suit needs, but all are quite viscous.

Numerous density gradient procedures have been devised, and protoplast technology contains its own variety. In this series of experiments, gradients were prepared using Percoll.

The usefulness of colloidal silica as a gradient medium has come largely from the work of Hakan Pertoft and his collaborators in Sweden,(1966). First reported in 1959 by Mateyko and Kopac, Pertoft and colleagues have investigated colloidal silica for its iso-osmolarity, density separation, toxicity, stability with changes in temperature and pH, lack of permeation into particles being separated and solubility or dispersibility in aqueous solutions. Some of these properties are improved by using polysaccharides to stabilise the gradients.

Gradients are frequently prepared using some form of gradient forming machine or by layering. Some pre-trials are normally carried out in order to assemble a gradient which meets specific separation

requirements. In work with protoplasts these might include an ability to identify sub-populations of protoplasts within a population of leaf mesophyll protoplasts and fusion products from somatic hybridisations.

In order to achieve this, the gradient must not cause cells or protoplasts to aggregate or clump together. Some gradient media enhance clumping. A detailed review of colloidal silica centrifugation is available. (Wolff, 1975).

Within protoplast technology, numerous attempts at separation of protoplasts from debris, separation of mixed populations and separation of fractions from disrupted protoplasts have all been reported. Each of these initial aims in turn may be achieved by one of several methods.

In many studies it is of advantage to have the protoplast population free from micro-organisms, sub-cellular debris, (especially chloroplasts), vascular elements, undigested cells and broken protoplasts.

The first step in purification is the removal of large pieces of undigested tissue. Many workers filter through sieves or muslin, although in this study, protoplasts and isolation enzyme mixture were removed using 60 ml Brunswick syringes. Since large pieces of tissue cannot easily enter the syringe, a crude but effective purification was achieved and the need for filtration removed.

Other workers use other steps which may include flotation on dense sucrose solutions, (Gregory and Cocking, 1965; Power and Cocking, 1970; Evans et al, 1972; Davey et al, 1974), flotation on Ficoll solutions, (Schenk and Hildebrandt, 1971), repeated centrifugation and resuspension, (Bui-Dang-Ha and Mackenzie, 1973; Kartha et al, 1974; Pelcher et al, 1974) or repeated sedimentation without centrifugation and resuspension, (Bala Bawa and Torrey, 1971; Kameya and Uchimiya, 1972; Eriksson, 1974). All of these techniques give inconsistent results and often only partial purification, (Kanai and Edwards, 1973; Watts et al, 1974).

Other developments were to follow. Hughes et al, (1978) reported that published purification procedures generally gave inadequate

purification and recovery efficiency. They further demonstrated that macerozyme-cellulase solutions used for isolation were highly contaminated with deoxyribonuclease (DNAase) which could be reduced by an improved purification technique involving discontinuous gradient centrifugation which also gave selective recovery of intact viable protoplasts in high yields.

In 1978, Harms and Potrykus reported yet another method. Protoplasts were separated by low speed centrifugation in an iso-osmotic, discontinuous density gradient on the basis of their buoyant densities. The major advance in this method was the ability to fractionate mixed populations of protoplasts by use of a simple gradient system with no reduction in potential to subsequently produce colonies and regenerate whole plants. Further systems were reported by Lin, (1980) and Fitzsimons and Weyers, (1983), but both systems required more than one centrifugation.

Most of the reports in the literature describe the use of discontinuous rather than continuous gradients. Briskin and Leonard, (1980) used a discontinuous gradient of sucrose for the isolation of protoplast vesicles, whilst Scowcroft and Larkin's, (1980) discontinuous gradient used five gradient steps of Percoll to separate debris and non-viable protoplasts from viable protoplasts.

A gradient using Ficoll rather than Percoll for the purification of corn root protoplasts and subsequent investigation of ion transport, was used by Willy Lin, (1980). This method used a flotation technique and a four-phase non-linear discontinuous gradient. Protoplasts were observed at other interfaces, but vital staining showed these to be non-viable. In addition, as no micro-organisms were found in the protoplast suspensions after gradient purification, Lin suggested that surface sterilisation of leaves and incorporation of antibiotics into isolation and culture media became unnecessary, a major change at that time to existing protocols.

Hampp's, (1980) method for the rapid separation of the plastid, mitochondrial and cytoplasmic fractions from intact leaf protoplasts of Avena demonstrated still further variation in the use of discontinuous gradients. The isolated fractions were used to determine in vivo ATP pools. Protoplasts were enzymatically isolated

and purified on a sucrose-sorbitol gradient. Relatively pure chloroplast fractions were separated from intact protoplasts within seconds by an integrated process of protoplast homogeneation and selective silicone oil filtration. (Robinson and Walker, 1979: Wirtz et al., 1980).

Besides those gradients described above, other methods and types of gradient are mentioned in the literature. Harms and Potrykus, (1978) used a discontinuous iso-osmotic gradient they called the KMC/S density system. Essentially, this system consisted of two solutions, one KMC and the other S mixed together in various ratios. About the same time, and perhaps of more interest, was the method proposed by Hughes et al, (1978) using three different gradients for purification of protoplast preparations. The first two techniques involved either sedimentation, or flotation, for protoplasts prepared in either mannitol or large volumes of sucrose as previously reported (Hughes et al,1977). The third method involved zonal centrifugation for purification of small volumes of sucrose prepared protoplasts.

In these methods, sedimentation onto the sucrose "pad" did not increase the viability of tobacco protoplasts, but did give the important advantage of selecting against "dead" protoplasts relative to their intact counterparts. It also discriminated against polar protoplasts and vacuoplasts. The whole procedure involved four centrifugation steps following protoplast release in enzyme solution. In some cases the protoplasts were also centrifuged whilst still in enzyme solution, prior to flotation.

A further important development was made by the method devised by Fitzsimons and Weyers, (1983) which used a Percoll discontinuous gradient for the separation of protoplast types from the leaf epidermis of Commelina communis. These types were mesophyll, guard cell, epidermal cell and subsidiary cell protoplasts. The procedure involved two separate centrifugations (A & B) on Percoll gradients. The first of these used a four part gradient system consisting of 0, 22, 67 and 90% Percoll to separate guard cell protoplasts from mesophyll and epidermal/subsidiary cell protoplasts. In the second centrifugation, the epidermal cell/subsidiary cell protoplasts were separated on a two-phase Percoll gradient using a 0% and 45%

mixture. For the first time separate suspensions were obtained which enabled investigations of their individual properties.

All the reports in the literature described the production of gradients, which whilst efficient, involved complicated mixtures of buffers, sucrose, mannitol, sorbitol and in one instance mixtures of oils. In addition, the procedures involved several centrifugations and multiple transfers, increasing the risk of contamination, and prolonging the "purification" stage of the preparation of cleaned protoplasts or protoplast fractions. With these problems in mind an attempt was made to produce a simpler gradient system and a protocol which would reduce the number of centrifugations required to produce a clean preparation to a minimum.

The large number of manipulations required seriously affects the possibility of maintaining pure, uncontaminated protoplast preparations. For this reason the method proposed by Piwowarczyk, (1979) offered great promise since it required just a single centrifugation. Further the method was claimed to produce spherical protoplasts, i.e. non-polar, separated from tiny cell fragments and bacteria which remain in the supernatant, and from cells and damaged protoplasts which pass into the sediment.

The gradient proposed by Piwowarczyk was investigated in the first instance as it presented the opportunity to achieve cleaning and separation of protoplasts in a single centrifugation. Since the procedure did not claim to identify possible sub-populations within a protoplast population, continuous and discontinuous gradients were prepared using Percoll and investigated with a view to using a suitably prepared gradient following production of protoplasts by Piwowarczyk's method.

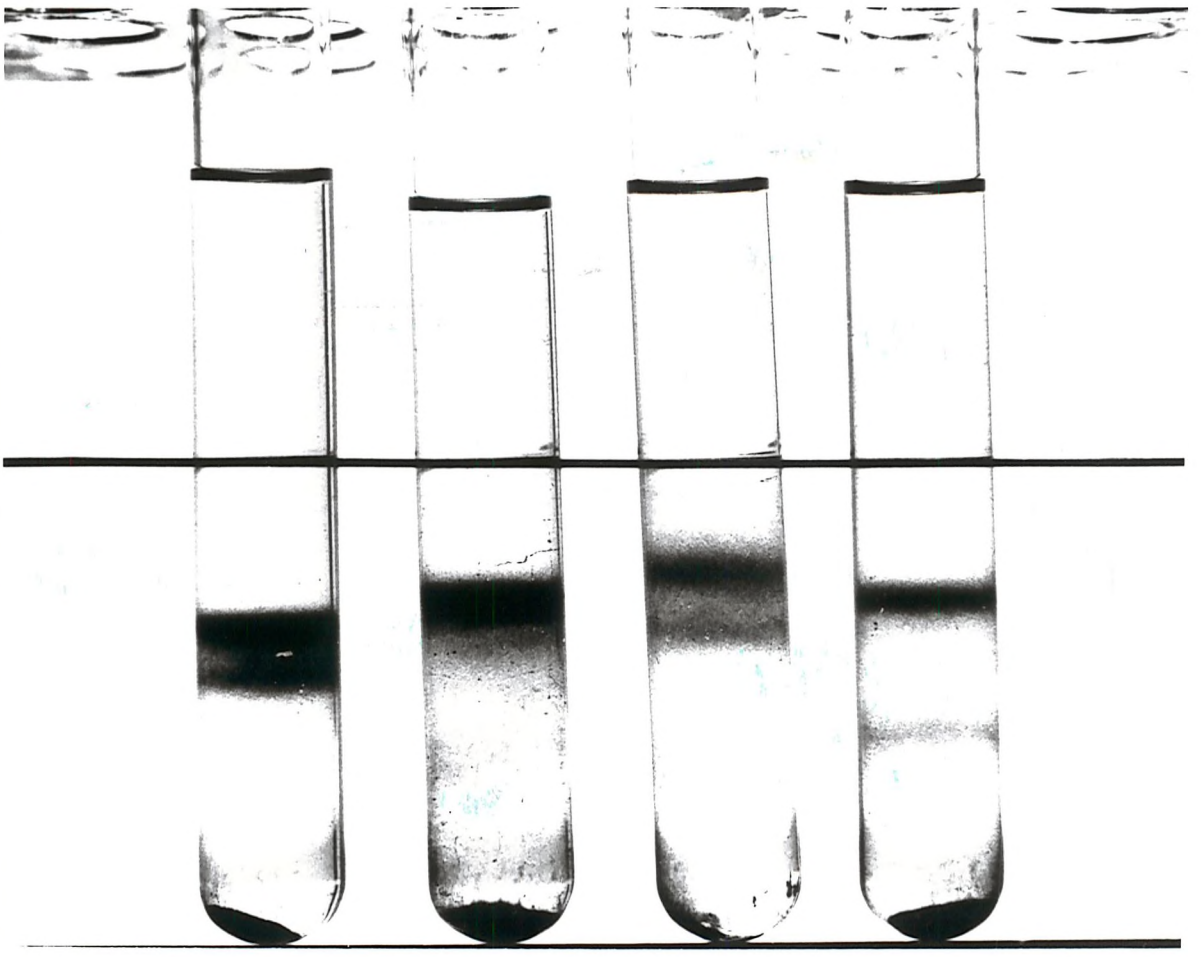
4.2 Variations to materials and methods

Percoll gradients were tested by allowing protoplasts to interact with Percoll for a time in excess of thirty minutes, the time required for centrifugation. Samples were removed and examined under the microscope.

Protoplasts were obtained in the usual manner and cleaned ready for gradient treatment. The population was randomly divided into two

FIGURE 4.1

Protoplasts following centrifugation on an eight-phase discontinuous gradient. Preparation centrifuged at approximately 100 x g for 30 minutes. Tubes show separation of protoplasts into distinct bands at junction between phases four and five and between six and seven and non-viable protoplasts as a pellet at the base of the tube.



portions, one to be used as a control population, the other as an experimental population. Both populations were given gradient treatment on the discontinuous gradient described.

Following centrifugation, the two bands which formed, (Fig. 4.1) but not debris, were retained separately for the experimental population, but were mixed to form a single population again for the control population. Counts were made and the necessary dilutions made so that a final density of 5×10^4 per ml would be achieved in MSP 9M liquid culture.

Following dilution, the control population was divided into two equal lots, one lot for culture in darkness for two weeks, the other for culture in light. The two bands from the experimental population were likewise divided and subjected to the same culture regime. At the end of the culture period each culture was assessed and the number of dead, expanded and dividing protoplasts counted,(Fig. 4.6).

4.3 Results and Discussion

In the method reported by Piwowarczyk, (1979), protoplasts were obtained from an isolating medium containing 2% cellulase, 1% Driselase and 1.5% pectinase dissolved directly in culture medium in which 0.3M sorbitol had been substituted for mannitol and the $CaCl_2$ content was augmented to 0.01M, which improves the mechanical resistance of the protoplasts. The density gradient was prepared in a centrifuge tube which was filled in turn with 0.5M sucrose in culture solution, a layer of 0.14M sucrose and 0.36M sorbitol in culture solution and finally with the layer of protoplasts in enzyme solution. The tube was centrifuged for 5 minutes at 400 x g. Following this, six phases could be clearly seen (see Fig. 4.2). Although Piwowarczyk, used *Petunia hybrida* protoplasts it has not been possible to reproduce these results using both *Petunia* and tobacco protoplasts. This was disappointing, as the method as reported offered a simple one-stage process whereby protoplasts could be cleaned and separated. Piwowarczyk claimed the results of this process are as shown, with cleaned protoplasts separating into a distinct band within an extensive transparent layer. Above this layer were cell fragments and bacteria suspended in the isolating enzymes and below a sediment consisting of whole cells and starch grains.

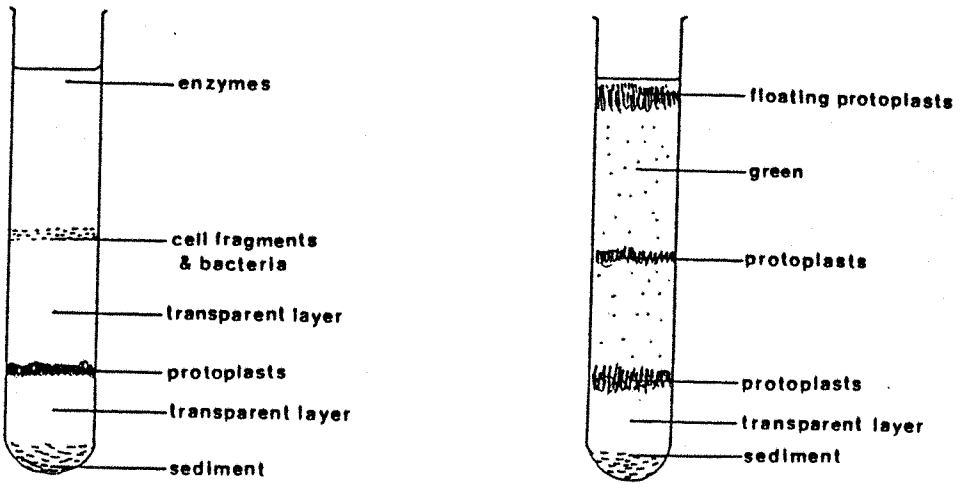


FIGURE 4.2

Preparation of protoplasts using the single centrifugation method of Piwowarczyk. Left, results as reported and right, results obtained from several trials of the method.

TABLE 4.1

DENSITY DETERMINATION OF CONTINUOUS GRADIENT

Sample number 1	Reading on cylinder	Density ($g\ ml^{-1}$)
1	160	1.050
2	150	1.053
3	115	1.062
4	90	1.067
5	80	*
6	65	*
7	50	*
8	**	*
9	**	*
10	**	*

* Density beyond limits of calibration curve

** Drops fell to bottom of cylinder without coming to equilibrium.

Sample number 2		
1	195	1.043
2	185	1.045
3	180	1.046
4	175	1.047
5	155	1.051
6	143	1.054
7	135	1.056
8	133	1.057
9	113	1.061
10	90	1.067

TABLE 4.2

DENSITY DETERMINATION OF DISCONTINUOUS GRADIENT

Band No	Reading on Cylinder	Density From calibration curve	Density by calculation
1	268	1.027	1.0266
2	240	1.033	1.0318
3	220	1.038	1.0369
4	196	1.043	1.0421
5	172	1.048	1.0472
6	148	1.053	1.0525
7	126	1.058	1.0576
8	100	1.064	1.0628

The density was calculated using the following formula:-

$$D = \frac{(\varrho_m \times V_m) \times (\varrho_p \times V_p)}{V_{m+p}}$$

Where ϱ_m = density of 8% mannitol (1.0266 g ml^{-1})

V_m = volume of mannitol used

ϱ_p = density of percoll (1.156 g ml^{-1})

V_p = volume of percoll used

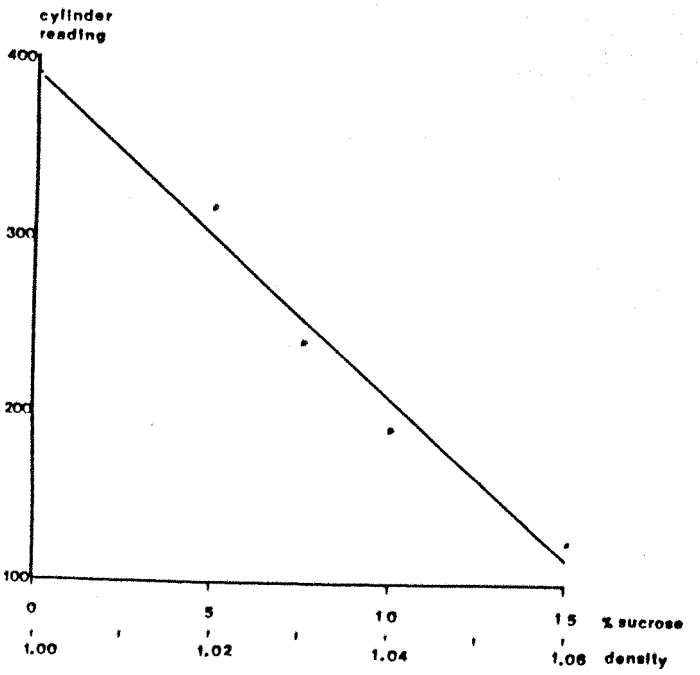
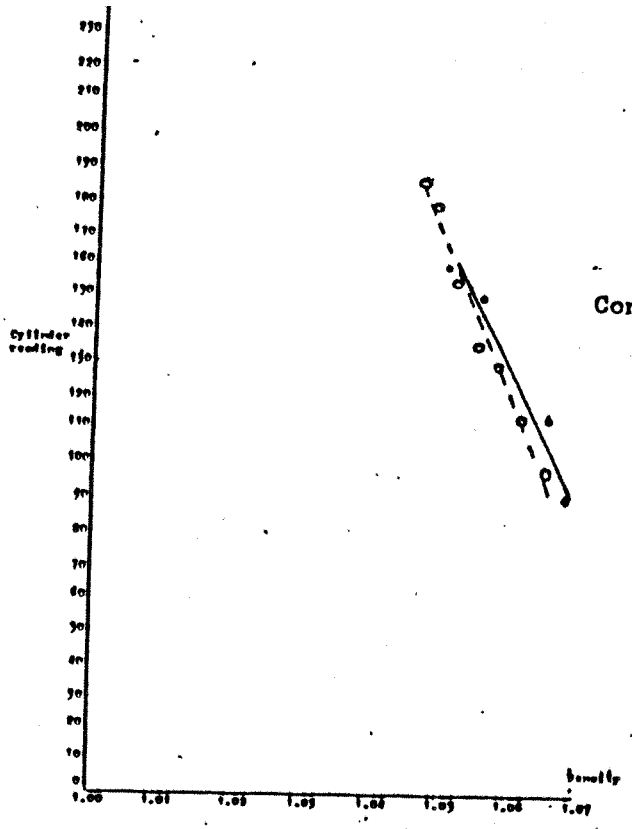


FIGURE 4.3

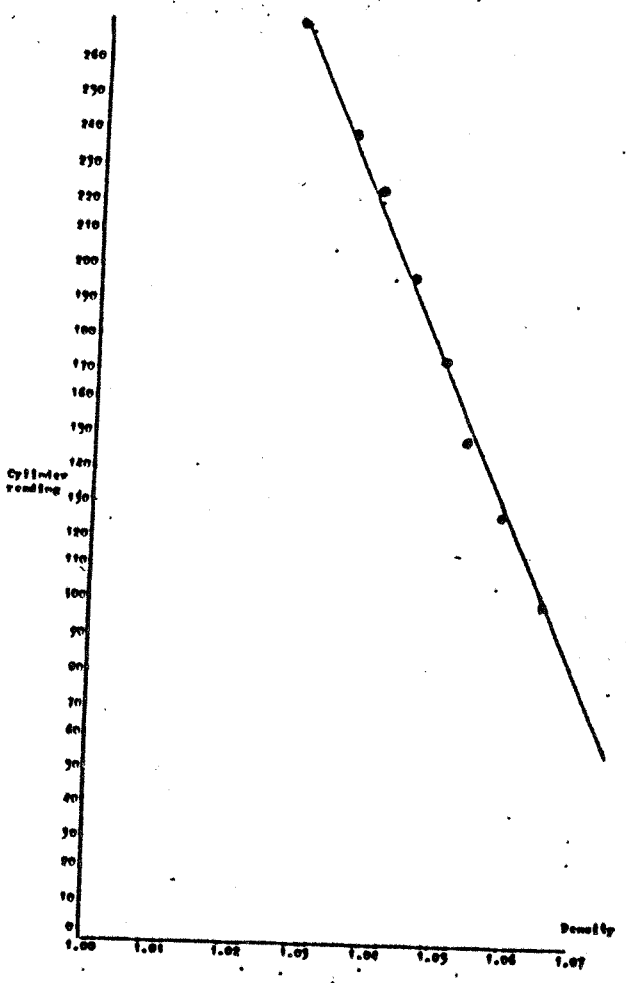
Calibration curve used to calibrate density gradients used for attempts at separation of possible sub-populations within a given protoplast population.



Continuous gradient

● Sample I

o Sample II



Discontinuous gradient

All samples

FIGURE 4.3.1.

Regression analysis of calibration data for continuous and discontinuous gradients

On any number of trials, at different times of year, it was not possible to produce these results, instead, the results recorded produced three bands of protoplasts not one. Of these three bands, one was in a similar position on the gradient as reported by Piwowarczyk, with a transparent layer and debris below. The situation above this band however, was very different. In the position supposed to contain cell fragments and bacteria, there was another band of protoplasts, and close to the meniscus of the gradient a further band. In addition, the gradient between the lower and upper bands was not transparent but green due to the presence of free chloroplasts.

After five days culture in MSP 9M only those protoplasts obtained from the floaters band were still alive, all others having collapsed and died. These protoplasts also failed to regenerate plants, dying after 19 days in culture. Furthermore, the protoplasts did not behave in a similar way to those separated by discontinuous gradient and cultured in either the dark for two weeks or one week in the dark followed by one week in the light.

The failure to achieve results in the method above provided the impetus to investigate the possibilities of producing an alternative gradient system for cleaning and/or separating protoplast preparations. Percoll in mannitol, and mannitol were used to produce both continuous and discontinuous gradient systems. The results of interaction between Percoll and protoplasts were good, with little or no clumping.

First examination of the results in Table 4.1 seemed to suggest that the gradient forming machine was producing inconsistent continuous gradients. Sample 1 produced a gradient which could only be calibrated for its upper 4 ml. of the total of 10 ml.. The remaining 6 ml. exceeded the calibration range of the organic solvent density column and sank to the bottom of the column without reaching an equilibrium. However the second sample did produce a gradient capable of being calibrated for its entire 10 ml. volume, but the density achieved in the last ml. was the same as that achieved in the fourth ml. of the first sample. The data obtained from calibration was plotted as reading on the cylinder against density, (Fig. 4.3.1) having used the calibration curve (Fig 4.3) plotted from data

obtained using the method described in chapter 2 (Materials and Methods).

The plot of this data made trends in the gradient much clearer. The slopes of the two lines fitted by regression analysis had similar values, 0.0002437 and 0.0002279 respectively. The only difference between the two samples was the range of densities achieved. This was 0.17 units for sample 1 and 0.24 units for sample 2, a difference of some 40% between the two samples. Thus the gradient forming machine produced a Percoll/mannitol gradient of uniform density change per ml., but an inconsistent gradient in regard to spread or range of density change.

When these particular types of gradient were used for separating populations of isolated protoplasts, no separation was achieved and protoplasts distributed themselves throughout the gradient with no clear peaks or bands forming. No protoplasts were found at all in the last two ml. of any trial. Fig. 4.4 shows the relative distribution of protoplasts in each successive ml. of the continuous gradient. Whether this lack of separation was caused by the poor spread of density in the gradient, or at least, in part, reflects the true effect of a range of protoplast densities in a population is not clear.

The lack of separation achieved on a continuous gradient led to an investigation of a discontinuous gradient for its ability to separate protoplast populations. A linear discontinuous gradient was designed using mannitol and Percoll in mannitol in various ratios to produce eight phases. The calibration gradient was sufficiently sensitive to detect the 0.005-0.006 g ml^{-1} density difference between each phase. It was possible to calculate the density of each phase using the density of each component and the volume of each used to produce it.

The discontinuous gradient produced more consistent results as Fig. 4.3.1 and 4.5 show. The samples referred to in Fig. 4.5 were from four separate isolations and each protoplast population was placed on a separate discontinuous gradient. Protoplasts on this gradient produced clear bands of protoplasts with debris and collapsed protoplasts collecting at the bottom of the tube. (Fig. 4.1). The protoplasts were found to band at densities between 1.04

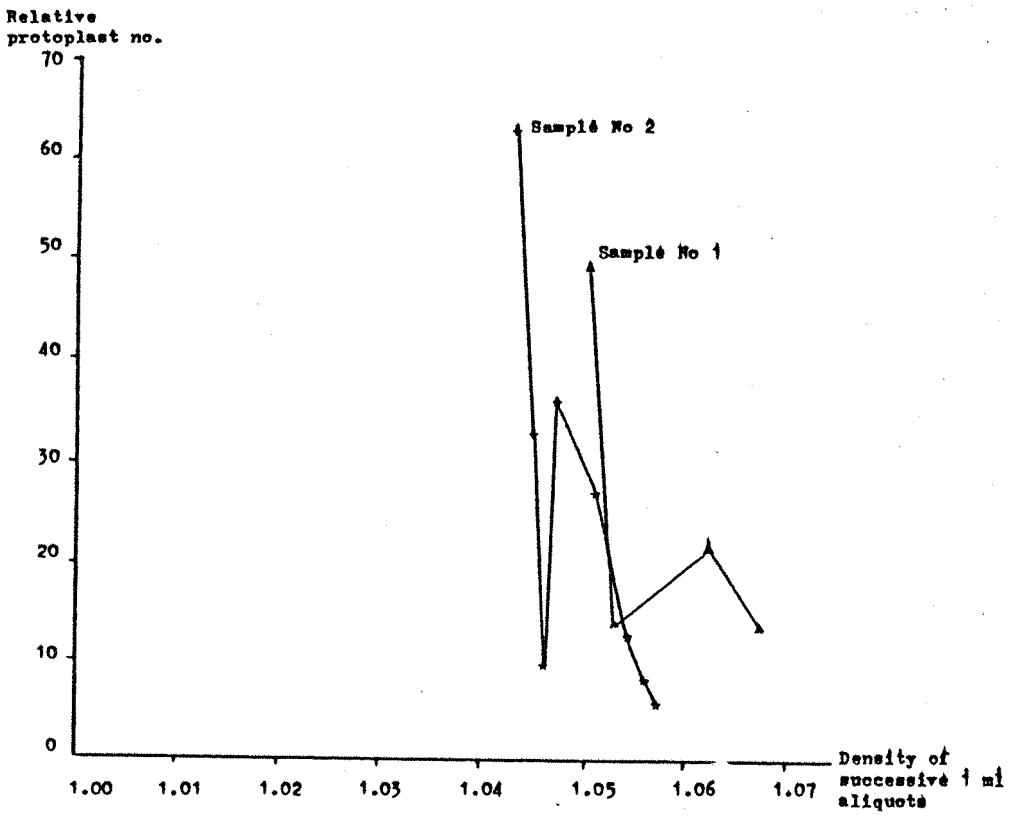


FIGURE 4.4

Separation of protoplasts by continuous gradient

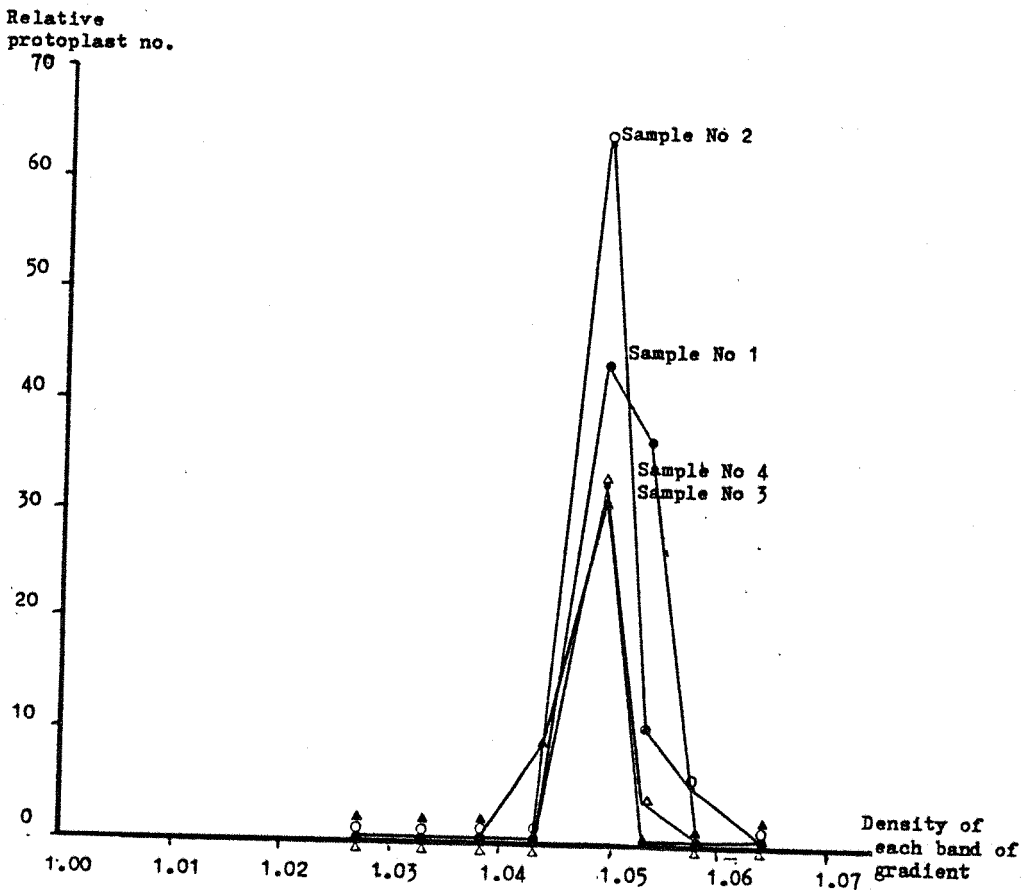


FIGURE 4.5

Separation of protoplasts by discontinuous gradient

and 1.055 g ml^{-1}. The two bands of protoplasts obtained from the gradient were examined qualitatively and quantitatively, and the debris examined for the presence of protoplasts also.

Using light microscopy there were no readily discernible differences between protoplasts from the two bands. An obvious suggestion was that the gradient had succeeded in separating protoplasts of palisade and spongy mesophyll origin. However, on lysing an equal number of protoplasts from both bands and counting the chloroplasts liberated it was found that the mean number of chloroplasts was 35 and 37 from band 1 and 2 respectively. It was expected that palisade mesophyll would produce the greater number of chloroplasts per protoplast. From these results it was reasonable to conclude that both types of mesophyll were present in each band.

The relative number of protoplasts present in each band was also determined, and it was found that the greater relative number of protoplasts was present in the upper band (1), although this ratio was not constant and varied from a 1:1 ratio to 10:1 ratio depending on time of year. (Table 4.3). These bands also usually separated in the fifth and sixth ml. of the discontinuous gradient, equivalent to a density of between 1.04 and 1.05 g per ml., although in one isolation, the protoplasts separated in bands four and five. Protoplasts were distributed throughout the continuous gradient with no clear separation into bands. The discontinuous gradient produced clear banding, with protoplasts entering a band and passing through it to the interface with the next band, which made harvesting of protoplasts particularly easy. No protoplasts were found in the pellet which collected at the base of the gradient.

The viability of the whole protoplast population was determined before gradient treatment and the viability of each band after treatment. This too, was very variable and was related to other factors, most notably again the time of year. However, for any given population the viability before and after was very consistent showing gradient treatment had no immediate effect on viability and was not separating living and dead protoplasts. Similar sets of results were obtained when protoplasts were isolated using a purified enzyme mixture.

TABLE 4.3

RELATIVE DISTRIBUTION PROTOPLASTS FOLLOWING GRADIENT TREATMENT

Band No	Continuous gdt		Discontinuous gdt			
Sample	1	2	1	2	3	4
1	49.0	62.4	0	0	0	0
2	13.9	32.8	0	0	0	0
3	21.3	9.6	0	0	0	0
4	13.1	35.8	0	0	9.2	0
5	10.2	27.2	44.3	63.8	31.7	32.6
6	9.0	12.5	37.3	10.4	0	3.8
7	6.1	8.1	0	5.2	0	0
8	0	4.9	0	0	0	0

Sample numbers refer to separate isolations

Numbers refer to mean numbers of protoplasts counted in known sample volumes from successive ml. of the respective gradient.

Viability was little different for the protoplast bands after 9 days in culture, however, there was a difference in the behaviour of the protoplast bands in culture.

The results in Fig. 4.6 show the responses of protoplasts placed in culture after no gradient, or discontinuous gradient treatment. The results are those obtained after two weeks in culture, either two weeks in the dark, or one week in the dark followed by one week in the light. Those protoplasts shown as having no gradient treatment were exposed to Percoll gradients, centrifuged and then recombined to produce a single control population. At the end of the two week culture period, the cultures were assessed for divided, expanded but not divided and dead protoplasts.

The plating efficiency was higher in the control culture which had been in the dark for the two week period. However, the proportion of expanded but non-dividing protoplasts was also higher than in the dark/light culture, 53% compared to 41%. In this culture, (light/dark), the proportion of dead protoplasts was also higher.

The other cultures consisted of protoplasts which had been separated on a discontinuous gradient system, which produced two distinct bands on the gradient. These were divided and given the same culture regime as the control population. The upper band from the gradient (band 1) produced expanded protoplasts, an almost equal number of dead protoplasts and less than 20% divided protoplasts when cultured for two weeks in the dark. This proportional relationship was maintained when the protoplasts were cultured for one week in the light and one week in the dark, although more protoplasts expanded in culture at the expense of those dividing. Overall, more protoplasts expanded, more died and fewer divided in this culture when compared to the control culture.

The lower band from the gradient (band 2) produced the fewest number of expanded protoplasts and about the same number of dead protoplasts for both sets of culture conditions. The number of dividing protoplasts exceeded 50% in both culture conditions and for the culture given one week in the dark and one week in the light, approached 60%. The reasons for this were not clear, but it possible that the discontinuous gradient had succeeded in separating protoplasts derived from cells at different stages of the cell

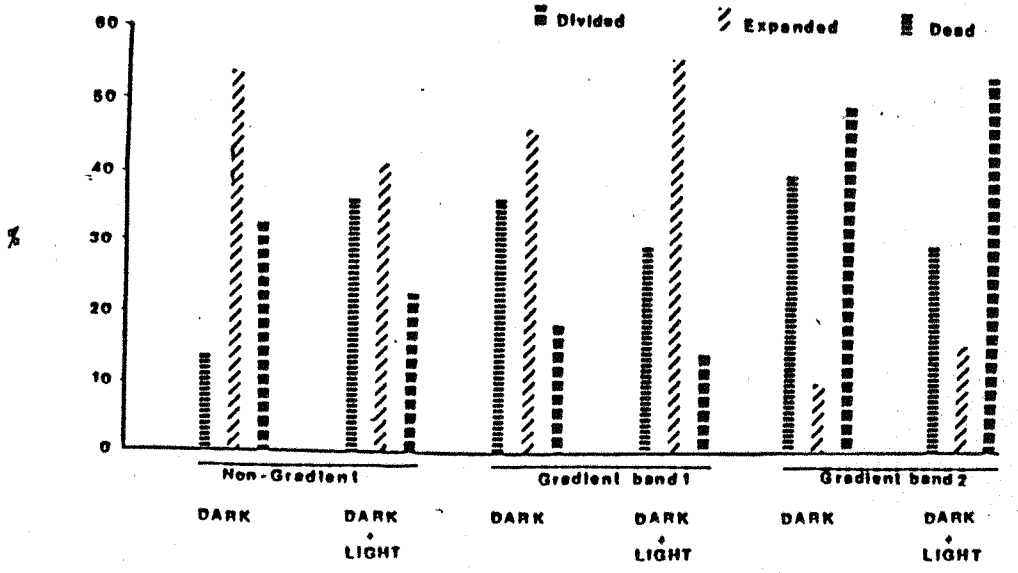


FIGURE 4.6

Histograms showing behaviour of protoplasts after no gradient and discontinuous gradient treatment. Protoplasts were cultured in the dark for two weeks or one week dark followed by one week light.

cycle, notably pre-S and post-S. The protoplasts from post-S cells would be of a greater density than those from pre-S cells and would therefore equilibrate lower down on the gradient. It is also reasonable to suggest that, as these have already undergone replication, they are primed ready for division following a period of growth and expansion in culture. Results seem to confirm this, with the fewest numbers of expanded but not dividing protoplasts being present. The protoplasts derived from cells in G2 of the cell cycle being those, which having expanded, go on to divide. It also follows that those which were present in the culture, may have still been in the process of expanding, and given sufficient time in culture, may have also gone on to divide, and raised the plating efficiency further.

The fact that chromosomal replication may already have taken place may have meant that protoplasts were less demanding of the conditions provided in culture and were more readily stimulated into a period of growth (G2) followed by division.

This is unlikely to be the entire story, but is an idea which is worthy of further investigation. It is also noted that under normal circumstances mature plant cells do not divide, so why should cells replicate chromosomes ready for cell division? On the other hand, polyploidy in plant cells is not uncommon.

The single step centrifugation method of Piwowarczyk appears unachievable at the present time and it is necessary to compare the protocol devised in this study with those which already exist. It seems that it will continue to be necessary to centrifuge more than once, although the ultimate aim remains, that of a single step procedure. The greater the number of centrifugations in a protocol, the greater the risk of contamination to the protoplast preparation. The advantages of using some kind of gradient to separate protoplasts are clear. Some of these advantages wane if the gradient is unable to identify the protoplasts which are likely to divide in culture and merely separates protoplasts from debris. Less complicated and more efficient procedures exist. Kanai and Edwards, (1973) developed a density buffer system which, when mixed with 0.13 volumes of crude protoplast suspension and centrifuged at 300 x g for 6 minutes partitioned into two phases. The debris and broken protoplasts were left suspended in the lower phase whilst the intact

protoplasts collected at the interface. The density buffer contained polyethylene glycol, (MW 6,000), Dextran, (MW40,000), sodium phosphate and sorbitol. Despite a requirement to subject protoplasts to a pH of 8.0 and despite the sensitivity of the method to brand of Dextran, this method was reported to be a vast improvement on earlier methods.

The protoplasts used by Hughes et al, (1978) were purified by one of three different centrifugation techniques. The first two involved either sedimentation or flotation for protoplasts which had been prepared in either mannitol or large volume sucrose, as described previously by Hughes et al (1977). The third method involved zonal centrifugation for purification of protoplasts prepared in small volume sucrose.

Mannitol (or sorbitol) prepared protoplasts are usually more dense than the medium and, therefore, can be sedimented to form a pellet on the bottom of a centrifuge tube, (Takebe et al, 1968). When this process was repeated by other workers, including Hughes et al,(1978) the recovery efficiencies were low and numerous damaged non-spherical protoplasts, organelles and debris were observed in the pellet. However, when they sedimented protoplasts on to a sucrose pad, intact spherical protoplasts were recovered at the mannitol-sucrose interface, while damaged protoplasts, organelles and other cellular debris passed through the sucrose pad and pelleted at the bottom of the centrifuge tubes. This procedure was repeated four times to dilute isolation enzymes and gave recovery yields of about 50% for barley and safflower, of which 5% were damaged protoplasts. With tobacco protoplasts, yields were only about 10%, increasing to about 40% by adjusting the concentration of mannitol used in the isolation from 0.45M to 0.7M. Again about 5% of the protoplasts were damaged. Protoplast viability was not decreased, and it was claimed that the technique was able to select against "dead" protoplasts relative to intact counterparts. For example, about 20% took up stain before centrifugation and would be "dead" as defined by Widholm, (1972), whereas only 9% did so following centrifugation.

In the alternative flotation technique, Hughes et al (1978) used 100 ml. Babcock bottles and centrifugation at 2°C. Protoplasts floated to the sucrose-mannitol interface, whilst damaged protoplasts, organelles and cellular debris sedimented. Four or five

repetitions were required to dilute the isolation enzymes sufficiently. Yields were typically about 60% with 6% damaged.

The zonal centrifugation method of Hughes et al, (1978) which was the third method used produced increased purification efficiencies. Protoplasts were floated as a narrow zone through fresh sucrose medium. In order to do this, protoplasts were first collected on a sucrose pad as described above and in a second centrifugation, floated as a narrow zone up through sucrose to a mannitol overlayer. This two-step method gave about 50% yields with only 2% damaged. Subsequent culture of these protoplasts gave a plating efficiency of 35%. Overall, although the methods adopted produced a "cleaner " protoplast preparation, and one with a high percentage of undamaged protoplasts, only the latter method succeeds in reducing the number of centrifugations required.

In Scowcroft and Larkin's method,(1980) the five density steps were 1.020, 1.029, 1.048, 1.066 and 1.085 (i.e. a non linear discontinuous gradient). This particular protocol is the most similar to the one devised here. It is different in that the gradient produced was non-linear, with a change of 0.009 between the first two phases, but 0.019 between second and third phases, 0.018 between third and fourth and 0.019 between fourth and final phase. Fractured protoplasts, cell debris and some intact protoplasts collected at the 1.029/1.048 interface, and intact, debris free protoplasts collected at the 1.048/1.066 interface after centrifugation at 100 x g for 5 minutes. The densities of the two phases producing the interface at which intact protoplasts collected concur with those achieved on the gradient devised in this present study. Whilst this gradient separated protoplasts of density 1.05 and 1.06 Scowcroft and Larkin's gradient failed to do so. They collected at the interface because they were too dense to be held in the 1.048 phase, but too buoyant to penetrate the 1.066 phase. Their gradient succeeded in isolating intact protoplasts but failed to separate different protoplasts types within the population due to too greater density differences between fractions of the gradient.

The method proposed by Willy Lin, (1980) isolated and cleaned protoplasts were resuspended in 7ml. of protoplast suspension medium to which had been added 10% Ficoll. Further 7ml. of 8%, 5% and 0% were then layered on top in sequence. Centrifugation at 300 x g for

20 minutes produced a band of intact protoplasts at the 0/5% interface. A further claim was made for this particular method because no bacteria were found in the cleaned protoplast preparation, it was suggested that surface sterilisation of leaves and incorporation of antibiotics in enzyme mixtures used for isolation and culture media became unnecessary.

In Fitzsimons and Weyer's method, (1983) the first centrifugation caused 100% of the epidermal cell and subsidiary cell protoplasts to collect in the upper (0-22%) layer of the gradient after centrifugation at 400 x g for 5 minutes. Guard cell protoplasts were collected from the 67/90% interface. This particular gradient was used at the end of a full incubation period in enzyme. The second method involved centrifugation at 400 x g for 5 minutes, mid way through the standard incubation period with enzyme, and was used to separate the epidermal and subsidiary cell protoplasts, but was also found to remove mesophyll contamination present, since these were not required in the subsequent studies. Results showed that epidermal/subsidiary cell protoplasts had the lowest density, usually appearing above the 22% Percoll layer, but if older plants were used as source material, the protoplasts were more dense and some penetrated to the 67/90% boundary. Most guard cell protoplasts banded at the 67/90% interface but appreciable amounts also appeared at the 22/67% interface. The larger numbers and higher purity preparation occurring at the former. Epidermal and subsidiary cell protoplasts could be harvested from the 0/22% interface in this method, but the preferred second method (B) for these types allowed pure preparations to be obtained by a simpler system in which guard cells were not involved, because of a shorter incubation period. The protoplasts present at the 0/45% interface were a mixture of epidermal cell and subsidiary cell derived protoplasts (three kinds) and mesophyll protoplasts were present as a pellet at the base of the gradient.

Hampp, (1980) claimed that the method he used removed some of the problems of artefact creation due to long time intervals between start of homogeneation of tissue and the investigation of more or less contaminated organelles, especially with regard to mitochondria. Hampp adapted the technique in such a way that it delivered mitochondria, in addition to pure plastid fractions and a cytoplasmic supernatant at the same time. The system developed was a non-linear discontinuous micrograd ient prepared in "polypropylene microtubes"

(450 µl) which contained 5 layers. Starting at the tip these were 20 µl 0.6M sucrose, 30 µl silicone oil type CR, 50 µl 0.4M sucrose, 50 µl silicone oil type AR, 70 µl air space and finally a 20 µM nylon mesh. Into the space above the mesh were pipetted 50 µl of protoplasts in 0.39M sorbitol. Following centrifugation fractions were collected by cooling tubes in ice and then slicing through the tubes in the region of the oils and the air layer.The three fractions so collected were supernatant, middle and pellet.Intact protoplasts were obtained from the lower oil/sucrose interface.

Harms and Potrykus, (1978) obtained their KMC solution by mixing together equal volumes of 0.35M KCl, 0.245M $MgCl_2$ and 0.254M $CaCl_2$ at pH 6. The S solution was 0.56M sucrose. After mixing together in appropriate ratios the gradient was formed by layering 1.5 ml. aliquots in glass centrifuge tubes. Eight layers, producing seven interfaces, were used. Gradients were run at various centrifugal forces for 5 -20 minutes. Recovery efficiency was as high as 90% but with a mean of 69%. Protoplasts subjected to such a gradient treatment were cultured and successfully regenerated. This method of fractionation requires that the protoplasts are obtained and cleaned before loading onto the gradient. Harms et al,(1978) floated their protoplasts in a 3:1 1.6M mannitol and 0.56M sucrose mix and then washed in a 0.25M $CaCl_2$ and sedimented by low speed centrifugation. This was not unlike the system of flotation in 20% sucrose (0.58M) and washing in CPW salts followed by sedimentation described earlier in chapter 2, (Materials and Methods).

In the method of Hughes et al, (1978), dilution of protoplasts into 0.5M mannitol in 15ml. centrifuge tubes was followed by sedimentation at 400 x g for 5 minutes through the mannitol onto a sucrose "pad" (0.5M). The protoplast band was transferred to a new tube with the addition of sucrose (0.8M) to 0.53M. After centrifugation at 400 x g for 5 minutes, the protoplasts had floated from the 0.53M band and passed through to an overlayer of 0.4M sucrose to its interface formed with 1ml. 0.5M mannitol. When this method was used for tobacco, yields of 10% intact protoplasts were obtained. Of these 95% were non-polar (even distribution of chloroplasts) about 5% were damaged and about 4% were vacuoplasts (generally devoid of organelles). Yields of intact protoplasts could be increased to 40% by adjusting the concentration of mannitol used in isolation and purification procedures from 0.45M to 0.7M.

All of these methods succeeded, to a greater or lesser extent, in producing a cleaned protoplasts preparation. In acheiving this, some of the methodologies involved a number of centrifugations and transfers between isolation mixtures and cleaning solutions, of varying complexity in both terms of manipulative procedures and composition of isolation and cleaning solutions. The yields of intact protoplasts from these procedures ranged from 10% to about 50% when tobacco protoplasts were produced. The protoplasts isolated were successfully cultured and plants regenerated, but only Hughes et al (1978) give any indication of the plating efficiencies achieved, and then only for the last of their three methods.

The method devised in the current study makes no claims to reduce the number of centrifugations required to produce a cleaned protoplasts preparation compared to other methodologies. However,the gradient system devised required only one further centrifugation and identified those protoplasts which were most likely to divide in culture, and as the results in Fig. 4.6 show produced a culture in which almost 60% of protoplasts were dividing after two weeks in culture.

Chapter 5

SOME FACTORS AFFECTING THE ISOLATION OF STABLE PROTOPLASTS

5.1 Introduction

There have been many reports of problems associated with the isolation of stable protoplasts from tobacco and other plants, (Watts et al, 1974; Kassanis and White, 1974). This has led to attempts at development of an empirical determination of the plant growth conditions considered optimal for the production and isolation of stable protoplasts. However, studies have shown that optimum growth conditions vary considerably between laboratories, treatments given prior to isolation are often contradictory and include glasshouse growth, (Motoyoshi et al, 1973) or controlled growth room treatment, (Kubo et al, 1975). Growth in soil-less compost is recommended by some, whilst others use John Innes compost, (Coutts et al, 1972). Other workers stress the importance of relative humidity during growth, others give it no mention.

The pre-wilting of plants and detached leaves prior to isolation is also mentioned and used in many laboratories in order to facilitate leaf peeling, (Coutts et al, 1972). However, wilting causes stress to plants and increases internal abscisic acid levels, (Wright and Hiron, 1969). It is these very conditions which are to be avoided if successful isolation is to be achieved, (Watts and King, 1976). Finally, very little information is given as to the reason for a particular choice of plant growth conditions.

One of the most detailed studies was that carried out by Cassells and Cocker, (1982). They found that the viability of tobacco protoplasts was affected by membrane calcium, total calcium, plant age and supplementary lighting. Feeding calcium nitrate and calcium chloride helped to increase protoplast stability. Further, whatever optimal feeding regime was chosen, it must be used in conjunction with the correct harvesting procedure, which depends on whether supplementary lighting was given. This particular study emphasised the complexity of the relationship between the plant and its nutrition and the changing environment. Intact plants will continue to be the preferred source for large scale isolation of genetically uniform and characterised protoplasts. Consequently the

development of standardised procedures for isolation of protoplasts from intact plants remains a primary objective.

Plants used as source materials for protoplasts are usually grown in greenhouses, and with some attempt at control of daylength. However, this controlled daylength is not as simple as perhaps it first appears. The period of light to which plants are exposed is made up of several different phases, which also change as the seasons change. In any examination of the light regime to which source plants are exposed, it might be necessary to distinguish between the various components in a given daylength period. These could include the proportion of the daylength during which natural light is present, artificial light is present, or the ratio between the two. When natural light is present as part of the daylength, then whether this also includes sunshine might also be important.

In an attempt to ascertain whether such components do indeed need consideration in defining a set of growth conditions for plants to be used as sources of protoplasts, daylength data and sunshine hours records were kept for a period of time approaching three years. During this time, the viability of any protoplasts isolated from source plants was assessed, in order to examine whether a relationship exists between daylength, daylight regime of source plants, and the viability of protoplasts isolated from these source plants.

5.2 Results and Discussion

The plants used in this series of experiments were maintained on a 16 hr daylength, which, in winter, was characterised by two separate phases, at least superficially. One of these phases was the time during which the outside environment was dark, but the greenhouse was illuminated, and the other phase occurred when the outside environment was in daylight. During this phase, the greenhouse lights might be on or off, and the daylight outside characterised by sunshine or not. In summer, it was possible to have the entire 16hr day characterised by the presence of natural daylight and thus rendered artificial illumination unnecessary. Again, this daylight may, or may not, have also included sunshine. During the change from winter to summer, and summer to winter, the changes in the relationship between the various daylength components changed

almost on a daily basis, especially if daily sunshine hours were considered.

Table 5.1 shows the sunshine hours at Mayflower Park, Southampton during three successive years. It was during this period of time that the effects of daylength, sunshine hours and changes associated with these factors were being specifically investigated. In some of the tables the data has been manipulated in an attempt to identify relationships, if any, between protoplast viability and light regime at various intervals prior to protoplast isolation.

Table 5.1 shows the monthly and yearly totals of sunshine hours for a major period of this study. As the table shows, the yearly total is variable, as is the month of highest sunshine hours, being August in 1981, June in 1982 and July in 1983. Similarly the months recording the least sunshine hours are variable, January 1981, February 1982 and January 1983. The results are also presented graphically in Fig. 5.1

The One Sample Chi-Square Test.

This non-parametric test requires only that the data be a random sample. The null hypothesis is that there is no difference in the viability of protoplasts with the time of year. The data was first classified into mutually exclusive categories of interest, months of the year in this case, and the expected frequencies for these categories were then computed. Expected frequencies are those which would be obtained if the given hypothesis were true.

Once the expected frequencies were obtained, the chi-squared statistic was computed as $X^2 = (O_i - E_i)^2/E_i$ where O_i is the observed frequency for the ith category, Ei is the expected frequency for the ith category and k is the number of categories. If the hypothesis is true, the chi-square statistic has approximately a chi-square distribution with k-1 degrees of freedom. This statistic will be large if the observed and expected frequencies are substantially different. Tables of critical values were consulted and significance assessed. The 1981 results and all results combined were significant at the 5% level and the null hypothesis was rejected in favour of the alternate hypothesis which states that there is a statistically significant difference in viability of protoplasts with time of year.

TABLE 5.1

MONTHLY SUNSHINE HOURS AT SOUTHAMPTON

	1980	1981	1982	1983
Jan	—	64.2	57.8	51.7
Feb	—	81.3	46.9	96.7
Mar	—	79.6	161.1	92.6
Apr	—	145.0	203.3	153.7
May	—	140.3	213.5	160.6
Jun	—	161.5	223.7	188.8
Jul	—	162.5	194.8	276.4
Aug	—	233.0	174.8	260.8
Sep	—	154.2	154.3	—
Oct	124.4	95.2	75.9	—
Nov	72.8	40.3	62.9	—
Dec	79.1	42.3	47.8	—
Yearly Total		1399.4	1616.8	—

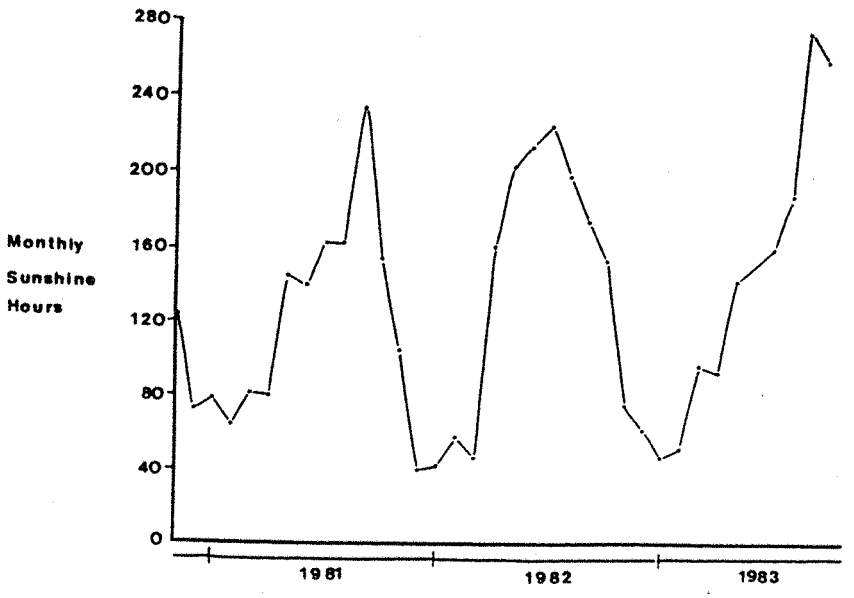


FIGURE 5.1

Changes in sunshine hours
at Mayflower Park, Southampton.

TABLE 5.2

Statistical analysis of protoplast viabilities at various times of year and different years

1981

Observed	Expected	$(O-E)^2/E$
93.80	80.33	2.26
93.55	80.33	2.18
89.40	80.33	1.02
74.32	80.33	0.45
75.50	80.33	0.29
49.10	80.33	12.14
78.40	80.33	0.05
85.20	80.33	0.30
83.70	80.33	0.14

$X^2=18.82$

1982

Observed	Expected	$(O-E)^2/E$
85.70	72.10	2.57
68.70	72.10	0.16
71.00	72.10	0.02
74.20	72.10	0.06
69.00	72.10	0.13
64.00	72.10	0.91

$X^2=3.85$

All Results

Observed	Expected	$(O-E)^2/E$
93.80	77.04	3.65
93.55	77.04	3.54
89.40	77.04	1.98
74.32	77.04	0.10
75.50	77.04	0.03
49.10	77.04	10.13
78.40	77.04	0.02
85.20	77.04	0.86
83.70	77.04	0.58
85.70	77.04	0.97
68.70	77.04	0.90
71.00	77.04	0.47
74.20	77.04	0.10
69.00	77.04	0.84
64.00	77.04	2.21

$X^2=26.39$

Expected viability was computed on the basis that it did not vary with time of year and compared using a one sample X^2 test with observed viabilities for any significant variation

The results for 1982 were not significant but here the analysis was based on a small set of results. This poses the question why protoplast viability should vary according to time of year and what factors if any, might be involved in producing such an effect.

The chi-squared test indicated a statistically significant difference in protoplast viability with time of year. For this reason, the lighting regime to which the plants had been exposed prior to use as sources of protoplasts was investigated using meteorological data collected by the Southampton Weather Centre at Mayflower Park, Southampton. The data consisted of daylengths and sunshine hours on a daily, weekly and monthly basis. This was used to try to identify any relationship between these parameters and protoplast viability.

Table 5.3 shows the monthly changes in sunshine hours, a plus sign indicating an increase in sunshine hours compared with the preceeding month, an minus sign a decrease. These figures are shown graphically in Fig. 5.2. As the table shows no two years were the same and in 1982 there was an increase of 114.2 sunshine hours between the months of February and March. (February 46.9 and March 161.1). Also in that year the "summer" months were particularly poor, declining by some twenty or so hours a month from June onwards and recording fewer sunshine hours than April or May. Results presented graphically are shown in Fig. 5.2 which illustrates this large increase in sunshine hours between February and March 1982 and the poor "summer" months. It is also worth noting the decrease in sunshine hours from one month to the next of nearly 80 hours between August and September 1981 and September and October 1982.

Using this information, the number of sunshine hours to which source material had been exposed prior to its use for isolation of protoplasts was also examined. The sunshine hours for 7, 14, 21 and 28 days prior to isolation were calculated. The figures are given in Table 5.4.

The data given in Table 5.4 was subjected to various analytical and statistical procedures. In particular a search was made for possible correlation between viability on isolation and the sunshine hours regime prior to isolation of protoplasts from the source material. No correlations were found between protoplast viability

TABLE 5.3

MONTHLY CHANGES IN SUNSHINE HOURS

	1980	1981	1982	1983
Dec - Jan	—	-14.9	+15.5	+3.9
Jan - Feb	—	+17.1	-10.9	-45.0
Feb - Mar	—	-1.7	+114.2	-4.1
Mar - Apr	—	+65.4	+42.2	+61.1
Apr - May	—	-4.7	+10.2	+6.9
May - Jun	—	+21.2	+10.2	+28.2
Jun - Jul	—	+1.0	-28.9	+87.6
Jul - Aug	—	+70.5	-20.0	-15.6
Aug - Sep	—	-78.8	-20.5	—
Sep - Oct	—	-59.0	-78.4	—
Oct - Nov	51.4	-54.9	-13.0	—
Nov - Dec	+6.3	+2.0	-15.1	—

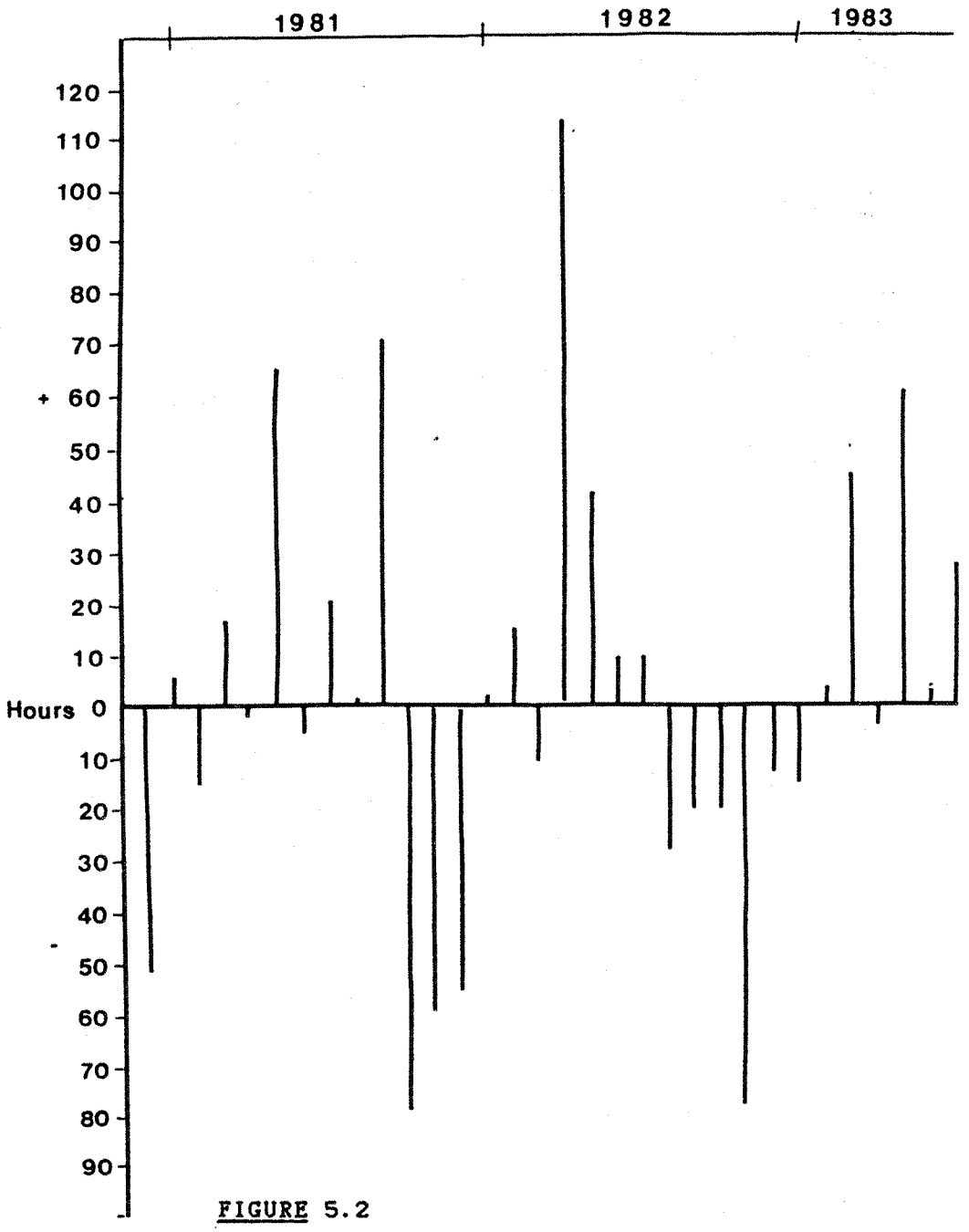


FIGURE 5.2

Monthly changes in sunshine hours at Mayflower Park, Southampton.

TABLE 5.4

Viability (%)	Date	Sunshine hours for days prior to isolation			
		7 days	14 days	21 days	28 days
93.8	24/3/81	20.6	46.8	52.5	66.4
93.5	23/4/81	60.0	103.1	25.5	149.3
88.9	7/5/81	25.8	41.9	101.9	145.0
89.9	28/5/81	40.9	79.9	106.2	131.2
73.3	11/6/81	23.7	62.8	103.7	133.3
75.4	25/6/81	37.0	74.2	97.9	137.0
75.5	2/7/81	37.7	77.8	115.0	138.7
49.1	29/9/81	28.6	49.4	90.0	134.5
78.4	6/10/81	13.2	49.8	70.6	111.2
82.0	20/10/81	13.3	31.7	44.9	78.3
74.8	27/10/81	33.8	47.1	65.5	97.9
85.2	17/11/81	5.3	22.0	40.1	73.9
83.7	8/12/81	14.8	26.4	33.0	38.3
85.7	19/1/82	19.7	27.1	29.6	30.9
62.0	16/2/82	18.0	27.8	46.3	57.3
75.3	23/2/82	6.1	24.1	33.9	52.9
71.0	9/3/82	30.6	45.1	51.2	69.2
74.2	8/4/82	30.5	82.4	115.7	152.7
73.0	27/4/82	52.0	118.8	157.8	189.7
69.0	8/5/82	44.0	96.4	163.2	195.1
64.0	8/6/82	66.8	124.8	143.1	203.7

Table shows the viability of isolated protoplasts at various times of year and the sunshine hours to which plants were subjected 7, 14, 21 and 28 days prior to their use as sources of protoplasts.

and sunshine hours 7 days, 14 days, 21 days or 28 days prior to isolation.

Changes in daylength were also investigated by collecting data from marine almanacs. Although published data was for London and an adjustment for Southampton was necessary by virtue of latitude, for the purposes of this study no such adjustment was made. The data is given in Table 5.5.

Table 5.5 shows the daylengths based on sunrise and sunset for London in 1983. As the table shows the shortest day of 7 hours and 49 minutes occurred on the 22nd of December and the longest day of 16 hours and 39 minutes on the 22nd of June. These results are shown in graphical form in Fig. 5.3. Thus daylength varies from about 8 hrs at its shortest to about 16 1/2 at its longest. The data presented in Table 5.6 shows the approximate monthly changes in daylength using the daylengths of the 1st day of the month and the last. The data in Table 5.7 shows the changes in daylength at weekly intervals.

The data in both the above tables reveal something of the nature of daylength changes over the course of a year. In January, the days were not only short, but the weekly increments in daylength were also small, ranging from 5 minutes at the start of the month to 22 minutes at the end. From beginning to end the daylength in January increased by 63 minutes. It could be argued therefore that the days were fairly consistently short.

February saw a consistent increase in daylength of about 22 minutes a week, with a monthly increase of 73 minutes. March, the time of the equinox was a somewhat different story. For this month, and to some extent both April and May, the weekly increment in daylength was 28 minutes or so, which resulted in an increase in daylength from beginning of the month to the end, of 111 minutes for March and 106 minutes for April. Therefore, although weekly increments in daylength were consistent, this resulted in an increase in daylength in a two month period, of more than three and a half hours.

During the months of May and June, the scenario changed yet again. During these two months, the weekly increments change very rapidly, being 22 minutes a week at the start of May, (about the same

TABLE 5.5

Daylengths for 1983 based on sunrise and sunset for London

Month	Daylength (hours and minutes)				
	1st	8th	15th	22nd	29th
Jan	7 55	8 04	8 19	8 36	8 58
Feb	9 08	9 32	9 57	10 24	—
Mar	10 51	11 18	11 46	12 14	12 42
Apr	12 54	13 20	13 48	14 15	14 40
May	14 48	15 12	15 34	15 54	16 11
Jun	16 18	16 29	16 36	16 39	16 36
Jul	16 35	16 26	16 13	15 56	15 37
Aug	15 26	15 05	14 41	14 20	13 49
Sep	13 38	13 11	12 45	12 17	11 49
Oct	11 41	11 14	10 47	10 20	9 54
Nov	9 43	9 18	8 56	8 35	8 17
Dec	8 13	8 00	7 52	7 49	7 52

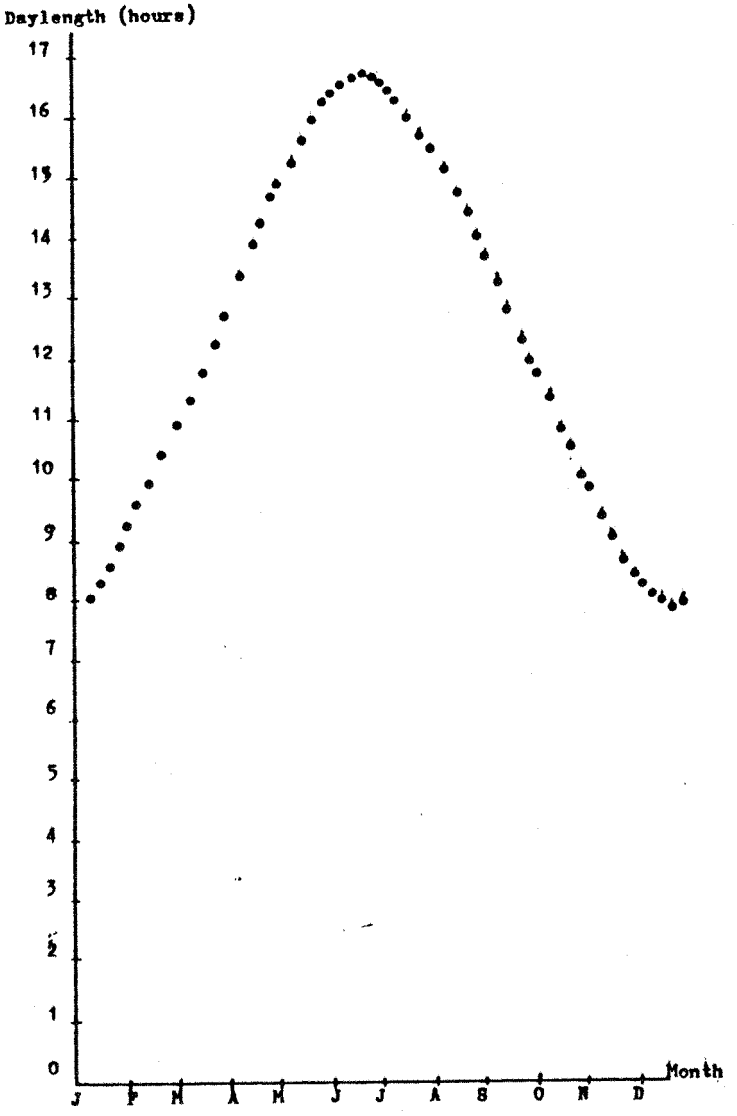


FIGURE 5.3

Changes in daylength with time of year

TABLE 5.6

Monthly changes in daylength

Month	Shortest day	Longest day	Change (in minutes)
Jan	7 55	8 58	+63
Feb	9 08	10 24	+76
Mar	10 51	12 42	+111
Apr	12 54	14 40	+106
May	14 48	16 11	+88
Jun	16 18	16 36	+18
Jul	15 37	16 35	-58
Aug	13 49	15 26	-97
Sep	11 49	13 38	-109
Oct	9 54	11 41	-107
Nov	8 17	9 43	-86
Dec	7 52	8 13	-21

TABLE 5.7

Weekly change in daylength

Month		Change in daylength (minutes/week)				
		1st	8th	15th	22nd	29th
Jan		–	05	14	17	22
Feb		–	24	25	27	—
Mar	+	–	27	28	28	28
Apr		–	26	28	27	25
May		–	24	22	20	17
Jun		–	11	07	03	03
Jul		–	07	13	17	19
Aug		–	21	24	24	28
Sep	–	–	27	26	28	28
Oct		–	27	27	27	26
Nov		–	25	22	21	18
Dec		–	13	08	03	03

as for March and April), but by the end of June had decreased to just 3 minutes a week. Thus the days were now consistently long.

This meant that although overall the daylength increased between January and June, for the first three months the weekly daylength at first increased quite rapidly, from a small weekly increase, through a large, but consistent weekly increase about the time of the equinox. From then, for the next three months to June, although daylength continued to increase, the weekly increments decreased, so that by the end of June, daylength varied by a matter of a few minutes a day.

This was repeated for the months July to December. The first three months of this period were characterised, not by increasing daylength, but decreasing daylength. However, how this was achieved is the same as for the first six months of the year. During July the weekly change in daylength increases once again, so that at the beginning of the month, the days shortened by less than they did at the end of the month, 7 minutes per week at the beginning and 19 minutes at the end. By August, the weekly decrease had changed from 21 to 28 minutes. This was similar to the situation in February and March. This month and the equinox in September again produced a consistent weekly decrease in daylength of 28 minutes, which resulted in a decrease in daylength of 97 minutes in August, 109 minutes in September and 107 minutes in October. A total decrease in daylength of some five and a quarter hours.

As with the first part of the year, although the days continue to shorten, the weekly changes to daylength again change and during November and December the weekly change reduced from 25 minutes at the start of November to just three minutes at the end of December. As with the first part of the year, although this time daylength was decreasing, the decrease in daylength followed the same pattern as for increase i.e. a period when weekly change increased, a period with a large but consistent change followed by a final period when weekly change decreased. The changes in protoplast viability which accompanied these changes in daylength are shown in Fig. 5.4. The graphical representation of weekly changes in daylength is given in Fig. 5.5.

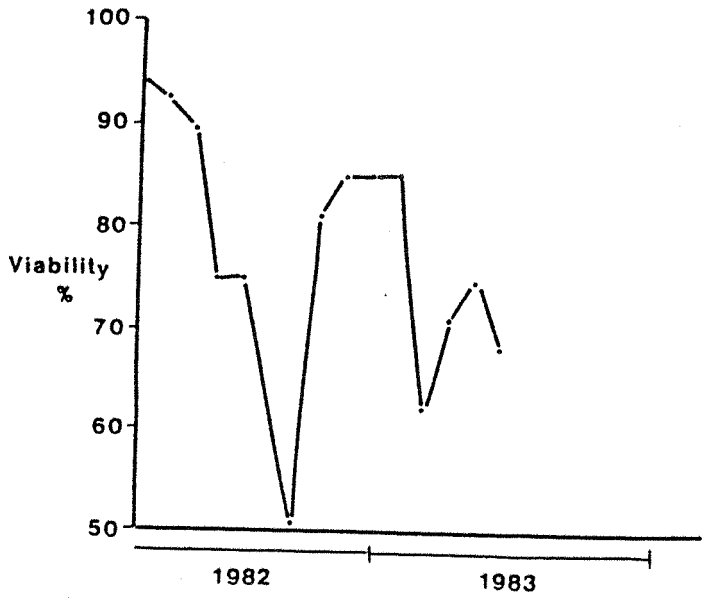


FIGURE 5.4

Changes in protoplast viability with time of year.

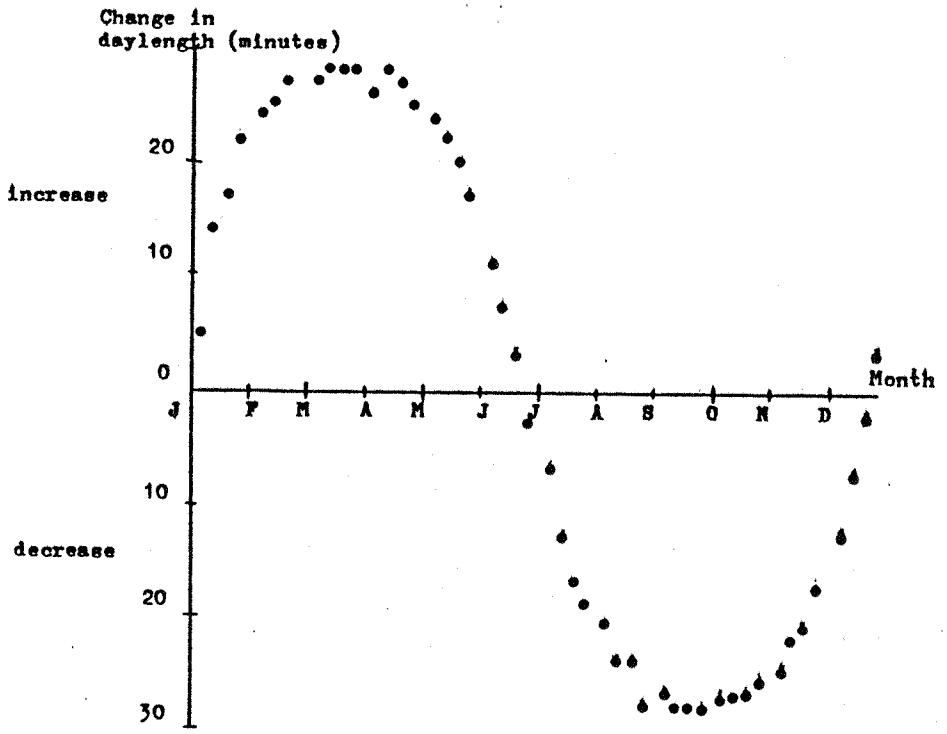


FIGURE 5.5

Approximate weekly change in daylength

Fig. 5.6 combines the changes in protoplast viability with time of year and the changes in daylength with time of year.

Although the figure appears complex, certain trends appear. Whilst not a perfect relationship, it appears that protoplast viability changes in accordance with change in daylength. As daylength increments increase so does viability, stabilises as daylength increases stabilise and falls as daylength increments decrease. Following the summer solstice daylength shortens, as these increments increase, protoplast viability remains low until the autumn equinox. Although days continue to shorten the incremental change decreases and protoplast viability begins to increase.

Further analysis of these trends would have proven useful, but was impossible as some of the data was incomplete and additional data was needed to enable a full analysis to be made.

Finally, an examination of the 16 hour daylength on which the plants were maintained was made. The data for this is shown in Table 5.8.

Although plants were maintained on a 16 hour daylength, this photoperiod was very variable. In the long days of summer the plants were in fact exposed to a daylength in excess of 16 hours, composed entirely of natural light. In the short days of winter, this same 16 hours is composed of about 8 hours of natural light and 8 hours of artificial light. This meant that the contribution made by natural sunlight to the daylength to which the source material plants were exposed ranged from some 52% in December and 100% in June and July based on mid-month daylength.

There were two other components also involved. As previous data shows the year was characterised by periods of change in natural daylength, sometimes a matter of 2 or 3 minutes a week, at other times 25 minutes a week. Between January and June these resulted in an increase in the contribution to daylength, but by no means a uniform increase, and between June and December, a decreasing contribution to daylength. It must be noted that during these periods of natural light, there may have been more or lesser amounts of sunshine. Indeed, some of the so-called winter months may better the summer months in the actual hours of sunshine recorded.

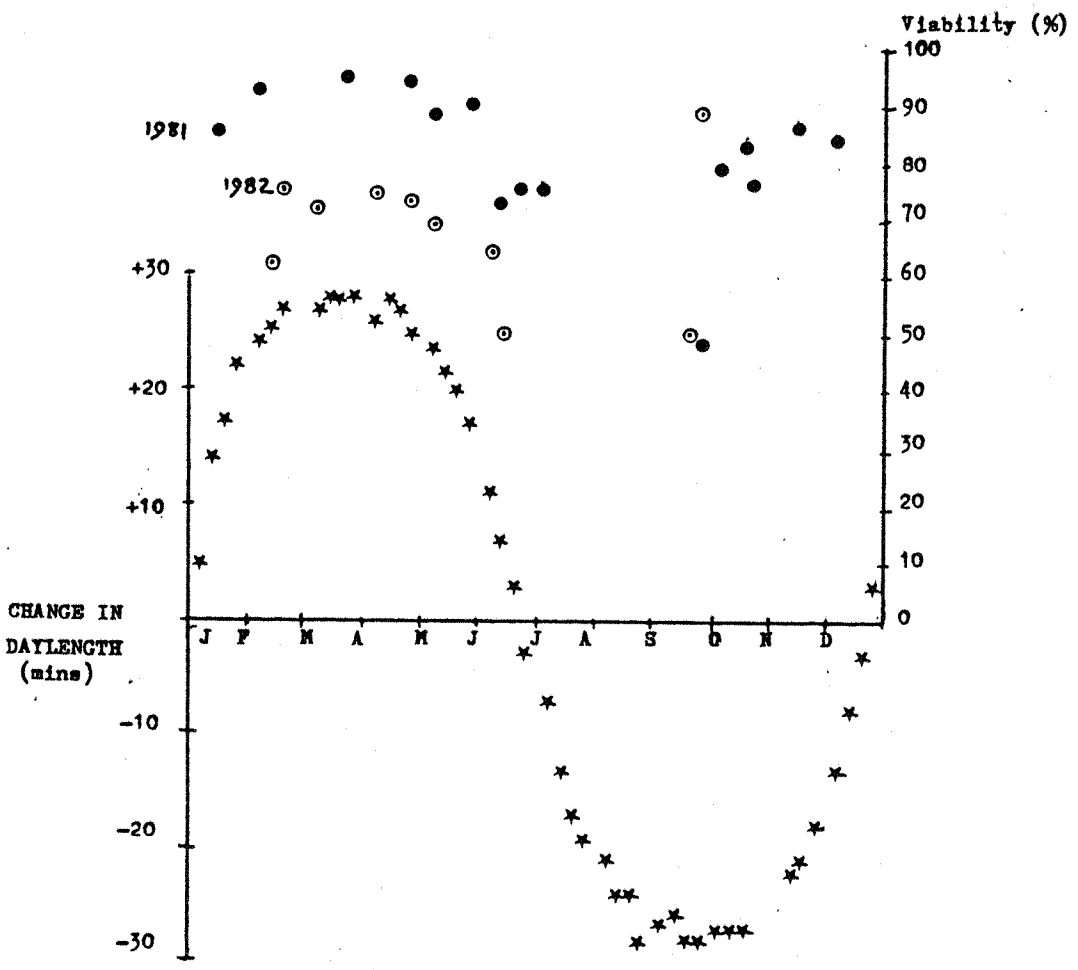


FIGURE 5.6

Changes in protoplast viability
with changes in daylength increments

TABLE 5.8

Contribution to 16hr daylength made by daylight

Month	Mid-month daylength	%
Jan	8 19	52.00
Feb	9 57	62.19
Mar	11 46	73.56
Apr	13 48	81.25
May	15 34	84.81
Jun	16 36	100.00
Jul	16 13	100.00
Aug	14 41	91.75
Sep	12 45	79.69
Oct	10 47	67.38
Nov	8 56	55.81
Dec	7 52	49.19

Thus it might be necessary, when investigating the growth conditions of source material to make some kind of distinction between daylength i.e daylight and the proportion of that daylight that included sunshine.

It is therefore apparent, that the light regime to which source material plants are exposed is multi-factorial and a carefully controlled examination of the effects of each factor needs to be assessed both independently and in combination, for any possible negative or positive interactions. However, this is easier said than done, it is not possible during the course of an experiment to control absolutely the sunshine hours component of the experiment.

If, as has been suggested, protoplast viability changed in response to changes in daylength, then source material maintained at a constant daylength in an environmental chamber might be expected to maintain viability at a given level, whilst those that are exposed to decreasing or increasing daylength might be expected to change.

The complexity of the light regime to which source plants are exposed is not likely to be the only factor affecting protoplast viability. Cassells and Barless, (1978) demonstrated that calcium was important for stabilising membranes. The feeding of additional calcium to tomato plants, and controlling light provided for plant growth, could improve protoplast survival in vitro. Using this study as a model, Cassells and Cocker, (1982) initiated an investigation to determine the range of seasonal variation in tobacco protoplast yield and viability in their laboratory. Light and supplementary feeding were manipulated in experiments on autumn and winter grown and spring and summer grown plants, to study the relationship between plant nutrition, total and membrane calcium and plant growth parameters, in relation to protoplast yield and stability. Seasonal variations in protoplast yield and viability from tobacco plants grown under glasshouse conditions were recorded with weather data. Sunshine hours were measured by a Campbell-Stokes recorder.

The tobacco seedlings they used were grown in peat supplied with a complete balanced fertiliser, Bio P base (Pan Britannica Industries, Herts UK). Plants were grown under glass throughout the year, with a daylength of 15 hours in winter produced by supplementary illumination from Phillips mercury vapour lamps.

Isolations were made from plants 40-70 days old. Balanced factorial experiments ensured each experiment contained eight feeding treatments, four age levels of plants and two light levels. Autumn and winter feeding experiments were conducted from November to January and summer feeding experiments from April to June.

The results recorded show that the change in percentage viability of protoplasts appears to be associated with changes in leaf shape, as plants age from 49-63 days, The leaf shape index (LSI) increased because leaves became narrower, and as the index increased, viability decreased. There was no noticeable change in cell release from one season to another, but protoplast release decreased during winter months. An increase in cellulase in the isolation enzyme mixture ensured 90-100% protoplast release with no detrimental effect on protoplasts. Protoplast viability changed during the twelve month period, with an optimum period from August to October when light intensities began to fall. As the number of sunshine hours per day increases from May, protoplasts from 50 day old plants did not show greater viability than those from winter grown plants, and leaves from 50 day old plants were difficult to peel during the summer months of May, June and July.

Separate examination of winter and summer experiments, coupled with an analysis of variance of results from factorial experiments showed interesting trends but were by no means conclusive.

In winter experiments, with and without supplementary lighting, there were significant, positive correlations between total calcium, membrane calcium and percentage viability for days 2, 3 and 4 after isolation V_{2-4}. Overall correlation was 0.32 which is significant at the 2% level. However, it is necessary to raise a query with this experiment, if no supplementary lighting was given, how was a 15 hour day length maintained in winter?

In later discussion it was reported that 78% of all viability values were below 60% and the wide scatter of points indicated that other factors were affecting protoplast viability independently of those being tested. When supplementary lighting was given then plant age, plant height, leaf number and leaf shape index have a significant correlation with percentage viability. Older plants gave more stable protoplasts and as LSI decreased, stability increased.

Winter feeding produced a correlation between feeding with $Ca(NO_3)_2$ and $CaCl_2$ and production of more stable protoplasts. Here plants grown under supplementary lighting tended to produce protoplasts of significantly lower percentage viability at both isolation and after.

Analysis of variance of results showed light had a strong interaction with plant age and plants grown under supplementary lighting improved with age and at 70 days produced more stable protoplasts compared to plants of similar age grown without supplementary lighting. During winter, harvesting of plants for protoplasts was recommended from plants up to an age of 50-60 days grown with no supplementary lighting.

In summer experiments, there was no significant correlations between total calcium, membrane calcium and percentage viability of protoplasts measured on days 0-5 post isolation. Plants were grown either shaded (5.3 MJ^{-2} m^{-1} day) or unshaded (16-25 MJ^{-2} m^{-1} day). For unshaded plants, plant height, plant age and LSI all negatively correlated with percentage viability for days 0-5 post isolation. The correlation coefficient was -0.6, significant at the 1% level, for the overall relationship between LSI and percentage viability and, therefore, to ensure a reasonable viability it was necessary to harvest leaves with a LSI of 0.009 or less, i.e. wide leaves. Summer feeding produced no significant results, except the ammonium sulphate feed (0.1M) which reduced both total and membrane calcium. The only feed to affect viability was $CaCl_2$, producing a reduction in viability to less than 34%. The feed treatments did not have any effect on LSI.

Feeding/light interactions were interesting, producing a significant interactive effect on viability. Shade grown controls and shade grown $Ca(NO_3)_2$ fed plants produced the protoplasts with the greatest longevity. Feeding plants ammonium under shade conditions decreased viability significantly. This interaction of light and feeding on viability also remained significant if membrane calcium was entered as co-variate in the analysis of variance. Therefore, the fact that protoplasts from shaded plants survived longer must be due to factors influenced by light and age, apart from membrane calcium.

Plant age alone had a very significant relationship to percentage viability, 17% for 63 day old plants and 62% for 42 day

old plants. Although membrane calcium increased during this time, co-variate analysis showed the effect to be independent of membrane calcium. The presence or absence of shading had no effect either. There was a significant light/age interaction, 63 day old plants, grown under shading were able to produce protoplasts which survived longer compared to plants grown without shading. Light and age also had a significant effect on LSI. Unshaded plants had relatively higher LSI values (narrower leaves). As the plants aged, the LSI continued to increase, and a significant increase in LSI occurred from days 56-63. This increase coincided with a significant increase in percentage viability of protoplasts. For both lighting conditions a LSI value of 0.008 for plants 42-49 days old corresponds with significantly higher protoplast viability. Co-variate analysis showed these effects to be independent of calcium. Their feeding experiments also showed that, irrespective of which feeding regime was used, it had to be used in conjunction with a suitable harvesting procedure.

If no supplementary light was given, plants could be harvested between 8-9 weeks after emergence. If supplementary lighting was given, older plants (with or without calcium feeding) could be used. Presence or absence of supplementary light affected leaf shape index (LSI). Spring and summer feeding has no effect on increasing total or membrane calcium. There was also no significant correlation between total membrane calcium and percentage protoplast viability from day 0-5; shade grown plants fed with calcium were no better at producing stable protoplasts than control plants. Thus, irrespective of feed treatments given, spring and summer plants, older than 50 days and grown under shaded and unshaded conditions, produced poor protoplasts despite the fact that there was more calcium present in the membranes compared to protoplasts of younger plants.

It was this study that revealed some of the true nature of the problems associated with the isolation of stable, viable protoplasts, capable of being stimulated to regenerate a cell wall in culture and subsequently divide. Longevity in culture is of little use if protoplasts do not divide. The results of the investigation carried out and discussed in this chapter demonstrate that there might be more fundamental factors affecting protoplast viability, and that before investigating the nature of the interaction between lighting regime and supplementary feeding it is necessary to determine the

nature of the plant's response to the changing light regime to which it is exposed and the reasons for a particular response or set of responses.

Chapter 6

HEAT STABILITY OF MEDIA COMPONENTS

6.1 Introduction

The necessity for investigating the effects of autoclaving on protoplasts media arose as a result of reports in Van Bragt(1971b). Scientists from very different branches of tissue culture were interested in the problems arising from the sterilisation of nutrient media. Successful plant tissue culture was not achieved until 1939, when, thanks to the discovery of the first plant growth regulator, auxin, and a fortunate choice of plant material, Gautheret, (1939), Nobecourt, (1937) and White, (1939) succeeded in culturing plant tissue. Table 6.1 shows the nutritional and hormonal requirements of a plant tissue or organ culture medium. It shows the components which may be used, but all the components given need not always be present.

A relatively simple medium consists of water, macro and micro elements, a sugar and a few growth regulators (an auxin and a cytokinin). On occasions amino acids, yeast extract, coconut milk, plant extracts or casein hydrolysate are added to the culture medium although workers prefer to avoid adding substances which may render the medium undefined.

Large volumes of culture media are made and used daily in plant tissue culture laboratories, though often with scant attention being paid to their quality and performance, except perhaps when poorer than expected results are obtained. The performance of a given medium depends on many variables, some of which may be easily controlled, others will be controlled only with difficulty and some perhaps not at all. It is generally recognised that the most important parameters are those connected with the medium itself, in other words its compositional or intrinsic factors. In addition, there are extrinsic factors such as temperature/time relationships during preparation and autoclaving and gaseous atmosphere. Finally, the culture tissue itself may influence the functioning of the medium.

In addition to control of its composition, careful attention to the mode of preparation is also necessary. Attention must be paid to

TABLE 6.1

Components which may be used in culture media

Water

Inorganic Substances		Organic Substances
Elements		
Macro	Micro	
N	Fe	Sugars
P	Zn	Vitamins
K	B	Amino acids
Ca	Mn	Growth regulators
Mg	Cu	Auxins
S	I	Cytokinins
		Gibberellins
		Abscisic acid

Undefined mixtures: Yeast Extract
Coconut Milk
Casein Hydrolysate

the quality of glassware and water to be used in preparation, to the mode of dissolving dry ingredients and the way in which any labile compounds are to be dissolved and added.

The usual method of sterilisation for culture media is thermal, using wet heat i.e. steam under pressure in an autoclave. The consequence of thermal sterilisation on components of culture media is well known, (Foster, 1952; El Miladi et al, 1969; Bridson and Brecker, 1970). The main type of reaction occurring during heating is the Maillard reaction in which NH compounds are coupled with carbonyl groups. Such primary Maillard complexes and their decomposition products are not without effect. Also, related to this heat induced coupling is the caramellisation of sugars, leading to higher molecular weight, often brown, compounds (Peer, 1971). Hence the mode of heat sterilisation may greatly affect the functioning of sugar containing protoplast culture media through these two reactions.

Sterilisation by heat also inactivates B-group vitamins. The extent to which this occurs depends on the type of vitamin and the various components of the medium (Ford et al, 1969). (Table 6.2).

Some of the chemical changes in media may continue during storage. Although the rate of reactions will be very much slower due to temperature differences of upwards of 100°C or more, long storage may compensate for this. Even more important are the reactions which occur due to the addition of compounds after sterilisation.

Differences in pH and composition of media after sterilisation are therefore unavoidable. The problems with pH might be solvable if buffers are used. Acetate, phosphate, ethanolamine and TRIS might be suitable, but all of these will participate in heat activated reactions which implies that only relatively high buffer concentrations can be used, which then alters the composition of the medium. They may also prove directly toxic to protoplasts at the concentrations used.

Sterilisation at room temperature is not without problems. The filtration of liquid media may, in principle at least, also lead to changes in composition, affecting the cultural value of the sterile filtrate. All filters allow some adsorption of polar components, the

TABLE 6.2

Stability of growth regulators

	pH		UV light	Oxidation	Reduction	Heavy metals	Heat
	<7	>7					
Thiamine HCl ($Vit B_1$)	+	—	—	—	—	—	0
Pyridoxine HCl ($Vit B_6$)	+	—	—	—	—	—	0
Nicotinic acid	0	0	0	0	0	0	0

— poor stability 0 fair stability + good stability

degree of adsorption depending on the structure and thickness of the filter used, the composition of the medium and the rate of filtration; in practice these effects will vary but only slightly, at least with currently used types of filter, (van Bulck, 1971).

Similar considerations may still apply for methods of sterilisation other than heat. Protoplast culture media contain vitamins, which have differing physico-chemical properties. (Table 6.2) As the table shows these vitamins have differing stabilities to heat, UV light, presence of heavy metals and pH, and the addition of vitamins to culture media prior to sterilisation by heat is not advisable. Filter sterilisation of the vitamin and addition after sterilisation is to be preferred, although this too has limitations.

Plant growth regulators incorporated into protoplast culture media also need considering. Sterilisation by filtration has no effect as far as auxins are concerned, but sterilisation by UV light is disastrous for IAA, as it rapidly destroys it. Auxins are not destroyed by autoclaving at 110-120°C during 15-30 minutes or even an hour, but only if the medium is not strongly acid or alkaline. For this reason NAA and 2,4 D, which are less destruction sensitive than IAA are usually used if sterilisation by UV is involved. Similarly, cytokinins, compounds that induce cell division in plant cells in co-operation with an auxin, are also included in culture media but are considered stable to autoclaving.

In the succeeding experiments attention has been focused on autoclave cycle time, filter sterilisation and pH, and how these effect the subsequent performance of protoplast culture media.

6.2 Variations to materials and methods

Tobacco mesophyll protoplasts were obtained in the usual way. These were subsequently cultured in variously prepared MSP 9M cultures. Initial viability was determined and several replicates of each culture medium preparation method set up. After one week in culture the plating efficiency of each culture was determined. In addition, the proportion of protoplasts dividing of those alive at the start of the culture period was also determined.

The MSP 9M medium used in this experiment was prepared as follows. Basic Murashige Skoog medium plus 9% (w/v) mannitol subjected to a short autoclave cycle time of less than 25 minutes in a small portable autoclave. A second portion of the same medium was subjected to a long autoclave cycle time in excess of 75 minutes in a large automatic autoclave. A third portion was given no heat treatment at all and filter sterilised. The final two culture media consisted of a sample of medium which had been autoclaved in a long cycle to which was added filter sterilised thiamine hydrochloride to give an expected final concentration of 0.5 mg per litre. The other was non-heated medium to which was added filter sterilised thiamine hydrochloride to give a final concentration of 0.6 mg per litre assumed to comprise 0.5 mg per litre addition and 0.1 mg per litre present in the original MSP 9M culture.

A second experiment was run simultaneously. Filter sterilised culture medium which had not been subjected to any heat treatment was divided into small aliquots so that 0.1 ml of concentrated protoplast suspension could be added to them to give a final culture density of 5×10^4 per ml. This meant there was no significant carry over of culture medium on plating out. A pre-trial was carried out to determine how long it took these aliquots to reach 100°C in a steamer. Sufficient aliquots were then placed in the steamer for the number of replicates required. As soon as a time equal to that of the pretrial time had elapsed the required number of aliquots were removed and rapidly cooled. This was considered to be zero time. The remaining aliquots were left in the steamer and a number removed at 10 minute intervals and also rapidly cooled. The final aliquots were removed after 100 minutes in the steamer, (excluding pre-trial time). These steamed liquid MSP 9M samples were then used as culture media for protoplasts.

A similar procedure was repeated with samples of MSP 9M containing Oxoid No 3 agar. It was not possible to prepare the agar medium without some addition of heat to melt the agar. To reduce this heat treatment to as little as possible, the following procedure was adopted. Double working strength MSP 9M was prepared and filter sterilised. A newly opened container of Oxoid No 3 agar was used to aseptically prepare double working strength agar, which was then heated to melting point. Once melted, the two were rapidly mixed and dispensed into working volume aliquots and allowed to set. The

aliquots were then placed in a steamer and allowed to melt. The first required numbers were removed and concentrated protoplast suspension added when the agar had cooled to 50°C to give a final culture density of 2.5 x 10^4 per ml. Further aliquots were removed at 10 minute intervals and similarly treated. These steamed agar samples were used as culture medium for protoplasts.

As an internal control, medium which had been subjected to a short cycle time was used. The medium was placed in an autoclave with steam already present, and brought rapidly to pressure and held at 121°C for 15 minutes. The autoclave was rapidly depressurised, the medium removed and rapidly cooled to 45°C in a water bath. A long autoclave cycle time was achieved by placing medium in a cool autoclave and subjecting it to a full cycle of heating, holding time and slow cooling. This time was in excess of two hours. Filter sterilised medium was not heated at all. Three further media were obtained by using media which had been subjected to a long cycle time and to which was added concentrated filter sterilised thiamine hydrochloride to give final concentrations of 0.1 mg l^{-1}, 0.5 mg l^{-1} and 0.6 mg l^{-1}, the final medium comprising 0.5 addition and 0.1 assumed to not have been lost from the medium during filter sterilisation. It was of course not possible to prepare MSP 9M agar media without using some heat.

6.3 Results and Discussion

Experience in a busy tissue culture laboratory soon indicated that no two autoclave loads were subject to the same temperature/time cycle during sterilisation in an autoclave. Indeed the loading level itself affected the total time that the autoclave took to reach 100°C, (heating), the time taken to reach 121°C and the time taken to cool. In addition, the introduction of an autoclave incorporating additional safety devices meant that the culture media could not be removed and cooled more quickly until the temperature had fallen to below 80°C.

The course of temperature versus time of a sterilisation shows (Fig. 6.1), that the medium is subjected to a number of conditions during a complete autoclave cycle. For each of these conditions a distinct equilibrium and a set of specific temperature dependent reaction rates apply. The reactions involved include, precipitation

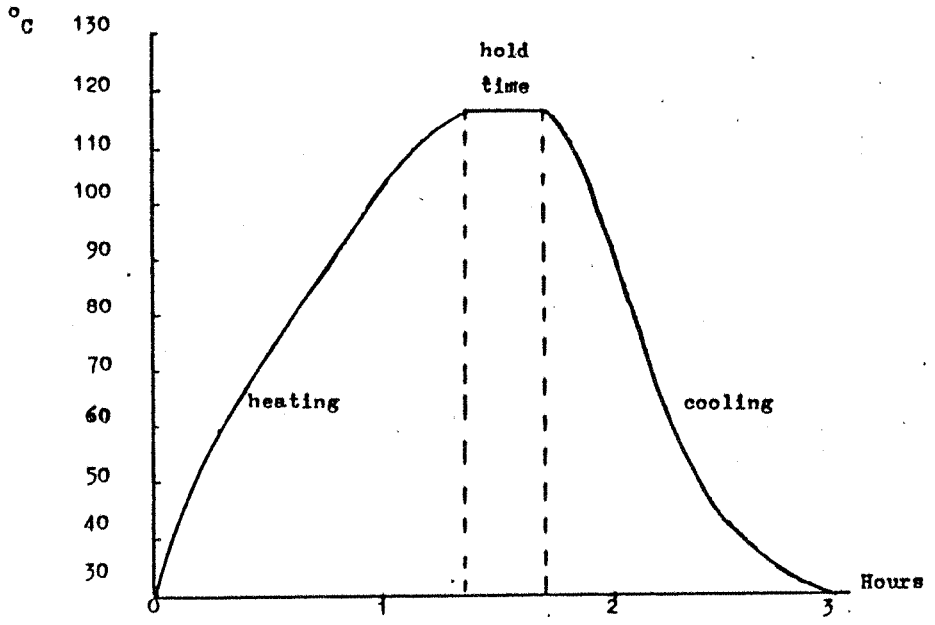


FIGURE 6.1

Typical temperature/time
cycle in an autoclave

of salts, neutralisation or esterification of acids, and hydrolysis of proteins, polysaccharides and esters. Further reactions could involve polymerisation or depolymerisation of alcohols, aldehydes and saccharides. The reactions are pH dependent and result in a pH change. The total sum of what has happened at the end of sterilisation is thus dependent on the course of the temperature, the rate of heating and cooling and exact holding temperature and time.

Further, the temperature inside an autoclave is not homogeneous, especially during heating and cooling, which means two bottles of media in the same autoclave do not get quite the same temperature course or heat treatment. The temperature course will not be the same for two different loads and more so if different autoclaves are used.

The results in Tables 6.3, 6.4 and 6.5 show the effects on plating efficiency when MSP 9M liquid or agar culture medium is subjected to a period of heat treatment in a steamer. The results from replicate A of Table 6.3 are shown graphically in Fig. 6.2. In both liquid and agar culture medium, the plating efficiency was progressively reduced as length of the exposure period increased. In liquid culture medium the plating efficiency was reduced from 74.6% in medium which had been prepared and sterilised using no heat, to 68.0% in medium which had been exposed to a short period of heat at 100°C and reduced still further to 43.0% if the heat treatment period was extended to 100 minutes. In agar culture, a similar situation occurred, a reduction from 58.9% in unheated medium to 35.8% in medium held at 100°C for a short time and finally reduced to 2.5% when the agar was held at this temperature for 100 minutes. A regression analysis of the data for both experiments produced a statistically significant correlation coefficient. The results were therefore worthy of further investigation.

A graph of plating efficiencies obtained in steamed MSP 9M agar against those achieved in MSP 9M liquid was plotted, (Fig. 6.3 and 6.4) and a regression analysis performed. This produced a straight line of gradient 0.712 and intercept -17.49. The correlation coefficient was 0.48. This suggested that the fitting of a straight line to the data as plotted was not statistically reliable. The scatter of points about this line was then investigated using the Durbin-Watson statistic.

TABLE 6.3

Effects on plating efficiency of autoclaving culture media

Medium	Plating Efficiency % Replicate			Plating Efficiency as % of viability. Replicate		
	A	B	C	A	B	C
Autoclaved short cycle (i)	52	32	30	71	43	37
Autoclaved long cycle (ii)	39	22	23	54	30	29
Filter sterilised (iii)	61	-	32	84	-	40
Autoclaved long cycle (iv) plus thiamine (0.1)	63	39	34	85	52	43
Autoclaved long cycle (v) plus thiamine (0.5)	68	33	30	91	44	38
Filter sterilised (vi0 plus thiamine (0.6)	57	49	29	79	65	37

TABLE 6.4

Effect on plating efficiency of steaming of MSP 9M liquid

Steaming Time (Minutes)	Plating Efficiency(%)
0	68.0
10	61.0
20	56.0
30	53.0
40	50.0
50	46.0
60	46.0
70	43.0
80	39.0
90	38.0
100	43.0

Viability of protoplasts 88.9%

Plating Efficiency in unheated medium 74.6%

Regression Analysis:-

Slope = -0.265

Intercept = 62.63

Correlation coefficient = -0.938

TABLE 6.5

Effects on plating efficiency of steaming of MSP 9M agar

Steaming Time (Minutes)	Plating Efficiency(%)
0	35.8
10	29.0
20	23.0
30	19.0
40	13.5
50	10.3
60	8.0
70	6.3
80	4.0
90	3.0
100	2.5

Viability of protoplasts 93.5%

Plating Efficiency in unheated medium 58.9%

Regression Analysis:-

Slope = -0.326

Intercept = 30.32

Correlation coefficient = -0.959

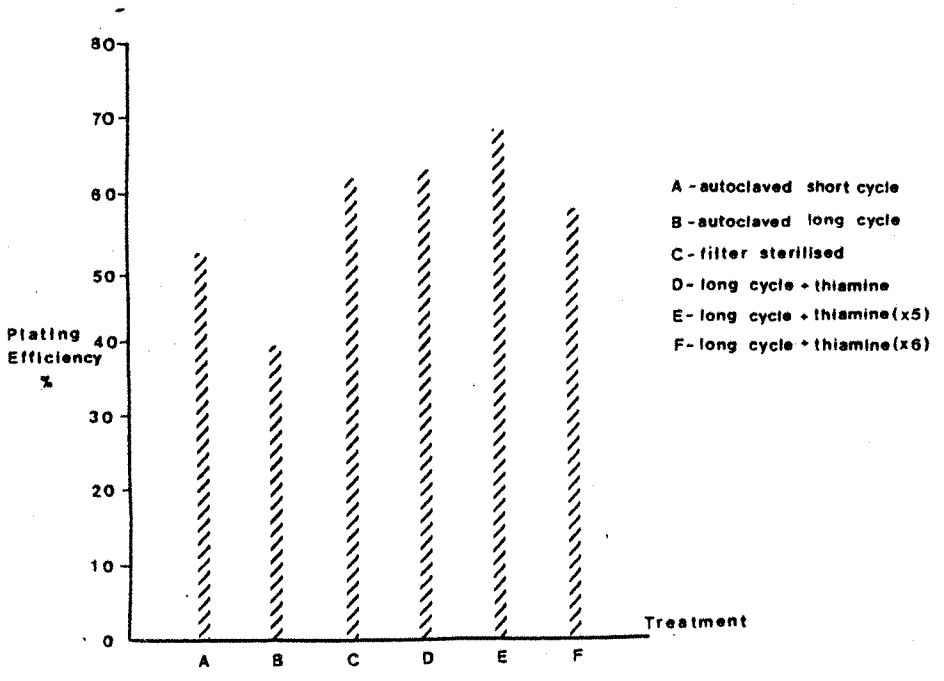


FIGURE 6.2

Effects of culture medium preparation conditions on plating efficiency.

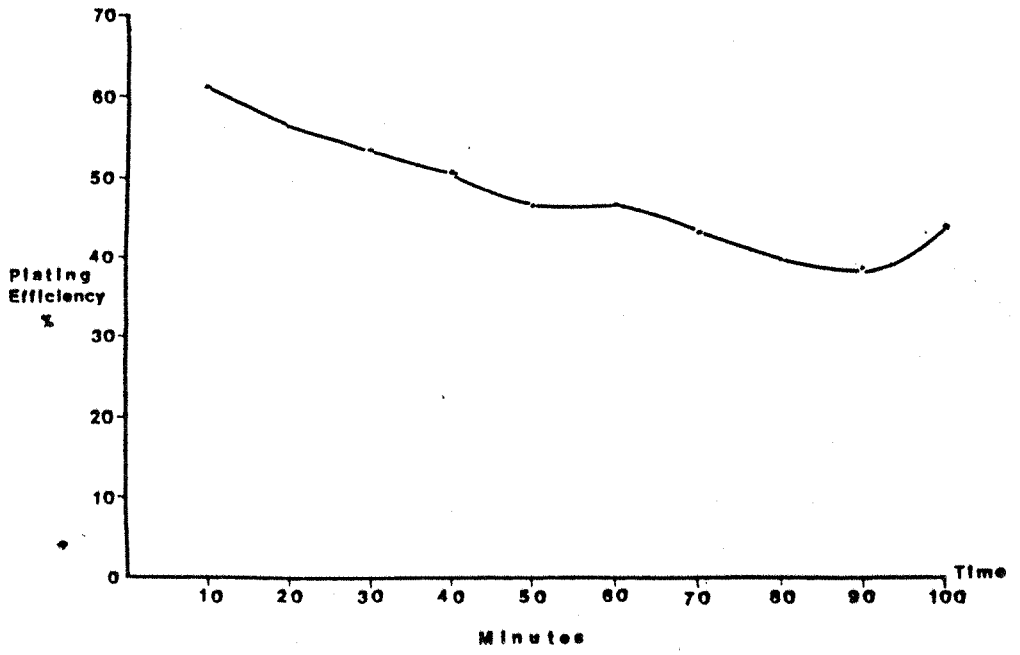


FIGURE 6.3

Effect on plating efficiency
of free steaming of MSP 9M liquid culture medium.

Small volumes of culture medium were placed in a steamer and free steamed. Media was then removed at regular intervals and rapidly cooled to room temperature. This steamed culture medium was used for the culture of protoplasts.

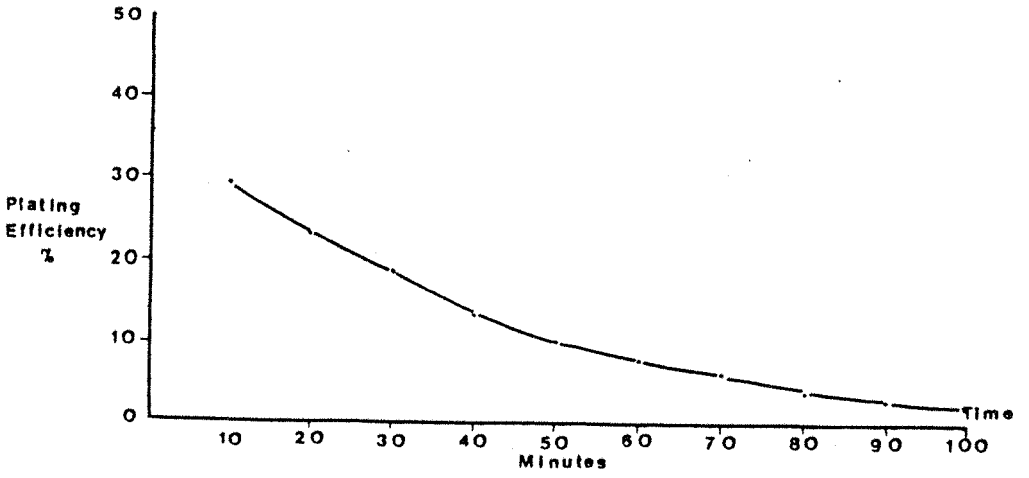


FIGURE 6.4

Effect on plating efficiency
of free steaming of MSP 9M agar culture medium.

The same procedure was adopted as for liquid culture medium (see Figure 6.3). However, the medium was cooled only to 50°C and then protoplasts were added, plates poured and then rapidly cooled.

The Durbin-Watson statistic d for auto-correlation examines the residuals e_i for evidence of non-randomness. The test was carried out by an initial least squares regression calculation so that the values of the residuals could be determined for the experimental data.

Using the equation of the regression line, the predicted value of y for each value of x was calculated. This predicted value of y was subtracted from the observed value of y to generate residual values. Each of these values was then used to calculate the statistic d. The value of d should be as close to 2.0 as possible. If the value obtained was below 2.0 the calculation was complete. If greater than 2.0 then the calculated value was subtracted from 4.0. In general, the closer to 2.0 the value of d, the less cause there was to suspect non-randomness of scatter of points. Critical value tables are available for large samples but it was possible to calculate a rough critical value for small samples using the relationship 1-d/2 and comparing the value obtained with the correlation coefficient for the appropriate sample size.

A least squares regression line was also fitted to the data points generated by a plot of plating efficiencies from replicate B against replicate A.(Fig. 6.5). This line should ideally pass through zero, or at least not deviate significantly from zero. If this result occurred, then effects occurring in A have also occurred in B. It was also necessary to test the scatter of points about this line of best fit. The scatter should be random, but there may be a pattern to the scatter of points, and so-called autocorrelation may have been taking place. It could also have been an artefact generated by fitting a straight line to a set of data which would have been better described by a curve.

These figures calculated in Table 6.6 were used to calculate the sample slope (b).

$$b = \frac{251.0}{498.8} = 0.503$$

The sample intercept (a) was then found by substituting in the formula:-

$$a = 35 - (0.503 \times 55.8) = 6.933$$

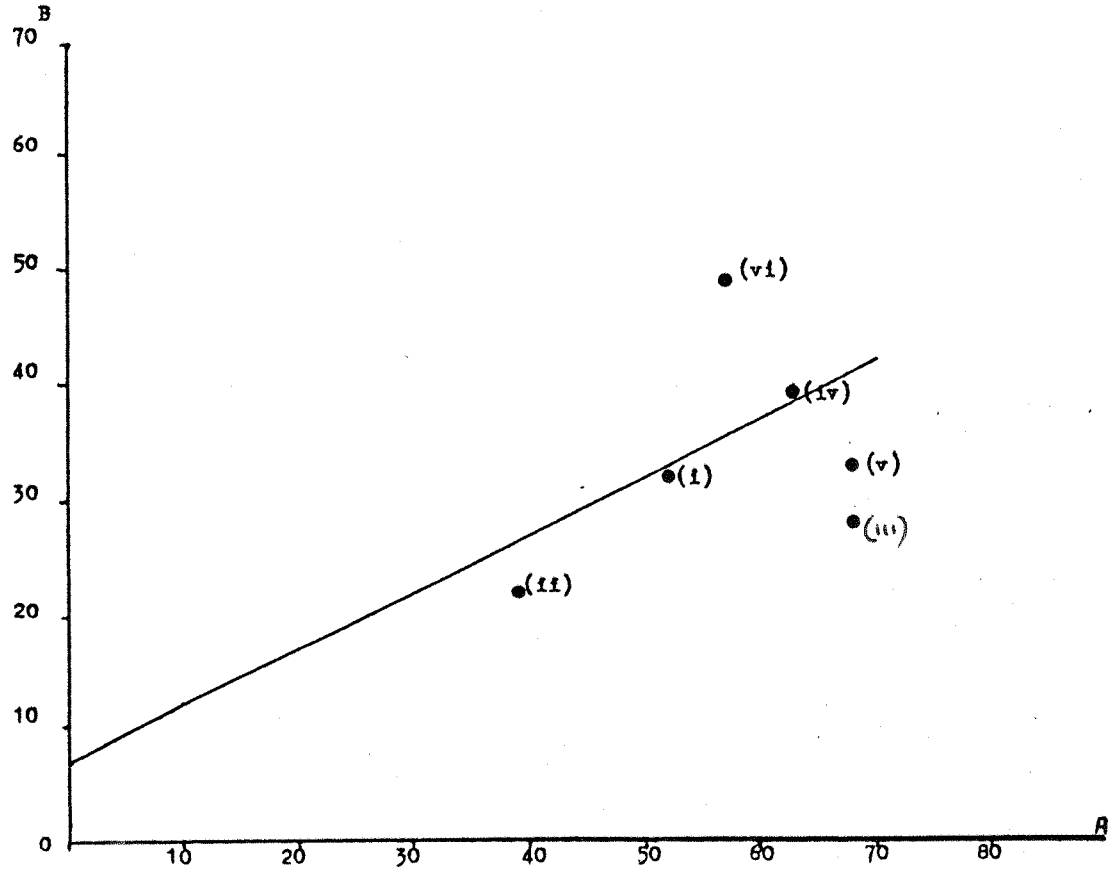


FIGURE 6.5

Regression analysis of replicate B against replicate A

This figure shows the analysis of the results obtained from replicates A and B for the five heat treatment regimes imposed on liquid MSP medium. A straight line has been fitted to the points using regression analysis and scatter of the points about the line was investigated using the Durbin-Watson statistic d.

TABLE 6.6

Sample calculation

A(x)	B(y)	xy	y (obs)	y (pred)	e_i
39	22	858	22	26.550	−4.550
52	32	1664	32	33.089	−1.089
57	49	2793	49	35.604	+13.396
63	39	2457	39	38.622	+0.388
68	33	2244	33	41.137	−8.137

$\Sigma x=279$ $\Sigma y=175$ $\Sigma xy=10016$ $n=5$

$\Sigma x^2=16067$ $\Sigma y^2=6519$ $\bar{x}=55.8$ $\bar{y}=35.0$

$SS_x=\Sigma x^2 -(\Sigma x)^2/n$

$SS_y=\Sigma y^2 -(\Sigma y)^2/n$

$SS_{xy}=\Sigma xy - (\Sigma x.\Sigma y)/n$

$(S_x)^2 = SS_x/n-1$

$(S_y)^2 = SS_y/n-1$

Sample slope $= SS_{xy}/SS_x = b$

Intercept on y axis = $y - b.x$

$$SS_x = 16067 - \frac{(279)^2}{5} = 16067 - 15568.2 = 498.8$$

$$SS_y = 6519 - \frac{(175)^2}{5} = 6519 - 6125 = 394.0$$

$$SS_{xy} = 10016 - \frac{(279 \times 175)}{5} = 10016 - 9765 = 251.0$$

$$(S_x)^2 = \frac{498.8}{4} = 124.7$$

$$(S_y)^2 = \frac{394.0}{4} = 98.5$$

TABLE 6.7

Using the data for replicate B against replicate A.
(Table 6.6)

The terms required

Numerator $[-4.550-(-1.089)^2]$
$+[-1.089-13.396^2]$
$+[13.396-0.388^2]$
$+[0.388-(8.137)^2]$

Denominator $(-4.550)^2$
$+(-1.089)^2$
$+(13.396)^2$
$+(0.388)^2$
$+(-8.137)^2$

$$d=\frac{11.9785 + 209.9195 + 169.2081 + 72.6756}{20.7025 + 1.1859 + 179.5493 + 0.1505 + 66.2108}$$

$$=\frac{463.7817}{267.7990}$$

$$=1.7318$$

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Using these values, the predicted value of y was calculated for each value of x in the experimental data. This predicted value was subtracted from the observed value of y to generate residual values. Each of these values was then used to calculate the statistic d.

$$d = \Sigma(e_i - e_{i+1})^2/\Sigma e_i^2$$

Since this value was less than 2.0 there was no reason to suspect a non-random distribution of points about the line of best fit to the data. Using the equation 1 - d/2 the observed value of 0.1341 was obtained, the critical value of the correlation coefficient is 0.878. Since the observed value was less than this, the initial conclusion of random scatter of points about the line of best fit was confirmed.

The statistical procedure was repeated for replicate C against A, and replicate C against B. The following results were obtained.

Replicate C against A. (Fig. 6.6). Regression analysis produced a line of slope 0.3018, intercept 12.565 and correlation 0.827. Examination of residuals and calculation of the Durbin-Watson statistic produced a value of 2.3464. Since this value was greater than 2.0 the desired value was obtained by subtracting the calculated value from 4.0, which produced a value for d of 1.6536. This indicated that the scatter of points about the line of best fit was random. Confirmation of this conclusion was obtained by comparison with the correlation coefficient for n = 6.

Replicate C against B. (Fig. 6.7). Regression analysis produced a line of slope 0.2360, intercept 20.94 and correlation 0.591. Examination of residuals and calculation of the Durbin-Watson statistic produced a value of 0.6955. This value deviated considerably from the desired value of 2.0, however, there was still no reason to suspect non-randomness. This was confirmed by examination and comparison with the correlation coefficient critical value of 0.878 for n =5.

The analysis of results from the graph of plating efficiencies obtained in steamed agar against those of steamed liquid were less straight forward. The correlation coefficient of 0.48 means the line of best fit was not statistically reliable, or expressed

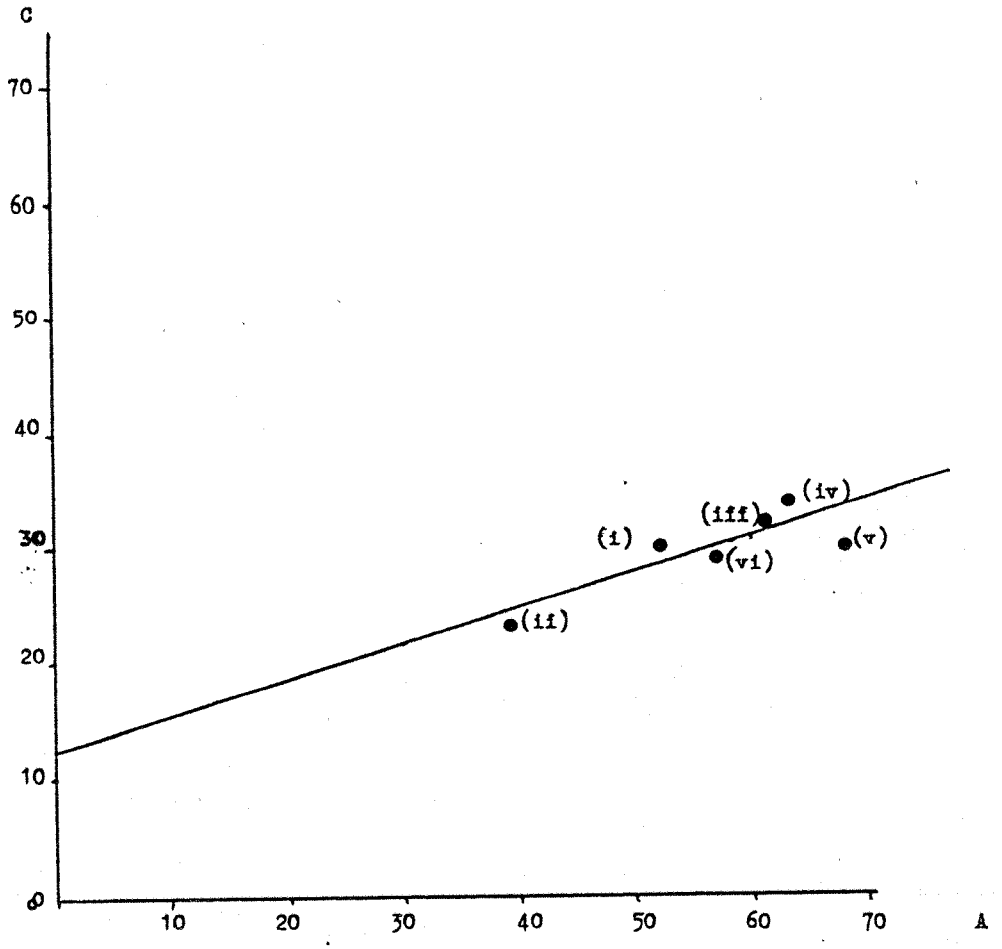


FIGURE 6.6

Regression analysis of replicate C against replicate A

This figure shows the analysis of the results obtained from replicate A and C for the five heat treatment regimes imposed on liquid MSP medium. A straight line has been fitted to the points using regression analysis and scatter of the points about the line was investigated using the Durbin-Watson statistic d.

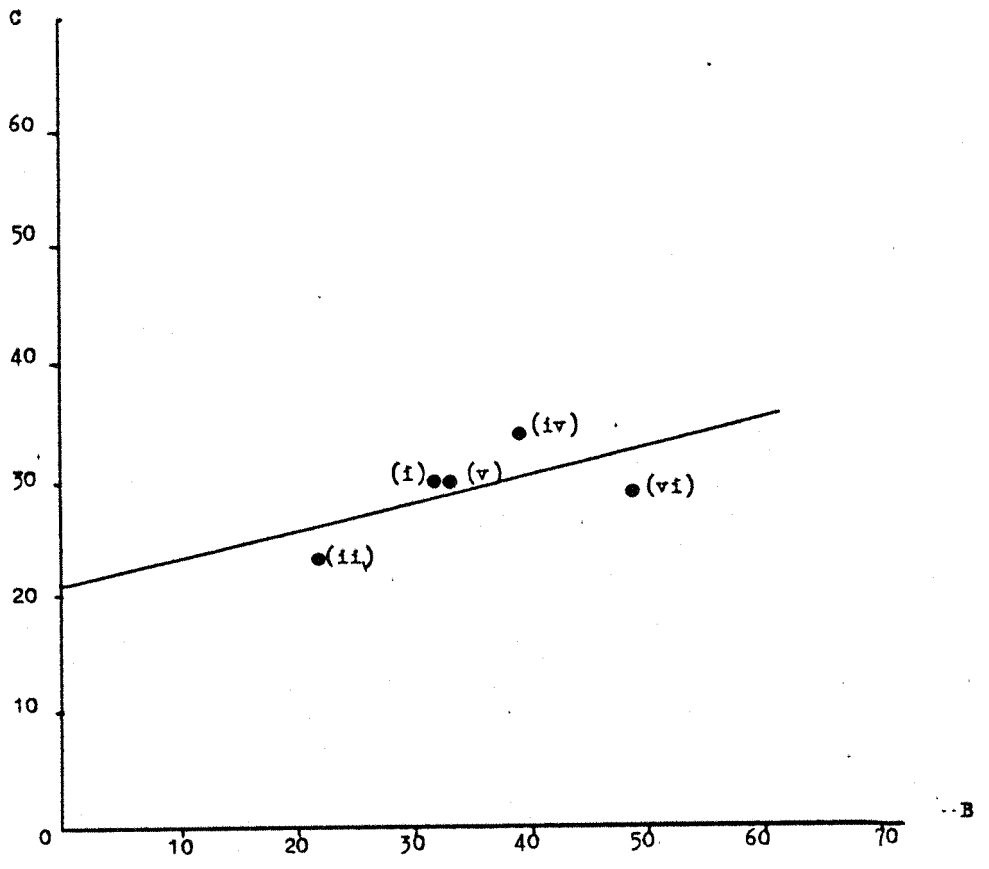


FIGURE 6.7

Regression analysis of replicate C against replicate B

This figure shows the analysis of the results obtained from replicate B and C for the five heat treatment regimes imposed on liquid MSP medium. A straight line has been fitted to the points using regression analysis and scatter of the points about the line was investigated using the Durbin-Watson statistic d.

another way, the scatter of points about the straight line was not random.

This may have occurred for one or both of two reasons, firstly, auto-correlation was taking place, or secondly, an artefact had been created by fitting a straight line to a set of points better described by a curve. The Durbin-Watson test when applied to these results showed that the scatter of points about the straight line was not random. The calculated value of d was 0.124 and using the approximation 1 -d/2 a critical value of 0.938 was obtained. As this is larger than the correlation coefficient for n=10, the non random scatter of points about the line was confirmed and auto-correlation and/or artefact creation had occurred. Closer examination of the points on the graph suggested a curve better described the points, and artefact creation was the more likely explanation.

Replicates analysis proved that the effects that occurred in one liquid batch also occurred in another liquid batch, i.e. real effects. Similar results occurred if the culture medium was agar. Thus steaming was having an effect on MSP 9M culture medium and in a way which produced repeatable results in a number of replicates.

As discussed earlier, the fitting of a straight line to the points was not statistically reliable and a curve better described the results. This curve was broadly 'S' shaped, suggesting that the effects of steaming were indeed different in liquid and agar. In liquid culture, a steaming period of 100 minutes produced a 25% decrease in plating efficiency, an average of about 0.25% decrease per minute. In agar culture, the protoplasts, although from the same isolation batch, behaved differently, and produced a lower plating efficiency even in unsteamed (though warmed) agar. The 100 minutes steaming in this experiment produced a 33% decrease in plating efficiency, an average of about 0.33% per minute. Although these decreases per minute were of the same order of magnitude, the effects in agar were some 33% more than in liquid culture.

Further examination of the figures in tables 6.3 and 6.4 also showed that the decline in plating efficiency is not uniform. In the case of liquid culture more than half the eventual decline in plating efficiency occurred in the first 30 minutes of steaming, i.e. during the heating phase of the autoclave cycle. This also held true for the

agar culture. Thereafter, the decline in the plating efficiency obtained was much more regular in both cultures although there were still differences.

In the liquid culture, the first 30 minutes of steaming appeared to have caused most of the changes possible in the culture to occur, and thereafter a gradual and steady decline in plating efficiency was obtained until the end of the experiment. This was not the case in the agar culture.

In the agar culture, a further substantial decline in plating efficiency was obtained from the agar subjected to a further 10 minutes of steaming, and only after 60 minutes of steaming did a steady and gradual decline in plating efficiency occur. These results perhaps reflect in some way the differences in the physical nature of the medium.

The statistical analyses of replicates showed these effects to be real. These effects, whatever they might be, occurred to the same extent and the same way in replicates treated to the same heat exposure regime. This was true of replicates of liquid culture medium and agar culture medium. The effects occurring in liquid medium and those in agar medium were, however, not the same. This was the reason that a graph of agar plating efficiencies against liquid plating efficiencies did not produce a statistically reliable straight line when analysed by the Durbin-Watson statistic. The 'S' shaped curve revealed in part some aspects of the nature of these events.

The first twenty minutes of steaming of agar caused a decrease in the plating efficiency achieved of 12.8% whilst that period of steaming of liquid caused a decrease of 13.0%. This suggested that whatever changes were being induced in the culture medium by heat were taking place in both types.

For the next thirty minutes, (20 - 50 minutes), the effects of heat were not the same. The heat treatment caused a further 12.7% decrease in plating efficiency achieved with agar culture medium whilst only a 6.0% further decrease was caused in liquid culture medium. This suggested that the changes occurring in the culture medium as a result of heat treatment occurred at different times. The

physical nature of the culture medium here appeared to play some role, liquid medium responding to heat treatment faster than agar culture. The agar culture responding slower to the heat treatment but for longer.

The results for the remaining period of time were also worthy of note. From 50 to 100 minutes steaming, the heat effects on the agar caused a further 11.0% decrease in the plating efficiency obtained, whilst this period of time caused only a further 3.0% decline in plating efficiency in liquid. This would tend to confirm the suggestion made earlier that whilst liquid cultures change rapidly in response to heat treatment, agar cultures respond less fast, but for longer.

For both types of culture the 100 minutes heat treatment was often within the period of time required for the autoclave to reach the required sterilisation temperature. It was possible that still further changes occurred during sterilisation and cooling which this particular series of experiments did not investigate.

The decline in plating efficiency with increasing time of exposure to heat may have been due to any number of reasons. As a means of demonstrating just one possible effect, the experiment outlined earlier was run. In the experiment culture medium was used which had been subjected to various heating regimes, or no heat at all. As Table 6.2 shows, there are a number of substances in culture media which are susceptible to changes induced by pH, light and heat. One such substance is thiamine hydrochloride, (Vit B_1), a constituent of MSP culture medium. The results of this experiment are shown in Fig. 6.2.

Treatments A and B were both subjected to a period of treatment in an autoclave. Treatment A was a short period of heat treatment in a small portable autoclave, whilst treatment B consisted of treatment in a large autoclave incorporating a number of safety features so that material could not be removed from it until the temperature had cooled to 80°C. This meant that the long cycle was between three and four times longer than the short cycle.

As the results show, the plating efficiency obtained in the long cycle treated culture medium was 39% compared to 53% in the short

cycle treated medium, an effective decrease of 25%. If these results were compared with those for treatment C, which were for a culture medium given no heat treatment, but filter sterilised, then both heat treated cultures gave a reduced plating efficiency. Treatment C gave a plating efficiency of 62%, indicating that even a short period of heat treatment given to treatment A altered the culture medium sufficiently to produce a decrease in plating efficiency. In absolute terms, a short period of heat produced a 15% reduction in plating efficiency and a long period of heat a 37% reduction.

Treatment D consisted of culture medium subjected to the same long cycle time as that in B, but to which had been added, after sterilisation, filter sterilised thiamine hydrochloride equivalent in amount to that present in MSP medium. (It was assumed that heat had destroyed that originally present, although thiamine hydrochloride is said to have fair heat stability). As the results showed, the plating efficiency obtained from this culture was 64%, the same as that for treatment C, the filter sterilised culture.

Treatments E and F were included to ascertain whether any further increase in plating efficiency could be achieved by increasing the amount of thiamine present in the culture. As the results showed, only when thiamine was increased to a level five times that normally present in the culture was any further increase in plating efficiency achieved, an increase from 64% to 68%. Interestingly, when the amount present was increased to six times that normally present the plating efficiency achieved decreased. This suggested some inhibitory effect, as filter sterilised medium, (no heat treatment, and therefore no degradation) produced a similar decrease in plating efficiency when additional thiamine was added to it, to produce a level of six times that normally present.

These findings suggested it would be worth investigating the effects of heat treatment and autoclaving on some of the other materials present in MSP culture medium.

When, in 1981, Roscoe and Bell reported the use of a pH indicator, bromocresol purple, in protoplast culture media, the effects of autoclaving on the pH of medium adjusted prior to autoclaving where investigated incidentally to the main experiments in progress. Bromocresol purple possesses a number of attractive

features. A high sensitivity to pH changes in the region of 5.5 - 6.0, stability on autoclaving, no detectable effect on protoplast colony forming efficiency and offers a means of detecting metabolic activity of the cultured protoplasts, by changing from purple to yellow. Bromocresol purple exhibits colour changes from yellow through grey to blue as acidity decreases, and it is possible to detect differences in pH of 0.1 units. The colours are stable to autoclaving at both 115°C and 121°C for 20 minutes. Colours are masked if the dye concentration is too high and a concentration of 8 ug/ml was routinely used. A further advantage of incorporation of the indicator is that of a colour change if an incompletely rinsed bottle, pipette or Petri dish is used. Also, the usually grey agar turning yellow within a few days of incubation, indicating metabolic activity of protoplasts. This colour change is an indication of at least 0.5 unit change in pH within the culture.

As they pointed out, protoplast culture media containing an osmoticum such as mannitol or sorbitol, plant growth regulators, an energy source and low concentrations of several salts, have essentially no buffering capacity. Therefore,the pH is adjusted to a value in the region 5.6 - 5.7. This may be done before or after sterilisation. In the former, the medium is adjusted to the desired pH and then autoclaved to eliminate contaminating organisms. In their experience autoclaving of pre-adjusted solutions can induce significant alterations in pH. This is unsatisfactory when seeking to prepare and culture protoplasts under well defined and standardised conditions. Aseptic adjustment of pH after sterilisation is desirable, but less easily achieved, and increases the risk of contamination.

Roscoe and Bell adapted their media preparation protocol to take advantage of the colour changes of bromocresol purple around pH 5.5 - 6.0. Their results showed that 10 samples of 11% mannitol at pH 5.72 had values ranging from 8.14 to 8.72 after autoclaving for 20 minutes at 115°C. Under the same conditions complete media B5 changed from pH 5.74 to a range 5.20 to 5.38. The results of autoclaving MSP 9M produced similar changes in pH. This is unsatisfactory and it becomes necessary to adjust media pH after autoclaving by the aseptic addition of sterile acid or alkali.

Whilst this method may remove some of the problems regarding pH, there are other problems still remaining related to pH. Firstly, pH may have a direct effect on the effectiveness of the actual sterilisation procedure and secondly, the influence of both pH and sterilisation on the quality of the medium after sterilisation. At extreme values of pH, there may be enough of an effect to result in a reasonable sterilising effect. The pH value may also accelerate denaturation of proteins, especially those involved in new cell wall synthesis, considered to be a prerequisite for cytokinesis. It may further affect the activity of potentially toxic substances when the toxicity of the substance depends on the degree of dissociation. Many substances are toxic only in the un-dissociated state.

There are two main reasons why the pH of a medium must have a given value after sterilisation. Firstly, the successful growth of the cultured cell, tissue or organ depends on this value and secondly, the pH value plays a role in suppressing the growth of undesirable contaminating micro-organisms. Whether one or other, or both of these apply in protoplast culture is not clear. As Roscoe and Bell also showed, a pH change of 0.5 units can occur within 2 days of starting protoplast culture and this too may produce subtle or major changes in the composition and quality of the medium.

These findings suggest that the preparation of media to be used for protoplast culture needs careful attention. Not only did autoclaving produce changes in the nature of the culture medium in terms of its composition but also with regards to its pH. The exact nature of these changes depended on factors which were themselves controlled by the nature of heating, holding and cooling events in an autoclave. It will be especially difficult to ascertain the exact nature of these changes as experience showed that no two loads in an autoclave were exposed to exactly the same temperature/time course in an autoclave.

Chapter 7

THE EFFECTS OF RAPID DILUTION OF LIQUID CULTURE MEDIA ON THE CULTURE OF TOBACCO MESOPHYLL PROTOPLASTS

7.1 Introduction

In 1975, Shepard and Totten reported a method of isolation and regeneration of protoplasts under low osmotic conditions. The initial osmoticum for isolation of these protoplasts was 0.2M (6.84%) sucrose, using "Macerozyme", cellulase and 2% PVP. The viability of these isolated protoplasts was confirmed through regeneration of fertile plants. However, plating and regeneration studies revealed that both qualitative and quantitative modifications in the plating and differentiation media used for culture, were required. Over-all, the procedure was claimed to be a simplified alternative to previously described regeneration protocols. They concluded that protoplasts could be induced to divide and regenerate plants with high efficiency over a broad range of osmotic conditions, provided that reduction in osmotic pressure were not made at the outset, by a method which was over-all, a simplified alternative to those previously described for tobacco protoplast regeneration.

When this simpler alternative was examined in more detail, there were major areas where considerable changes to published procedures had been made and far from simplifying procedures, it seemed the complexity and number of procedures had increased and the time course of culture, lengthened. These changes included subjecting the isolation enzyme mixture to a pH change from a non-disclosed value to 9.5 and from this value to 5.4. Also, protoplasts were subjected to different rinsing solutions containing between 0.25 and 0.4M sucrose. Each new batch of ionagar required a pre-trial to determine its gelling ability before it could be used. Plating efficiencies were not, or could not be determined until three weeks after plating and dilution plating did not start until four weeks after culture commenced. Changes to plating osmotica were made at two day intervals, which began after two weeks, if prescription bottles were being used, but not until four weeks,if dilution plating was involved. Colonies were only accessible if the plating agar used to produce the thin layer in the prescription bottles was disrupted using a glass rod. Finally, retrieved colonies were diluted ten fold with

medium and a further four transfers were necessary to produce plants growing in soil.

With these complexities in mind, an attempt was made to devise a protocol which would reduce the number of stages, reduce the time required to achieve a reduction in osmotic strength of the culture medium, yet achieve similar or better results in terms of plating efficiency.

7.2 Variations to materials and methods

Protoplasts were isolated, cleaned, counted and adjusted to give a density of 5 x 10^4 per ml in the usual manner. Isolation was carried out at a level of 11M. The protoplasts were divided into ten equal portions, two for each of five treatments. One of the pair was to serve as a control, the other to serve as experimental material which would be subjected to a dilution regime over a period of five days. Both treatments were maintained in liquid culture at 27°C in medical flats in the dark. The following cultures were set up by centrifuging all tubes at 100 x g for 15 minutes to pellet the protoplasts. The 11M mannitol was removed using a sterile Pasteur pipette and replaced with MSP medium containing differing levels of mannitol, namely 5% (5M), 7% (7M), 9% (9M), 11% (11M) and 13% (13M). Two tubes were produced for each mannitol level, one control and one experimental.

On each of the next five days, the tubes were removed from the culture room and treated in the following manner. A calculation had been made to determine how much culture medium had to be removed and replaced by MSP 0M (no mannitol) to produce a reduction in mannitol level in the culture of 1% with respect to actual mannitol present i.e. 1M (Table 7.1). In reality, the reduction in mannitol content from say, 10M to 9M, represented a 10% change, whilst a reduction from 3M to 2M would represent a 33% change in mannitol content. This reduction in mannitol was achieved by allowing the protoplasts to settle for half an hour in upright medical flats and then removing the appropriate volume with a sterile pipette.

The control bottles were subjected to the same mechanical procedures, except that the removed volume was replaced by an equal volume of fresh culture medium of the same mannitol level, thus

TABLE 7.1

Dilution Schedule of volumes to be removed to reduce mannitol content of MSP liquid culture by 1M per day

Day•	13M	11M	9M	7M	5M
1	0.77	0.91	1.11	1.43	2.00
2	0.83	1.00	1.25	1.67	3.33
3	0.91	1.11	1.43	2.00	-
4	1.00	1.25	1.67	3.33	-
5	1.11	1.43	2.00	-	-
6	1.25	1.67	3.33	-	-
7	1.43	2.00	-	-	-
8	1.67	3.33	-	-	-
9	2.00	-	-	-	-
10	3.33	-	-	-	-

Volumes shown refer to a culture volume of 10 mls. Volume removed was replaced by a equal volume of MSP 0M.

Protoplasts were isolated and placed in culture at various levels of mannitol. The mannitol content was reduced by 1M each day by removal of culture medium and replacement by MSP culture medium containing no mannitol. This procedure continued until all cultures had been reduced to a level of 3M.

giving no dilution effect. The schedule adopted produced a drop in mannitol content of 1% per day in the experimental culture.The dilution continued until a final level of 3M had been reached. In the case of the 5M culture this was reached on the third day. The same mechanical procedures were continued by removing old culture medium and replacing it with fresh 3M medium, in this way all cultures had equal amounts of fresh culture medium each day. After five days plating efficiencies were determined for undiluted controls and dilution treatments.

7.3 Results and Discussion

Protoplasts were isolated using a medium containing mannitol at 11% (11M). Protoplasts were divided and placed in culture media containing 7%, 9%, 11% and 13% mannitol. Therefore some protoplasts were subjected to an immediate osmotic shock with a reduction in mannitol content of up to 4%. These protoplasts were put into culture and examined regularly for periods up to two weeks. At the end of the first and second weeks plating efficiencies were determined for each culture. The highest plating efficiency was achieved in the culture with the lowest mannitol content and lowest in that with the highest content. (Fig. 7.1).

The range of plating efficiences was from 8% (11M) to 34% (7M). The protoplasts put into culture at 13M, an increase in mannitol content from the isolation level, failed to survive. The 11M culture represented culture at the same mannitol level as that used for isolation. The fact that reduction in mannitol content, immediately following isolation, did not kill protoplasts and they subsequently regenerated a cell wall and divided, to produce a plating efficiency of 34% in a 7M culture, served as the basis for the second series of experiments later. These cultures were maintained for another week, after which plating efficiences were determined again. The relative difference in plating efficiences had been maintained with the plating efficiency in 7M having increased to 60%, that in 9M to 46% and in 11M to 32%. This represented an additional increase in plating efficiency in each culture averaging 25%. Therefore the differences present in each culture at the end of the first week were still present, and to the same extent, at the end of the second week.

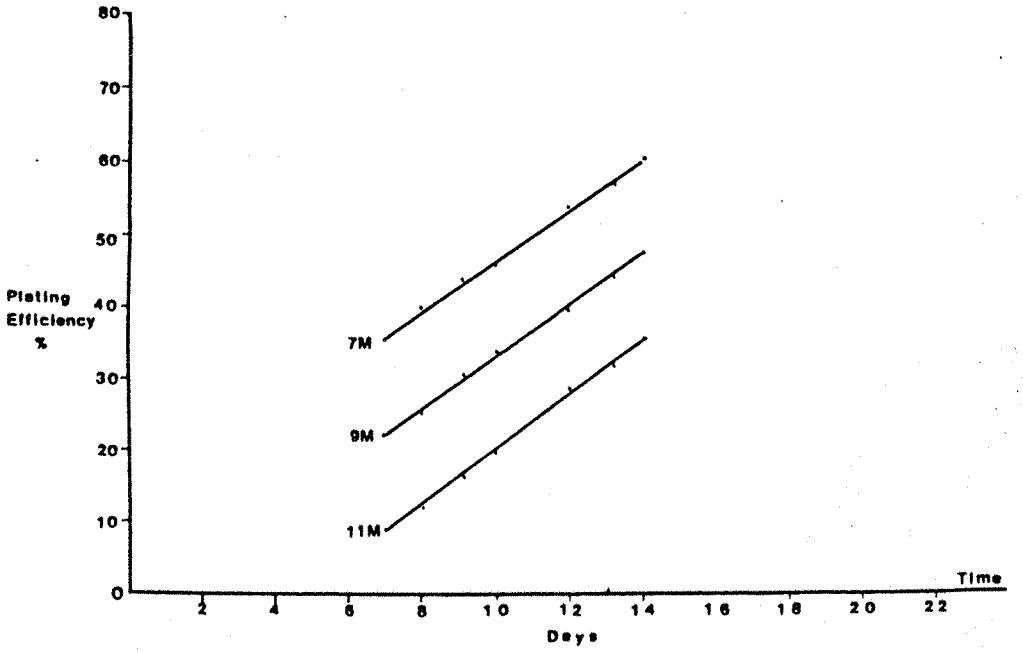


FIGURE 7.1

The effect on plating efficiency of the mannitol level in MSP medium in the first two weeks in liquid culture.

As this experiment indicated that immediate reduction in mannitol content appeared not to affect viability or plating efficiency, a second experiment was carried out which involved an immediate reduction in mannitol content followed by a progressive reduction of 1% actual mannitol per day down to a level of 3M. The procedure for this experiment is explained in variations to materials and methods.

The protoplasts for this experiment were not those for the first experiment so results are not directly comparable. This is particularly apparent in the results for the undiluted controls where plating efficiences achieved were particularly low, reaching only 2% in the culture maintained at 5M. The 5M culture which was diluted over two days to 3M failed to produce any protoplasts which generated a cell wall and divided, suggesting that the decrease from 11M to 5M and then to 3M was too a severe an osmotic shock in too short a time, although a small plating efficiency was also achieved in the undiluted control. (Fig. 7.2).

The highest plating efficiency was achieved in the culture immediately reduced to 7M and reduced to 3M over a period of 4 days. A plating efficiency of 40% was achieved. Lower and decreasing plating efficiencies were achieved in cultures started at 9M, 11M, and 13M respectively. This would suggest that osmotic potential of the culture medium has an effect on plating efficiency and that it is the initial osmotic potential and not the reducing or final osmotic potential affecting plating efficiency. Although the results are not directly comparable, the first experiment suggest that protoplasts can withstand a fairly large reduction in initial mannitol levels in culture and successfully regenerate a cell wall and subsequently divide.

The form of the cells produced in the dilution experiments is shown in Figs. 7.3 and 7.4.1 and 7.4.2 . The cells were characterised by an elongated and twisted form, with sparse chloroplasts, although this form was by no means the general morphology, as a large number of apparently normal cells were also present. This particular morphology may have indicated the protoplasts' response to osmotic shock. The elongated cell may be of use in selection procedures when cells from specific sources need to be identified.

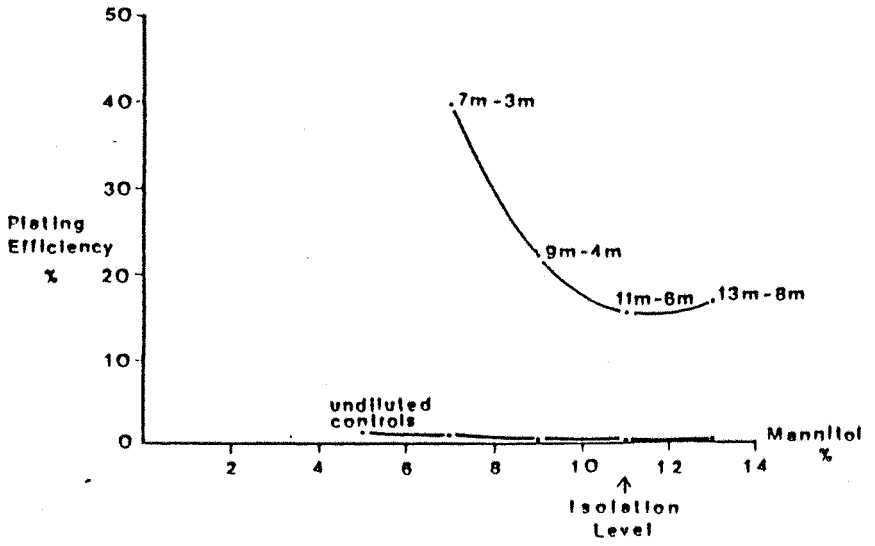


FIGURE 7.2

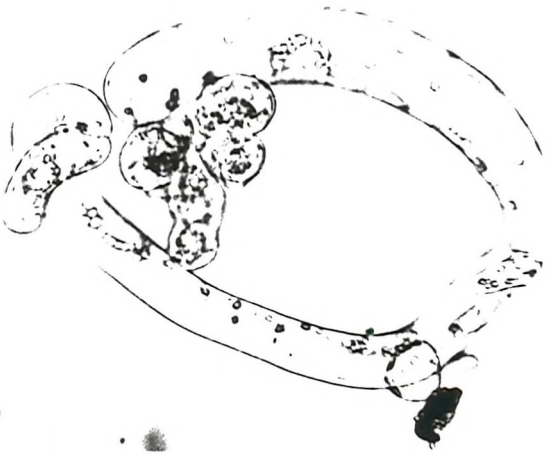
Effect on plating efficiency of progressive dilution of various strengths of MSP 9M liquid medium.

FIGURE 7.3

(a-e) Cells regenerated from protoplasts in diluted culture media.
Protoplasts were isolated at 11M level and immediately placed in different mannitol levels for culture. Each day the level was reduced by 1M until a level of 3M was reached and then held at this level.
Cells from 7M culture reduced to 3M in 4 days and held for 5 days at this level. (x290)

Callus cells produced from this dilution schedule would not be considered typical. Cells are both elongate and twisted into corkscrew shapes.

a



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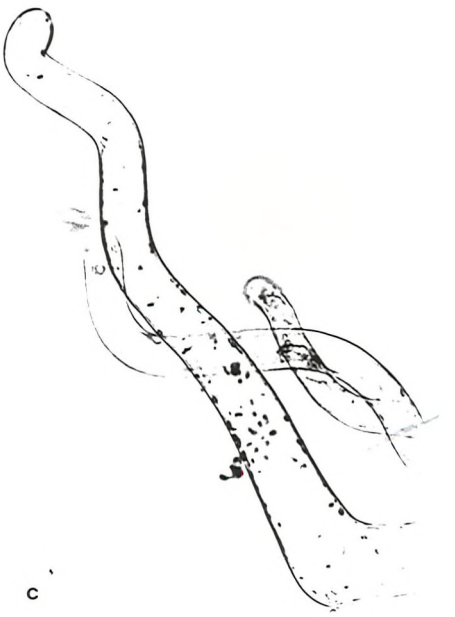

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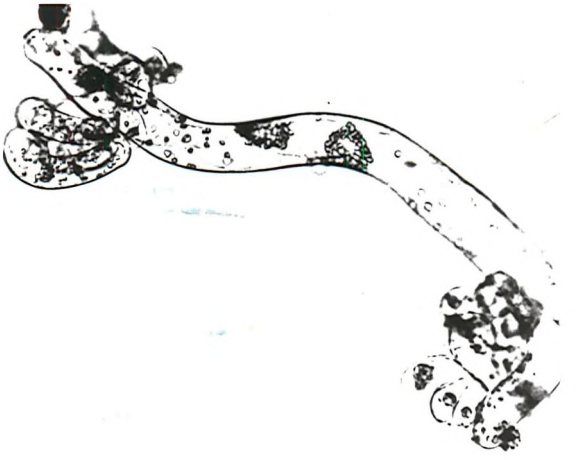
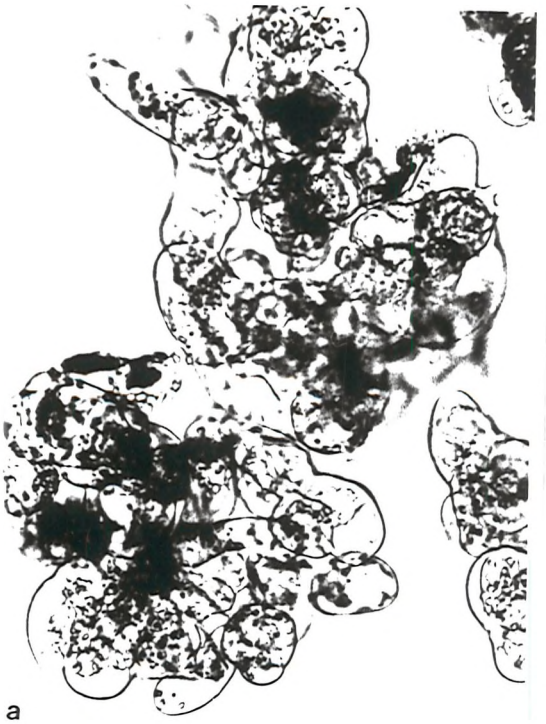


e

FIGURE 7.4.1

(a-d) Cells regenerated from protoplasts in diluted culture media.
Protoplasts were isolated at 11M and placed in culture at 11M. Each day the mannitol content was reduced by 1M until a level of 3M was reached and then held at this level.
Cells from 11M culture reduced to 3M in 8 days and held for five days at this level. (x290).

Dilution schedule was slower for this culture, reducing from 11M to 3M in eight days. Callus cells produced by this schedule would be considered typical, although atypical cells such as those produced by the dilution schedule used for the cells shown in Figure 7.3 were also produced and are shown in Figure 7.4.2.



a

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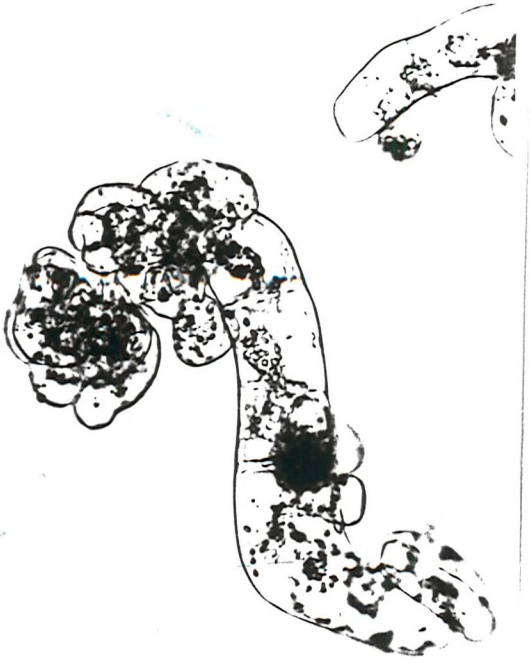


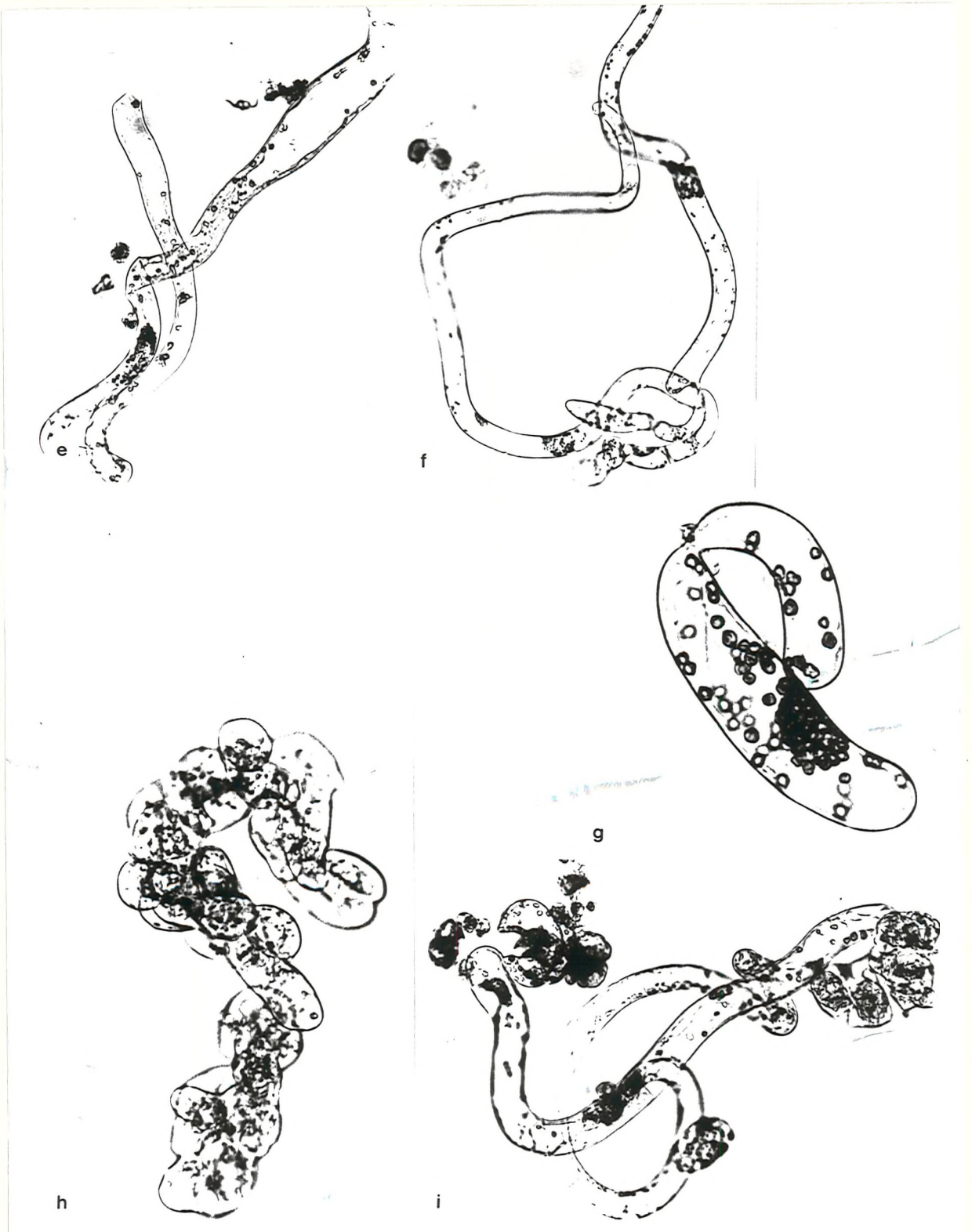
c

d

FIGURE 7.4.2

(e-i) Cells regenerated from protoplasts in diluted culture media.
Protoplasts were isolated at 11M and placed in culture at 11M. Each day the mannitol content was reduced by 1M until a level of 3M was reached and then held at this level.
Cells from 11M culture reduced to 3M in 8 days and held for five days at this level. (x290)

Atypical, elongated and twisted callus cells found amongst typical callus cells produced by the slower dilution schedule.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Shepard and Totten (1975), described the isolation of their protoplasts in detail, the major feature being the necessity to take the isolation enzyme mixture to pH 9.5 to enable the dissolution of significant amounts of otherwise insoluble material and returning the mixture to pH 5.4 with 0.1M HCl. Following isolation, the protoplasts were separated by filtration through layers of cheese cloth into Babcock bottles, and centrifugation. The intact protoplasts floated to the top of the solution and were placed in a rinse solution containing 0.25-0.4M sucrose, (depending on experiment) and 1/10 concentration medium, and centrifuged. Several culture media were then compared in their protoplast regeneration experiments, using variable plating densities.

In regeneration experiments which followed, dilution plating of colonies was performed approximately four weeks after protoplasts were plated. In initial experiments, they flooded the surface of the culture media with 0.05M sucrose, followed by three additional aliquots at two day intervals. However, flooding of the agar slowed or terminated colony growth. Different results were achieved when protoplasts were cultured in 236 ml glass prescription bottles (medical flats). A thin layer of agar medium was made using 5 ml. of protoplast-agar medium and placing the bottle in an horizontal position. When liquid media additions were started (2 weeks after culture), 2 ml of liquid medium were added and the bottles tilted slightly to prevent the liquid from covering the agar surface. However, in both series of experiments dilution plating only began after 4 weeks of culture, by which time the experimental material must surely be regarded now as plant cells and not protoplasts and there must be some contribution to the prevention of cells bursting by the agar.

In the discussion of their results which followed, Shepard and Totten recorded that the osmotic pressure of media, when used for dilution plating and colony transfers, seemed to influence colony survival and differentiation. Again, this can hardly be claimed as a procedure for the culture of protoplasts under conditions of low osmotic pressure. Colonies that had received no supplemental medium addition between plating of protoplasts and subsequent dilution plating were subject to osmotic shock in transfers to media containing less than 0.3M sucrose. Colonies became brown in colour within a few hours and growth ceased. Similar effects were reported

when colonies 3-4 mm in diameter were transferred from dilution plates to medium 3 containing 0.05M sucrose (1.71%) and those which survived remained as undifferentiated callus up to 10 mm in diameter. When 0.3M sucrose was present in media however, survival and differentiation rates were improved. This level of sucrose was significantly lower than those previously reported as optimal for tobacco mesophyll protoplasts (Cocking, 1972; Takebe et al, 1968).

Shepard & Totten

Further experiments by (1975), revealed that when prescription bottles (medical flats) were employed, and colonies repeatedly supplemented with a medium containing 0.05M sucrose, there was little evidence of osmotic shock either during dilution plating or subsequent transfer to media with 0.05M sucrose. No details were given as to which features exhibited by protoplasts were considered as evidence of osmotic shock. They described the features when the osmotic strength of the culture medium was increased, but not decreased.

Shepard and Totten investigated three different media, the first was the basic medium of Nagata and Takebe, (1971) which was unsatisfactory for plating protoplasts prepared by their protocol. At plating densities less than 1 x 10^4 per ml, the medium was toxic, and all protoplasts died within 4 days. At densities of 1 to 1.5 x 10^4 per ml, protoplasts did survive and a moderate plating efficiency was achieved. Reduction in strength of the medium to 1/2 or 1/3 strength permitted colony growth at progressively lower plating densities, but plating efficiencies were not high. No benefit was obtained further by varying the amount of organic, inorganic and hormonal constituents. One tenth strength medium 1 permitted growth of protoplasts but in in the form of elongate cells for 4-6 weeks but little or no colony formation. This particular growth form description would seem to be the same as that produced in this study. However in this study there were large numbers of cells which were not elongated and did grow to produce colonies.

The second and best plating medium was medium 2, essentially with no alterations, a combination of 90% modified White's medium and 10% medium 1, (Paul, 1970). Plating efficiencies ranged from between 60 to 80% at cell densities between 6 x 10^3 per ml and 2 x 10^4 per ml, and reached 93% three weeks after protoplasts were plated at 7.2

x 10^3 per ml. Between 2 x 10^3 and 4 x 10^3 per ml most protoplasts survived and percentage colony formation was highly variable.

However, this second medium did not provide for sustained growth of colonies. In initial studies, colonies were removed from the medium after approximately 4 weeks and were dilution plated in medium 1. However, more consistent results were obtained by adding small aliquots of liquid medium 1 containing 0.05M sucrose to prescription bottles after colony formation was well underway, (about two weeks after isolation). This was followed by dilution plating in medium 1 after 4 to 5 weeks in culture.

The procedure adopted in this study was certainly faster than that of Shepard and Totten (1975) who did not commence their dilution regime until at least two weeks in culture had elapsed, and in some cases four weeks and can genuinely claim to dilute media containing protoplasts and not cell colonies. The plating efficiencies achieved by their procedure were very variable, but in one case reached 93% after three weeks. The schedule adopted here produced a plating efficiency of 60% at the end of two weeks, by an immediate reduction in mannitol content from the 11M used for isolation, to 7M, followed by a further reduction to 3M over the next four days. This dilution regime was carried out on protoplasts and not on "protoplasts" which had already had a period of time in agar culture and really should be considered as cells, having had sufficient time to regenerate a cell wall. Although numerically a smaller plating efficiency was achieved with the true protoplasts, the results showed clearly that reduction in the osmotic strength of the culture medium can commence immediately on isolation and be reduced still further over a relatively short period of time.

Chapter 8

THE QUALITY OF AGAR AND ITS EFFECTS ON PROTOPLAST CULTURE

8.1 Introduction

Evidence has accumulated which suggests that agar used in the culture media for anthers of Nicotiana tabacum could affect the yield of embryos from such cultures, (Kohlenbach and Wernicke, 1978).

When a liquid medium completely lacking in agar was used, a significantly better yield was obtained than on any solidified agar medium. On the agar medium a high percentage of microspores were induced to divide and show the first androgenetic mitoses, but very few developed to complete plants, (Sunderland and Wicks, 1969;1971). Other workers showed that yield in anther culture could be significantly increased by the incorporation of active carbon into the culture medium (Anagnostakis, 1974). These results have been confirmed subsequently, although the exact action of the active carbon has not been found.

The technique of agar production has been fully described by Bridson and Brecker, (1970); Chapman, (1970); and Whistler, (1973). Although agar is a mixture of polysaccharides, it contains two major groups of compounds, low-sulphate, pyruvic acid free, agarose and high-sulphate, high ash, agaropectin. The proportion of agarose to agaropectin varies according to the seaweed of origin. These include the rhodophyceae genera Gelidium, Pterocladia, Gracilaria and Acanthopeltis. The highest ratio is 75% agarose to 25% agaropectin. About half the annual production of agar is for microbiological work, where high gel strength and freedom from toxic metals are important. It should also be free from impurities commonly found in commercial agars, such as non-agar gums, nitrogenous compounds, insoluble salts, free sugars, dead micro-organisms and thermoduric bacteria. These requirements are reflected in several agar types available.

Commercial agar, sometimes called technical agar, is produced primarily for the food industry. It is free from toxic materials but may contain high calcium and magnesium levels and has relatively poor clarity. Bacteriological agar is specially clarified and freed from pigments, although clarification may not reduce the cation levels and

phosphate and "floc" may appear in culture media after autoclaving. Purified agar has a reduced ash and metal content and is generally compatible with all the usual ingredients of culture media and exhibit minimal inhibition of diffusion of antibiotics. Agarose is the most specific agar fraction and is collected and dried. Chemical composition is still very variable but the specification should include an electroendosmosis factor (m_r) which should be less than 0.15. (Table 8.1). Plating of protoplasts in agar media is particularly attractive as it allows the development of particular protoplasts to be followed very easily, but whether similar effects as in anther culture exist for protoplast culture is not clear.

8.1 Results and Discussion

Much of the data regarding the effects of agar on the culture of protoplasts was generated during other experiments in this study. As the data accumulated it became apparent that success or failure to culture protoplasts depended on the brand of agar used to produce solidified culture media. As the figures in Table 8.2 show, isolated protoplasts randomly divided into lots and placed into culture media prepared with four different agars produced very different plating efficiencies after a period of time in culture. A difference in plating efficiency was also obtained if the isolation of the protoplasts was carried out using purified enzymes.

Irrespective of whether purified or non-purified enzymes were used, the plating efficiency was reduced, although not to the same extent with each agar. The cultures produced from protoplasts isolated using purified enzymes and which used the more crude agars had their plating efficiencies reduced to about a third of that in cultures produced from protoplasts isolated with non-purified enzyme. These were bacteriological agar where plating efficiency was reduced from 10.52% to 3.53% and New Zealand which reduced from 21.69% to 7.75%. The so called high grade agars had plating efficiencies with purified enzymes reduced to just a tenth of that for non-purified enzymes. Sigma agar reduced from 3.22% to 0.37% and Oxoid agar from 31.93% to 2.4%. Not only were the plating efficiencies with non-purified enzyme lower with high grade agars compared to crude agars, but the reduction in plating efficiency was greater when purified enzymes were used.

TABLE 8.1

Technical data for agars used in experiments

Average batch analysis	1 Sigma	2 Bact.	3 No 3	4 New Zeal.
Moisture		7.0%	7.0%	20.0%
Ash		2.0%	3.2%	4.0%
Acid insoluble ash		0.1%	0.1%	-
Cl as NaCl		0.1%	0.1%	-
P as P_2O_5		0.005%	0.005%	0.004%
CO_4		0.9%	1.7%	-
Total nitrogen		0.1%	0.1%	-
Ca		100 ppm	1000 ppm	1000 ppm
Mg		40 ppm	300 ppm	-
Fe		80 ppm	70 ppm	100 ppm
Precipitate with phosphate		none	medium	minor
Working strength		1.0%	1.2%	1.5%
M_r index		0.32	0.40	-

1 Sigma Chemical Company
2,3 Oxoid Ltd
4 Davis Gelatine Division

Although the results were not conclusive, one interpretation of the results would suggest that the undefined nature of both agar and enzyme mixture results in the provision of one or more materials or substances of benefit to the culture of protoplasts. The refining of agar and enzyme mixture removes these substances and reduces the potential for successful protoplast culture.

It is also possible that refining, instead of removing substances essential for successful protoplast culture, could be removing materials which, although not directly involved, somehow provide a function in the culture which is essential to protoplast cell wall regeneration and subsequent division. This could be as diverse as provision of buffering capacity, to provision of essential trace elements, to the amelioration of the effects of toxic materials. Further investigation of such effects would prove worthwhile.

The results from this particular series of experiments were not easy to analyse. The changes in plating efficiency could have been produced by a change in the quality of agar used, good quality compared with crude quality, a change in the quality of the enzyme used for the isolation of the protoplasts or an interaction of the two, agar and enzyme quality.

It was first necessary to investigate whether the latter condition had occured. The X^2 test is suitable statistical test for this. The results in Table 8.2 were used to generate the expected values for plating efficiencies if such an association had taken place. The null hypothesis was that agar quality and enzyme quality had acted independently in these experiments. The calculations for this test are shown in table 8.3. Applying the usual calculation for X^2 the value of $X^2 = 5.008$. The critical value for X^2 at the 5% level for 3 degrees of freedom is 7.81. Therefore the null hypothesis was retained and it was concluded that any changes in plating efficiency was caused either by a change in enzyme quality, or a change in agar quality, but not by an interaction between the two.

It is worth noting that the application of X^2 to this data may not have been entirely valid, for two reasons. Firstly in a test of association, the results which are obtained are integer values, i.e. either yes or no, diseased or not diseased and cured or not cured.

TABLE 8.2

Plating efficiencies obtained for protoplasts from a single batch cultured on various agars

Agar Type	Average Plating Efficiency(%)
Sigma	3.22
Bacteriological	10.52
Oxoid No 3	31.93
New Zealand	21.69

Plating efficiencies obtained for protoplasts from same source as above but isolated using purified enzyme

Agar Type	Average Plating Efficiency(%)
Sigma	0.37
Bacteriological	3.53
Oxoid No 3	2.40
New Zealand	7.75

Protoplasts from the same plant source were isolated using commercial enzymes or purified enzymes. The protoplasts were then placed in MSP 9M agar medium prepared using a number of propriatory agars.

TABLE 8.3

Statistical analysis of the results of experimental data from Table 8.2

	Observed	
	Non-purified	Purified
Agar Type		
Sigma	3.22	0.37
Oxoid	31.93	2.40
Bacteriological	10.52	3.53
New Zealand	21.69	7.75

	Expected	
	Non-purified	Purified
Agar Type		
Sigma	2.97	0.62
Oxoid	28.41	5.93
Bacteriological	11.63	2.45
New Zealand	24.36	5.08

Data was used to compute a one sample X^2 value as explained in the text.

This means that values must be whole numbers, it is not possible to have results other than in whole numbers. When X^2 was repeated using whole numbers produced by rounding up the plating efficiencies obtained to whole numbers a non significant result was still obtained and the null hypothesis retained. Secondly, even if this approximation was used, it produced a contingency table containing cells with less than five counts which may have further rendered the statistical test less valid, though this was by no means certain. Bearing in mind these reservations, it was felt that further investigation of the results would be worthwhile.

It was then possible to investigate if, agar quality or enzyme quality, or both had affected the plating efficiencies achieved. Examination of the results seemed to indicate that agar quality was the more likely candidate as the plating efficiencies obtained with the good quality agars were similar and those with the poorer quality agars also similar.

The agars used in this series of experiments were classified as follows; Bacteriological agar, a technical grade agar of high gel strength which was suitable for purposes where clarity and compatibility with added materials was not of prime importance. Oxoid No 3 agar, described as a specially purified agar of high gel strength, processed to reduce its mineral content below that of any comparable agar on the market. Apart from its clarity and compatibility with all culture media, it is unique in that liquid and solid formulations of the same medium will have very similar mineral contents. This is of extreme importance in some studies when complexing of substances is to be avoided. Full descriptions of Sigma and New Zealand agars were not available.

In this instance a one sample X^2 test was appropriate as the conditions for this test were met by the data. The value of X^2 obtained for the protoplasts isolated using non-purified enzyme was 28.31 which is significant at the 0.1% level. The value for the protoplasts isolated using purified enzyme was 8.28 which is significant at the 1% level. Whilst the statistics used on the results may not have been have been as precise as possible they are sufficiently reliable to suggest that the quality of agar used to plate protoplasts had an effect on plating efficiency. (Figs 8.1 and 8.2).

FIGURE 8.1

(a-d) Effects of agar type on plating efficiency. Protoplasts isolated at 9M level and cultured in MSP 9M for 9 days. Agar used at 1.2% and plating density was 2.5 x 10^4 per ml.

(a) Sigma agar.
(b) New Zealand agar.
(c) Bacteriological agar.
(d) Oxoid No 3 agar. (x40).

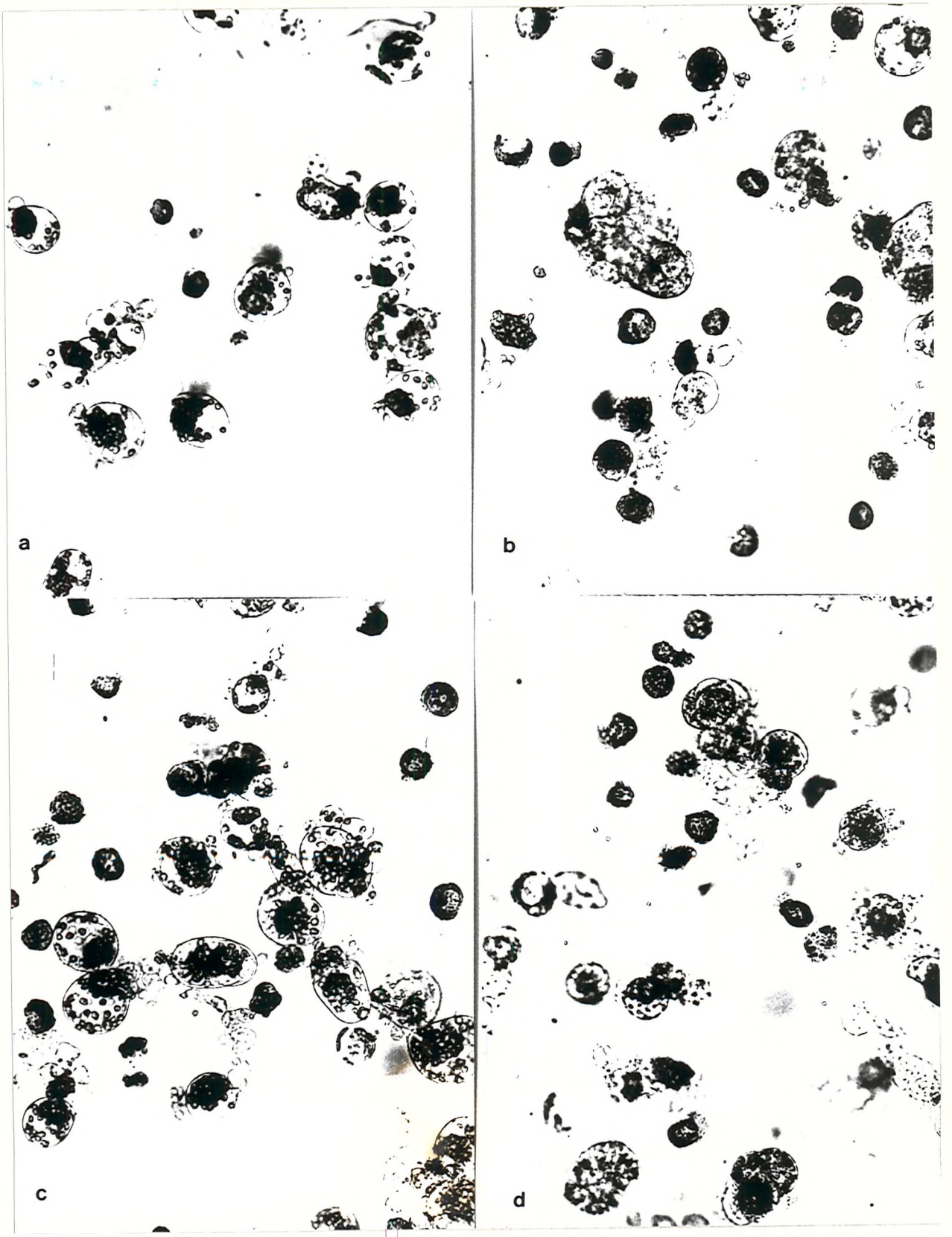
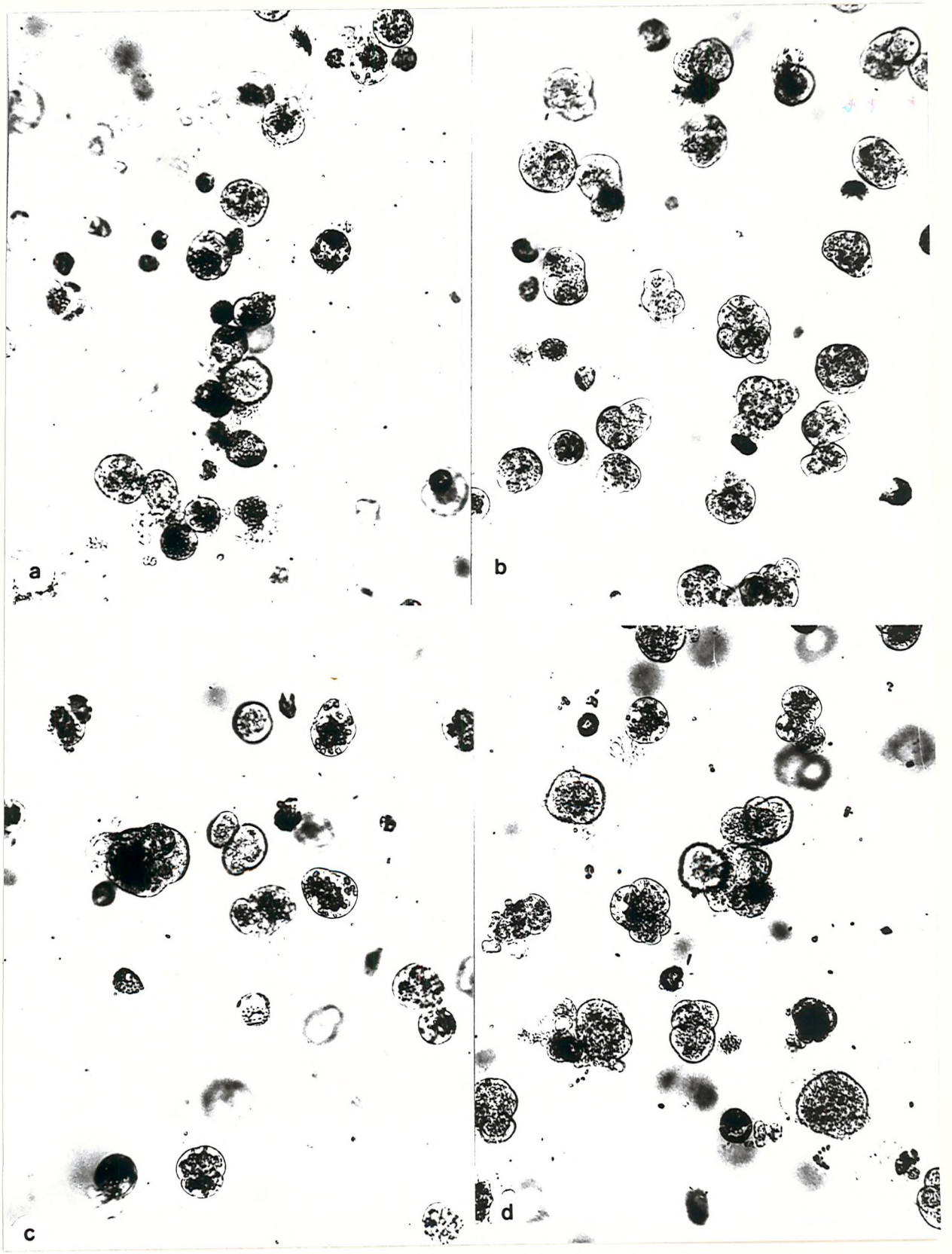

a
b
c
d

FIGURE 8.2

(a-d) Protoplasts isolated using purified enzyme at 9M and cultured in MSP 9M. Agar used at 1.2% and plating density was 2.5 x 10^4 per ml.

(a) Sigma agar.
(b) New Zealand agar.
(c) Bacteriological agar.
(d) Oxoid No 3 agar. (x40).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b
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d

As a result of experiments carried out, Kohlenbach and Wernicke, (1978) concluded that a number of possibilities existed which might have accounted for the increased yield of embryos in liquid anther culture. It may have been due to the physical nature of the medium itself, where the culture provided better nurture of the anthers in liquid medium, due to better contact with nutrients or, inhibitory compounds may have been better able to diffuse away from the developing microspores, since agar can act as a diffusion barrier. Or perhaps, were there substances present in the agar, but not in liquid, which were producing an effect on anther development?

To answer these questions, they devised further experiments in which anthers were cultured in liquid, liquid in contact with agar, and liquid containing agar wash water. Results showed that embryos aborted prematurely in agar culture and that agar contained substances inhibitory to anther culture. By equilibrium dialysis it was demonstrated that active carbon, already successfully used in anther culture, (Nakamura and Itagaki, 1973; Anagnostakis, 1974) functioned by absorbing these inhibitory substances. It was also found that increased embryo yields could be achieved by the use of more purified commercially available agar types and agarose. These and personal results suggest that whilst highly purified agars are required for anther culture, protoplast culture shows the reverse and better results are achieved with the so-called crude agars. Further, in species where liquid media are not favourable (e.g. Datura) yields were only increased if highly purified solidifying agents were used in the preparation of culture media, although Binding (1974) successfully cultured Petunia protoplasts using less purified agars in the culture medium. The results achieved in this present study - also suggest that protoplasts can be successfully cultured without the need to resort to purified enzymes and highly purified agars.

The plating efficiencies achieved in this set of experiments were considered to be sufficiently high that the addition of activated charcoal as a means of increasing these was considered unnecessary. The addition of activated charcoal to media for plant tissue culture has, in many instances been shown to have beneficial effects on the growth of explants. These effects are thought to be due to, removal of an inhibitor of development arising from autoclaving of sucrose (Weatherhead et al., 1978), removal of harmful phenolic/carboxylic compounds produced by growing tissues in culture

(Fridborg et al., 1978); removal of plant growth hormones (Constantin et al., 1977; Weatherhead et al., 1978) or removal of other substances necessary for growth (Fridborg et al., 1978). There are, however, also instances where such incorporation has been inhibitory to growth. It then becomes necessary to consider whether activated charcoal contributes in some way to growth by donating compounds or elements to the media. Indeed, mass spectrographic analysis of activated charcoal reveals a high level of impurities in samples which could substantially increase the amount of a particular element already present in a medium and donate others not already present.

It has been shown that activated charcoal adsorbs auxins and cytokinins and other media components to varying degrees. Thiamine HCl and nicotinic acid are strongly absorbed, whereas inositol and sucrose are not (Weatherhead et al., 1979). Thus it seems that the addition of activated charcoal to culture media can have a considerable effect on the composition of the medium, a situation to be avoided if protoplasts are to be cultured using standardised and well defined conditions. Weatherhead et al.(1979) concluded that where the addition of activated charcoal gave an increased response it was pointless to add to culture media, substances to replace those strongly adsorbed. However, adsorption of other compounds by the charcoal could lead to inhibition of growth, and the replacement of these to increase plating efficiencies, was more problematic.

Chapter 9

GENERAL DISCUSSION AND SUMMARY

Protoplast isolation, culture and regeneration was originally a technique that could only be applied to a small number of Solanaceous species. The first plants regenerated from protoplasts, the first somatic hybrids and the first effective mutant isolation procedures were all reported for Nicotiana.

When the techniques that were successful for tobacco were used for other species, notably crop species such as cereals and legumes, both the yield and viability of protoplasts were greatly reduced and plants could only be regenerated in a small number of species. Recent years have seen the development of a number of methods that have lead to the successful regeneration of plants from protoplasts of a wide taxonomic range of plant species, (Evans and Bravo, 1983). Much of this advance is the result of long and painstaking empirical investigation of the conditions and variables affecting the isolation, culture and subsequent regeneration of plants from particularly recalcitrant species.

Leaf mesophyll cells have been used most frequently for protoplast isolation, however it is possible to isolate protoplasts from virtually any plant tissue. It is necessary though, before isolation is attempted, to pay careful attention to one or more pre-isolation conditions. Since all plants have different growth requirements, it seems that no general optimal conditions can be stated. In this study protoplast viability changed according to time of year, which may be related in some way to changes in daylength, or components which made up the daylength period. It has been accepted that in general, any condition that increases the growth rate of the donor plant will increase the probability of isolating viable protoplasts.(Evans and Bravo, 1983).

Amongst a multitude of pre-isolation conditions affecting protoplast yield and viability, there are some which appear to be more significant than others. These include photoperiod experienced by donor plant, with short photoperiods of dim light giving consistently high protoplast yields, (Shepard and Totten, 1977), a period of low temperature incubation in salt solution, (ibid.) or a combination of

high humidity (82%) and low temperature (15°C), (Tal and Watts, 1979), both affecting protoplast yield and stability. Other reports suggest that leaf age,(Kao and Michayluk, 1980) and feeding with ammonium, (Cassells and Barlass, 1978) affect protoplast release of Lycopersicon esculentum, whilst supplementary feeding of calcium in winter but not spring or summer is required to enhance protoplast stability of Nicotiana tabacum, (Cassells and Cocker, 1982). Other workers even suggest the pre-treatment of leaves with senescence retardants, (Kaur-Sawhney et al, 1977) or pre-plasmolysis with cysteine to produce better digestion of the cell wall and greater stability of protoplasts,
(Senn and Pilet, 1980).

Clearly, the physiological state of the donor plant prior to its use as a source of protoplasts is crucial to obtaining high yields and viable, stable protoplasts. It is likely that it will continue to be necessary to empirically determine what factors affect which plant species, and in what way. The likelihood of a standardised, successful protocol for donor plant culture conditions is still some years away.

The enzymes used in mixtures to isolate protoplasts are required to degrade the three primary components of the cell wall: cellulose, hemicellulose and pectin. The percentages of these three components vary from species to species. Most frequently a cellulase is combined with a pectinase to achieve single step isolation of protoplasts.

Most of the commercial enzyme preparations contain toxic substances and other impurities which may damage protoplasts. Consequently, several laboratories routinely desalt enzymes prior to use, although in the present study, no advantage was gained from such a procedure. On the other hand, Schenk and Hildebrandt, (1969) tested highly purified enzymes for protoplast isolation. They determined that highly purified cellulases were less efficient than partially purified preparations in isolating protoplasts in both terms of yield and viability. This suggests that cellulase alone is insufficient for protoplast isolation and some additional, undefined enzyme activity is necessary.

The cell wall that is enzymatically digested away during protoplast isolation normally provides support for the cell. When it

is removed, the osmotic strength of the cytoplasm and the isolation medium must be balanced, or the protoplasts plasmolyse or burst. Protoplasts should be just slightly plasmolysed during isolation, as excess osmotic pressure has been reported to impair metabolism, (Ruesink, 1978) and cell wall regeneration, (Pearce and Cocking, 1973). The osmotic pressure of the protoplast medium is usually manipulated by the addition of sugars or sugar alcohols. Mannitol and sorbitol are the most common additions, with mannitol being preferred for leaf mesophyll protoplasts.

The osmoticum for a protoplast culture medium may be metabolically active, such as glucose or sucrose, or inert like mannitol. Active substances are metabolised by the culture and thereby gradually lower the osmotic strength of the culture medium. This is said to be necessary to eliminate the osmotic shock that would otherwise occur when regenerated cells are transferred to a shoot regeneration medium, (Eriksson, 1977). Since mannitol is inert and not metabolised, no such reduction in osmotic strength of the culture medium takes place.

A wide range of osmotic strengths have been used during the isolation of protoplasts, and particularly for mesophyll protoplasts, the environmental conditions under which the donor material is grown influence the osmotic concentration needed for successful protoplast isolation. (Shepard and Totten, 1975).

This begs the question as to what strength osmoticum should be used to isolate protoplasts for any given set of plant growth conditions, and if mannitol is used, how can the osmotic strength of the culture medium be reduced to avoid the osmotic shock identified by Eriksson (1985). Further, has this reduction to be made gradually and in small amounts, or are protoplasts able to withstand an immediate and substantial drop in osmoticum without exhibiting signs of undue harm, and regenerate cell walls and subsequently divide?

Generally, the same osmoticum used in the protoplast isolation medium is retained in the culture medium, but it has been demonstrated that protoplasts were able to endure a reduction in strength of osmoticum immediately following isolation of 4M, i.e. isolation in a medium containing 11% mannitol to culture in one containing 7% mannitol. In addition. this culture medium strength

could be reduced further by an amount equal to 1% (1M) mannitol per day for four days, bringing the culture medium strength to just 3% mannitol (3M). Protoplasts exposed to this rapid dilution regime achieved a plating efficiency in excess of 40% compared to undiluted controls which achieved a plating efficiency of less than 2% during the same culture period.

It was noted that some of the cells produced in this rapid dilution regime were not the usual callus type cells but the culture also produced cells that were in no way different, at least superficially, from those in the undiluted culture and would have been recognised as typical callus cells.

Following enzymatic isolation of protoplasts from leaf or cultured cells, the protoplasts must be "purified". The most commonly used methods for achieving this are filtration / centrifugation and flotation. Both procedures involve a number of manipulations and any procedure which reduces or eliminates any particular stage is to be encouraged. The method reported by Piwowarczyk, (1979) appeared particularly attractive as it claimed that the purification of protoplasts could be achieved in a single centrifugation procedure. Such promise was not fulfilled, and to date, it has not been possible to achieve the results reported.

A good purification procedure is required to separate intact viable protoplasts from the rest of the materials present, including enzymes, undigested plant material, debris and various plant cell organelles liberated from burst protoplasts, in the shortest possible number of steps. Even the two favoured procedures present problems, fragile protoplasts do not normally withstand filtration and if the alternative method is used, they need to be able to tolerate centrifugation in a concentrated sucrose solution. A successfully purified protoplast preparation still does not guarantee a successful culture.

Isolated protoplasts are usually cultured in either liquid or semi-solid media. Liquid culture allows easy dilution and transfers but has the disadvantage of not permitting the study of individual cells or the isolation of colonies derived from one parent cell. Immobilised protoplasts give rise to cell clones and allow accurate determination of plating efficiency, but once immobilised complex

hand manipulations are needed to transfer from one culture medium to another.

The techniques used for protoplast culture are basically the same as those for tissue and cell culture with the proviso that the size of the protoplast population to be handled determines the type of culture vessel and system used. If liquid medium is used then the available possibilities include culture in liquid in medical flats, various sizes of Petri dish and drop culture on the inner side of the lid of a Petri dish. More recently culture has been attempted in microchambers, and a technique called multiple drop array has been tried. In the former, culture takes place in the well of a cavity slide and the latter culture takes place in five to ten large drops on the bottom of an ordinary petri dish.(Harms et al, 1979).

A similar range of possibilities exists if semi-solid media is used. Agar of different qualities is the most frequently used material to solidify the culture medium. More recently there has been interest shown in other gelling agents besides agar. Agarose, K-carrageenan, alginate, gelatin and polyacrylamide have all been used. Agarose has given the best results in terms of retention of viability and secondary product production (Brodelius and Nilsson, 1980). Others report improvement of plating efficiency with agarose instead of agar (Lorz et al, 1983; Shillito et al, 1983).

These studies showed a similar effect to those discovered in this study, namely that the agar component itself plays a role in the solidified culture medium. The studies of Lorz et al and Shillito et al also showed that the improved plating efficiency achieved with agarose is not due just to the absence of contaminating toxic substances. One aspect here which would be worthy of further investigation is the effect on protoplasts due the greater negative charge on agar compared to agarose. SeaplaqueR agarose which has been used in some studies is a chemically modified agarose with additional hydroxyethyl groups introduced into the agarose molecule. The superior ability of agarose to support protoplast culture may relate to the essentially neutral characteristic of the polymer.

If these new plating techniques could be combined with the use of a discontinuous gradient technique to identify, what has been suggested are protoplasts from cells which have gone through a

S-phase of the cell cycle, then the prospects for protoplast culture would appear to be much improved. Implicit in this statement is an assumption that the method of culture media preparation can be controlled sufficiently well to reduce or even eliminate some of the heat induced changes identified as occurring in this study.

Whilst many minor modifications of the most frequently used protoplast culture media have been reported, usually in response to an empirical determination of the requirements of a particular species, most culture media separate into two broad categories. These are chemically defined media, also sometimes called regular, and complex culture media, also called rich media. The actual requirements of isolated protoplasts in terms of the composition and nature of the culture medium is complex and poorly understood. It appears that even if time and trouble is taken to carefully define the culture medium, it can easily become an undefined medium as a result of heat induced changes during its preparation.

There is no easy way to remove these problems and alternatives to autoclaving, such as filter sterilisation, bring with them their own sets of problems. It may be necessary ultimately to assess the heat stability of each culture medium component in turn, and to treat each one in the most appropriate and least damaging way to effect sterilisation and construct the required culture medium, post-sterilisation. This would be a major task, and probably one that would not be welcomed, but at this present time would seem to be the only alternative if the true role of the components in the culture medium for protoplasts is ever to be fully described.

It appears that some generalisations can be made for the isolation and culture of protoplasts. Conditions which optimise growth of the plant will increase the probability of isolation of metabolically active protoplasts. Conditions most often manipulated to achieve this are light, temperature, humidity and supplementary feeding. These factors are difficult to control without an environmental growth chamber. Fluctuating daylengths and light intensities affect the isolation and growth rate of protoplasts. Control of environmental conditions would allow greater reproducibility between experiments.

If the crude protoplasts preparation contains a large amount of debris and undigested cells, a suitable procedure for cleaning must be found which pays due regard to the mechanical strength of the protoplasts. On purification a procedure such as discontinuous gradient centrifugation may be able to separate those protoplasts more likely to divide.

When culturing, it is often necessary to experiment with a number of plating densities before an optimum is found. Also, if it is known that the protoplasts to be cultured produce large amounts of harmful products then agar culture may assist in separating clones from each other, and thereby restricting the effects of these harmful products by acting as a diffusion barrier. However, if the culture technique reqires the addition of fresh culture medium at stages in culture, then liquid culture is to be preferred.

Once cell division has been initiated and callus initiated the prospect for regeneration of whole plants is good. The most critical stage in the culture is the period from protoplast to callus. This period is particularly susceptible to environmental conditions and medium components, especially if these have in some way changed from those present in the medium prior to autoclaving.

If greater attention to these aspects is made, it may be possible to add to the list of species responding to attempts at regeneration of whole plants and to no longer be required to accept the idea of a recalcitrant species.

Final Conclusions

Tobacco mesophyll protoplasts can be readily isolated using both commercial and purified enzyme preparations, and at various times of the year. The time of year, rather than enzyme purity, is more important in securing a preparation of protoplasts capable of regenerating a cell wall and dividing in culture. Cell wall regeneration and division with a high plating efficiency are imperative if any use is to be made of the potential of protoplasts as sources for plant production, somatic hybrids and other genetically altered plants.

As a means of achieving the maximum plating efficiencies, there are certain techniques which can be used, and factors which need special attention before any procedures using protoplasts are considered.

The time of year is an important factor as far as tobacco protoplast plating efficiency is concerned, and this may well apply to other species and should be investigated before any major work is commenced. Some workers report increased benefits using purified enzymes, but this was not the case with tobacco. However, the combination of suitable time of year and enzyme purity may prove beneficial for other species.

Isolated protoplasts derived from tobacco leaves were found to consist of two distinct populations when subjected to

centrifugation on a discontinuous gradient consisting of eight phases made up of mannitol and mannitol/percoll. Two distinct bands formed on the gradient, the lower of which, produced significantly higher plating efficiencies in culture at all times of the year. The use of such a gradient readily identified those protoplasts more likely to divide in culture.

The media used to culture the protoplasts was found to be affected by the treatment given during autoclaving, which resulted in the loss of activity and it was found necessary to reduce the amount of time spent in the autoclave to the minimum. Thiamine hydrochloride present in MSP medium was found to be adversely affected immediately by temperatures above 100°C, and continued to be for periods up to 100 minutes. Careful attention to autoclaving regimes is therefore necessary in any work, to reduce the changes which may occur on autoclaving.

Whilst the culture of protoplasts in agar medium is particularly attractive as it allows the development of individual, immobilised protoplasts to be followed, it was found, that in addition to the changes induced in media by autoclaving, the quality of the agar used to produce the solid or semi-solid media also affected overall plating efficiency. So-called high grade agars were not necessarily the best for protoplast culture, and the lower grade agars were better at nurturing protoplasts during culture.

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