PLANT CELL LINES RESISTANT TO ENVIRONMENTAL STRESSES

by

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To my parents for many years of encouragement and support.

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Some of the work included in this thesis has already been published as follows:

Dix, P.J. and Street, H.E. (1974).

    Effects of p-fluorophenylalanine (PFP) on the growth of cell lines

    differing in ploidy and derived from Nicotiana sylvestris.

        Plant Sci. Letters 3, 283 - 88.

Dix, P.J. and Street, H.E. (1975).

    Sodium chloride resistant cultured cell lines from Nicotiana

    sylvestris and Capsicum annuum.

        Plant Sci. Letters (in press).

This thesis is my own work unless otherwise acknowledged and has at no

time been submitted for another degree.

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I certify that this statement is correct.

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CONTENTS

ABBREVIATIONS

DNA	Deoxyribonucleic acid
ADP	Adenosine diphosphate
NADH	Nicotinamide adenine dinucleotide, reduced form
BUdR	5-Bromodeoxyuridine
NTG	N-Methyl-N'-nitro-N-nitrosoguanidine
EMS	Ethylmethanesulfonate
PFP	p-Fluorophenylalanine
BSA	Bovine serum albumen
EDTA	Ethylenediamine tetra-acetate
EDDHA	Ethylenediamine di-O-hydroxyphenylacetate
2,4-D	2,4-Dichlorophenoxyacetic acid
NAA	Naphthalene acetic acid
IAA	Indole-3-acetic acid
p-CPA	p-Chlorophenoxyacetic acid
GLC	Gas liquid chromatogram
S.E.M.	Standard error of the mean
$\bar{G}$	Mean generation time

GENERAL INTRODUCTION

The recognition by Haberlandt in 1902 of the potentialities of culturing individual plant cells "in vitro" preceded by many years the advent of plant tissue culture (Gautheret, 1939; White, 1939) and by half a century the initiation of culture lines from single cells (Muir, Hildebrandt & Riker, 1954). In the last two decades intense interest has been focussed on the growth "in vitro" of plant tissues and cells, and methods have been evolved using them for the study of a wide variety of problems, from basic studies on cellular metabolism to such practical dilemmas as finding techniques for the rapid propagation of valuable species or varieties (Street, 1974). Somewhere about halfway along this line between basic research and immediate application is the use of "in vitro" cultures for the genetic improvement of plants; applied in its goal yet still requiring an extension of basic knowledge before practical application will become feasible.

The use of plant tissue cultures for mutation and the selection of variants has certain prerequisites if it is to have any practical potential. Firstly the plant material must be cultured in a form which makes it amenable to mutation and selection, and secondly the cultures must have, and retain the ability, under suitable conditions, to reconstitute the intact plant by one of several developmental sequences (Konar, Thomas & Street, 1972). The latter criterion has been met using an ever-increasing number of species, and a wide range of media and methods, but no single reliable system has been found which can be universally used to initiate shoots or embryos in plant tissue cultures. The requirement for the tissue to be available in a suitable form for mutation and selection depends on the nature of the mutant under consideration. Culture systems are likely to be at least as convenient as intact plants for the application of mutagenic agents, and the large number of dividing units involved should improve the chances of selection without

recourse to the use of such agents. For these reasons, if one considers

mutants which require only a very small number of gene changes, their

induction should cause no real problems. The critical stages are likely

to involve the method used to select mutant cells from a mixed population.

Where mutants resistant to growth inhibition by an antimetabolite or a

hostile environment are required the selection pressure can be applied to

callus cultures and those regions of culture showing survival and growth

can be removed from the dead or dormant sectors and grown up as independent

callus lines. In these the continued resistance can subsequently be

examined. This method has been used by a number of workers, particularly

to obtain mutants resistant to antimetabolites (e.g. Binding, Binding &

Straub, 1970; Maliga, Sz. Breznovits & Marton, 1973; Maliga, Marton &

Sz. Breznovits, 1973; Lescure, 1973), and has been suggested as a means of

selection for chilling tolerance (Steponkus, 1972). If the primary aim of

the work is to obtain mutants these methods can be used, but they lead to

problems if quantitative aspects of mutant induction and isolation are to

be studied. Calli may contain variable proportions of viable cells and it

is difficult to determine how many cells in a single callus piece are

contributing to regrowth after selection. Generally quantitative work with

callus cultures is difficult, fresh weight determinations require the sacrifice

of large quantities of tissue (wholly unsuited to the study of small

quantities of mutant callus) and non-destructive methods (e.g. Fowler &

Janick, 1974) are tedious and require the callus to grow in a regular form.

Generally suspension cultures are more suited to the selection of

resistant mutants and since the development by Bergmann (1960) of a means of

cultivating single cells on agar plates, attention has been paid to this

type of approach (e.g. Widholm, 1974; Mansfield, 1973; Gathercole,

unpublished report). A plating method has advantages over selection within

agitated suspension cultures as discrete colonies are likely to arise from

single mutant cells, whereas a mutating suspension culture may comprise the

derivatives of a number of mutant cells. Often, however, it is difficult
to plate single cells with a high efficiency of colony formation, and the
plating of small aggregates from filtered suspensions must be used instead.
This is a compromise method, allowing a semi-quantitative consideration of
the mutant isolation procedure and retaining a fairly high probability that
mutant lines arise from single cells.

The advent of the successful enzymic isolation of higher plant proto-
plasts (Cocking, 1960) put a new perspective to the problem which many
workers were quick to grasp (Cocking, 1973a; Carlson, 1973; Melchers &
Labib, 1973). The potentials of protoplast culture are many and varied,
but include, if they can be plated at a high level of efficiency, a much
improved system, from the quantitative point of view, for the selection of
resistant mutants. In some species, plants can be regenerated rapidly from
protoplasts, with a minimal callus stage (e.g. Takebe, Labib & Melchers,
1971; Bajaj, 1972) thereby reducing the possibility of multiple chromosome
mutations in culture (Sacristan, 1971; Sunderland, 1973; Bayliss, 1973) and
the chances of reversions occurring. Successful use of these methods have
been reported by Carlson (1973a).

So far we have considered the means of isolation of resistant mutants,
as probably the easiest type of mutant to select for, judging by the
success that has already been reported, of obtaining such mutants stable
through a plant stage and hence amenable to genetic analysis (Maliga,
Sz. Breznovits & Marton, 1973; Marton & Maliga, 1975; Carlson, 1973a;
Widholm, 1974). Other classes of mutant, such as auxotrophic mutants,
require the use of tricks to allow identification of the mutant clones.
Replica plating methods cannot be used for plant cell cultures because of
the high level of adhesion between cells; even very friable colonies cannot
be relied upon to give a good and repeatable transfer using a stamp. Carlson
(1970) succeeded in using the destruction of growing cells by BUdR for the
selection of vitamins and amino acid requiring mutants of tobacco, but this

method has not yet proved widely applicable to plant tissue cultures. This

consideration of the means of isolation of mutants can be summarised by

saying that several approaches are available for the acquisition of

resistant mutants, and for some of these approaches the technical difficulties

have been overcome, but a great deal more work has to be done to surmount

basic difficulties in the selection of auxotrophs. There are other means

by which hereditary changes may be induced in plants using plant tissue,

cell or protoplast culture techniques. These include somatic hybridisation

by protoplast fusion (e.g. Power, Cummins & Cocking, 1970; Cocking, 1973b;

Melchers & Labib, 1974) and the incorporation into cells or protoplasts of

foreign DNA, either in the form of isolated nuclei or organelles (Potrykus

& Hoffman, 1973; Potrykus, 1973), bacteria (Davey & Cocking, 1972), virus

(Takebe & Otsuki, 1969) transducing phage DNA (Doy, Gresshof & Rolfe, 1972;

1973 a and b; Johnson, Grierson & Smith, 1973) and naked DNA (e.g. Ohyama,

Gamborg & Miller, 1972; Ledoux, Huart & Jacobs, 1974). Most of this work is

in its infancy and although these methods probably have a great deal to offer,

both from the practical viewpoint of crop improvement and for the improvement

of our knowledge of plant genetics, their detailed consideration is beyond

the scope of the present work.

So far tissue, cell and protoplast cultures have been considered in

general terms with no consideration of the source of material to be used. As

early as 1958, Melchers and Bergmann pointed out the great potential of

haploid plant cell cultures for studies on the induction and isolation of

mutants. Many workers have since realised the significance of haploid

cultures, particulatly for the selection of recessive mutants, and the bulk

of research in this field, including much that has already been mentioned,

now involves the use of haploid callus, cell suspensios, or protoplasts. For

certain plant species haploids can be obtained by such methods as parthogenesis,
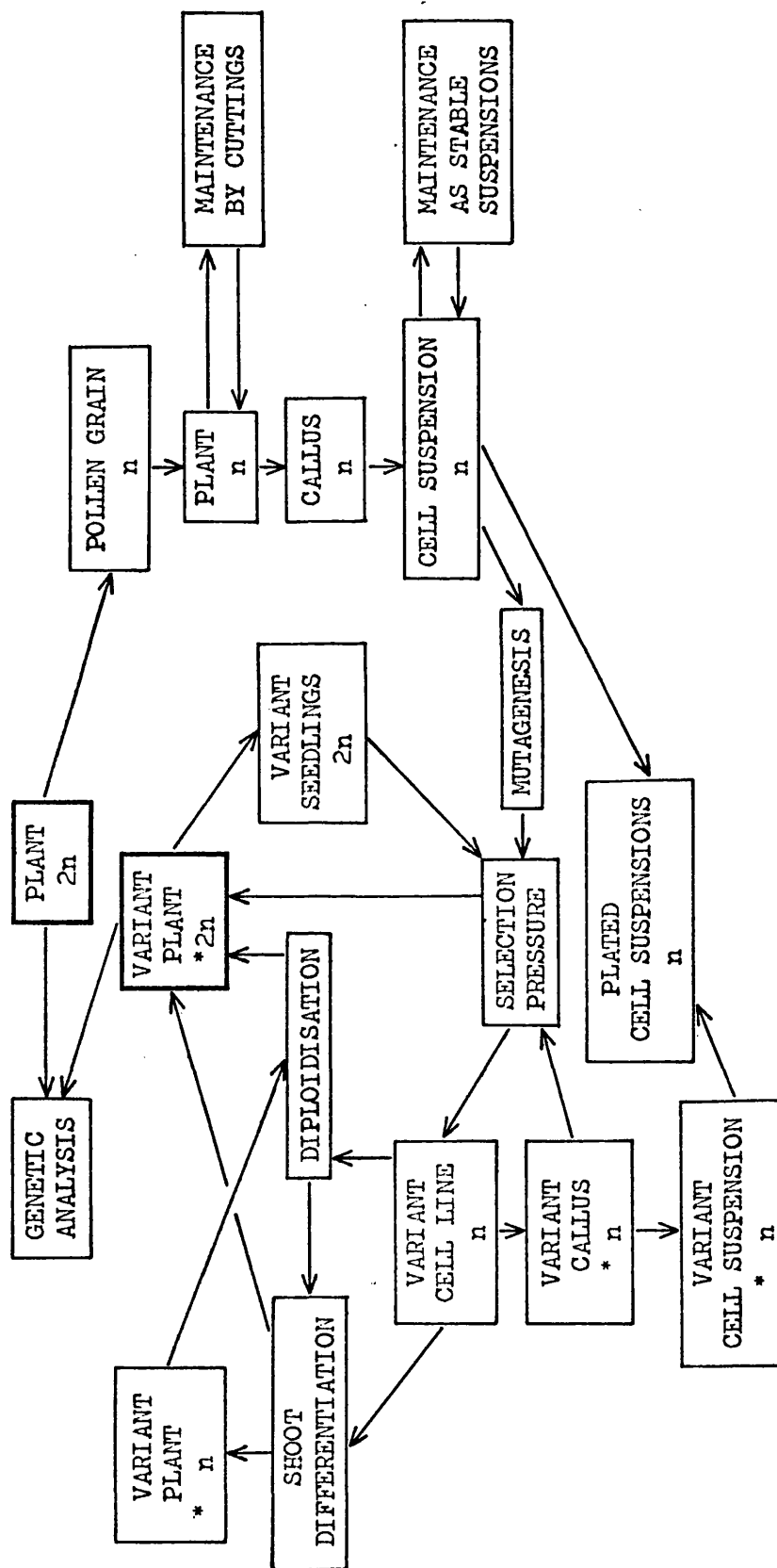
androgenesis and chromosome elimination (Kasha, 1974) but the advent of

anther culture (Guha & Maheshwari, 1964) increased the range of species

for which this was possible. So far the technique has not proved readily

applicable to all species, but the number is increasing year by year

(Sunderland, 1974). It appears to work well for certain families of plants

and success has been obtained with a wide range of Solanaceae including

various species of Nicotiana (e.g. Nitsch & Nitsch, 1969; Nitsch, 1970;

Sunderland, 1971), Datura (Guha & Maheshwari, 1964; Kohlenbach & Geier,

1972), Lycopersicon (Sharp, Dougall & Paddock, 1971; Gresshoff & Doy, 1972),

Solanum (Harn, 1972; Zenkteler, 1973; Dunwell & Sunderland, 1973; Raina &

Iyer, 1973), Atropa (Rashid & Street, 1973), and Petunia (Raquin & Pilet,

1972). The products of pollen mitosis vary giving rise to embryos, callus

masses, or both. Embryos may develop into haploid plantlets which can be

used to initiate haploid callus cultures or grown to a suitable stage for

the isolation and culture of haploid protoplasts. In certain cases shoot

and root initiation on haploid callus from anther cultures can be induced

by suitable modification of the cultural conditions. The production of

pollen derived plants is desirable even where tissue cultures are to be

used for mutation and selection studies as they can be used to provide uniform

tissue originally derived from a single pollen grain. The technique of

isolated pollen culture developed by Nitsch and Noreel (1973) and Debergh

and Nitsch (1973) may provide a useful system for mutant selection, allowing

selection pressures to be applied directly to plated pollen grains. The

survivors would give rise to embryos and plantlets completely bypassing a

callus stage.

The present project was set against the backcloth of the researches

outlined above. The aim was to examine the potential of plant tissue or cell

culture as a system for mutation and selection capable of giving rise to

plants with an improved genotype. Clearly there is a wide range of
starting materials from which such a study could be made. As the work
was to be carried out in a laboratory highly orientated towards the growth
and study of cell suspensions and in which considerable progress had been
made in the plating of such suspensions (Mansfield, 1973) this system was
chosen as the most readily applicable to selection studies. It was also
desirable to use haploid material, so a true diploid species (Nicotiana
sylvestris) capable of giving rise to haploids by anther culture (Bourgin
& Nitsch, 1967) was chosen. This species was also highly morphogenic under
suitable conditions of culture (Okonkwo, unpublished observations) and
therefore promising from the point of view of reconstituting plants from
variant cell lines. At the same time, it was proposed to examine the
application of these methods to a plant species on which less background
work had been done. Capsicum annuum (sweet pepper) was chosen as a species
of economic importance, and a member of Solanaceae, a family which had
proved generally very amenable to anther culture. At the outset of this
study no successful anther culture or tissue culture had been reported for
this species. It was proposed to examine the cultural requirements of the
species, including the possibility of reconstituting plants from tissue
cultures, and at the same time to try to obtain haploid material by anther
culture. In order to improve the chances of the latter five varieties (all
F1 hybrids) were obtained, after evidence had been presented that there
might be a varietal effect on the response of a species to anther culture
(Gresshoff & Doy, 1972 a and b). If the cultural criteria for this species
could be met, it was intended to subject it to the same programme of mutant
induction and selection as was used for N. sylvestris.

The selection pressures chosen for application to the cultures were a
range of environmental stresses. These were easy to apply and it was hoped
that resistant variants could be obtained showing modifications at the

Plan for the proposed isolation of variants resistant to environmental stresses. To be applied to *Nicotiana sylvestris* and *Capsicum annuum*.

MAINTENANCE BY CUTTINGS

MAINTENANCE AS STABLE SUSPENSIONS

POLLEN GRAIN n

PLANT n

CALLUS n

CELL SUSPENSION n

PLANT 2n

GENETIC ANALYSIS

VARIANT SEEDLINGS 2n

MUTAGENESIS

VARIANT PLANT *2n

SELECTION PRESSURE

PLATED CELL SUSPENSIONS n

DIPLOIDISATION

VARIANT CELL LINE n

VARIANT CALLUS * n

VARIANT CELL SUSPENSION * n

VARIANT PLANT * n

SHOOT DIFFERENTIATION

*Stages at which characterisation of variants might be feasible.

2n = diploid   n = haploid

cellular level which might permit resistance in the reconstituted plants.
The only work on the selection, at the cellular level, of variants resistant
to environmental stresses was presented in a short report by Steponkus (1972).
He obtained calli of Hedera helix with increased chilling tolerance and
pointed out the potential of such a technique for the improvement of
chilling tolerance of crop plants. C. annuum is one species the chilling
sensitivity of which causes its range to be closely delineated by climate,
so small changes in its level of tolerance could have a substantial commercial
application. Chilling tolerance was selected for using both species, and
high temperature and high salt resistance chosen as other environmental
stresses worthy of examination.

This strategic research plan which formed the framework to which the
various experimental programmes were to contribute is set out opposite. The
extent to which this plan was achieved or modified in the light of technical
difficulties and limitations of time is considered in the General Discussion
which terminates this work.

MATERIALS & METHODS

(A) Plant material

Seeds of <u>Nicotiana</u> <u>sylvestris,</u> Speg. and Comes from a stock of plants maintainedatLeicester University Botanic Gardens for several years, were germinated and grown in trays in the greenhouse. At a rosette diameter of 1 to 3 inches, they were potted independently in Levington's potting compost, under which conditions they flowered after 2 to 3 months.

. Seeds of five varieties of <u>Capsicum</u> <u>annuum</u> (Bell Boy, Canape, New ace, Propa and Westlandia) were obtained from A.L. Tozer Ltd., Pyports, Cobham, Surrey, and grown to flowering in the greenhouses at the Unilever Research Centre, Colworth House, Sharnbrook, Bedfordshire.

(B) Initiation and maintenance of cultures

(i) Glassware

Glassware used in the preparation of media, and Erlenmeyer flasks, 1 litre bottles, Universal bottles and test tubes, used for the maintenance of cultures were cleaned as described by Street (1973).

(ii) Culture media

Media were prepared from "Analar" grade chemicals and the pH adjusted by dropwise addition of 1N NaOH to the liquid medium. To solidify medium 0.8% Difco "Bacto-agar", or Oxoid, Ionagar No. 2, was added to the medium, after adjustment of the pH, and melted by placing the preparation vessel in a steamer. Media were dispensed into appropriate culture vessels and autoclaved at $121^{\circ}C$ (15 psi) for 15 minutes.

Two types of culture media were used throughout most of the work, modifications are described as they arise. For initiation of callus cultures and routine maintenance of callus and suspension cultures, the medium of Linsmaier and Skoog (1965) was used. Except where otherwise stated, this

contained 2,4-dichlorophenoxyacetic acid (2,4-D), 0.4mg/l and

6-furfurylaminopurine (kinetin) 0.03 mg/l. For anther culture and the

maintenance of sterile plants (Appendix 1), the "H" medium of Nitsch and

Nitsch (1969) was used, the hormone and sucrose levels being modified as

appropriate.

(iii) Initiation of cultures

Callus cultures were normally initiated from petioles or leaves. These

were surface sterilised in 20% "Domestos" for 10 minutes before being cut

into 2 to 3 mm segments and placed on solid medium in 100 ml Erlenmeyer

flasks (25 ml medium) or Universal bottles (10 ml medium). Flower buds

were surface sterilised in the same way prior to dissection for anther

culture. Suspension cultures were commenced by dispersion of friable callus

in liquid medium (60 ml) in 250 ml Erlenmeyer flasks.

(iv) Maintenance of cultures

Culture flasks were sealed with autoclaved squares of aluminium foil

(single thickness). Flasks containing callus cultures or plant material for

callus initiation were incubated in a constant temperature room at 25°C under

constant illumination. Flasks containing suspension cultures were maintained

on a platform shaker (L.H. Engineering Co. Ltd., Stoke Poges, England) at

120 r.p.m., with a 2 inch diameter of rotation. The flasks were maintained

under constant illumination at 25°C in a controlled temperature room.

Callus cultures were subcultured by removal of portions of the callus

to fresh medium in 100 ml Erlenmeyer flasks. Suspension cultures were

subcultured, using automatic pipettes (Arnold R. Horwell Ltd., London) with

3 mm diameter canulae, into 250 ml Erlenmeyer flasks containing 60 ml fresh

medium. The usual initial cell density on subculture was 10^{5} cell ml^{-1}.

Cultures were normally subcultured every 28 days (callus cultures) or every

21 days (suspension cultures) but with the wide range of cell lines

under consideration, these times were subject to modification.

(C) Measurements on cultures

(i) Cell number

A chromium trioxide treatment was used to disperse cell aggregates

sufficiently for counting, as described by Henshaw et al. (1966). 2 ml

samples of suspensions were normally removed to Universal bottles and 10 ml

10% (w/v) chromium trioxide solution added. The bottles were then incubated

for 15 min. in a $70^{\circ}C$ water bath and shaken at the maximum speed on a

flask shaker (Baird & Tatlock Ltd.) for 3 min. The lengths of the chromium

trioxide and shaker treatments occasionally had to be modified to give a

high level of cell separation. Special cell counting slides were prepared

as described by Henshaw et al. (1966) except that the depth of the chambers

was fixed at 1 mm. This gave a volume of 0.8 μl when viewed at 100x magnif-

ication. Chromium trioxide treated cell preparations were diluted to give

5 to 20 cells per field when samples were placed on the counting slide and

viewed at 100x magnification. The cells in each of 10 random fields in each

chamber were counted at this magnification using a Watson Microsystem 70

microscope, and this procedure was repeated for 10 chambers per sample. The

cell number per ml of culture was determined by multiplying the mean number

of cells per field by the appropriate correction ($\frac{10,000}{8}$) to give an

expansion of the volume counted (0.8 μl) to 1 ml and multiplying by the

dilution from the culture. The mean counts of the 10 sets of 10 fields

were determined and the standard error of this mean (S.E.M.) was calculated.

S.E.M. values >5% of the mean were considered unsatisfactory and the cell

counts were repeated.

(ii) <u>Dry weight</u>

Glass fibre pads (25 mm diameter, Whatman GF/C) were heated for at

least 6 hours in an 80°C oven. They were then placed in a desiccator for

60 min. and weighed individually. Cell samples were removed from

suspensions and the cells collected on the weighed pads by vacuum filtration.

The volume of the culture used depended on the age of the culture and the

density of the cells in it, but was adjusted to give about a 1 mm depth

on the filter pads. The cells were washed twice on the pad, with distilled

water, and the pads returned to the oven for 12 hours. They were then placed

in the desiccator for 60 min. before re-weighing. The original dry weight of

the pad was subtracted to give the dry weight of the cells which could be

expressed on a per ml. of culture or per 10^6 cells basis.

(iii) <u>Packed cell volume</u>

Samples (10 ml.) were removed from cultures and transferred to 10 ml.

graduated glass centrifuge tubes and spun at 1,500 x g for 10 min. The

volume of the pellet obtained could be expressed as a proportion of the

sample volume or as packed cell volume per 10^6 cells.

(iv) <u>Cytology</u>

The following procedure was used to prepare slides for the determination

of mitotic indices, and usually for microdensitometry. For chromosome counts

it was preceded by a pretreatment in which the cells were incubated in the

presence of 0.1% (w/v) colchicine (BDH) for 4 hours. Cell samples were

transferred to 10 ml. centrifuge tubes. This allowed the solutions used in

the subsequent stages to be rapidly decanted after a 2 min. spin at 2,000 -

3,000 x g. The samples were fixed in 50% aqueous formic acid for 8 - 12 hours

and washed 3 times with distilled water. The material was then hydrolysed

by resuspension in 1 N aqueous HCl preheated to 60°C and incubated at this

temperature for 12 minutes. The acid was decanted after a rapid spin
(total time for removal = 2 min.) and the material resuspended in basic
fuchsin solution (Darlington & La Cour, 1969) and kept at room temperature
for 2 hours. It was then washed 3 times (5 min. per wash) in sulphur
dioxide water (50 ml. 1N HCl, 5g. potassium metabisulphite made up to 1 l.
with distilled water) before being resuspended in 25% acetic acid.

Permanent squash preparations were prepared as follows. A small amount
of material was placed on each slide in 25% acetic acid. A cover-slip
coated with silicone "repelcote" was placed over the material and tapped
with the handle of a needle to disperse the cells. The preparation was
then squashed by firm vertical pressure of the thumb through several layers
of filter paper. The slide was then placed on a block of solid carbon
dioxide until frozen. This allowed the cover-slip to be prised off with a
razor blade. All slides were placed in absolute alcohol and then processed
through 1:1 alcohol:xylol, 1:3 alcohol:xylol to absolute xylol, and mounted
in XAM neutral mounting medium (Searle Diagnostic).

Microdensitometer profiles were compiled based on a suitable number of
measurements made using a Vickers M85 scanning microdensitometer using a
100x objective and a wavelength of 565 nm. Arbitrary values were plotted
on a linear or logarithmic scale and considered to represent the distribution
of DNA content in the nuclei, based on the assumption that the density of
Feulgen staining is directly proportional to the DNA content of the nucleus
(Leuchtenberger, 1958).

(v) Viability

The viability of cells in suspension cultures was estimated using the
technique of Widholm (1972). A stock solution of fluorescein diacetate
(National Biochemicals Corporation) was prepared (0.5% in 100% acetone) and
stored at $-20^{\circ}C$. 0.5 ml. of this was added to 25 ml. culture medium in ice.

This solution was freshly made up when needed and replaced every 30 min. One drop was added to 2 to 3 drops of cell suspension on a microscope slide and the proportion of flüorescent cells determined using a Zeiss photomicroscope.

(D) The plating of cell suspensions

Cell suspensions were filtered through nylon mesh (Nybolt, Swiss silk bolting cloth MFG Co. Ltd., or Henry Simon Ltd., Stockport, England) in the filter tower shown on Plate M.1. Material retained by the mesh was flushed twice with fresh medium and the accumulative supernatant transferred to sterile 250 ml. Erlenmeyer flasks. The medium used for plating comprised the usual culture medium containing 0.9% agar and dispensed either into 100 ml. (45 ml. per flask) or 250 ml. (90 ml. per flask) Erlenmeyer flasks. For plating the medium was melted in a steamer and the flasks placed in a water bath at 38°C to equilibrate at this temperature. The cell number in the filtered suspension was determined and the appropriate volume calculated to give the required inoculation density when diluted to a total of 50 or 100 ml. Occasionally this involved dilution of the suspension with fresh liquid medium. These volumes of suspension were then added to the flasks of molten agar medium together with sufficient liquid medium to make a total of 50 ml. or 100 ml. This was immediately dispensed into sterile plastic Petri dishes (9 cm. diameter, Sterilin Ltd.) 10 ml. per dish. Dishes were sealed with Nescofilm (Bando Chemical Ind. Ltd., Japan) and incubated in a controlled temperature room at 25°C in the dark.

(E) The use of isolated mitochondria

(i) Isolation procedure

The solutions used closely resemble those of Raison and Lyons (1970) and are given in Table M.1. Cells were removed from suspension by filtration

PLATE M.1. Tower used for sterile filtration of cell suspensions prior

to plating.

F: Position of filter
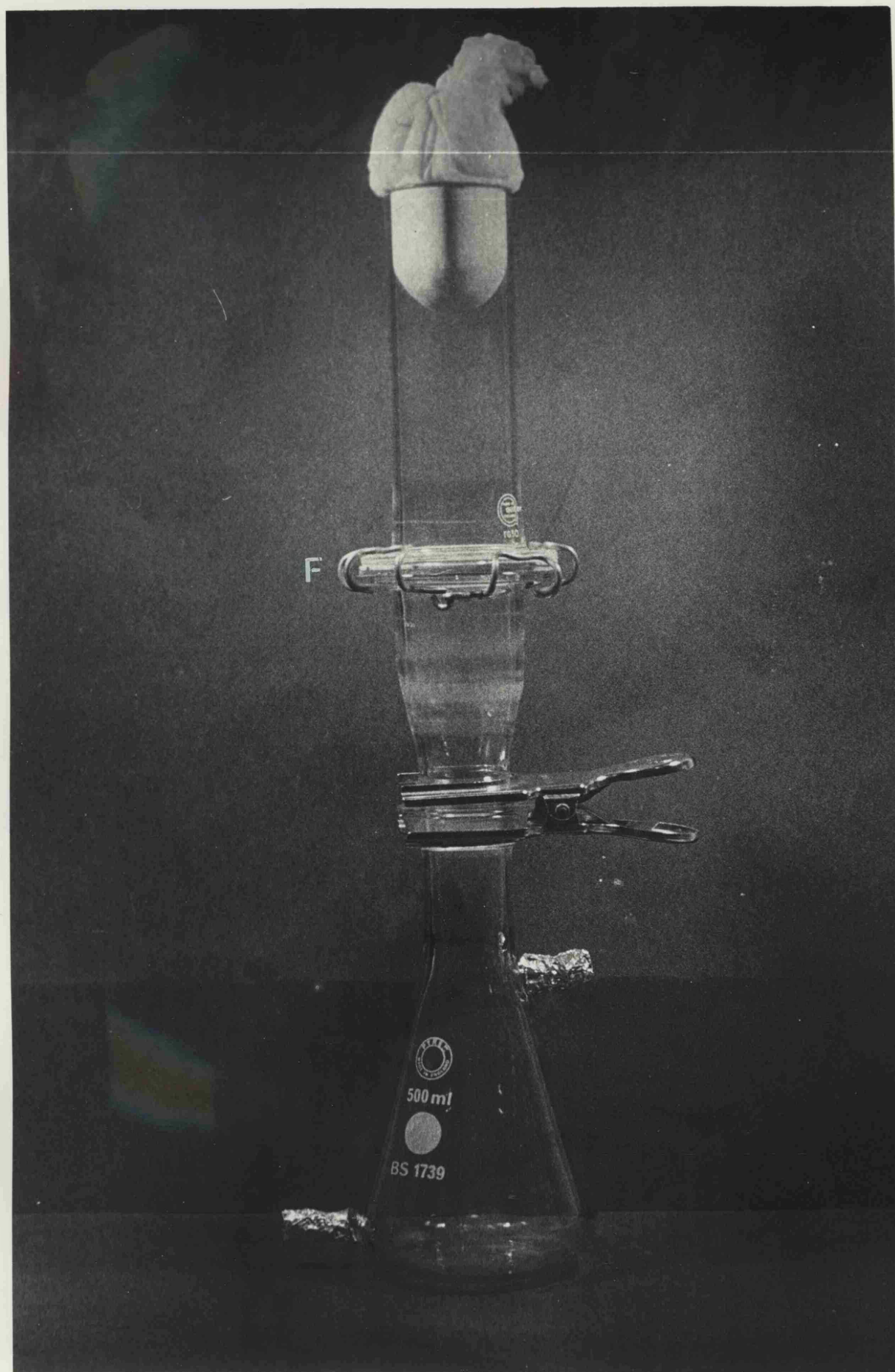
TABLE M.1.

Media used in the isolation of mitochondria from cell suspensions of
C. annuum

	Component	Concentration
Isolation medium	mannitol	0.5 M
	KCl	0.01 M
	EDTA	0.005 M
	tris	0.025 M
	$MgCl_2$	0.002 M
	cysteine hydrochloride·	0.004 M
	BSA (Sigma,London)	0.5 mg/ml
	pH: 7.6	
Wash medium	mannitol	0.5 M
	KCl	0.01 M
	$MgCl_2$	0.001 M
	tris	0.01 M
	KH_2PO_4	0.01 M
	pH: 7.2	
Reaction medium	sucrose	0.25 M
	tris	0.01 M
	K_2HPO_4	0.01 M
	$MgCl_2$	0.005 M
	EDTA	0.005 M
	BSA	0.5 mg/ml
	pH: 7.2	

All pH adjustments were made using 1N KOH.

through 4 layers of muslin and washed with distilled water and 0.3M

mannitol, both of which had been precooled in ice. They were then

resuspended in the isolation medium and macerated by a 10 second, full

speed treatment with a Polytron homogeniser. The resulting cell debris

was largely removed by a further filtration through muslin, the pH of the

supernatant was adjusted to 7.6 using 1N KOH, and it was transferred to

50 ml. polypropylene centrifuge tubes in ice. These were spun at 2,500 x g

for 15 min. using an MSE High Speed 18 centrifuge cooled to $5 - 10^{\circ}$C, and

the pellets discarded. The supernatants were spun at 12,000 x g for 20 min.

and the resulting supernatants discarded. The pellets were resuspended each

in 20 ml. wash medium and the contents of each pair of tubes pooled. These

were subjected to a further low speed spin at 2,000 x g for 10 min. and the

supernatants finally spun at 8,000 x g for 15 min. The supernatants were

discarded and the pellets, containing mitochondria, resuspended each in

2 to 5 ml. wash medium. The contents of the tubes were then pooled and

returned to 25°C prior to use.

(ii) Protein estimation

The concentrations of mitochondria in the preparations were considered

to be directly proportional to the protein concentration. The method of

protein estimation is based on the Folin method described by Layne (1957).

The reagents used are given in Table M.2. A standard curve was prepared

using serial dilutions of a 1mg./ml. solution of bovine serum albumen (BSA).

For each dilution 0.2 ml. protein solution was added to 3 ml. reagent C in a

test tube and allowed to stand at room temperature for 10 min. The tube

was then mixed thoroughly and 0.2 ml. reagent E added. The tube was again

thoroughly mixed and allowed to stand at room temperature for 30 min. after

which time the optical density was read at 750 mµ using a Unicam SP600

TABLE M.2.

Reagents used in the estimation of protein concentration

Reagent	Components
A	2% Na_2CO_3 in 0.1 N NaOH
B	0.5% $CuSO_4$. $5H_2O$ in 1% sodium tartrate
C	Alkaline copper solution. 50 ml. reagent A + 1 ml. reagent B
E	Folin-Ciocalteu reagent (BDH) diluted 1:1 with distilled water

Reagent C is prepared immediately before use from reagents A and B.

spectrophotometer. 0.2 ml. samples of appropriate dilutions of the

mitochondrial preparations were treated in the same way and protein

concentrations determined from the standard curve.

(iii) Apparatus used in determinations of respiration rate on chilling

The complete assembly is shown on Plate M.2. It consists of a Clark

type polarigraphic oxygen electrode (Rank Brothers, Bottisham, Cambridge)

in a cuvette with hollow walls through which the cooling or warming water

could be continuously passed. This water was circulated using an electric

pump (Stuart Turner Ltd., Henley-on-Thames, England) from a glass reservoir

containing a heater-thermostat temperature regulator (Circotherm II,

Shandon Scientific Co. Ltd., London) and the probe of a "U-cool" cooling

device (Neslab Instruments Inc.). Further cooling was accomplished using a

bucket containing water and ice which was circulated, using a second electric

pump, through the glass reservoir. The temperature within the electrode

chamber was determined using an electronic thermometer comprising an NiCr/NiAl

thermocouple (Type 1604, Comark Electronics Ltd., Rustington, Sussex.) Oxygen

concentration was recorded using a model KM Oxygraph (Gilson Medical

Electronics, Middleton, Wisconsin, U.S.A.). The temperature within the

electrode chamber was adjusted by balancing the contributions of the various

heating and cooling elements.

(iv) Calibration of the apparatus

Both the solubility of oxygen in the reaction medium and the sensitivity

of the oxygen electrode are dependent on temperature. A calibration curve

was prepared to counteract all temperature effects by recording the deflection

caused by a known concentration of oxygen at a range of temperatures. This

was achieved by incorporating an excess of catalase (Sigma Chemical Co.) in

3 ml. reaction medium in the electrode chamber and addition of a constant

PLATE M.2. Apparatus used in studies on the respiration of isolated mitochondria

    e.   oxygen electrode

    s.   magnetic stirrer

    sc.   control box for magnetic stirrer

    t.   electronic thermometer

    tp.   thermometer probe inserted into electrode chamber

    r.   glass reservoir

    h.   heater-thermostat temperature regulator

    c.   cooling device

    b.   bucket containing a mixture of water and ice

    g.   Gilson oxygraph

    c.   chart

PLATE M.2
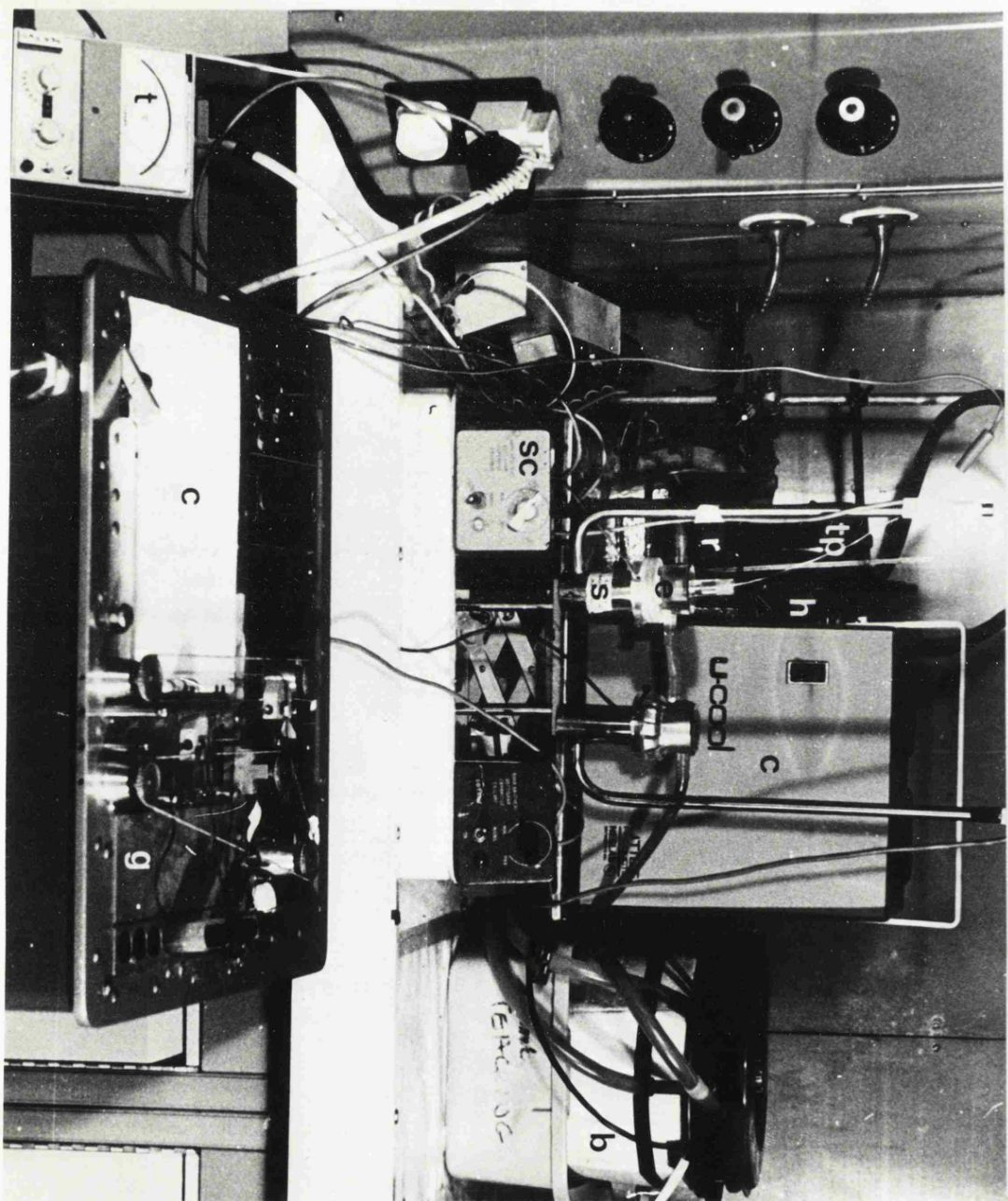
volume of hydrogen peroxide (20 volumes, Analar reagent) diluted 10 x with

reaction medium. From the deflection achieved at 20° (solubility of

oxygen = 240 μ mole/ml.) the concentration of the oxygen added could be

determined. The same volume could then be added at each of a number of

temperatures and from the deflections recorded the deflections corresponding

to a change in oxygen concentration of 100 μ mole/ml. could be calculated for

the complete range of temperatures. The experiment was repeated several

times and from the data a calibration curve was ɔe drawn from which

gradients on the polarigraphic trace at a range of temperatures could be

converted to absolute respiration rates.

The full experimental procedures,performed with isolated mitochondria

are given in the appropriate experimental section (Section IV.A. (ii) and

(iii)).

(v) <u>Chemicals</u>

ADP, NADH and succinate were all obtained from Sigma Chemical Company.

ADP was stored at -20°C and solutions prepared fresh immediately before use.

A fresh batch of ADP was obtained every 3 weeks. NADH was stored desiccated

at room temperature.

(F) <u>Lipids and fatty acids</u>

Except where stated Analar grade solvents were used throughout.

(i) <u>Extraction of lipids</u>

Method A: -

The cells were extracted at room temperature with chloroform:methanol

(2:1 v/v) 20 volumes solvent to 1 volume cells, for 12 hours. The liquid

was then collected by filtration and 1/5 its volume of saline (0.75% aqueous

NaCl) added. The mixture was shaken and allowed to settle. The chloroform

Fig. M 1. Calibration curve to compensate for the changes in solubility

of oxygen and sensitivity of the apparatus used for determinations

of oxygen concentration, associated with temperature changes.

Fig. M. 1.

Deflection corresponding to 100 μ mole/ml oxygen (squares)

Temperature (°C)
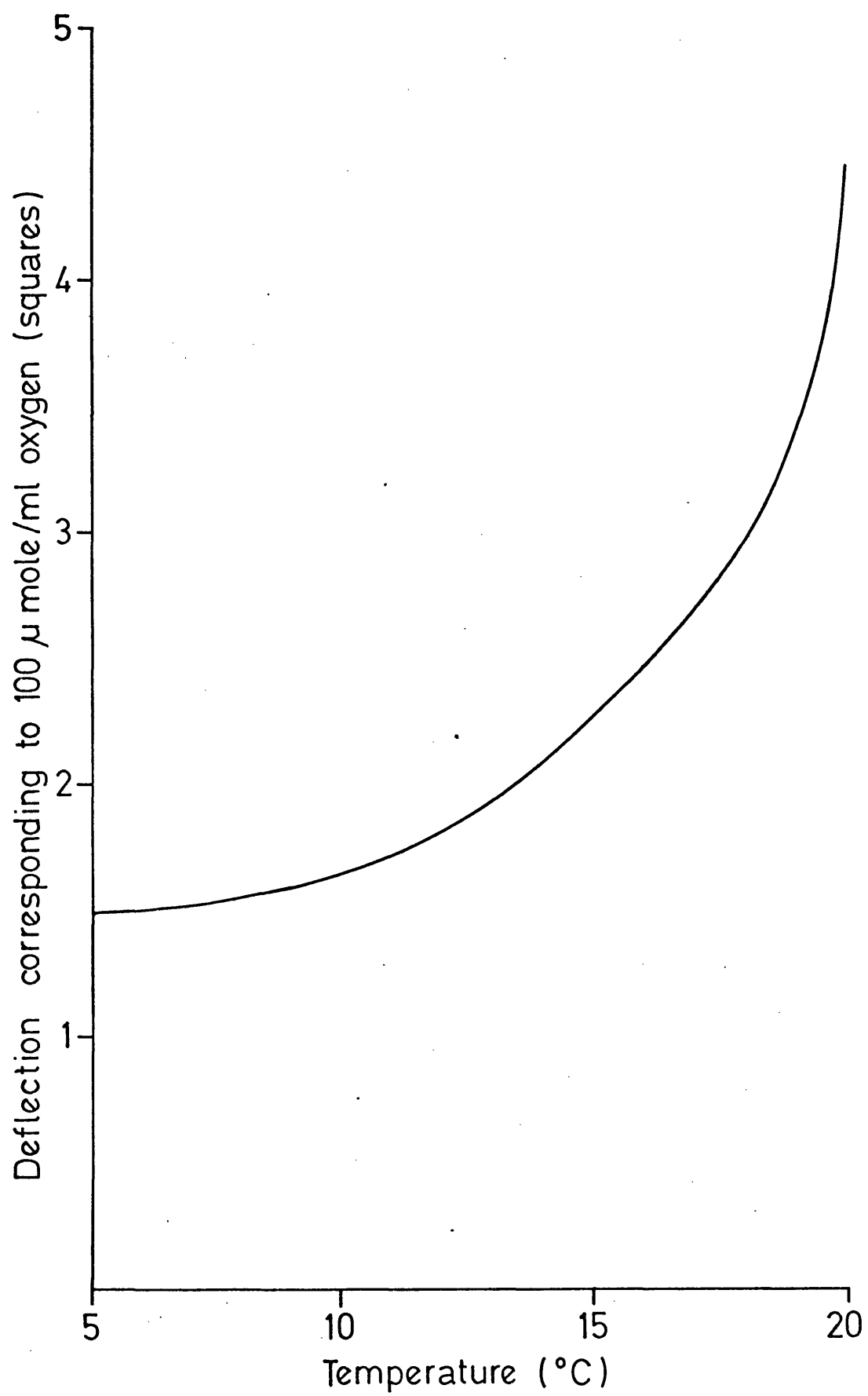
phase (lower layer) was removed and evaporated to dryness (rotary evaporator).

The residue was taken up in 5 ml. 3% methanol in chloroform and stored at

0°C under nitrogen prior to transmethylation.

Method B:-

This method incorporated a pre-extraction with iso-propanol. The cells

were homogenised for 10 seconds (Polytron homogeniser) in iso-propanol

and extracted for 2 hours. The cell debris was then collected on a filter

(Whatman No.1) and transferred to chloroform/methanol in which it was extracted

as described in method A. The filtrate from the iso-propanol extraction was

evaporated to dryness at 40°C and the residue taken up in 5 ml. 3% methanol

in chloroform. The iso-propanol and chloroform/methanol extracts were

combined and subjected to transmethylation.

(ii) Transmethylation

This step involves the hydrolysis of the lipids and the methylation of

the component fatty acids to form their methyl esters. The extracts in 3%

methanol in chloroform were transferred to test tubes and evaporated to

dryness in an 80°C water bath. As soon as the liquid had boiled off the

tubes were plunged into ice. This allowed the solvent to condense to make

a total volume of approximately 200 µl. 3 ml. transmethylating mixture

(methanol:benzene:concentrated sulphuric acid: 20:10:1) were added to each

tube and the mixtures were refluxed for 1 hour. 3 ml. diethyl ether (Fisons,

Distol reagent) and 3 ml. distilled water were added and the ether/benzene

phase (upper layer) removed. The aqueous phase was discarded and anhydrous

sodium sulphate was added to the ether/benzene phase until the solutions

became completely clear, when they were transferred to fresh tubes and

evaporated to dryness (using bumping granules) in a water bath. The residues

(methyl esters of the fatty acids) were taken up in 5 ml. ether and stored

at 20°C for subsequent analysis by gas-liquid chromatography.

(iii) <u>Gas-liquid chromatography</u>

A Pye 104 Model 64 twin F.I.D. GLC was used with a standard flame

ionisation detector. The column was a 5 ft. glass coil packed with 10%

phosphoethyleneglycol-succinate (PEGS) on 80 - 100 selite. The column was

run isothermally at 175°C with a detector temperature of 300°C. The carrier

gas, nitrogen, was passed over the column at 60 ml./min. The detector was

connected to a digital data processor with a PDP.8. computer link. For

injection samples of methyl esters of fatty acids in ether were evaporated

down to a suitable volume (100 - 500 μl) for injection of 5 or 10 μl into

the injection port of the GLC to give a loading which resulted in approximate

full scale deflection of major fatty acid peaks on the recorder.

(iv) <u>Argentation thin layer chromatography</u>

This method separates straight chain fatty acids on the basis of their

degree of unsaturation. TLC plates were prepared, coated with a 0.25 mm layer

of silica gel G containing 5% silver nitrate. 20 - 50 μl. samples of fatty

acid mixtures containing substantial levels of unknown acids were spotted

adjacent to spots containing a known mixture of standards. The TLC was

run first in 35% ether in petrol (to separate the trienes and dienes) and

then, in the same direction, in dichloromethane to a point halfway to the

first solvent front (to separate saturated fatty acids and monoenes). The

chromatagrams were dried and the spots located by UV flourescence. Spots

corresponding to saturated acids, monoenes, dienes and polyenes were eluted

with chloroform and samples of each passed through the GLC. The retention

times gave an indication of the lengths of the carbon chains of the component

acids. Matching these traces with the test traces containing peaks correspond-

ing to the unknown acids allowed a tentative estimation of the degree of

unsaturation and carbon chain length of these unknowns.

(G) Electron microscopy

The method used for the preparation of specimens for electron microscopy was based on that of Withers and Cocking, 1972. The details are as follows. Cell samples were removed from cultures and fixed in 6% glutaraldehyde in 0.1M phosphate buffer at pH 7 (overnight), washed three times (10 min.) with 0.1M phosphate buffer and post-fixed in 1% osmium tetroxide in phosphate buffer (1 hour). They were then dehydrated by an ethanol series (30,50,70, 90%, 20 min. each) to absolute ethanol (10 min.). The specimens were then stained with 1% uranyl acetate in absolute alcohol (1 hour) and washed three times (total of 2 hours) in absolute ethanol before infiltration with a 50:50 mixture of absolute ethanol and embedding medium (1 hour). A hard embedding medium was used of the following composition: 3 vol. styrene, 3.5 vol. butyl methacrylate, 3.5 vol. methyl metacrylate and 1% benzoyl peroxide (the medium was stored over anhydrous calcium sulphate). Infiltration was continued by two changes (at least 1 hour each) of pure embedding medium before polymerisation, in fresh embedding medium in dry gelatin capsules, in an oven at 54 - 60°C for 40 hours.

Sections were cut on a Tesla ultra-microtome using glass knives. They were then mounted on formvar coated copper grids, stained with Reynold's lead citrate (Reynolds, 1963) and examined in an AEI EM802 electron microscope operating at 80kV with a 50 μ objective aperture.

(H) Miscellaneous

(i) Chilling cabinets

For incubation at -3°C, a Gallenkamp illuminated cooled incubator, and for other temperatures, a custom-built (Wathes Ltd.) bank of incubators, were used.

(ii) Mutagens

N-methyl-N'-nitro-N-nitrosoguanidine (NTG) was obtained from
Ralph N. Emmanuel Ltd., Wembley, England, and ethyl methane sulfonate (EMS)
from Sigma Chemical Company, U.S.A. For removal of mutagens, after short
term exposures, from cell suspensions, the washing apparatus designed for
the purpose by Mansfield (1973) was used.

(iii) Other chemicals

Macerozyme and cellulase "onozuka" SS were prepared by Yakult
Biochemicals Co. Ltd., Japan and distributed by R.W. Unwin and Co. Ltd.,
Welwyn, England. DL-p-fluorophenylalamine (PFP) was obtained from Ralph
N. Emmanuel Ltd., Wembley, England. These substances, being heat labile,
were sterilised as concentrated stocks, by filtration through a Millipore
filter (0.22 μ) and appropriate volumes added to autoclaved medium.

EXPERIMENTAL

SECTION I

PROBLEMS OF HIGH AGGREGATION AND LOW GENETIC STABILITY IN SUSPENSION
CULTURES.

INTRODUCTION

As discussed in the general introduction a fine cell suspension containing entirely or predominantly haploid cells, capable of being plated in agar medium with a high efficiency of subsequent colony formation would be the natural choice as the most suitable material from which to select variants resistant to environmental stresses. In the case of N. sylvestris haploid plants could easily be obtained and callus cultures obtained from them (Appendix 1). Five varieties of C. annuum were used in anther culture studies, but no haploid callus was found (Appendix 2). Callus could be initiated from diploid plants in the same way as for N. sylvestris. Friable callus of both species could be dispersed in liquid medium to make suspension cultures.

A number of workers have reported the occurrence of a wide range of ploidies and karyotypes in callus (e.g. Sacristan, 1971; Niizeki & Grant, 1971; Guo, 1972; and Wright & Northcote, 1973), and suspension (e.g. Kao et al., 1970; Okonkwo, unpublished observations; and Bayliss, 1975) cultures. The complexity of this cytological variation and its possible origins in plant tissue cultures have also been discussed at length (Sunderland, 1973; Bayliss, 1973). Polyploidisation in culture has been reported as occurring more rapidly in initially haploid tissue than in initially diploid tissue (Sacristan, 1971) and this has generally been confirmed by work in this laboratory. This, however, varies greatly between species, and relatively stable haploid cultures have been reported of Lycopersicon esculentum (Gresshoff & Doy, 1972) and Atropa belladonna (Rashid & Street, 1973). Endo-polyploidisation and other chromosome mutations were therefore envisaged as likely to make difficult the aim of using haploid cultures of N. sylvestris in studies on mutation and selection. It was therefore of considerable

interest that Gupta & Carlson (1972) had indicated that p-fluoro-phenylalanine (PFP), an amino acid analogue, might be used to prefer-entially select haploid cells from a mixed population. This possibility was examined exhaustively for N. sylvestris. Other approaches to this problem were also examined, including the use of alternative auxins to 2,4-D since reports by Sunderland (1973) and Sharnina (1966) had suggested that 2,4-D might act as a mutagenic agent, accelerating polyploidisation. The possibility of isolating a stable haploid line by cloning was also studied.

Another major problem was whether the suspension cultures available could be regarded as providing uniform inocula of small meristematic units for selection studies. In many cases suspension cultures are highly aggregated and heterogenous, containing a wide range of aggregate sizes (King & Street, 1973). This variation is caused by a cycle of formation of cell aggregates by cell division, and their dissociation into free cells and smaller aggregates by cell expansion as cell division slows down (Henshaw et al., 1966; Wallner & Nevins, 1973). The concentrations of growth hormones, such as NAA, 2,4-D and kinetin, may affect the degree of cell separation in suspension cultures (e.g. Torrey et al., 1962; Simpkins et al., 1970; and Davey et al., 1971) and manipulation of the relative concentrations of these and other components of the medium may yield a more finely dispersed suspension. In many cases, however, the suspension may still be too highly aggregated and other methods must be used to increase cell separation. Methods that have been tried include the use of cell separating agents such as cell wall degrading enzymes (Mansfield, 1973; Street et al., 1971) or chelating compounds (El Hinnawy, 1974), and the gradual selection brought about by subculturing only the most finely dispersed part of the suspension (Veliky & Martin, 1970; Mansfield, 1973).

Before proceeding to use cultures of N. sylvestris for selection

studies it was considered important to examine the ploidy stability and

aggregation of the suspensions and to try to overcome the problems outlined

above. On the basis of this preliminary work the experimental plan was to

devise a standard protocol for subjecting the cultures to appropriate

selection pressures and selecting surviving cells. This protocol is

described in the discussion which terminates this section. The data

presented here refers only to experiments carried out with N. sylvestris.

It was considered more important to overcome these problems (particularly

the problem of endopolyploidy) with this species as the cultures could be

initiated from haploid material and plants regenerated from them. Where

appropriate, notes are included on parallel work carried out with cultures

of C. annuum.

EXPERIMENTAL

(A) Changes in ploidy and aggregation over a number of suspension passages, from initiation.

(i) Chromosome stability of the callus cultures

The callus was initiated and maintained on the medium of Linsmaier and

Skoog (1965) supplemented with 0.4 mg/l 2-4 dichlorophenoxyacetic acid

(2-4, D) and 0.3 mg/l 6-furfurylaminopurine (kinetin). In later work it

was found that the kinetin level could be dropped to 0.03 mg/l without

affecting the growth of the callus. These hormone levels gave callus of

varying consistency, but a high proportion was sufficiently friable for the

initiation of suspension cultures.

The ploidy of a callus initiated from the petiole of a haploid plant

and subcultured every 28 days was examined in its first three passages (the

initiation passage was designated P1). Squash preparations were made of

actively growing callus pieces, 12 to 15 days after subculture, pretreated

with colchicine and stained with Feulgen (see Materials & Methods).

50 metaphase plates were counted in each passage and the results are given

in Table I.1. Counting this number of plates from callus tissue meant

including imperfect metaphases where the chromosomes obscured each other

to some extent, often giving a slight underestimate of chromosome number.

For this reason, aneuploids were not recorded (no clear aneuploids were

seen) and limits were placed on the estimates of chromosome number,

i.e. haploid, n, means 9-12 chromosomes; diploid, 2n, means 18 - 24

chromosomes. It was easier to prepare good slides with high mitotic

indices, and a high proportion of countable metaphases, from suspension

cultures.

The results (Table I.1) are complicated by the fact that all the dividing

nuclei in individual nodules of callus growth were often of the same ploidy

and only a limited number could be examined. This phenomenon is demonstrated

in Fig.I.1. in which microdensitometer profiles of three actively growing

nodules from the same callus, in its third passage, are compared. The

results suggest that this particular haploid callus, at least, was largely

haploid after three passages, although it did also contain diploid cells,

largely restricted to particular regions of the callus.

In order to ensure a largely haploid inoculum for the suspension

cultures, these were established from freshly initiated callus, either removed

directly from the explant, or after one subsequent passage as callus. The

stock haploid material was maintained as haploid plants, propagated by

cuttings, rather than as callus cultures.

(ii) <u>Changes in ploidy over several suspension passages</u>

Suspension cultures were routinely initiated by breaking up 1 to 2 cm^3

friable callus, dispersing it in liquid medium (60 ml in 250 ml Erlenmeyer

flasks) and incubating at $25^\circ C$ under constant illumination on a rotary

shaker, rotating at 120 r.p.m. (see Materials & Methods) for 14 to 28 days,

TABLE I.1.

The ploidy distribution, in the first three passages after initiation of a
callus culture initiated from the petiole of a haploid N. sylvestris plant.

Values are percentages of nuclei scored at each ploidy level.

| | | Ploidy level | |
Passage number	n	2n	>2n
1	98	2	0
2	78	22	0
3	80	18	2

Fig.I.1   Microdensitometer profiles for actively growing nodules of growth from a third passage callus initiated from a haploid plant of N. sylvestris

Each profile is compiled from readings taken from 200 nuclei. Profiles A,B and C correspond to nuclei of different nodules of growth from the same callus.

Arrows correspond to G1 and G2 peaks from diploid root tips.
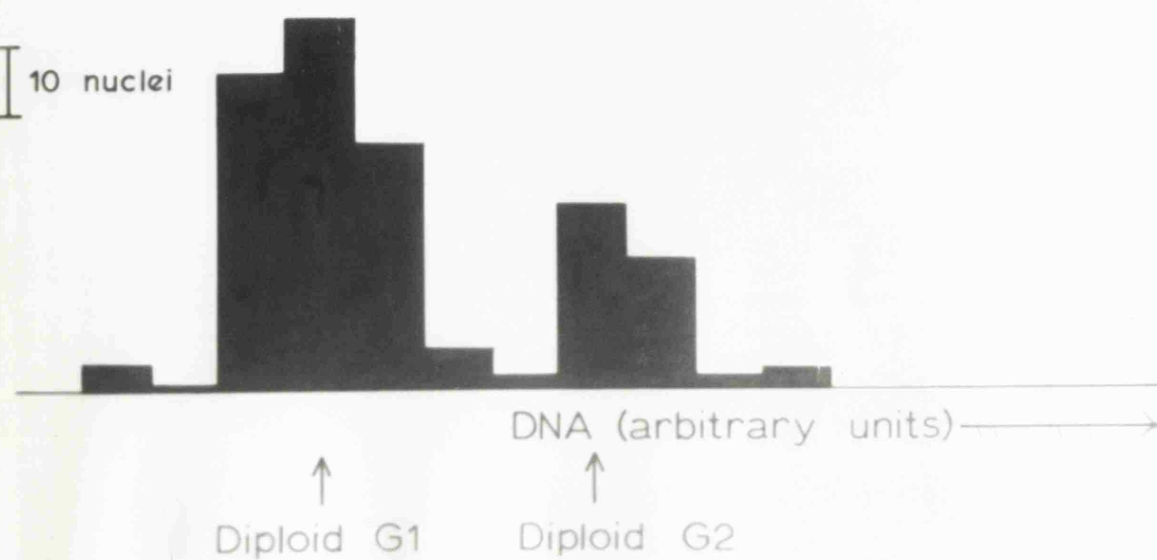
Fig. I.1
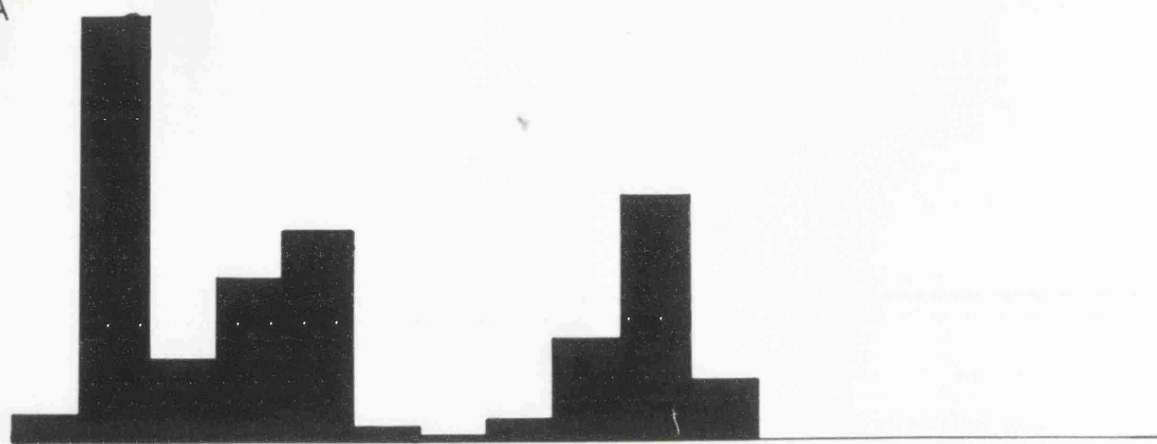
depending on how rapidly the callus became dispersed and started to grow.
They were then subcultured at 10^5 cells ml^{-1} into fresh medium and
subsequently routinely subcultured every 21 days by the end of which period
of incubation the stationary phase of the culture had normally been reached,
as shown by two typical growth curves, from very different, freshly initiated
cultures of N. sylvestris on Fig. I.2. The second passage callus from the
section (i) above was suspended in this way. For the first three passages
samples were removed at 21 days, just before subculture, and squash
preparations made for microdensitometry. 200 interphase nuclei were measured
for each profile and the peaks located by comparison with haploid G1 and
G2 peaks obtained from haploid root tips processed with the samples. The
results of this experiment are given in Fig. I.3.

Clearly the haploid, G1 peak has diminished over three passages and
the proportions of nuclei corresponding to diploid G_2/tetraploid G_1 and
tetraploid G_2/octoploid G_1 have gradually increased, suggesting that endo-
polyploidy occurs rapidly in suspension cultures of N. sylvestris. Even
after just one suspension passage, quite a high proportion of the nuclei
are at least diploid.

(iii) Changes in aggregation in suspension cultures

The general picture of aggregation in a suspension culture of
N. sylvestris is one of decreasing aggregation over the first three or four
suspension passages until a culture is arrived at which still contains a
wide range of aggregate sizes, but will not become any more finely dispersed
with continued subculture. The speed with which this level is reached, and
the degree of dispersion which is accomplished both vary dramatically
depending on the friability of the callus culture used to start the suspension,
which in turn varies between different plants and between different cultures
from the same plant. The data presented in Fig. I.4. indicated the shift,
during the first three passages in suspension culture in the size distribution

Fig.I.2   Growth of cultures of N. sylvestris during their second suspension

passage

    O   Culture of haploid origin, derived from haploid plant, H_1.

    ●   Culture of diploid origin, derived from a diploid plant, D.

Fig. I. 2.

Fig.I.3   Changes in DNA distribution of an initially haploid suspension of

N. sylvestris during the first three passages.

P1, P2 and P3 refer to the 1st, 2nd and 3rd passage respectively.

Arrows correspond to G1 and G2 peaks from haploid root tips.

Fig. I.3.

P1

P2

P3

10 nuclei

Haploid Haploid   DNA (arbitrary units)
G1   G2

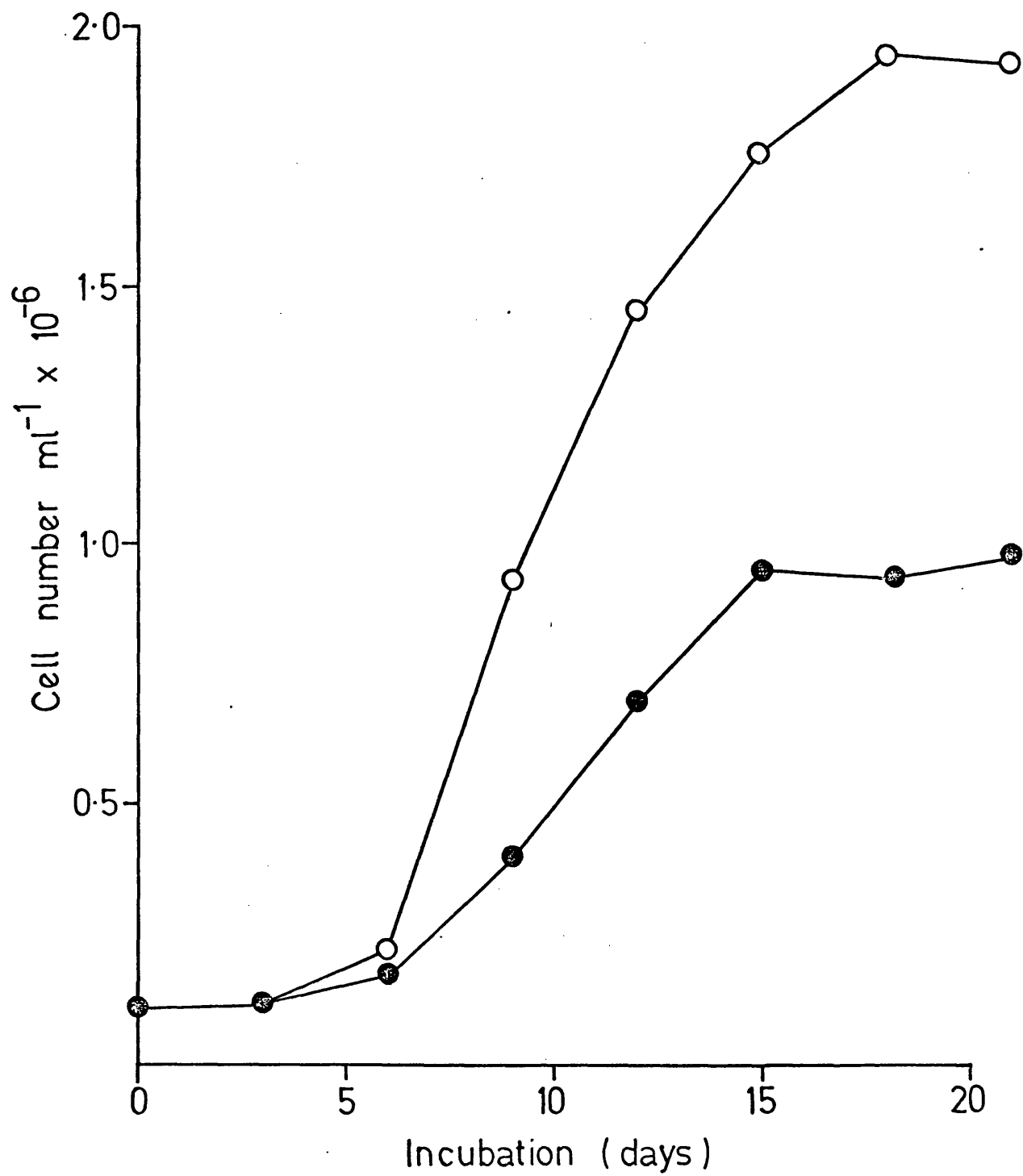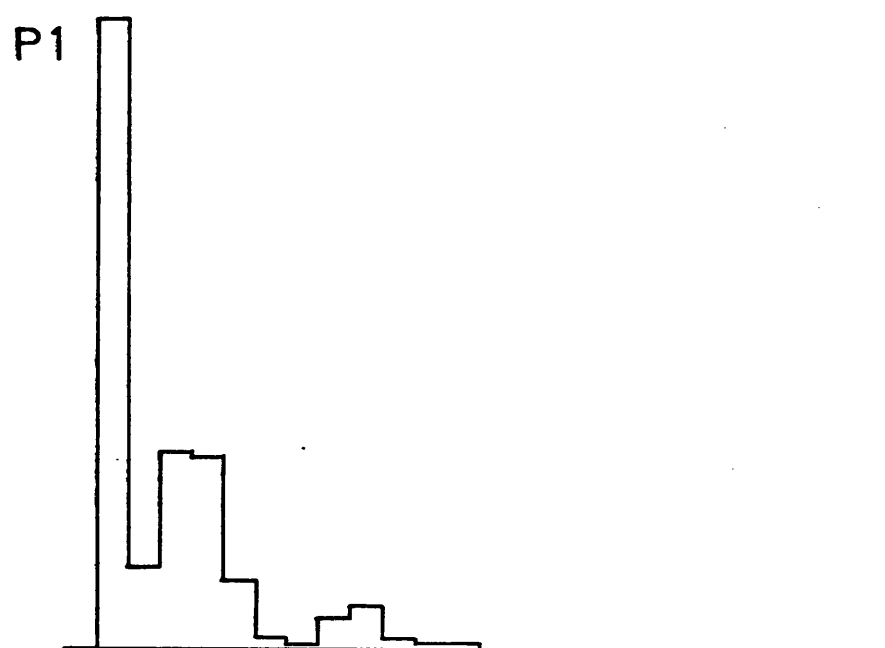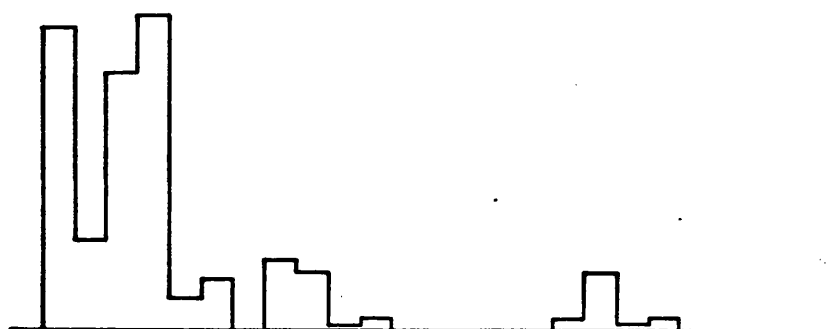Fig.I.4   Changes in aggregation of an initially haploid suspension of

N. sylvestris during the first three passages.

P1, P2 and P3 refer to the 1st, 2nd and 3rd passages

respectively.
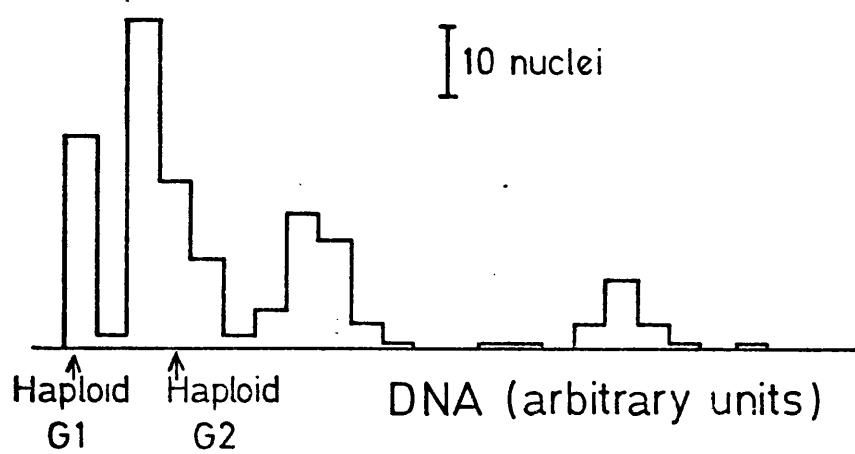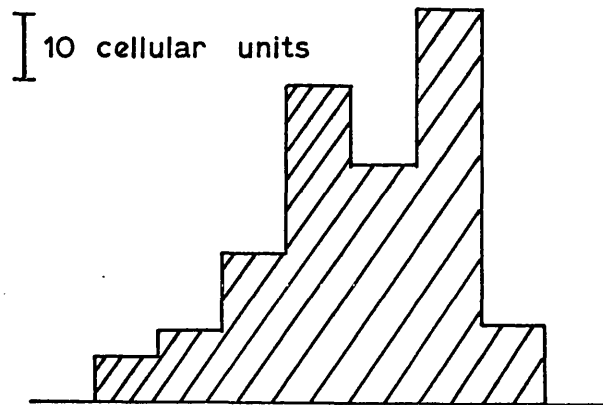
Values are the numbers of cellular units (free cells or

aggregates) falling within the arbitrary size limits, during

the 21st day of suspension.

Each profile is based on 200 cellular units.

Fig. I 4

P 1 (initiation passage)

P 2
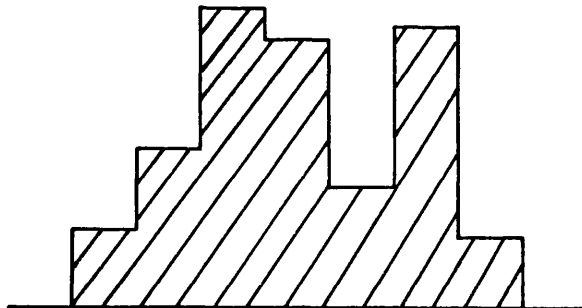
P 3
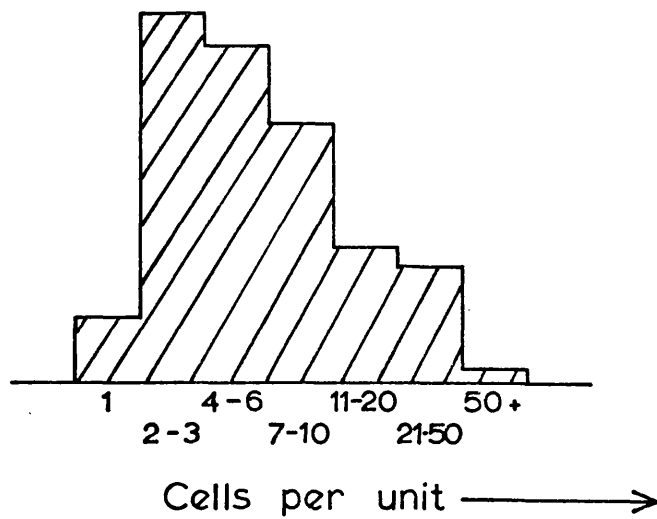
of aggregates towards lower aggregate size in a <u>N. sylvestris</u> culture,

initiated from haploid callus. This involved diluting samples of the cell

suspension at 21 days from subculture and examination under a cover slip

on a cell counting slide. The slide was examined at 100x magnification

and for each field the number of cells in each cellular unit (free cells

or aggregates) was estimated. This was performed on a number of fields

until 200 cellular units had been estimated. They were then grouped into

suitable size ranges and plotted as shown in the histograms on Fig. I.4.

Despite the shift towards lower aggregate size reported here, a high

level of variation remains, from single cells to aggregates containing more

than a thousand cells, and in most cultures this variation is retained

through an indefinite number of passages. Occasionally unpredictable

changes in aggregation have been observed leading to very clumped cultures

which may again revert to the usual level of dispersion in subsequent

passages. The factors responsible for this phenomenon have not been

identified. It has been observed more frequently in the cultures of

<u>C. annuum</u> which normally form a rather finer and more rapidly growing

suspension than <u>N. sylvestris.</u>

(iv) <u>Growth, aggregation and ploidy of a well-established suspension culture</u>
<u>of haploid origin, of N. sylvestris.</u>

In the above work the ploidy changes and changes of aggregation following

initiation of a suspension culture were examined. As a prelude to the use

of such suspensions for plating it was considered important that a more

rigorous study should be made of an established cell suspension. This

should include an examination of the growth curve and any relation between

this and the degree of aggregation of the culture.

A suspension culture, designated "A" of <u>N. sylvestris</u>, which had been

initiated from haploid callus and maintained in suspension for about 8 months

(11 passages) was used. It was inoculated at 10^5 cells ml^{-1} initial cell

density, into a number of 250 ml Erlenmeyer flasks containing 60 ml

medium and two replicate flasks were removed from the shaker at intervals

and used for measurements of cell number, dry weight, packed cell volume

and determination of the proportion of cellular units containing less

than 10 cells. In addition to this, samples were fixed, without a

colchicine pretreatment, and Feulgen stained squash preparations made for

the determination of mitotic index. From the final set of slides, prepared

after 21 days, a microdensitometer profile was prepared from the density

values of 200 interphase nuclei. The results of this study are presented

in Figs. I.5, I.6. and I.7.

The plots of cell number (Fig. I.5. (a)) and dry weight and packed

cell volume (Fig. I.6.) against time are typical, in shape, of those

obtained from most suspensions of N. sylvestris and C. annuum maintained in

this way. The plot of $\log_{10}$ cell number against time (Fig. I.5. (b)) shows

that with this rather high inoculation density the log phase is extremely

short and the data are compatible with exponential growth extending only

to day 6 of incubation. After this time the growth rate is always decreasing.

This is confirmed by the curve for mitotic index (Fig. I.5. (a)). The mitotic

index increases rapidly up to 4% in the first 3 days after subculture and

is still high after 6 days. It then falls off very rapidly as the rate of

increase in cell number decreases. The degree of aggregation of the culture

(Fig. I.5. (b)) can also be related to the growth curve. Aggregation

increases whilst the initial high growth rate is maintained reaching a

peak at about day 9 of incubation and then gradually falling off over the

remainder of the passage. The changes in the degree of aggregation through

the growth cycle can be clearly seen on examination of the culture (Plate I.1.).

As expected the culture is highly polyploid (Fig. I.7.) exhibiting a range of

ploidy levels, but no haploid nuclei were found.

Fig.I.5   The growth of N. sylvestris culture line, A, in its 12th passage
in suspension

A. Cell number (●) and mitotic index (O) against time after
inoculation.

B. Degree of aggregation (●) and log_{10} cell number (O) against
time after inoculation.

Fig. 15

A

B
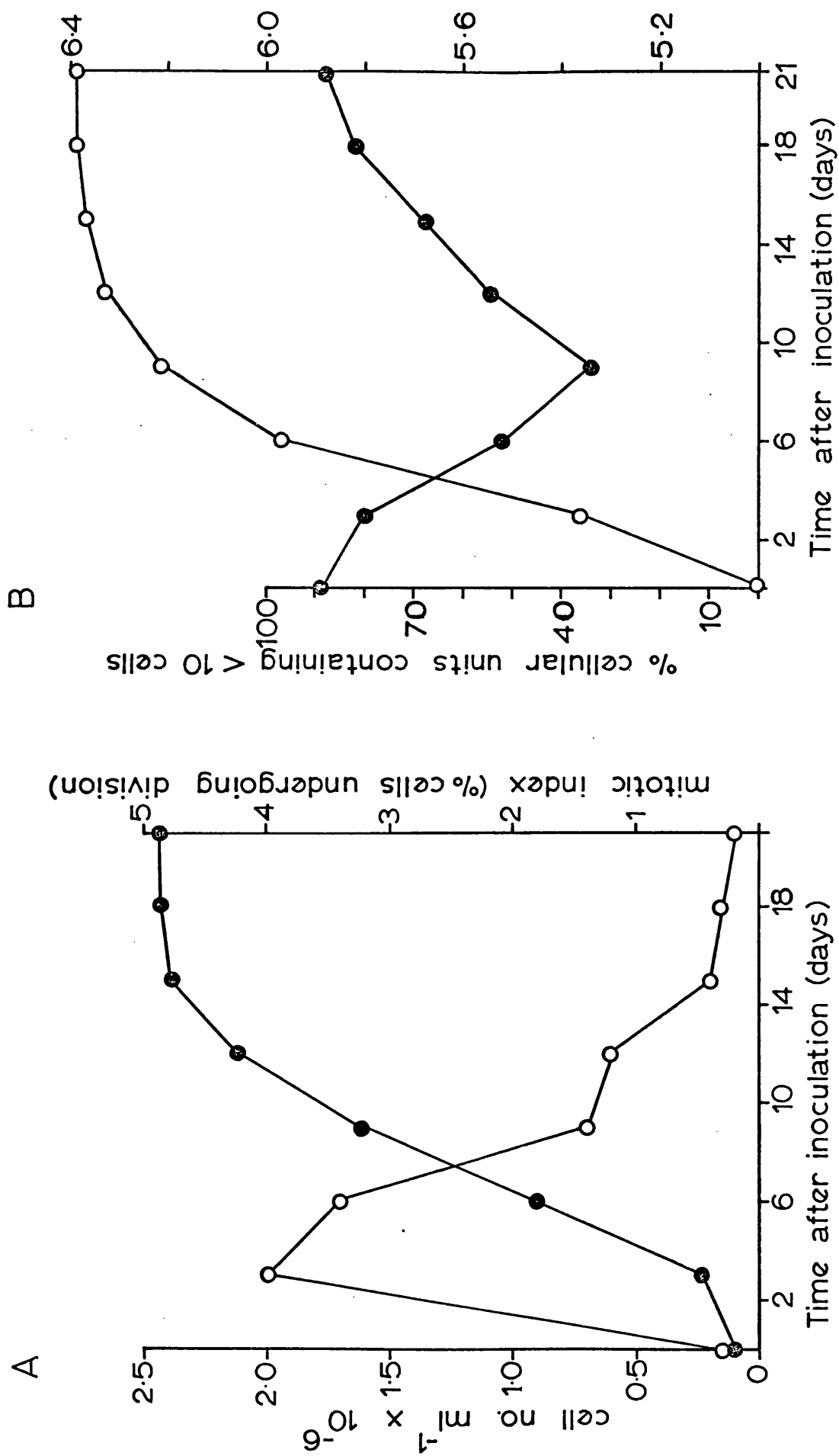
Fig.I.6   The growth of the N. sylvestris culture line, A, in its 12th

suspension passage

Dry weight (●) and packed cell volume (O) against time after

inoculation.

Fig. I 6
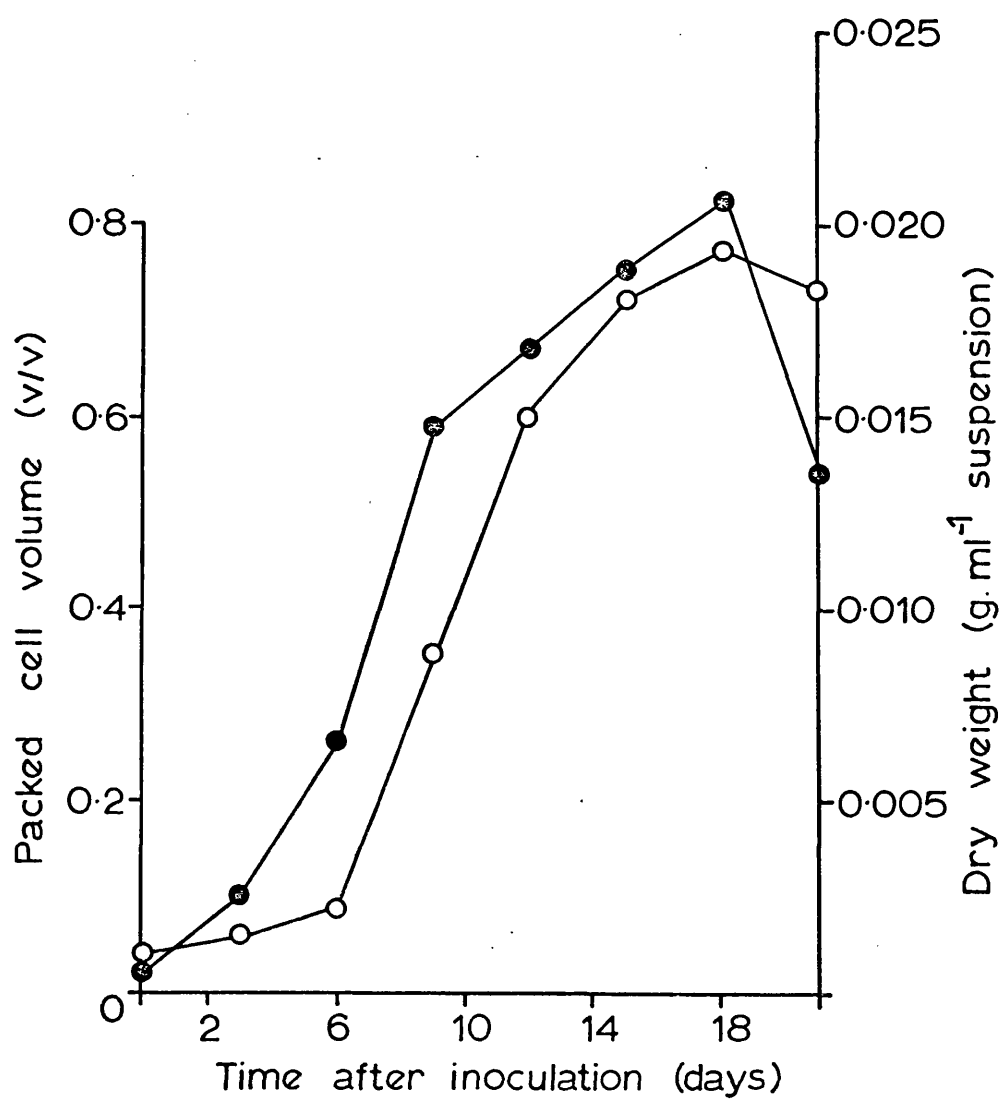
PLATE I.1. Changes in the level of aggregation during the growth curve of

a suspension of N. sylvestris (line A).

Samples were removed at 0 (A), 3 (B), 6 (C), 9 (D), 12 (E) and

21 (F) days after subculture.

x 60

PLATE I.1
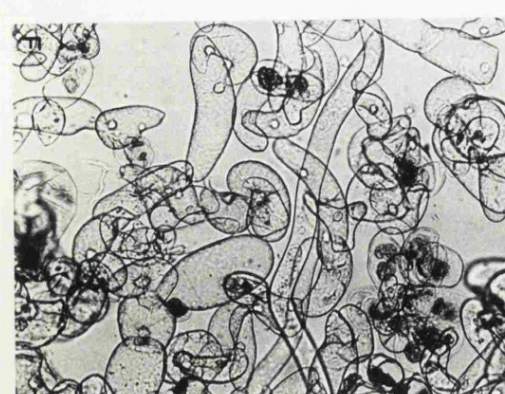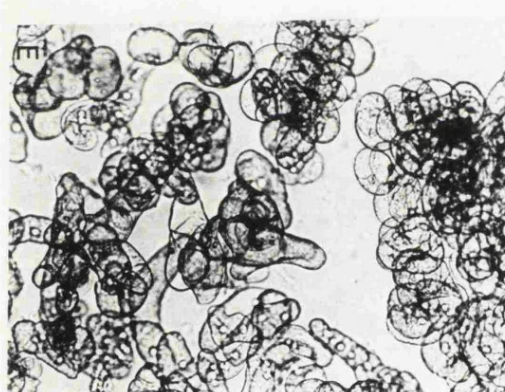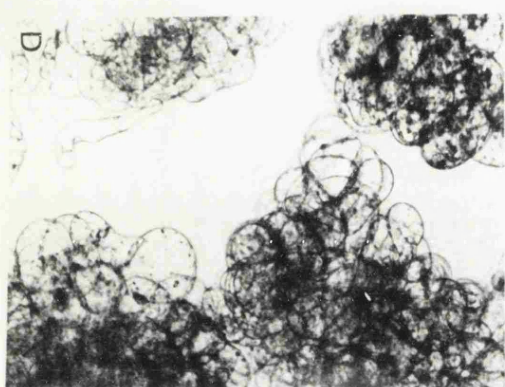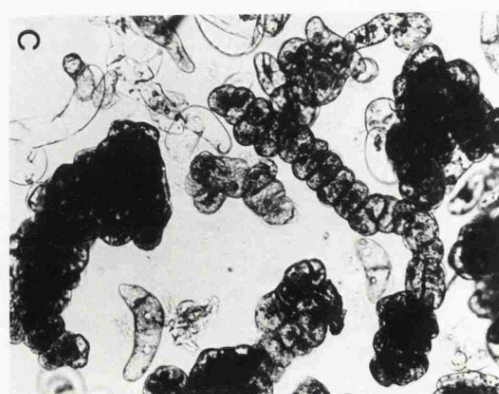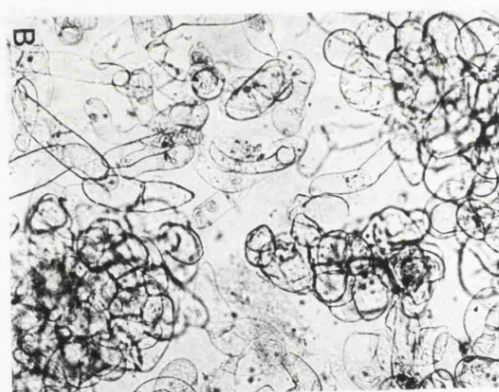
Fig.I.7   Density profile of nuclei of the N. sylvestris culture line, A,

in its 12th passage suspension.

The profile is based on readings from 200 interphase nuclei in a

sample taken 21 days after inoculation.

Fig. 17

28.
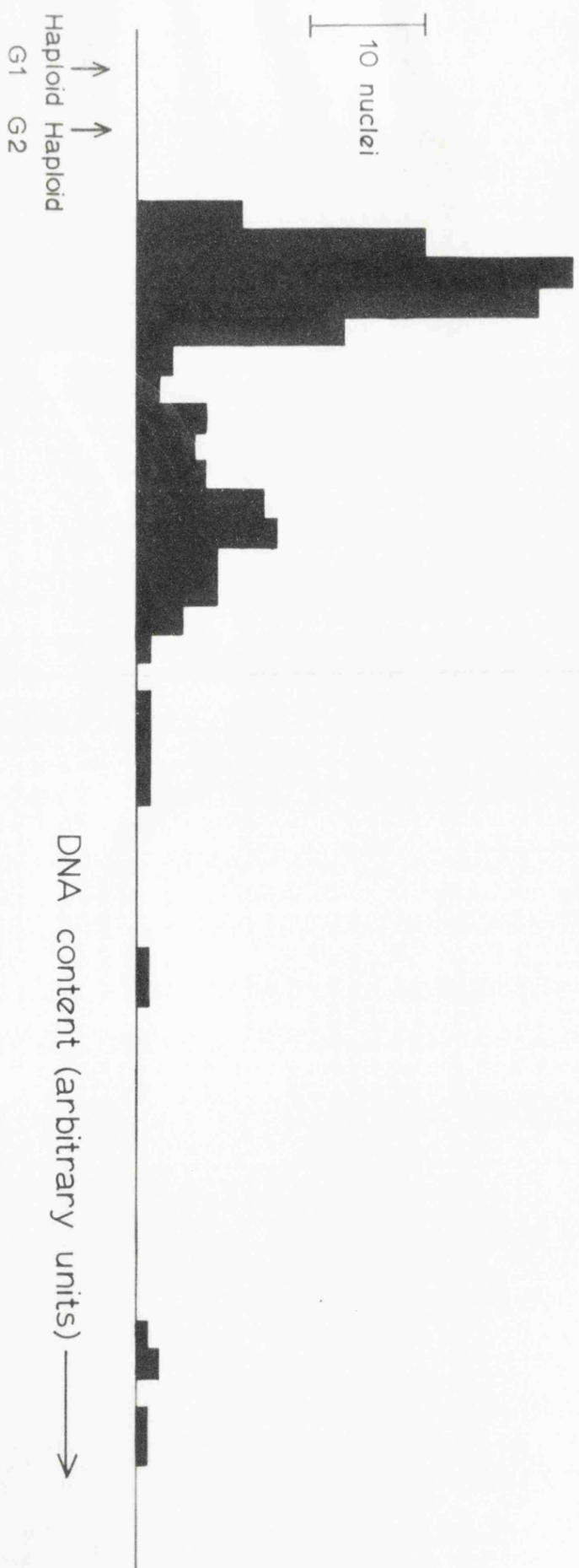
The relationship between the different parameters examined above
was similar for all cultures examined but the order of magnitudes of some
aspects, such as final cell number, growth rates, and the length of
exponential phase varied substantially between cultures, especially if
the inoculation density was varied. Variation between culture lines is
observed both in well established cultures such as A, and in relatively
freshly initiated cultures. This can be seen from the growth curves of
the second passages of cultures H_1 (haploid origin) and D (diploid origin)
plotted in Fig. I.2., and the semi-logarithmic plots for these cultures
(Fig. I.8). Both cultures exhibit a longer lag phase than culture A, and
the final cell numbers and maximum growth rates differ. From these data
mean generation times ($\bar{G}$) during exponential phase can be determined (see
Materials & Methods) and the values of these for the cultures discussed
above are as follows: -

for line A, $\bar{G}$ = 44.1 hr

for line H_1, $\bar{G}$ = 48.8 hr

for line D_1, $\bar{G}$ = 81.2 hr

The lines differ greatly in mean generation time; in this instance the
culture of diploid origin divides at little more than half the rate of the
other two.

The general situation can be summarised by saying that the values for
growth parameters vary between culture lines, but the pattern of growth is a
fairly standard one.

(B) The problem of high aggregation

(i) The effect of changes in the medium and shaker speed on aggregation

The data presented on Fig. I.5. (b) shows that at 21 days nearly 90%
of the cellular units comprise 10 cells or less, but this is less satis-
factory than it sounds as the remaining 10% are variable in size, up to a

Fig. I.8   Log$_{10}$ cell number against time after inoculation for two culture

lines of N. sylvestris in their second suspension passage

●   Culture of haploid origin derived from haploid plant, H$_1$.

○   Culture of diploid origin derived from diploid plant, D.

Fig. I 8
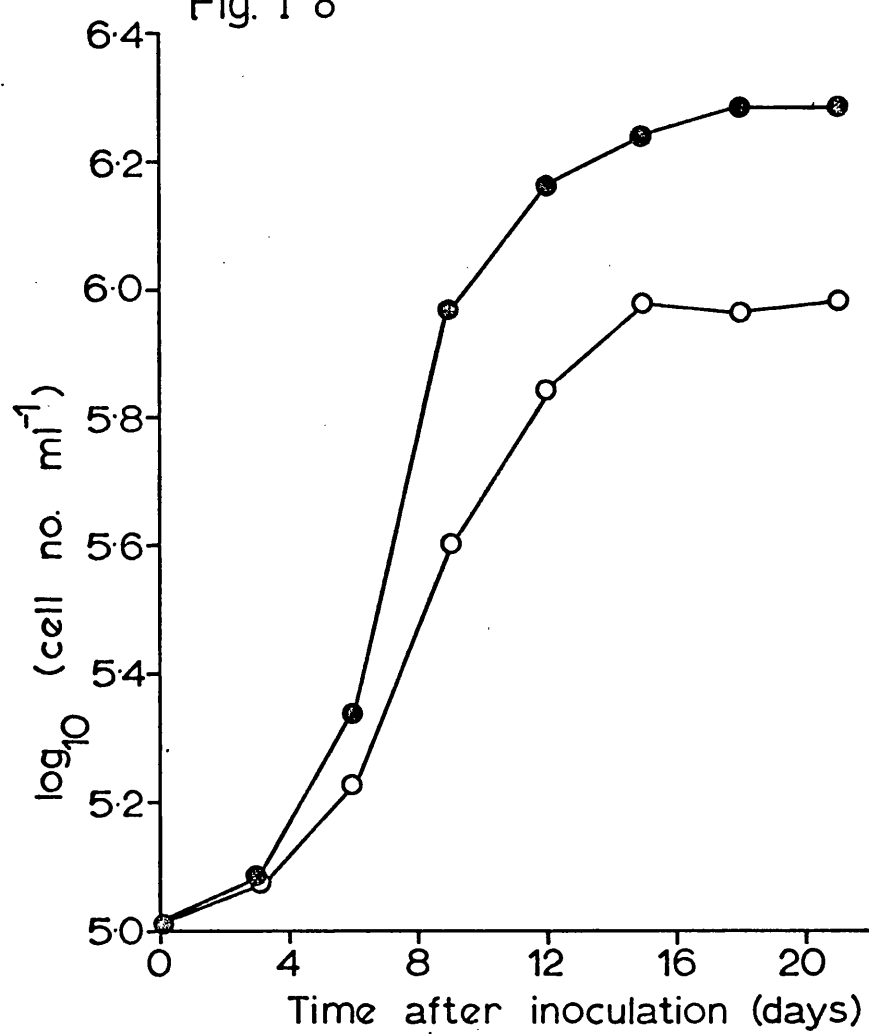
thousand cells, and being larger, have a growth advantage on plating.

A large increase in the proportion of very small aggregates, and in the

uniformity of these aggregates is required. A suitable inoculum for

plating would be one with a uniform aggregate size of 5 to 20 cells.

Initial attempts to achieve this involved modification of the culture

medium to try to establish an actively growing, finely dispersed

suspension. Most of the variations involved modifications in the relative

levels of 2,4-D and kinetin or the use of alternative auxins to 2,4-D

(NAA and p-CPA). An alternative chelating agent (NaEDDHA) was also tried

instead of NaEDTA. Using the usual medium, different shaker speeds were also

tried, 60 and 180, r.p.m. in addition to the usual 120 r.p.m., but 60 rp.m.

proved too slow to aerate the cultures, which quickly died. The various

treatments used are outlined and given code letters in Table I.2. N.sylvestris

culture H_3 (haploid origin) was used and inoculated into the range of media

from its 3rd or 4th passage in suspension. The distribution of aggregate

sizes was as shown on Fig. I.9. 85 - 90% of the cellular units comprising

less than 10 cells. The suspensions were incubated for two suspension

passages in each of the media A to S and after 21 days of the second passage,

the percentage of cellular units comprising less than 10 cells was estimated.

This method of comparison can be criticised in so far as it relies on all

the cultures being in stationary phase after 21 days, and in some media the

growth was slower than in medium A. In most of the slowly growing cultures,

however, the suspensions were clearly far more highly aggregated than control

cultures. The results are presented on Fig. I.9.

Without any detailed examination it was already clear from examination

of the flasks that none of the treatments had resulted in any significant

reduction in the level of aggregation. The data presented on Fig. I.9.

confirm this. Two treatments, C and D, containing 1.0mg/1 2,4-D gave a

slight increase in dispersion, but this was not significant and the suspensions

still contained a wide variety of aggregate sizes. Increasing the kinetin

TABLE I.2.

Modifications in the medium composition and shaker speed used in experiments designed to improve the dispersion of suspensions of N. sylvestris.

| Treatment | Concentration (mg/l) | | | | | | Shaker speed (r.p.m.) |
	24D	NAA	pCPA	Kinetin	Na-EDTA	Fe-EDDHA	
A (usual medium)	0.4	0	0	0.03	37.3	0	120
B	4.0	"	"	0.003	"	"	"
C	1.0	"	"	0.03	"	"	"
D	"	"	"	0.3	"	"	"
E	"	"	"	1.0	"	"	"
F	0.4	"	"	0	"	"	"
G	"	"	"	0.03	0	10	"
H	"	"	"	0.3	37.3	0	"
J	"	"	"	1.0	"	"	"
K	"	"	"	2.0	"	"	"
L	0.1	"	"	1.0	"	"	"
M	0	5.0	"	0.3	"	"	"
N	"	"	"	1.0	"	"	"
P	"	4.0	"	3.0	"	"	"
Q	"	0	1.2	"	"	"	"
R	"	"	0.4	0.03	"	"	"
S	0.4	"	0	"	"	"	180

Fig. I.9   The degree of dispersion of N. sylvestris culture line H$_3$ after

two suspension passages in a range of media

For details of all the treatments see Table I.2.

% cellular units containing < 10 cells

Fig. 19

30 40 60 80 90

A 0.4mg
B 4.0
C
D 1.0
E
F
G 0.4
H
J
K
L 0.1
M
N 0
P
Q 0
R 0
S 0.4

2.4.D (mg/ℓ)
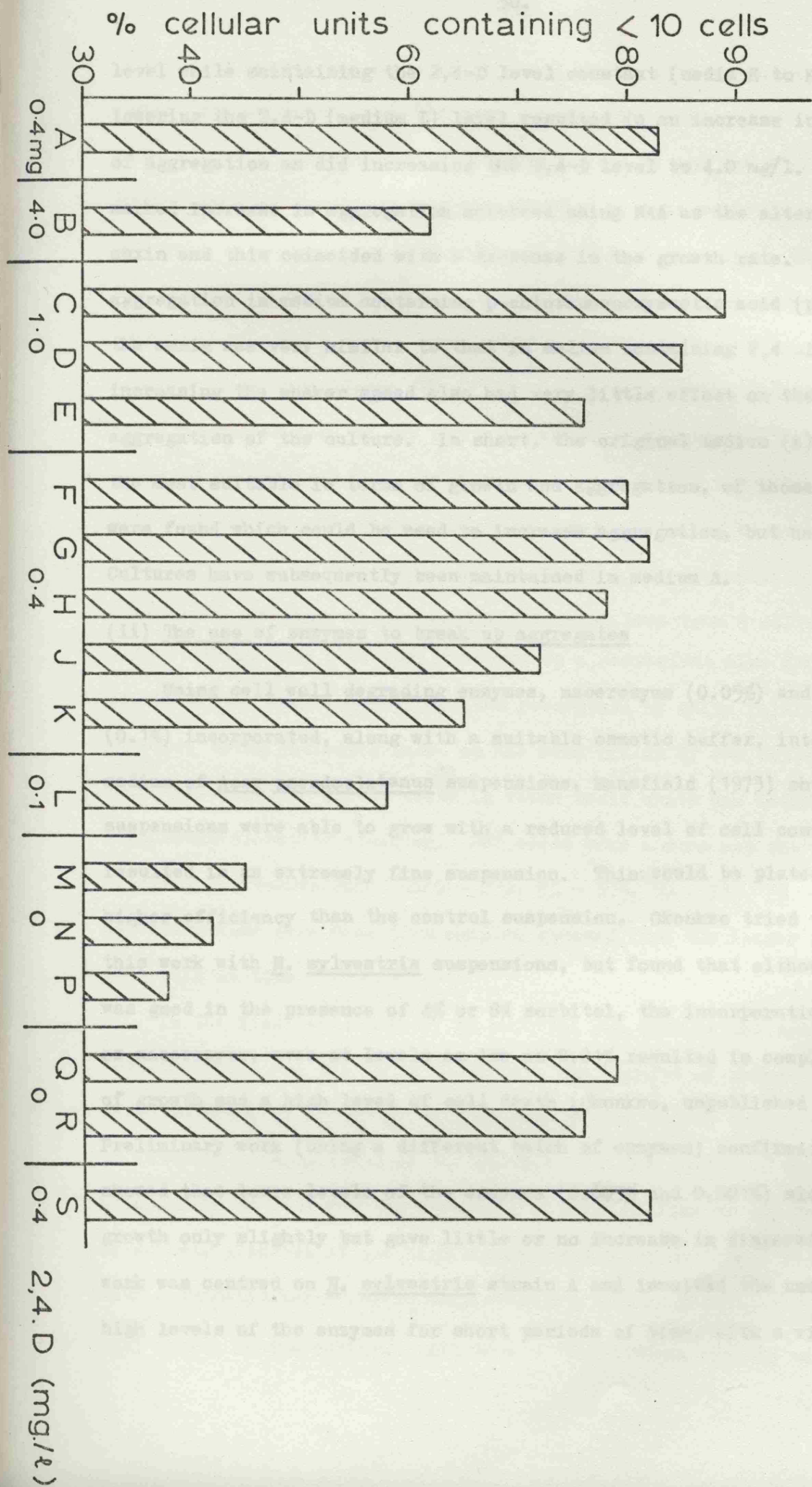
level while maintaining the 2,4-D level constant (media H to K) or

lowering the 2,4-D (medium L) level resulted in an increase in the level

of aggregation as did increasing the 2,4-D level to 4.0 mg/l. The most

marked increase in aggregation occurred using NAA as the alternative

auxin and this coincided with a decrease in the growth rate. Growth and

aggregation in medium containing p-chlorophenoxyacetic acid (p-CPA) as

the auxin was very similar to that in medium containing 2,4 -D and

increasing the shaker speed also had very little effect on the growth and

aggregation of the culture. In short, the original medium (A) was among

the most suitable in terms of growth and aggregation, of those tried. Media

were found which could be used to increase aggregation, but not decrease it.

Cultures have subsequently been maintained in medium A.

(ii) The use of enzymes to break up aggregates

Using cell wall degrading enzymes, macerozyme (0.05%) and cellulase

(0.1%) incorporated, along with a suitable osmotic buffer, into the growth

medium of Acer pseudoplatanus suspensions, Mansfield (1973) showed the

suspensions were able to grow with a reduced level of cell contact which

resulted in an extremely fine suspension. This could be plated with a

higher efficiency than the control suspension. Okonkwo tried to repeat

this work with N. sylvestris suspensions, but found that although the growth

was good in the presence of 4% or 8% sorbitol, the incorporation of cellulase

or macerozyme, even at levels as low as 0.01% resulted in complete inhibition

of growth and a high level of cell death (Okonkwo, unpublished report, 1972).

Preliminary work (using a different batch of enzymes) confirmed this and

showed that lower levels of the enzymes (0.005% and 0.001%) slowed the

growth only slightly but gave little or no increase in dispersion. Subsequent

work was centred on N. sylvestris strain A and involved the use of relatively

high levels of the enzymes for short periods of time, with a view to obtaining

a standard enzyme treatment which would give a fine inoculum for plating.

Short term exposures resemble more closely the techniques widely used to

obtain protoplasts from plant material. Here we are not trying to obtain

protoplasts but merely reduce the adhesion between cells so more attention

was paid to determining an appropriate level of macerozyme than cellulase,

but the use of cellulase alone, and mixtures of the two enzymes were also

examined.

In these experiments 100 ml Erlenmeyer flasks containing 20 ml medium

were inoculated from 14 day old suspensions and incubated in the usual way.

Sorbitol or mannitol were added to the medium to act as an osmotic buffer

and filter-sterilised enzyme solutions, made up in the medium, were injected

into the flasks of autoclaved medium, to give the appropriate final con-

centrations of enzyme. Aggregates containing less than 5 cells (i.e.containing

1 to 4 cells) were arbitrarily chosen as a convenient size for gauging the

effect of the enzyme solutions, the percentage of units falling into this

category being estimated based on the examination of 200 cellular units. In

turn the viability of the cells in these small units was determined by

fluorescein diacetate staining. 200 cells were scored and the viability

classed, for convenience, as 0 - 25%, 25 - 50%, 50 - 75%, 75 - 100%. These

determinations were made from samples removed from the flasks at suitable

intervals of time.

In the first experiments, 4% sorbitol was used as the osmotic buffer

and the incubation was up to 21 hours. The results of these experiments are

given on Figs. I. 10 and I. 11. In most of the enzyme treatments there was

an increase in dispersion of the culture with the length of the treatment,

although in no case was the resulting culture as fine as was required. This

effect was greatest at the high concentrations of cellulose but was associated

with a dramatic reduction in cell viability and an increase in the amount of

cell debris in the culture. All levels of cellulose and the enzyme mixtures

Fig.I.10   The dispersion of suspensions of N. sylvestris, culture line A,

brought about by cellulase treatments

Length of treatment: -

A	zero
B	15 min.
C	30 min.
D	2 hr. 30 min.
E	5 hr.
F	8 hr.
G	21 hr.

Viability: -

+	
+	
+	75 - 100%
+	
+	
+	
+	
+	50 - 75%
+	
+	
+	25 - 50%
+	0 - 25%

Fig. I. 10.
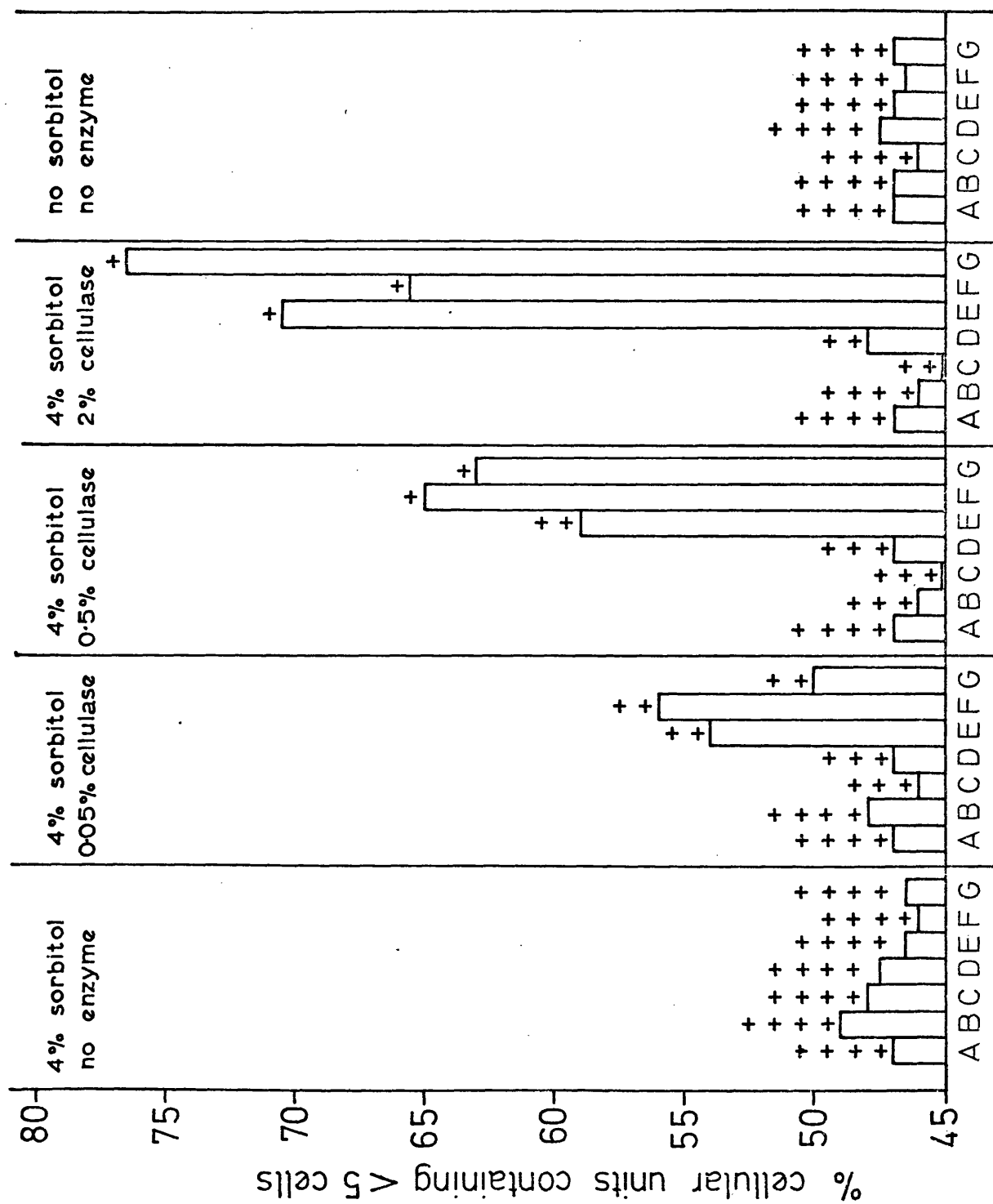
<u>Fig.I.11</u>   The dispersion of suspensions of <u>N. sylvestris</u> culture A,

brought about by cellulase or cellulase + macerozyme treatments

Length of treatment: -

A	zero
B	15 min.
C	30 min.
D	2 hr. 30 min.
E	5 hr.
F	8 hr.
G	21 hr.

Viability: -

+	
+	75 - 100%
+	
+	
+	
+	50 - 75%
+	
+	25 - 50%
+	
+	0 - 25%

For controls see Fig. I.10.

Fig. I. 11.

% cellular units containing <5 cells

80 — 75 — 70 — 65 — 60 — 55 — 50

4% sorbitol
0.01% macerozyme

A B C D E F G

4% sorbitol
0.05% macerozyme

A B C D E F G

4% sorbitol
0.5% macerozyme

A B C D E F G

4% sorbitol
0.05% cellulase
0.05% macerozyme

A B C D E F G

4% sorbitol
0.5% cellulase
0.05% macerozyme

A B C D E F G
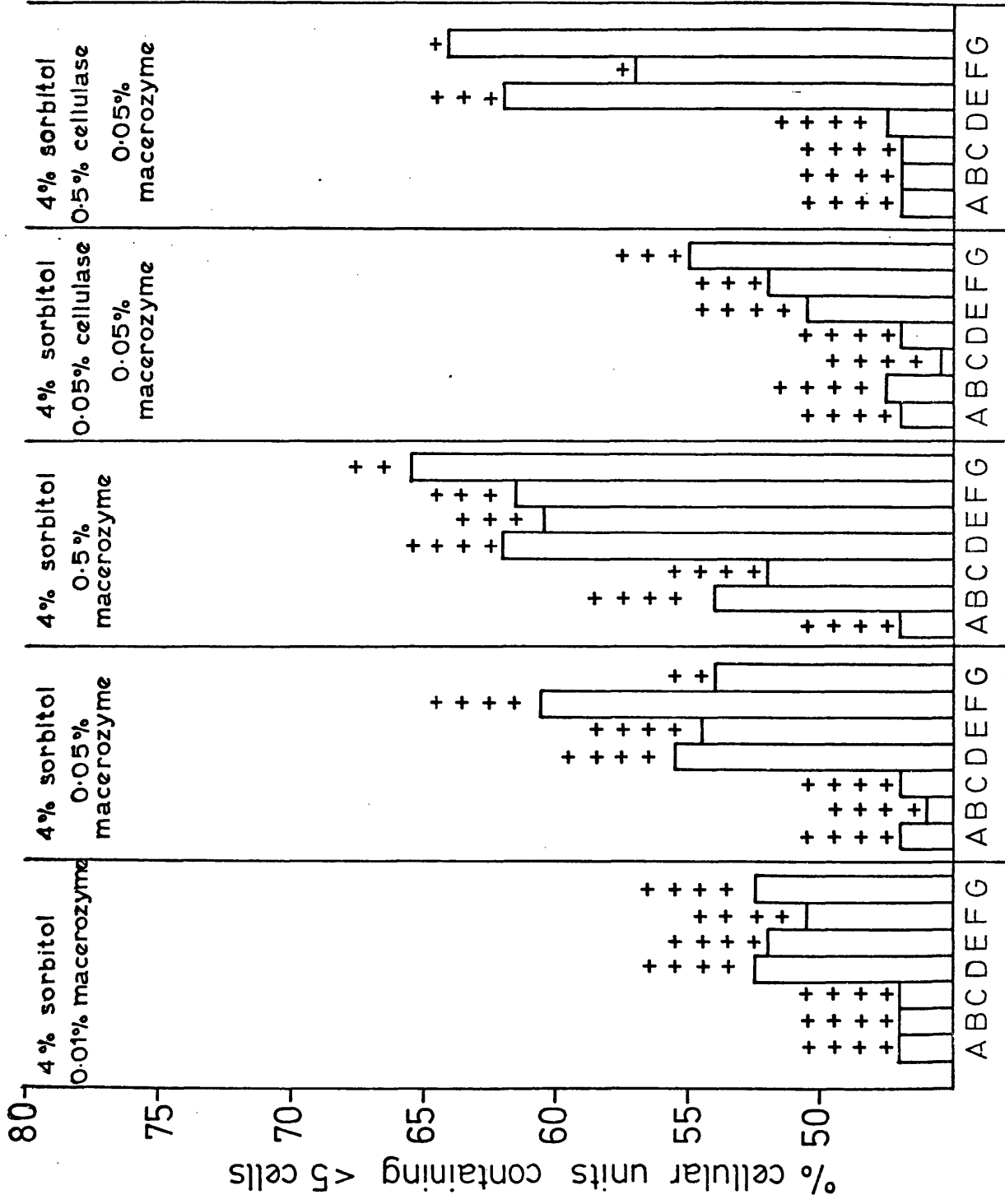
had a deleterious effect on the cells. Macerozyme alone gave a smaller increase in dispersion but effected this more rapidly and with less killing of the cells. Long exposures to macerozyme, however, also resulted in cell death. The lowest level of macerozyme used (0.01%) had very little effect on the suspension.

Further experiments were carried out using macerozyme concentrations the same as the two higher levels in the above experiments and one still higher level (2%) and using mannitol as the osmotic buffer at two different levels to see if the above results could be improved upon. Since the longest exposures in the previous experiments had only resulted in cell death, these experiments were terminated earlier (after 6 hr 30'). The results are shown on Fig. I.12. Again there is an increase in the dispersion with time but the longest treatments resulted in some cell death. Death was more rapid and more extensive in those treatments where 2% mannitol were used. 4% mannitol resulted in suspensions with higher viability than 2% mannitol, and slightly higher viability than 4% sorbitol. The most successful treatments in terms of increasing the dispersion and maintaining the viability of the culture were 0.5% and 2% macerozyme for 5 hours in medium containing 4% mannitol, but even these treatments fell far short of those required to provide a suitable inoculum for plating. They did little more than reduce the level of aggregation normally found 14 days after sub-culture to that normally found 21 days after subculture (see I.A. (iv)). It appeared that the use of these enzyme preparations on suspensions was not going to solve the difficulty of the variable and highly aggregated nature of the suspensions and this work was discontinued in favour of examination of other approaches to the problem.

(iii) <u>Filtration as a means of obtaining fine cell suspensions for plating</u>

The use of a filtration technique relies on the suspension having a very wide range of aggregate size. Despite the suspension being highly

Fig.I.12   The dispersion of suspensions of N. sylvestris culture A,

brought about by macerozyme, using two mannitol concentrations.

Fig. I. 12.

| 2% mannitol no enzyme | 2% mannitol 0.05% macerozyme | 2% mannitol 0.5% macerozyme | 2% mannitol 2% macerozyme | 4% mannitol no enzyme | 4% mannitol 0.05% macerozyme | 4% mannitol 0.5% macerozyme | 4% mannitol 2% macero-zyme |

Length of treatment

A zero
B 1 hr
C 2 hr
D 3 hr
E 4 hr 30'
F 6 hr 30'

for key to viability see Fig I.11

% cellular units containing <5 cells
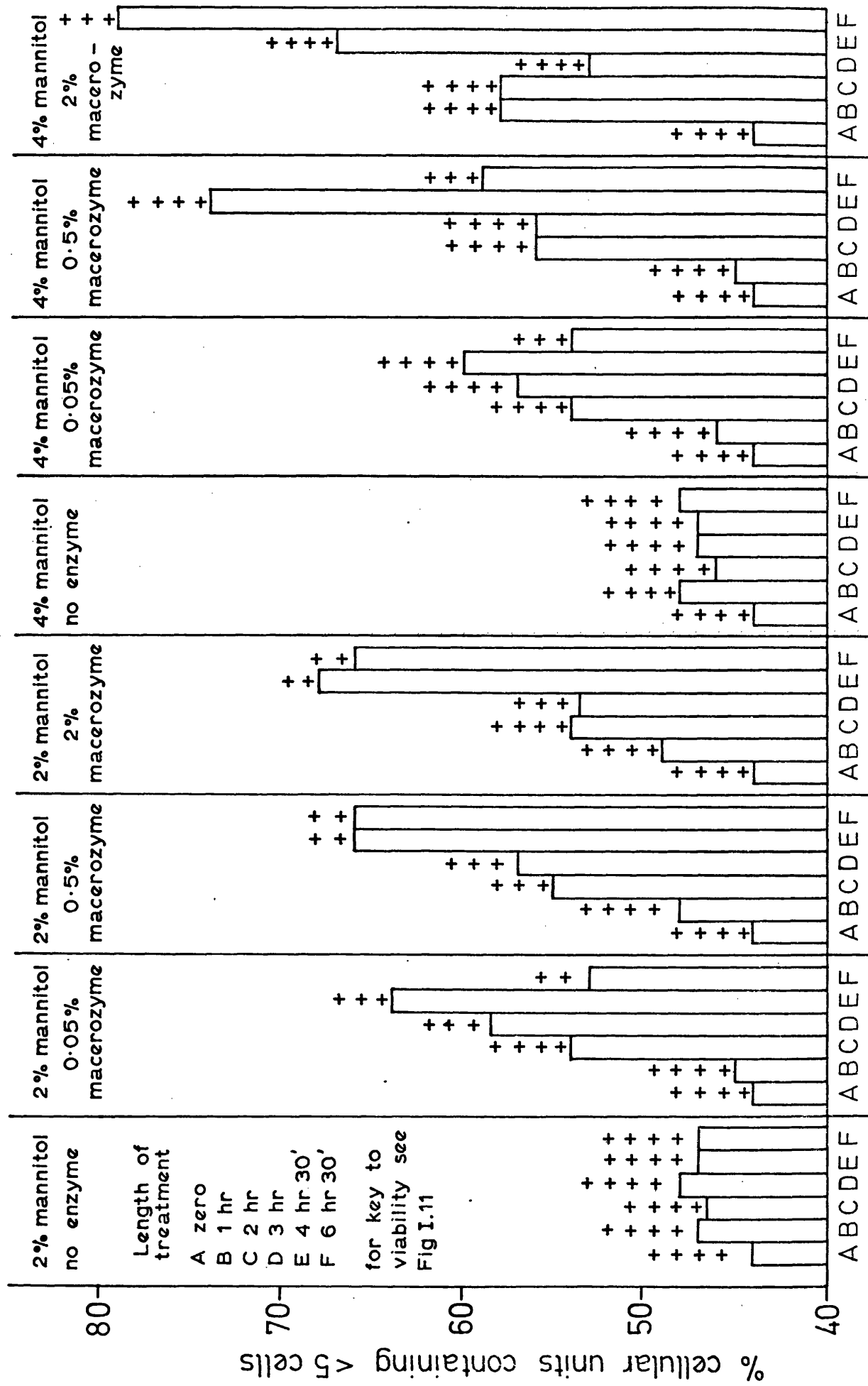
aggregated it must contain a substantial proportion of viable aggregates below a certain size. It has already been shown (I.A. (iii) and I.A. (iv)) that well established and freshly initiated suspension cultures of N. sylvestris both fulfil the criterion of having a high proportion of small aggregates. Further data presented on the control plot (no sorbitol, no enzyme) of Fig. I.10 show that aggregates containing less than five cells show greater than 75% viability. If we are prepared to use aggregates containing up to 20 cells a very high proportion of the aggregates should contain viable cells and therefore, at least potentially, be able to form discrete colonies on plates.

Two haploid plants (H_7 and H_9) and one diploid plant (D) of N. sylvestris were chosen as the stocks from which all cultures used in selection studies with N. sylvestris would be derived. These plants could be continually propagated, by cuttings, under aseptic conditions, and provide a source of plants of the same stock, which could be planted in soil and grown until they reached a suitable stage for their petioles to be used to initiate fresh callus and suspension cultures (Appendix 1). For this reason a freshly initiated suspension of one of these lines, H_9, in addition to the established culture line A, was used to examine the effect of filtration on providing a suitable inoculum for plating. Microscopic examination of strain A revealed that single cells tended to be larger (about 65 x 130μ to 80 x 150μ) than cells in aggregates of about 10 (diameter of aggregates was about 75μ to 200μ). There was, however, a great deal of variation in cell size. Nylon bolting cloth of a range of mesh sizes was used for the filtration. The filtration procedure was carried out under sterile conditions and the resulting suspension plated in agar medium at 10,000 and 30,000 cells ml^{-1} (see Materials & Methods for details of both procedures). At these densities non-filtered suspensions rapidly formed a lawn of growth, most of the growth coming from very large aggregates and

obscuring the colony formation from smaller aggregates. On the plates

of filtered suspensions colony formation usually occurred between 3 and

6 weeks from plating, or not at all, and, except where otherwise stated,

the visible colonies were counted after 6 weeks. Plates showing no

growth at this time were kept for 3 months and then microscopically examined

for any sign of active growth before being discarded.

The initial plating experiments carried out in this way involved the

use of both actively growing (9 days) and stationary phase (21 days)

suspensions which, as already discussed, differ in their extent of aggregation

in addition to their growth rate. For each mesh size used the distribution

of aggregate sizes was determined, the assessment being based on 200 cellular

units, and the mean unit size ($\frac{\text{cells ml}^{-1}}{\text{units ml}^{-1}}$) was also calculated. A summary

of the data is presented in Table I.3. and the results from plates made from

the filtered suspensions are presented in Table I.4.

The results suggested that a filtration technique of this type could be

used to provide a suitable inoculum for plating of cell suspensions. There

are distinct differences between the two cell lines, line A providing a

slightly finer inoculum and a greater yield of colonies on plates, than line

H_9. On the basis of these results a protocol was developed for the filtration

and plating of freshly initiated suspension cultures, which was used for most

of the subsequent work on the selection of variants. This procedure is

discussed at length in the discussion at the end of this section.

(C) The problem of low genetic stability

(i) The effect of alternative auxins on genetic stability

Two alternative auxins were used. These were 1-naphthalene acetic acid

(NAA), which had been shown by Shamina (1966) and Sunderland (1973) to

maintain more stable callus cultures than 2,4-D, and p-chlorophenoxyacetic acid

(p-CPA) which is a close structural analogue of 2,4-D. Preliminary studies

TABLE I.3.

A summary of data relating the size and distribution of aggregates to filtration mesh size in suspensions of N. sylvestris culture lines A and H_9 after filtration through nylon bolting cloth.

Time after sub-culture (days)	Mesh size used for filtration (μ)	Culture line H			Culture line H_9		
		% units containing <20 cells	% units containing <5 cells	Mean unit size (cells/unit)	% units containing <20 cells	% units containing <5 cells	Mean unit size (cells/unit)
9	56	100	100	1.04	100	100	1.26
	65	100	100	1.03	100	100	1.27
	185	100	87.5	1.57	100	82.0	2.07
	600	99.5	73.15	3.12	98.5	70.0	3.40
	1,000	93.0	71.5	3.15	98.0	67.5	3.52
	2,000	76.0	68.0	5.62	72.0	53.0	6.03
21	56	100	100	1.02	100	100	1.39
	65	100	100	1.02	100	100	1.05
	185	100	93.5	1.48	100	93.0	1.85
	600	100	80.5	3.15	100	86.0	3.35
	1,000	98.5	78.0	3.06	99	83.5	3.40
	2,000	81.0	69.5	4.98	78.5	65.5	5.18

TABLE I.4.

Colony formation after plating suspensions of N. sylvestris lines A and H$_9$ after filtration through various mesh sizes.

Data presented is mean colonies /plate for 5 plates

Cell line	Time after sub-culture (days)	Inoc-ulation density (cells ml^{-1})	Mesh size used for filtration (μ)					
			56	65	185	600	1,000	2,000
A	9	10,000	0	0	213	532	545	+++
		30,000			479	+++	+++	+++
	21	10,000	0	0	257	521	571	+++
		30,000			510	+++	+++	+++
H$_9$ (2nd passage)	9	10,000	0	0	0	38	58	214
		30,000			0	261	298	+++
	21	10,000	0	0	0	47	57	230
		30,000			0.4	249	279	+++

+++ = Plates overgrown, colonies could not be counted.

using various levels of both of these auxins suggested that the most

suitable concentrations for the growth of suspension cultures of

N. sylvestris were 0.4 mg/l NAA and 1.2 mg/l p-CPA. At these concentrations

the suspensions could be serially subcultured but in the case of NAA the

growth was slower than in 2.4-D, and the suspensions highly aggregated

showing frequent shoot formation. Suspensions grown in medium containing

p-CPA grew almost as rapidly as in 2,4-D, showed a comparable level of

aggregation and no morphogenesis. Cultures derived from the haploid

N. sylvestris plant H_7, were used to study the effect of these auxins.

Callus cultures were initiated on the usual medium and used to initiate

a suspension culture. After 21 days in the initiation passage, P1, Feulgen

stained preparations were made and the nuclei had the density distribution

shown on Fig. I.13.A. This suspension was subcultured at 10^5 cell ml^{-1}

into fresh medium containing either 0.4 mg/l 2,4-D or the above concentrations

of NAA or p-CPA. The cultures were incubated for 21 days and then sub-

cultured again at 10^5 cells ml^{-1} into the same medium. At the end of this

passage (P3) samples were removed and Feulgen stained preparations made for

microdensitometry. Density measurements were made on 200 interphase nuclei

for each treatment and semi-logarithmic plots of the results are given in

Fig. I.13. B,C, and D. Plotting the results in this way should give a

roughly equal spacing between the peaks if each peak corresponds to double

the DNA content of the previous peak. If there are x peaks there should be

at least x - 1 logarithmically increasing ploidy levels. That means, in this

case with 5 peaks, there must be approximately n, 2n, 4n and 8n nuclei present

but the lack of clarity of the peaks suggests that, as has been found in

other cultures of N. sylvestris, intermediate ploidy levels and aneuploid

nuclei may be present.

The results show an increase in the occurrence of higher ploidy levels

over three passages in liquid medium containing 2,4-D. This increase also

<u>Fig. I.13</u> Microdensitometer profiles for suspension cultures of <u>N.sylvestris</u>

<u>line H$_7$ (haploid origin) grown in media containing three different</u>

<u>auxins</u>

All samples taken after 21 days of culture.

A. Suspension passage P1 in medium containing 0.4 mg/l. 2,4-D.

B. Suspension passage P3 after two further passages in medium

   containing 0.4 mg/l. 2,4-D.

C. Suspension passage P3 after two passages in medium containing

   1.2 mg/l. p-CPA.

D. Suspension passage P3 after two passages in medium containing

   0.4 mg/l. NAA.

Arrows indicate the positions of peaks corresponding to haploid G_1

and G_2 root tip nuclei respectively.

The DNA values ranged from 15 to 500 arbitrary units and these

values were grouped into 30 classes defined by dividing $\log_{10} 500$ -

$\log_{10} 15$ into 30 equal spacings; the antilogs setting the class

limits for the densities recorded.

Fig. I. 13.
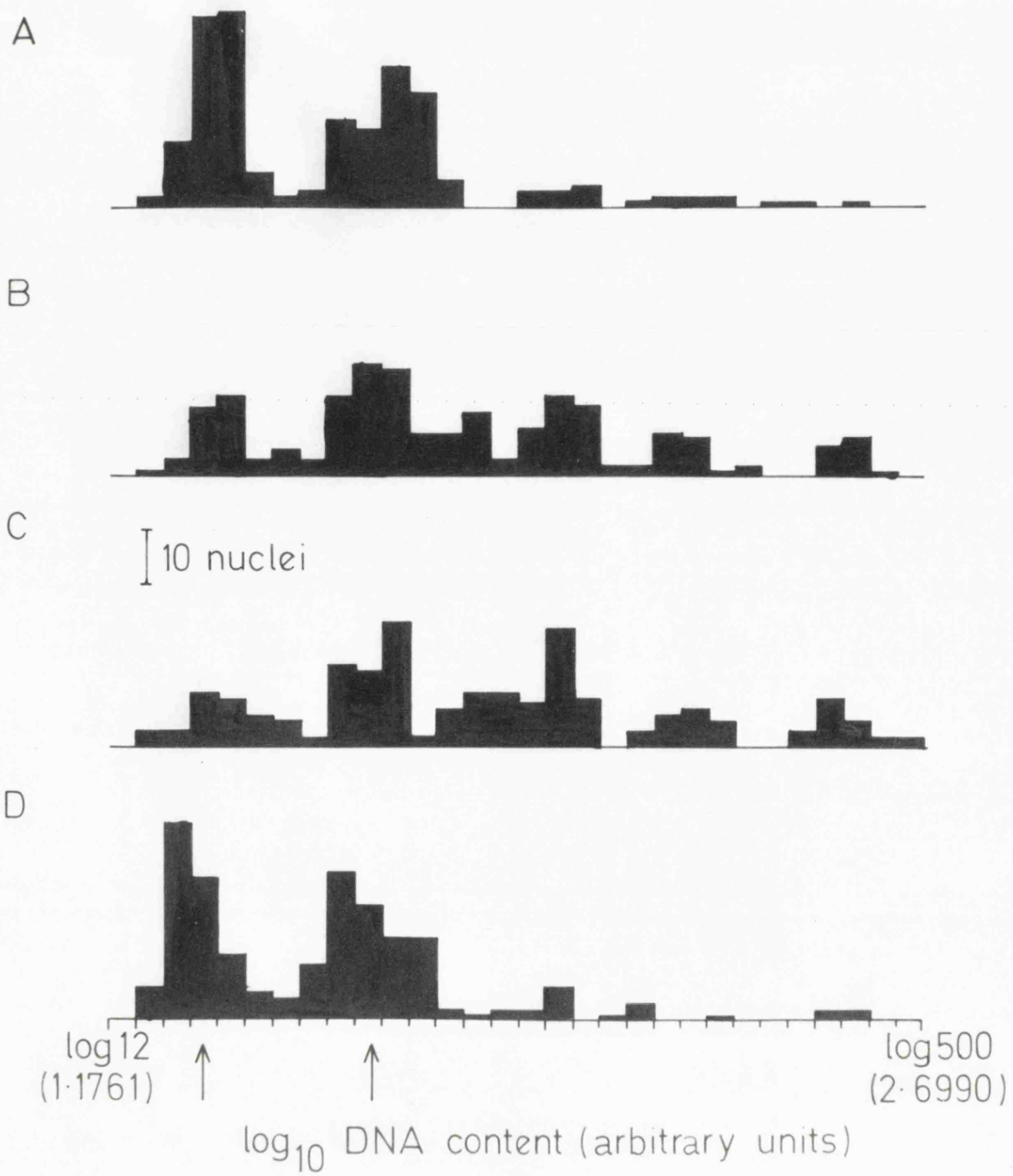
occurs if the suspensions are grown for two passages in medium containing

p-CPA instead of 2,4-D. In medium containing NAA however, this increase

in ploidy does appear to be checked, showing little change from the

initiation passage. Unfortunately this suspension is very highly aggregated,

extremely morphogenic, and difficult to subculture by pipetting in the usual

way. It is wholly unsuited to plating containing very few single cells or

small aggregates. It has not been possible to obtain a fine undifferentiated

suspension by growing suspensions for a number of passages in medium

containing various levels of NAA.

(ii) The use of p-fluorophenylalanine (PFP)

Gupta and Carlson reported a strongly inhibitory effect of PFP at

$9\mu g/ml$ on the growth of diploid callus of Nicotiana tabacum, whilst little

inhibition of haploid callus occurred at this concentration. This technique

was considered potentially very important and the possible application of it

to N. sylvestris was studied by looking at the effect of PFP, at various

concentrations, on the initiation of callus from haploid and diploid petioles,

and on the growth of freshly initiated suspensions of haploid and diploid

origin and well established, highly polyploid suspensions.

As a preliminary to this investigation, the toxicity at $10\mu g/ml$ on

freshly initiated diploid callus was investigated. The fresh weight increase

of callus pieces growing on medium containing 0 and $10\mu g/ml$. PFP was

examined, over 28 days, callus pieces from 5 replicate flasks for each

treatment being harvested every 7 days. The means of these are plotted on

Fig. I.14. PFP clearly had no effect at this concentration, on the growth

of diploid callus and it was concluded that higher concentrations of PFP
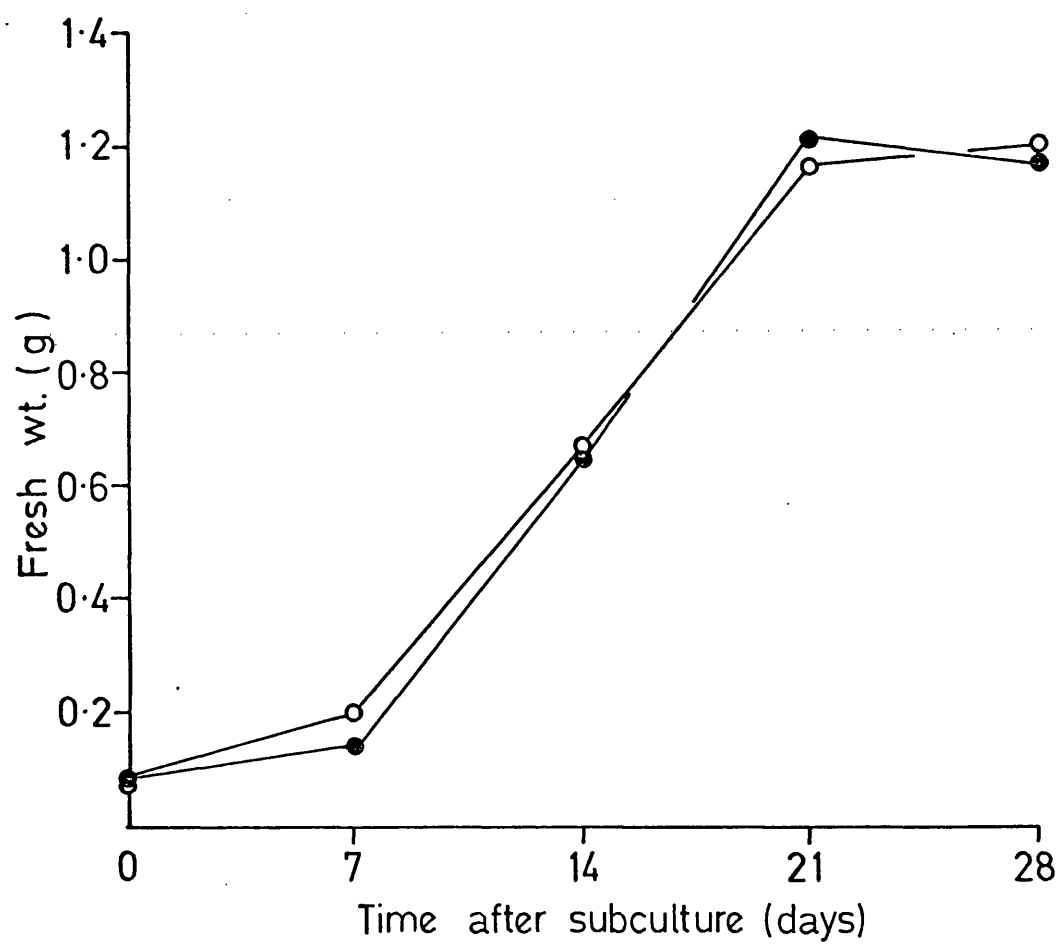
would be necessary.

The effect of PFP was examined, on the growth of suspension cultures of

line A and freshly initiated line H_3, of haploid origin. Line A was highly

Fig. I.14 The growth of a diploid callus of N. sylvestris on medium

containing 0 or 10 µg/ml. PFP

○ 0 µg/ml. PFP

● 10 µg/ml. PFP

Fig. I. 14.

polyploid and line H_3 still contained a proportion of haploid cells. If

the technique is to be of any use in selecting haploid cells from a

mixed culture, at some concentrations there should be complete inhibition

of line A but some growth of line H_3. Suspensions of A and line H_3, in its

initiation passage, were inoculated at 10^5 cells ml^{-1} into media containing

a range of PFP (0 - 50 μg/ml) concentrations. They were incubated in the

usual way for 21 days and then the cell numbers, dry weight and packed cell

volumes were determined. From these values the percentage inhibition of

growth compared with the control culture could be determined and these data

are presented on Fig. I.15.A. (cell count data) and B (dry weight and packed

cell volume data). For each point the value is based on assessment from

triplicate samples. Any difference in the growth between these cell lines

is slight and would be unlikely to result in the selection of haploid cells

in a mixture. Levels of PFP greater than 25μg/ml are required for inhibition

and it seemed worthwhile repeating this experiment using levels of PFP

ranging from 25 to 100μg/ml, a level which it was supposed would completely

inhibit both lines. The results of this experiment are plotted on Fig. I.16.

They suggest that line A might be more susceptible to 50μg/ml PFP than line

H_3, an effect which might be promising in terms of selecting a line of

higher haploidy. The lines under examination here, however, are widely

divergent in age, origin, and degree of dispersion, all of which might effect

the results.

It was decided that the best and most conclusive approach to this problem

would be to examine the effect of PFP on haploid and diploid derived cell lines

at the same stage of culture. To this end one diploid plant, D, and three

haploid plants, H_6, H_7 and H_9 were chosen. The effect of PFP on the initiation

of callus from petioles was examined for H_6, H_7 and D, and on the growth of

freshly initiated suspensions, was examined for each of the four plants.

Petiole segments (2 - 3 mm in length) were placed on medium containing 0,

Fig.I.15   The effect of PFP on the growth of suspensions of N. sylvestris

lines A and H$_3$.

Left: - Percentage inhibition of final cell number of line A (●)

and line H$_3$ (▲).

Right: - Percentage inhibition of final dry weight and packed cell

volume of line A (Dry wt: ● , PCV : O) and line H$_3$ (Dry wt: ▲ ,

PCV : Δ).

Fig. I.15.
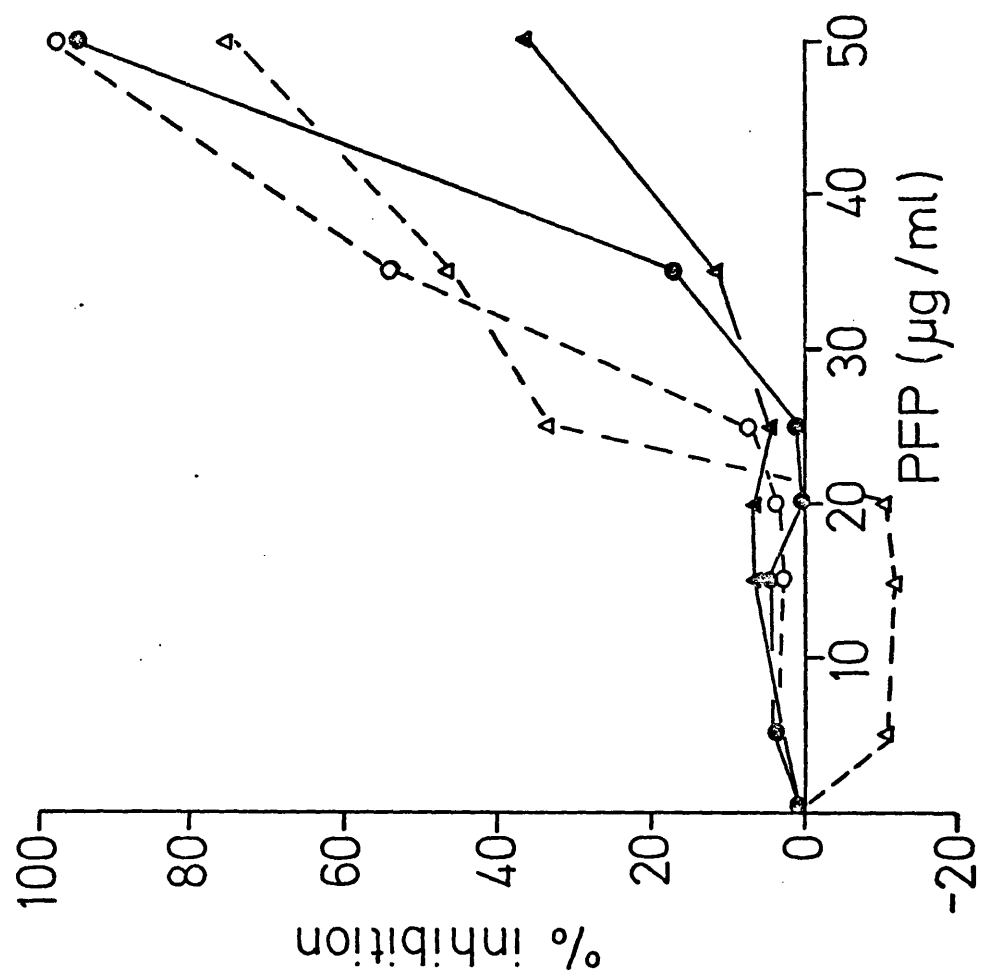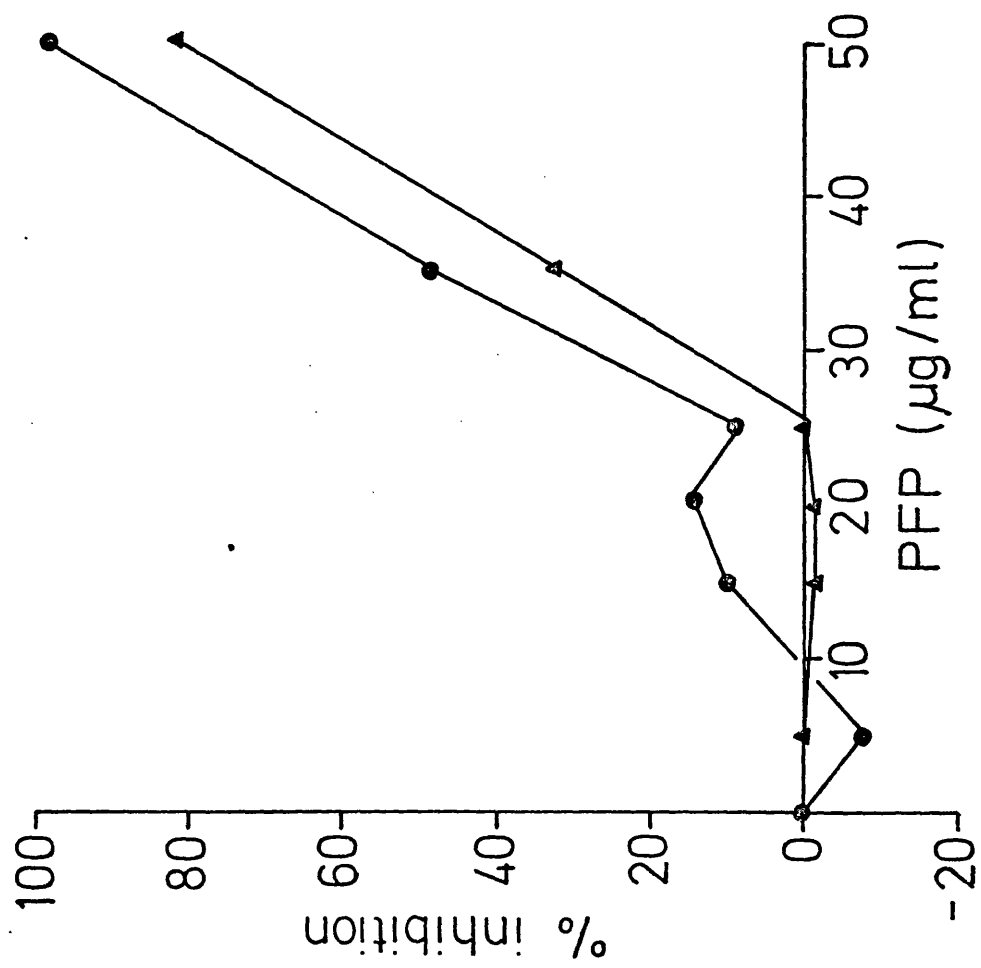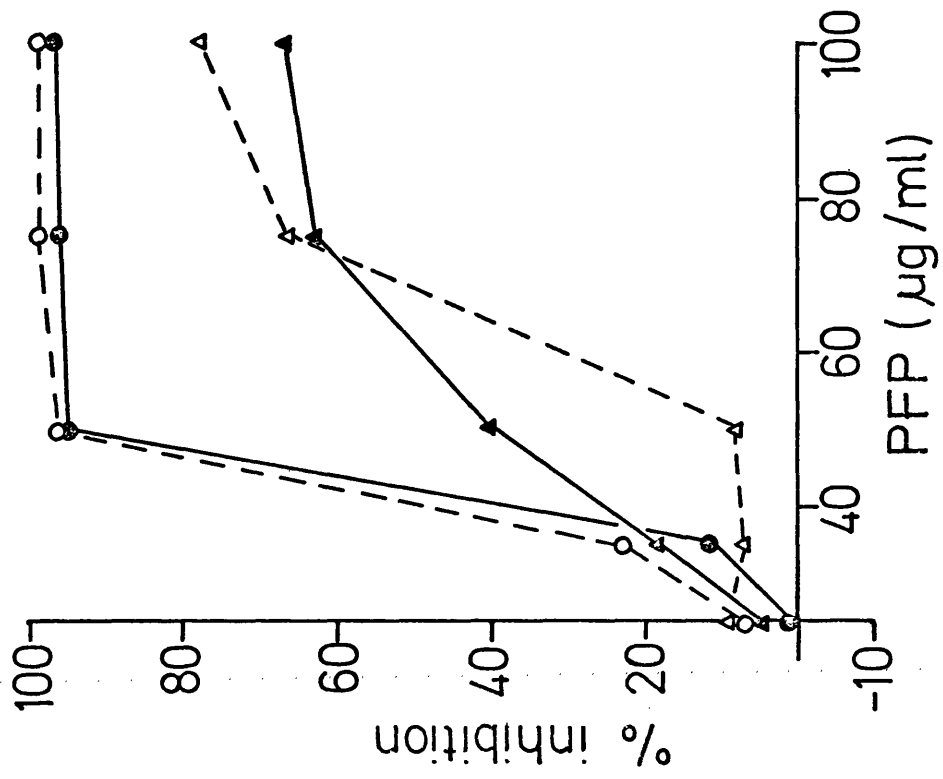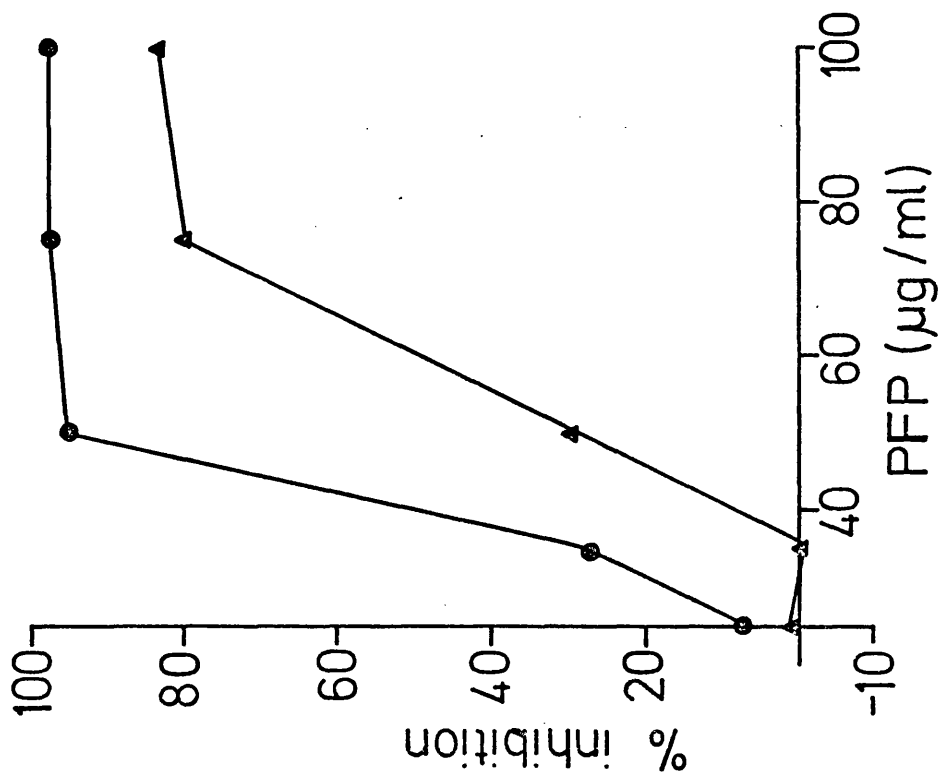
<u>Fig. I.16</u> <u>The effect of a wide range of PFP concentrations on the growth of</u>

<u>suspensions of N. sylvestris lines A and H_3.</u>

Left: - Percentage inhibition of final cell number of line A (●)

and line H_3 (▲).

Right: - Percentage inhibition of final dry weight and packed cell

volume of line A (Dry wt: ● , PCV : O) and line H_3 (Dry wt: ▲ ,

PCV : Δ).

Fig. I. 16.

37.5, 50, 75 or 100µg/ml PFP and incubated at 25°C in the light. After

28 days the number of petiole segments showing growth, and the extent of

growth was estimated. The results are given in Table I.5.

Despite the small number of samples in each treatment clear differences

can be seen between the diploid and the two haploid plants, the former

showing much less callus growth on medium containing PFP. This is also

shown on Plate I.2. in which representative samples for plants D and H_7

are compared. Again this is promising evidence in favour of the use of

PFP, but the true test must be carried out in the liquid medium in which

we would hope to apply it.

From the four culture lines, initiated and grown in the absence of

PFP, cell suspension cultures were initiated and tested for their sensitivity

to PFP inhibition after one or two culture passages in medium without PFP.

They were tested in passage 2 and passage 3 of culture. The cultures were

initiated at 10^5 cells ml^{-1} and the effect of PFP on growth was assessed by

cell counts after 21 days of incubation (no further significant increases

in cell number occurred when the incubation was extended for a further 7 days).

From the cell count data percentage inhibition of final cell number was

determined and these data are presented in Fig. I.17. The experiments from

both culture passages show clear differences between the lines, the line of

diploid origin again being the most susceptible. The three lines of haploid

origin also differed greatly from each other. This is particularly noticeable

in the third passage, in which line H_9 is clearly inhibited less than the

other two and is the only line to show any growth at 75µg/ml PFP. The

differences between the cell lines are seen most clearly on Fig. I.18. in

which the actual final cell densities at the key concentration, 37.5µg/ml of

PFP are compared to each other, and to the control cell densities in medium

containing no PFP.

TABLE I.5.

Callus initiation from petioles on medium containing various levels of PFP

Plant	PFP (ug/ml)	Segments in treatment	Segments showing callus initiation	Extent of callus formation
D (diploid)	0	5	4	+++
	37.5	7	4	+
	50	6	2	+
	75	5	2	+
	100	6	1	+
H_6 (haploid)	0	6	6	+++
	37.5	6	6	+++
	50	8	5	++
	75	8	4	++
	100	8	1	+
H_7 (haploid)	0	8	8	+++
	37.5	8	6	+++
	50	8	6	+++
	75	7	5	+
	100	8	2	+

Key: +   Callus growth just visible

++ Callus growth up to 3x size of explant

+++ Callus growth greater than 3x size of explant

PLATE I.2.   Callus initiation from petioles of haploid (H_7) and diploid (D)

plants of N. sylvestris on medium containing various levels of

PFP.

A. Line H_7

B. Line D

Values are concentrations of PFP in the culture medium ($\mu g/ml.$)

PLATE 1.2

A

B
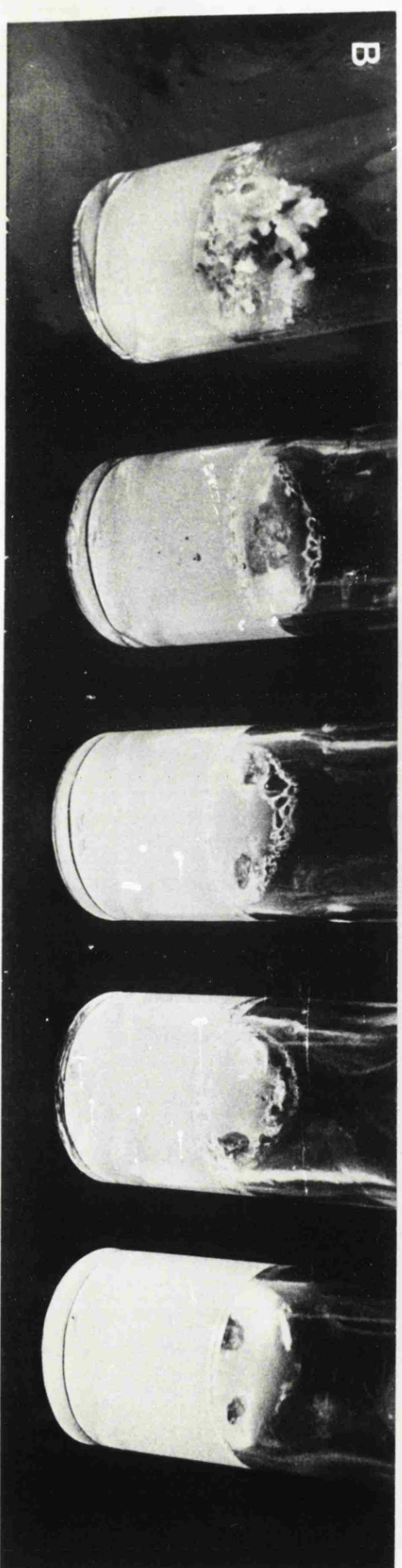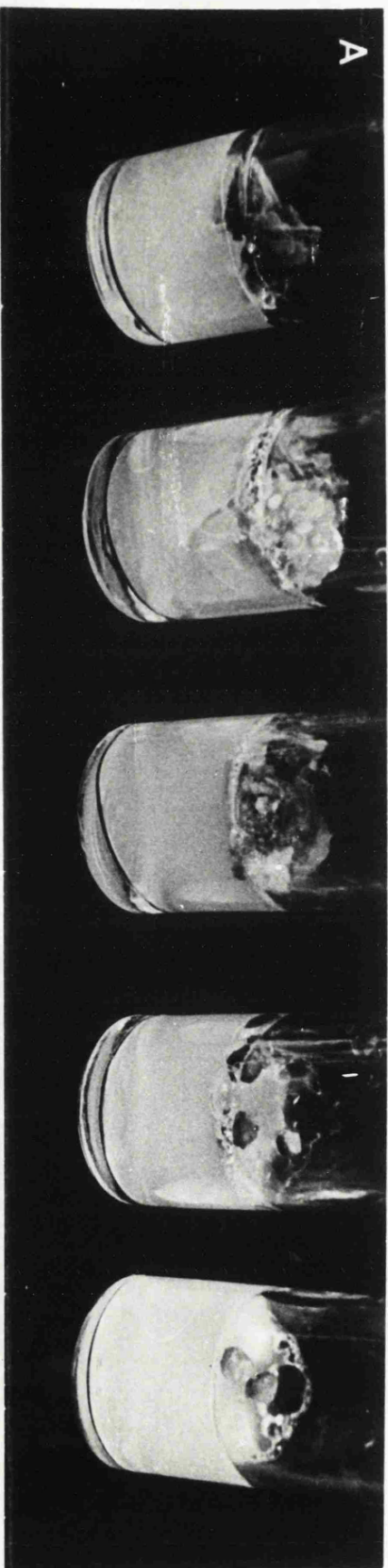
O   37·5   50   75   100

Fig. I.17 The inhibition of final cell number by PFP, in haploid and diploid

derived suspensions of N. sylvestris.

A. PFP incorporated into the second suspension passage. Control

cultures grown for two passages in the absence of PFP.

B. PFP incorporated into the third suspension passage, after two

passages in the absence of PFP. Control cultures grown for three

psaages in the absence of PFP.

Values are percentage inhibitions, compared with controls, after

21 days incubation.

Fig. 1. 17

A

Legend:
- ○ D
- ● H_6
- △ H_7
- ▲ H_9

Y-axis: % Inhibition cell no.
X-axis: PFP μg/ml.

B

Y-axis: % Inhibition cell no.
X-axis: PFP μg/ml
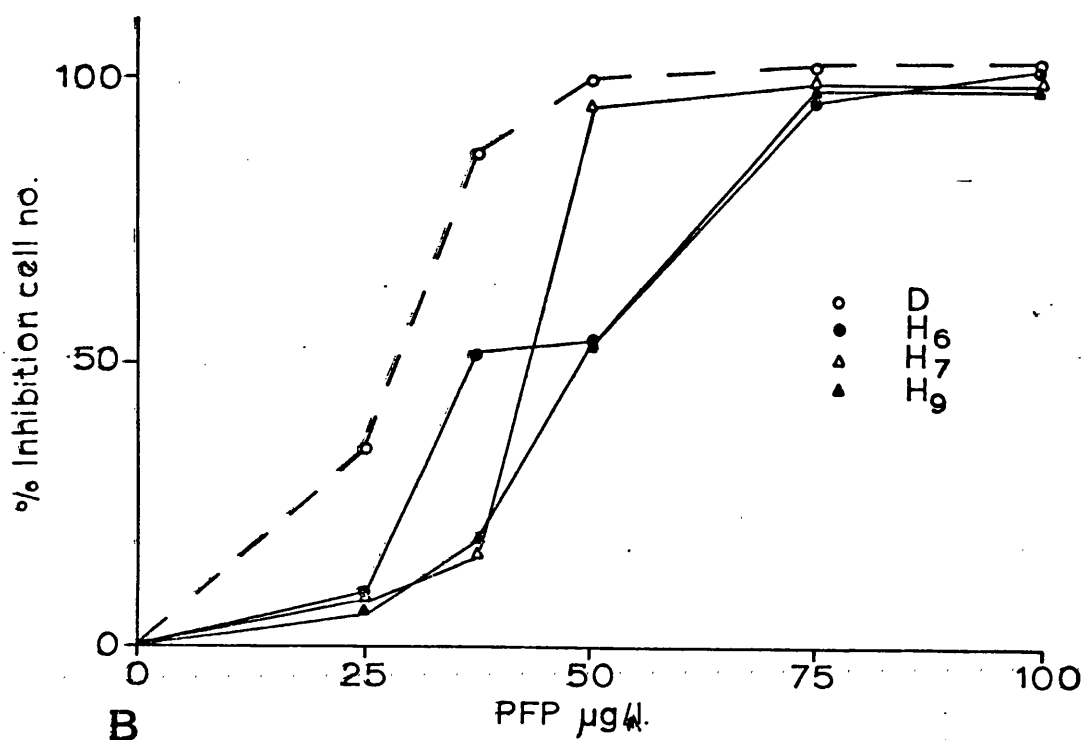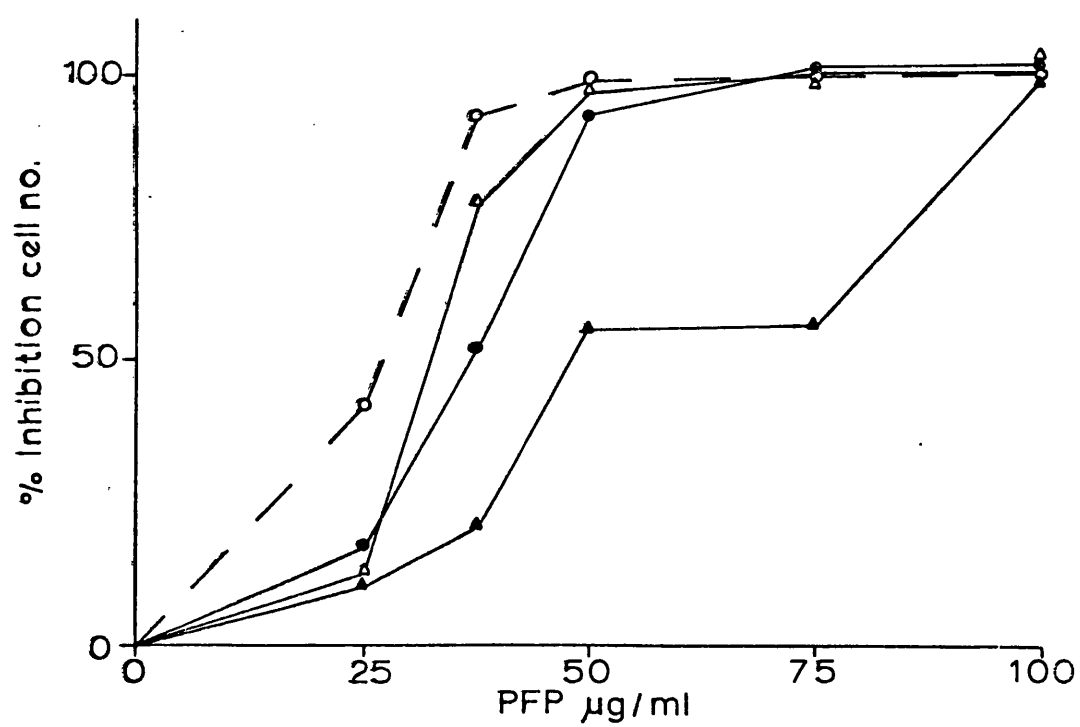
Fig.I.18   Values for cell number after 21 days incubation in medium containing

0 or 37.5 μg/ml. PFP.

A. After two passages in the absence of PFP.

B. After a passage in medium containing 37.5 μg/ml following the

first suspension passage.

C. After a passage in medium containing 37.5 μg/ml following the

second suspension passage.

The vertical lines equal twice the standard errors plotted with the

means as midpoints.

Fig. I. 18.
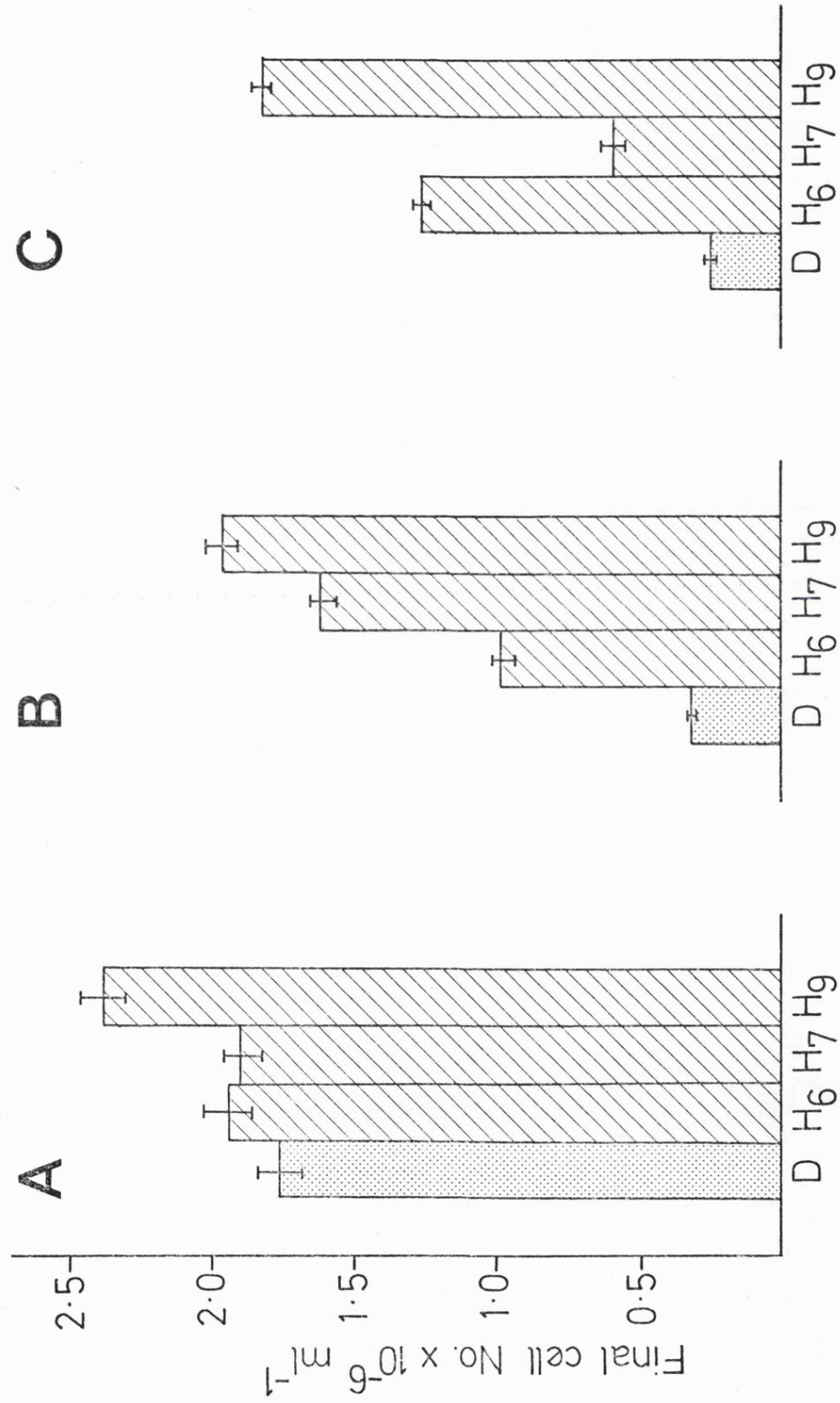
In addition to the samples required for the growth data, samples were removed on the 10th day of the passage, pretreated with colchicine and used to make Feulgen stained preparations for chromosome counts. At the end of each passage preparations were made for DNA estimation by micro-densitometry. The data compiled in this way allowed an assessment of the relative stability of the four lines over the first three suspension passages and revealed the effect of incubation in medium containing PFP on the ploidy distribution. The Feulgen staining density profiles are presented, as semi-logarithmic plots, on Figs. I.19 (line D), I.20 (line H_6), I.21 (line H_7) and I.22 (line H_9). Good preparations for chromosome counts were obtained from some of the treatments of lines H_9 and D, and the relative proportions of the different ploidy levels are given in Fig.I.23.

Several inferences can be drawn from these data. All the density profiles contained three or more peaks showing that at least two ploidy levels were present. This was true even in the initiation passage of the suspensions, and the familiar trend of an increase in the higher ploidy peaks over several passages was also evident. Comparison between the three haploid derived lines, however, showed marked differences in the rate at which higher ploidy peaks appeared. After three suspension passages line H_9 still only possessed three DNA peaks and appeared to retain a high proportion of haploid cells, while in line H_7 there were five DNA peaks and the haploid G_1 peak was greatly diminished. Just as the three lines had differed in their growth response to PFP, they differed in their rate of chromosome duplication in culture. When the profiles for lines actively growing in the presence of PFP are examined it is clear that there is no shift towards the lower ploidy peaks, as would be expected if PFP were inhibiting the growth of cells of higher ploidy than haploid. This suggests that while diploid and tetraploid cells in suspensions of line D are not growing in the presence of PFP, diploid and tetraploid cells in the other

<u>Fig. I.19</u> Microdensitometer profiles for <u>N. sylvestris</u> cell line D (diploid

origin).

A. 1st suspension passage, in medium containing no PFP

B. 2nd suspension passage, in medium containing no PFP

C. 3rd suspension passage, in medium containing no PFP

D. 4th suspension passage, in medium containing no PFP

E. 3rd suspension passage, grown in the presence of 37.5 μg/ml.

PFP after the first two passages grown in the absence of PFP.

<u>Figs. I.19 - I.22</u> Arrows indicate the positions of diploid G_1 and G_2 peaks

as assessed by 50 measurements of interphase nuclei from diploid

root tips.

All profiles are based on samples taken 21 days after subculture.

Fig. I. 19.

A

B

C

D

I 10 nuclei

E

log 15
(1·1761)

$\log_{10}$ DNA content (arbitrary units)

log 500
(2·6990)
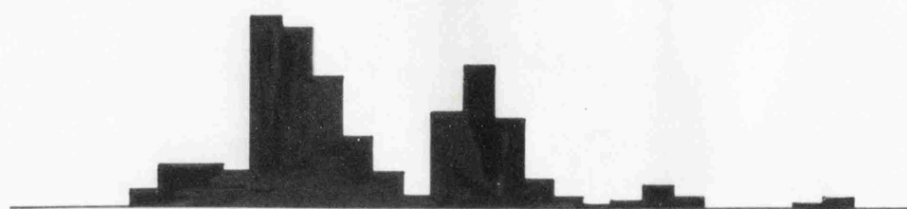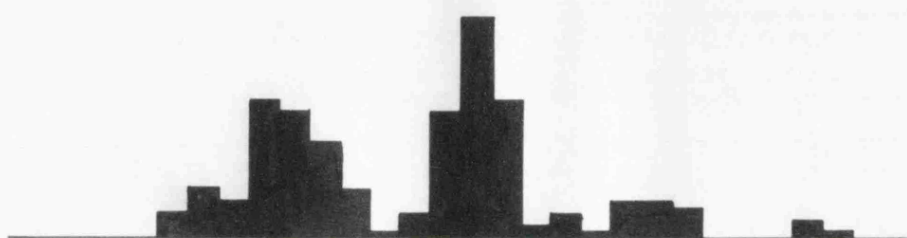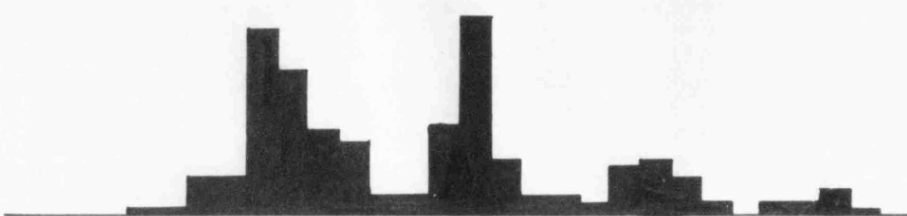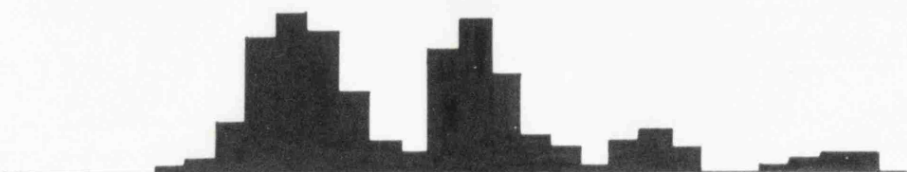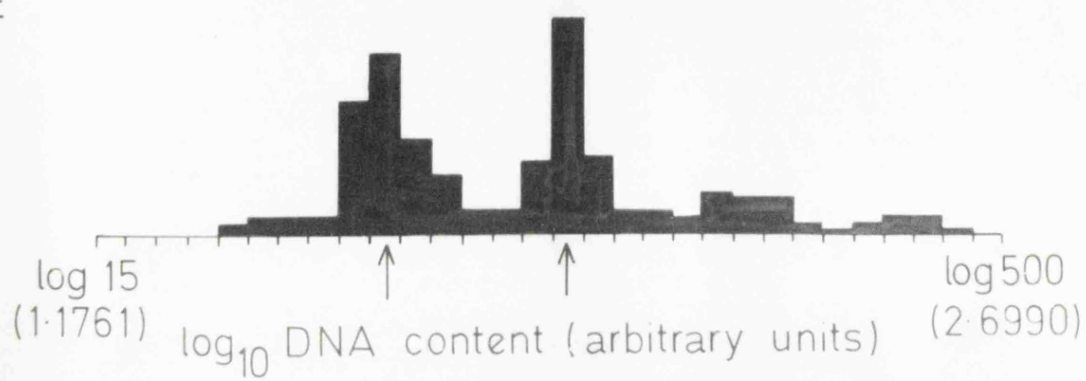
Fig. I.20 Microdensitometer profiles for <u>N. sylvestris</u> cell line H_6(haploid origin)

A. 1st suspension passage, in medium containing no PFP

B. 2nd suspension passage, in medium containing no PFP

C. 3rd suspension passage, in medium containing no PFP

D. 3rd suspension passage, in medium containing 37.5 μg/ml. PFP after the first two passages in the absence of PFP

Fig. I. 20.

A

B

C

D   10 nuclei

log 15   log 500
(1·1761)   (2·6990)

$\log_{10}$ DNA content (arbitrary units)
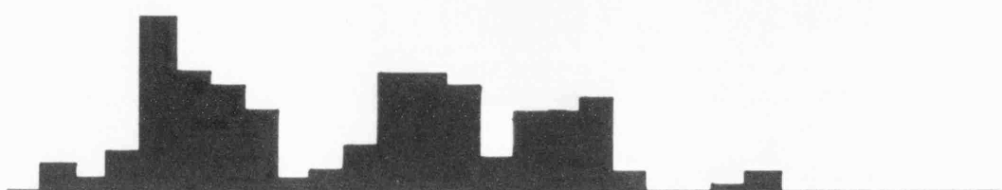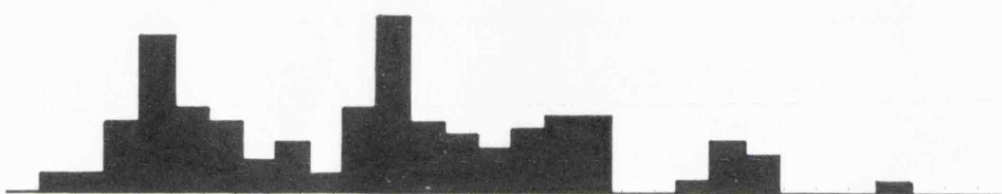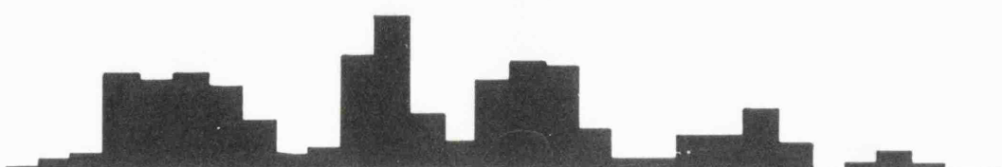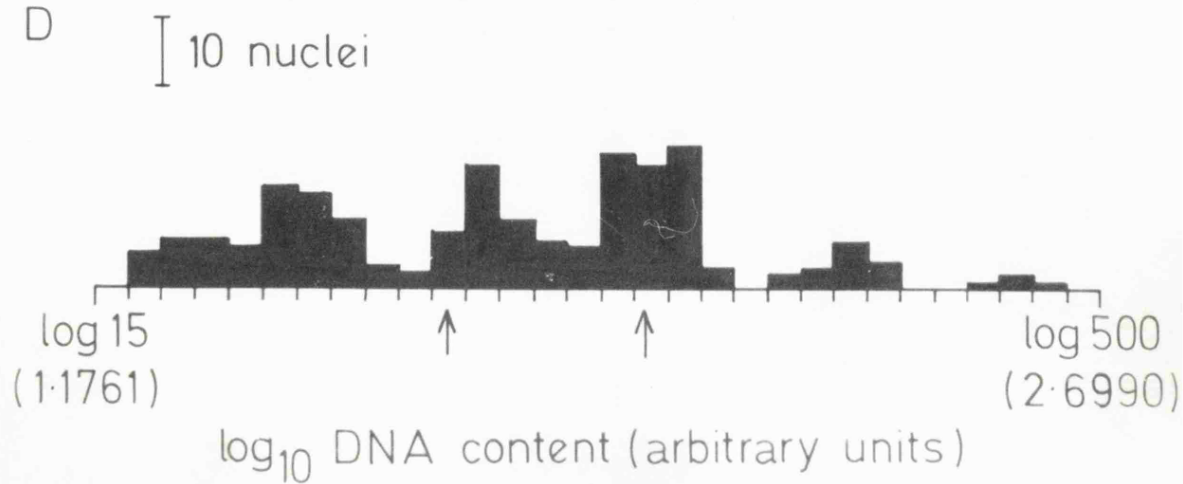
Fig. I.21 Microdensitometer profiles for N. sylvestris cell line H7 (haploid

origin)

A. 1st suspension passage, in medium containing no PFP

B. 2nd suspension passage, in medium containing no PFP

C. 3rd suspension passage, in medium containing no PFP

D. 3rd suspension passage, in medium containing 37.5 μg/ml. PFP

after the first two passages in the absence of PFP

Fig. I. 21.

A

B

C

D ⟮ 10 nuclei

log 15   log 500
(1·1761)   (2·6990)

$\log_{10}$ DNA content (arbitrary units)
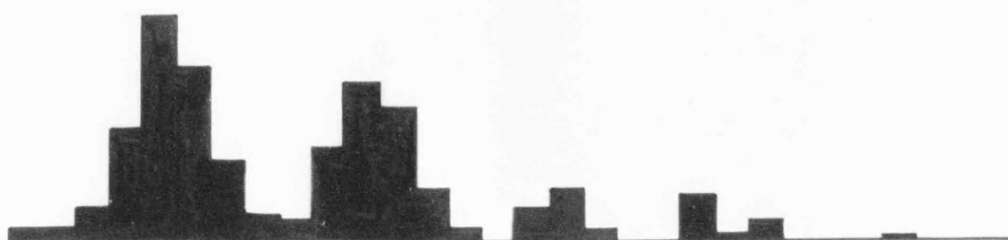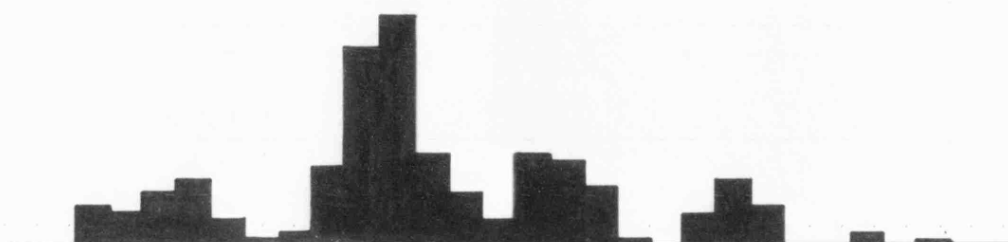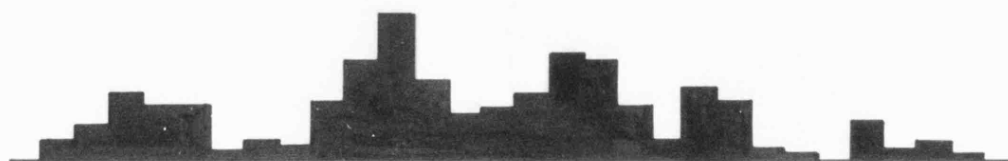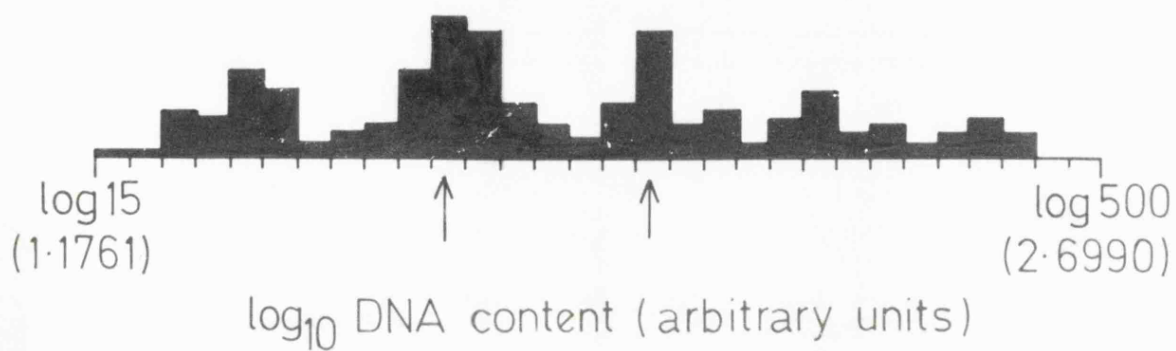
Fig. I.22 Microdensitometer profiles for N. sylvestris cell line H_9 (haploid origin)

A. 1st suspension passage, in medium containing no PFP

B. 2nd suspension passage, in medium containing no PFP

C. 3rd suspension passage, in medium containing no PFP

D.E. and F. 3rd suspension passage grown in medium containing 37.5, 50 and 75 μg/ml. PFP respectively, after the first two passages in the absence of PFP.

Fig. I. 22.

A
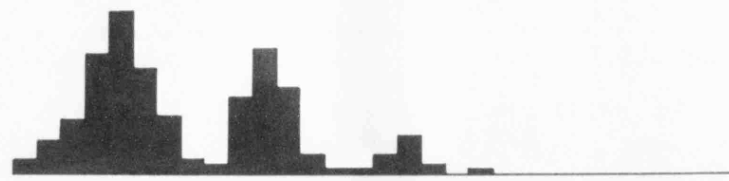
B
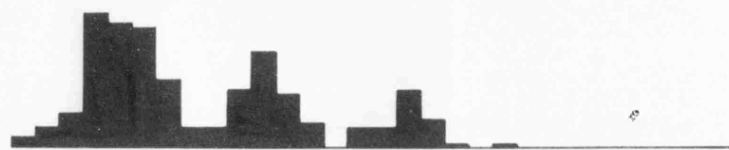
C

D

E
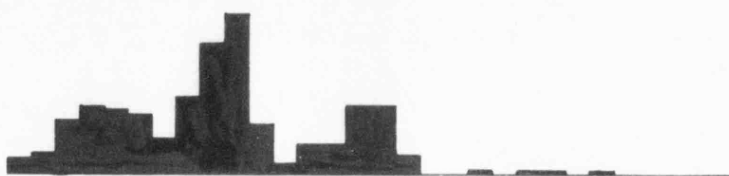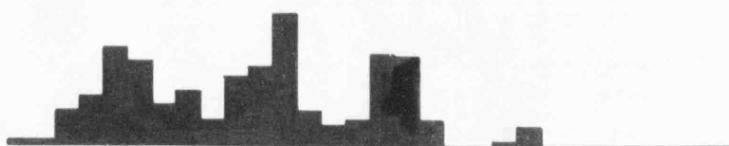
F   10 nuclei

log 15   log 500
(1·1761)   (2·6990)

log₁₀ DNA content (arbitrary units)

Fig. I.23 Ploidy distributions of N. sylvestris cell lines H_9 and D.

H_9 P_1 and H_9 P_3, in suspension cultures of line H_9 during passage 1 and 3 respectively.

H_9, 0/0/37.5, in a suspension culture of line H_9 during passage 3 in medium containing 37.5 μg/ml. PFP after the first two passages in the absence of PFP.

D P_2, in a suspension culture of line D during passage 2.

Fig. I. 23.

H_9 P_1

H_9 P_3

H_9
0/0/37·5

D P_2
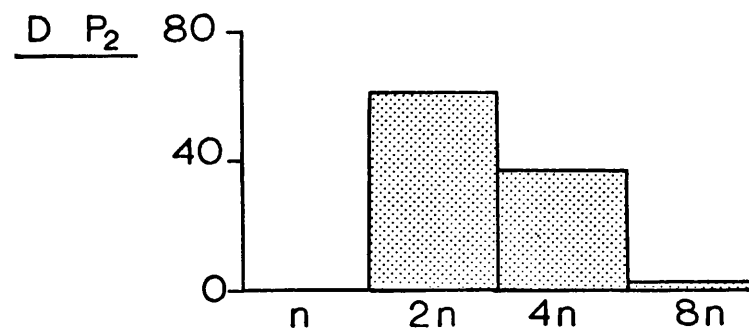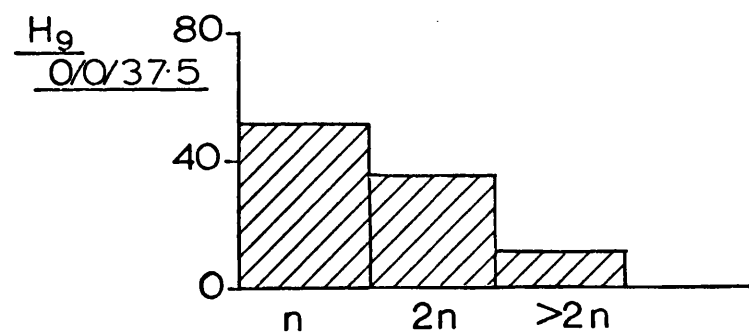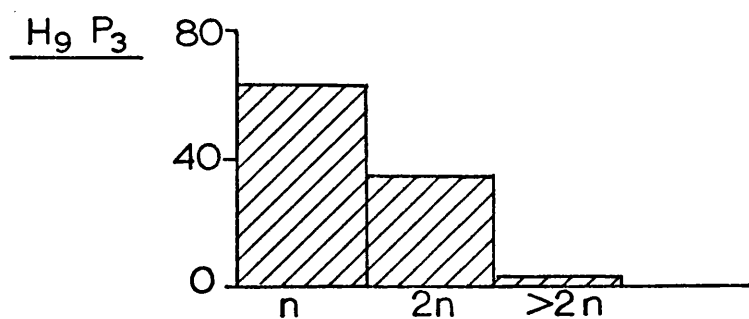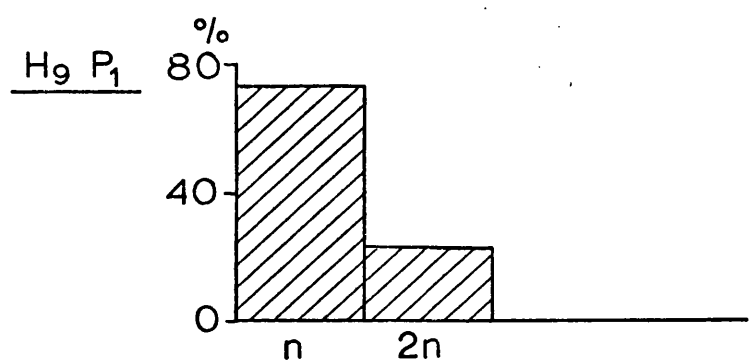
PLATE I.3.   Mitoses from N. sylvestris line H$_9$ (haploid origin) growing in medium containing 37.5 µg/ml. PFP.

A.   Haploid nuclei in division.

x 2600

B.   Diploid nucleus in division.

x 1300

PLATE I.3
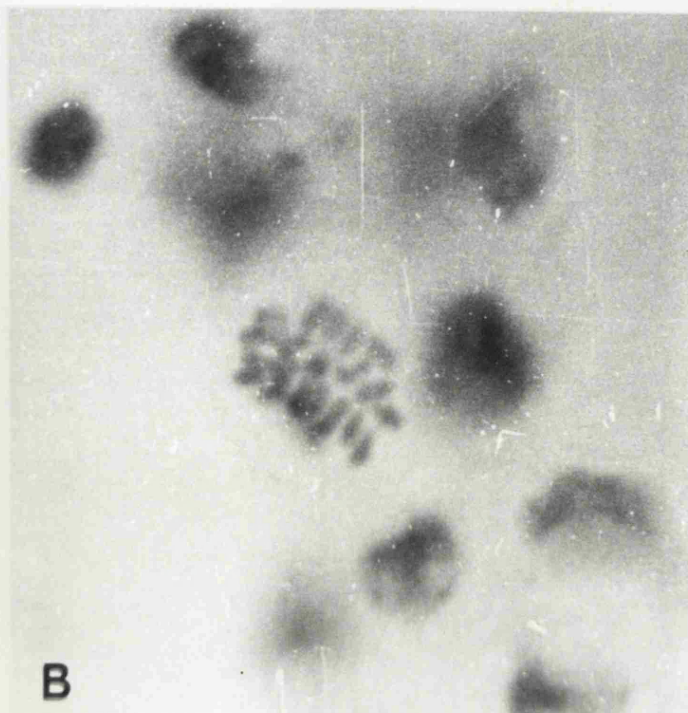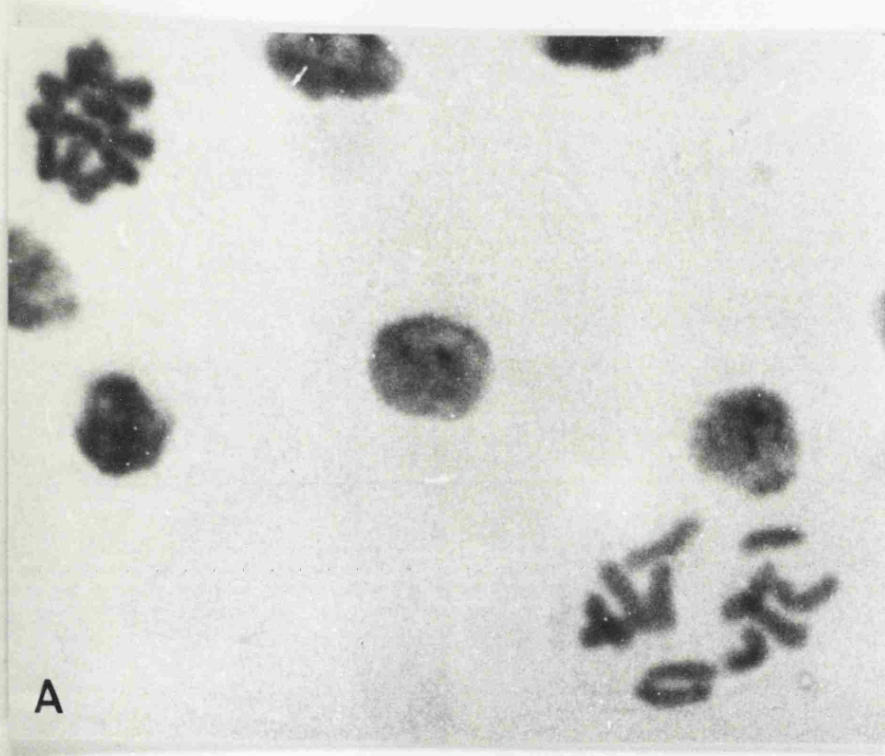
lines are. The latter is confirmed by the chromosome counts from line H_9 grown in the presence of 37.5 μg/ml PFP (Fig. I.23). This agrees favourably with the microdensitometer profile and shows that haploid, diploid, and higher than diploid cells are all dividing in the presence of PFP. Haploid and diploid cells in division in the presence of 37.5μg/ml PFP are shown on Plate I.3.

From the point of view of selecting a haploid line of N. sylvestris from a culture of mixed ploidy, these results suggest that PFP could not be used.

(iii) Cloning as a means of producing stable lines

If, in a culture of mixed ploidy, there are individual aggregates containing entirely or predominantly haploid cells, these must have resisted in some way the tendency toward endoduplication of DNA in the cultures. If this is due to a genetic characteristic of the component cells of these aggregates they might give rise to colonies capable of retaining their haploid nature on plating. To examine this possibility an assessment was first made of the ploidy distribution in different aggregates of a culture of mixed ploidy. 6th passage suspensions of haploid derived lines H_7 and H_9 were used. At this time a high proportion of the cells in both cultures were diploid or tetraploid, but some haploid cells were still present. Samples were removed 6 days after subculture, pretreated with colchicine, fixed and used to make Feulgen stained squash preparations. The only deviation from the usual method was that only a single large aggregate was placed on each slide. Slides were prepared of 6 aggregates for each of the two culture lines and these were labelled a to f. The mitotic indices of each aggregate were calculated, based on 1,000 nuclei, and are given in Table I.6. Line H_7 was rapidly dividing and clearly all the aggregates squashed had been contributing to the rapid rate of cell division in the culture. Line H_9 was dividing more slowly and there was a wider range of

TABLE I.6.

Percentage of nuclei undergoing mitosis in 6 aggregates in each of culture lines H_7 and H_9 at 6 days after subculture.

Figures are the percentage of nuclei in mitosis and are based on 1,000 nuclei counted.

Culture	Aggregate					
line	a	b	c	d	e	f
H_7	2.5	1.9	2.4	2.3	3.5	4.0
H_9	0.5	0.3	0.5	0.4	1.3	1.0

mitotic index between the aggregates. In each of the aggregates of H_7 enough cells undergoing mitosis could be found for 50 metaphases to be measured on the microdensitometer and profiles could be plotted for each aggregate. These are shown on Fig. I.24 and the composite profile presented on Fig. I.25. represents the overall ploidy status of the culture. Some of the metaphases could be counted and confirmed the allocation of the peaks to n, 2n and 4n using measurements made on interphase nuclei of diploid root tips. Fewer metaphases would be found in the aggregates from line H_9 so the profiles on Fig. I.26 are based on smaller and variable numbers of measurements. The composite profile, Fig. I.27 is based on only 175 readings.

The profiles show that the overall ploidy distribution in the culture is not reflected by the distribution in individual aggregates and often a single ploidy level is prevalent within an aggregate. In the case of line H_7, for example, of the six aggregates examined two were predominantly tetraploid, two predominantly diploid, one predominantly haploid and one a mixture of haploid and diploid cells. A similar variety was found among the aggregates of line H_9 except that in this case none of the aggregates were predominantly haploid.

These results suggest that plating of these suspensions might give rise to colonies a proportion of which are haploid and likely to remain haploid over an extended period in culture, and several attempts have been made to do this with line H_7. Unfortunately, no stable haploid lines have been obtained in this way, although the colonies have frequently been of a single ploidy, mostly tetraploid. Two diploid lines have been obtained in this way.

DISCUSSION

Cell suspensions of N. sylvestris were studied in terms of growth characteristics, ploidy variation and aggregation. The failings and advantages of the individual methods used to make this study are reviewed here.

Fig. I.24 Ploidy distributions in aggregates taken from a 6th passage

suspension of N. sylvestris line H_7 (haploid origin).

Each profile is compiled from density readings on 50 metaphase

nuclei in a single aggregate. 6 aggregates, designated A to F

were examined.

Fig. I. 24.

DNA (arbitrary units)
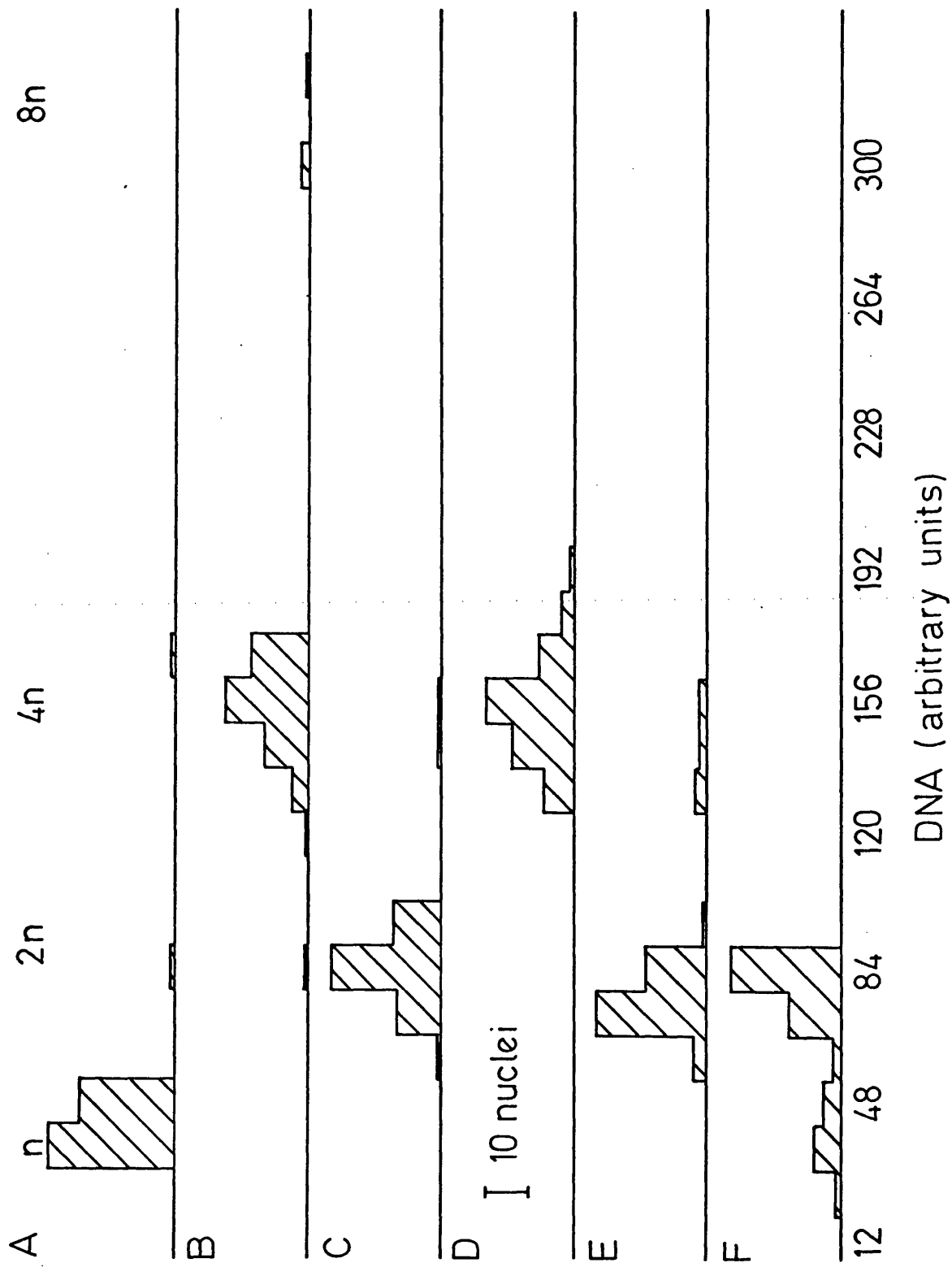
Fig. I.25   Overall ploidy distribution in a 6th passage suspension of

N. sylvestris line H$_7$ (haploid origin)

The profile is an accumulation of the data from 6 aggregates given

in Fig. I.24 (based on 300 metaphase nuclei).

Fig. I. 25.

n   2n   4n

[10 nuclei

12   48   84   120   156   192   228   264   300

DNA (arbitrary units)
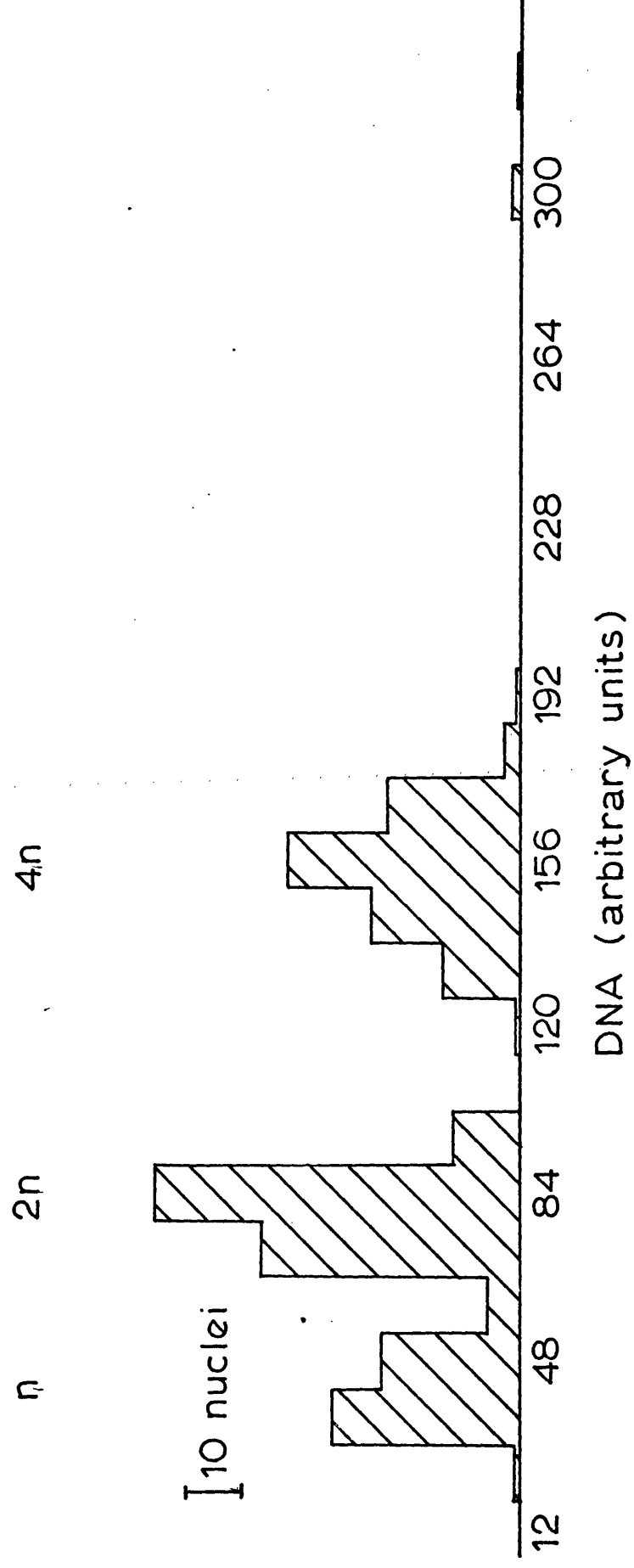
Fig. I.26 Ploidy distributions in aggregates taken from a 6th passage

suspension of N. sylvestris line H₉ (haploid derived)

Profiles are based on a variable number of metaphases nuclei. Each

profile is compiled from density readings on nuclei from one of

6 aggregates, designated A to F.

Fig. I. 26.

DNA (arbitrary units)
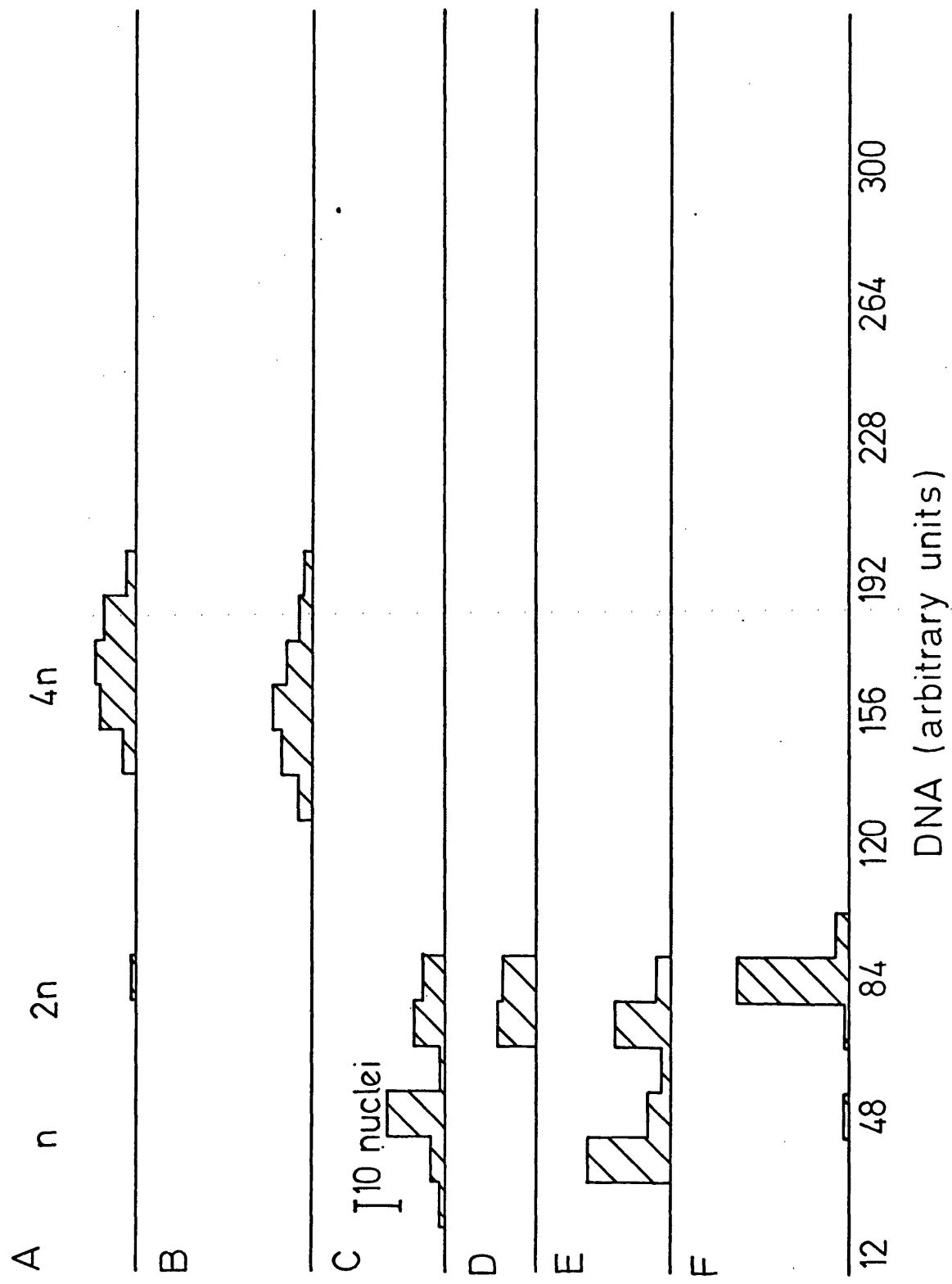
Fig. I.27 Overall ploidy distribution in a 6th passage suspension of

N. sylvestris line H_9 (haploid origin)

The profile is an accumulation of the data from 6 aggregates

given in Fig. I.26 (based on 175 metaphase nuclei).

Fig. I. 27.

n   2n   4n

I 10 nuclei

DNA (arbitrary units)

12   48   84   120   156   192   228
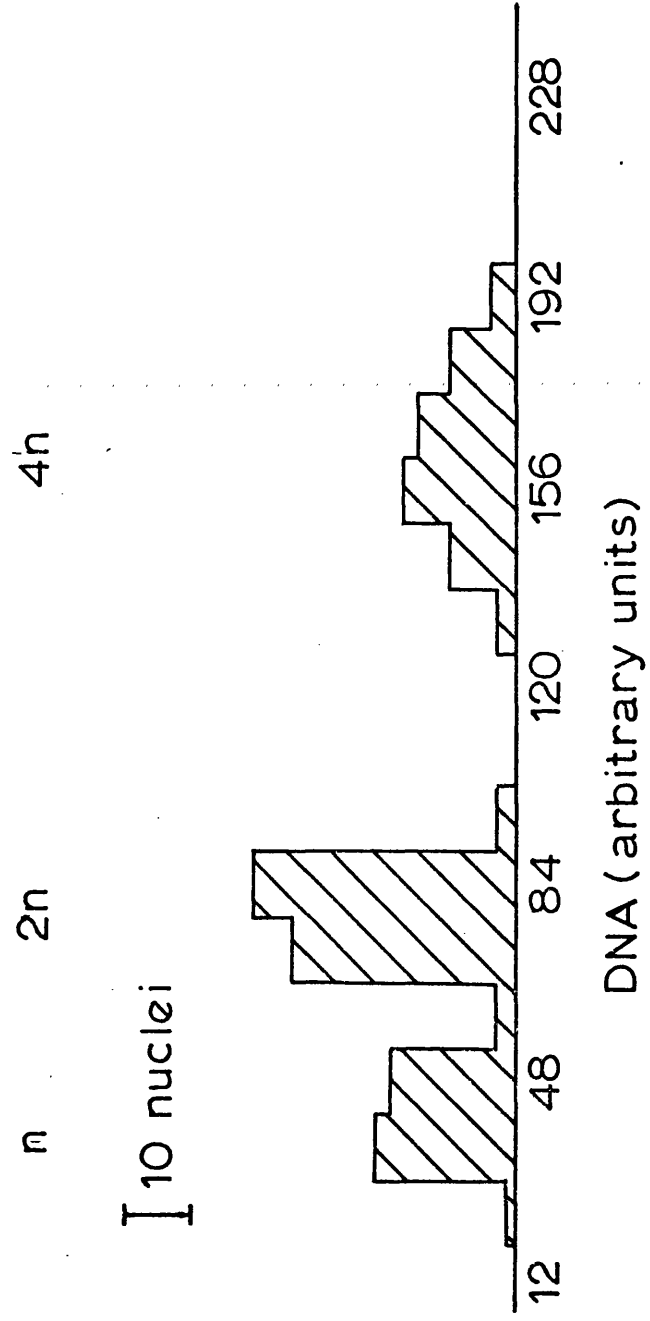
Two methods were used in the assessment of ploidy variation within

the cultures. Of these chromosome counting is the more difficult to carry

out satisfactorily. In order to prepare slides containing enough countable

metaphases, samples must be removed from the culture within a narrow region

of the growth curve when the growth rate is at its maximum. Even when

there is a high mitotic index, only a small proportion of the metaphases

might be squashed well enough to be clearly countable. If there is more

than one ploidy level it may be easier to count metaphases of the lower

ploidy cells, so that more of these might be counted, giving a distorted

picture of the true ploidy distribution. In addition to this, the relative

lengths of the cell cycle and particular regions of the cell cycle may

differ for different ploidy values, again giving rise to complications

when estimating the ploidy distribution.

Microdensitometer profiles of interphase nuclei enable a representative

sample of the nuclei to be examined, but give rise to confusion due to cells

of each ploidy level presenting two peaks, due to nuclei containing the DNA

contents corresponding to the G1 and G2 phases of the cell cycle. This

means that profiles compiled from the same region of the growth curve can be

compared to give a rough indication of the ploidy distribution and the

minimum number of complete ploidy levels can be determined, but an accurate

determination of the relative numbers of cells of each ploidy level cannot

be made. Also the technique is insufficiently sensitive to record the

slight differences that may occur between aneuploid and euploid nuclei.

Clearly neither of these techniques can be relied upon to give a

completely accurate picture. Some of the difficulties mentioned above can

be surmounted by making microdensitometer profiles based on readings taken

from nuclei in division. For a given ploidy these should all contain the

same quantity of DNA and therefore negate the problem of overlapping peaks,

giving profiles in which each peak corresponds to a ploidy level.

All three of these methods have been used here, the choice being

made depending on the nature of the information required. For example,

where an accurate record of the ploidy distribution was needed (e.g. in

the cloning work) density readings of nuclei in division were made, while

where general trends in the culture were under examination (e.g. in the

work with PFP) density readings on interphase nuclei were made, backed up,

where possible, by chromosome counts.

Using these techniques it was established that the ploidy distribution

in a suspension culture of haploid origin of $\underline{N}$. sylvestris tended to higher

ploidy levels over several passages in suspension culture and that it did

this at an unpredictable rate, varying from culture to culture. In addition

to this, although the degree of aggregation decreased with subculture it

did so insufficiently to give a fine inoculum for plating. These two

problems, both anticipated at the beginning of the experimental work, were

encountered and were not satisfactorily solved. More experiments might have

been performed to improve the ploidy status and level of aggregation of the

cultures. This is particularly true of the examination of the problem of

high aggregation. More success might have been obtained if a wider range

of medium variations had been used in the culture of the suspensions. A

finer suspension might have been obtained but there was some indication

(e.g. in the use of NAA as alternative auxin) that the problems of ploidy

level and aggregation might be linked and that conditions ideal for the

growth of a fine suspension might also encourage chromosome doubling. This

problem would not arise in the use of cell wall degrading enzymes and this

is a section of the work which might have been greatly expanded upon, both

by varying the nature of the treatments used and by looking at the purity of

the enzymes. Very crude preparations of cellulose and macerozyme were used

and no attempts were made to purify them. This may have been one of the main

reasons for the toxicity of the enzyme treatments, particularly severe

in those where cellulase was used. The possibility of selecting stable

haploid lines by cloning also needs further examination. The lack of

success obtained here might be partly attributable to the human element

involved in selecting colonies for removal from the plates. For example,

fast growing colonies will be larger and more prominent, yet may arise from

a proportion of the population in which we are not interested.

The results obtained from the work described in this section were,

despite their incompleteness, used to set out the most suitable, if far

from ideal, protocol which would enable the work to be developed along the

lines envisaged. By using freshly initiated suspensions of haploid origin,

the experimental inoculum is clearly at least partly haploid. By filtering

such suspensions prior to plating, the inoculum has been shown to contain a

high proportion of small aggregates capable of giving rise to colonies on

plates. Haploid material can be maintained in the form of the plant, serially

propagated by cuttings, and from this, fresh callus and suspension cultures

can be regularly initiated. With these points in mind, the following

protocol was devised for the plating of suspensions for selection studies on

N. sylvestris and was also found to be applicable to suspension cultures of

C. annuum.

Suspension cultures were generally used between 6 and 9 days after sub-

culture. Where haploid material was required, suspension cultures were used

in their first three passages after initiation. The suspensions were filtered

through 1 mm or 0.6 mm mesh nylon bolting cloth using the filtration tower

shown in Materials & Methods. The residue was resuspended in the tower with

fresh medium to allow a higher proportion of the small aggregates to pass

through. The filtrate was then transferred to a 250 ml Erlenmeyer flask from

which a sample was removed for cell counts. From the value obtained an

appropriate dilution could be determined for the incorporation into agar

medium and plating in Petri dishes (see Materials & Methods) at a final

density of between 1×10^4 and 5×10^4 cells ml^{-1}. The plates were

incubated for the length of time indicated in the appropriate experimental

sections, before scoring for colony formation.

The scoring method was as follows. For plates containing less than

100 colonies, all colonies per plate greater than 1 mm in diameter were

counted. In cases of higher levels of colony formation, the colonies

greater than 1 mm in diameter contained in 12 random squares (1 cm) were

counted and multiplied by: $\dfrac{\text{Area of plate } (= 57 \ cm^2)}{12}$ to give colonies per

plate. This method gives a good approximation ($\pm$ 5%) to the true number of

colonies per plate. The data was usually presented as mean colonies/plate

for 5 or 10 plates and except for the cases of very low colony formation

(mean colonies/plate $<$ 10) standard errors were always less than 5% of the

mean.

No attempt was made to deduce percentage plating efficiencies for these

figures, due to the complexity of the inoculum. Large aggregates (20 - 50

cells) grew more rapidly than smaller aggregates, and many single cells and

aggregates of several cells were inviable. Thus, at the arbitrary time

after plating, chosen for scoring plates, some viable small aggregates might

still be below the minimum size limit for scoring (1 mm) but if the plates

are left for a longer period, small colonies will be obscured by larger ones.

Such difficulties are inevitable in the absence of a truly uniform inoculum

such as that provided by bacterial or protoplast suspensions. Providing the

plating method is consistent, useful inferences can be drawn and different

treatments compared, but values for such parameters as plating efficiency and

mutation rate cannot be deduced. "Plating yield" will be used to describe

the mean number of colonies per plate, as determined by the scoring method

described above.

This routine of filtration, plating, incubation and scoring was used in the work described in Sections II and III wherever plating of cell suspensions was involved. Slight modifications included washing procedures to remove chemical mutagens, and the extended incubation of filtered suspensions and plates to allow cells time to recover from mutagen treatments and exposure to selection pressure. The details of such modifications are given as they arise.

SECTION II

MUTATION, AND THE SELECTION OF VARIANTS

INTRODUCTION

In this section the means are described by which variant cell lines

were obtained resistant to exposure to chilling temperatures, high

temperatures and high salt concentrations. Selection was achieved both

from cultured cells to which no external mutagenic agent was added, and

from cells exposed to chemical mutagens N-methyl-N'-nitro-N-nitrosoguanidine

(NTG, Ralph N. Emmanual, Wembley, England) and ethylmethanesulfonate (EMS,

Sigma Chemical Company, U.S.A.).

The mechanism of action of NTG is unknown, but it has been used as a

mutagen in bacterial systems (Adelberg, Mandel and Chein Ching Chen, 1965;

Hopwood, 1970) and in plant cell cultures (Veleminsky et al. 1967; Lescure,

1970), Mansfield (1973) looked at the toxicity of NTG on actively growing

suspensions of N. sylvestris and, on the basis of his results, suggested

exposure to 400 μg/ml NTG for 20 minutes as suitable conditions for

selection studies.

The action of EMS as a mutagen is better understood. It is one of

the family of alkylating agents each of which acts specifically on guanine

converting it to the appropriate 7-alkyl-guanine. This is spontaneously

released from the DNA backbone as the deoxyriboside linkage is labilised,

and may be replaced by any one of the four bases, giving rise to transition

or transversion mutations (Brookes and Lawley, 1960). This is not restricted

to a particular part of the cell cycle and occurs at a high rate with little

killing (Loveless and Howarth, 1959). It has been used effectively on

bacteriophage (e.g. Loveless, 1958), bacteria (e.g. Loveless and Howarth,

1959) and plant cell cultures (Carlson, 1970; Nabors et al. 1975)

In the present experiments, variants were selected without application of

chemical mutagens and hence it was possible to study the effect of mutagens

on the yield of variants. A large increase in the yield should indicate

a higher proability that the variants are truly genetic in nature. Mutagenic

treatments were used which killed a proportion of the cells as it was

considered that such a severe treatment is most likely to give rise to

genetic changes in a high proportion of the survivors.

EXPERIMENTAL

(A) Existing variation within the cultures

As discussed in Section I, and in Dix and Street (1974), there is

considerable variation in ploidy, even in quite a freshly initiated

suspension of $\underline{N}$. sylvestris. A first passage suspension of an initially

haploid line, H_7, of $\underline{N}$. sylvestris, was examined further for variation in

the friability and morphogenesis of colonies formed on plating. A 21 day

old suspension was filtered through a 1 mm mesh and plated at 60,000 and

120,000 cells ml^{-1}. After six weeks discrete colonies could be removed

from the 60,000 cells ml^{-1} plates. 100 colonies 1 - 3 mm in diameter

were removed and grown up individually in Universal bottles. At three

weeks from subculture, portions of each callus were removed to pieces of

aluminium foil and classed under the following headings describing their

reaction to being squashed under the end of a spatula.

Hard: - Remains intact. Can be cut with a scalpel.

Friable - wet: - Forms a smear which sticks to the foil.

Friable - dry: - Forms large fragments which can be poured from the foil.

These are convenient headings, but each in fact covers quite a range of

callus types. The lines were also scored for shoot formation on the usual

medium for maintenance of the callus, and for greening not associated with

morphogenesis. The results for the 100 callus lines are given in Table II.1.

TABLE II.1.

Callus types of 100 callus lines derived by plating from a first passage suspension of N. sylvestris cell line H_7.

Callus type	Number	Number showing shoot initiation	Number showing greening
Hard	12	2	0
Friable - wet	32	0	1
Friable - dry	49	12	2
Mixed	7	2	0

The results show a wide range of callus types obtained by plating a freshly initiated suspension derived from a single petiole of a haploid plant. With the exception of the green lines which did not retain their colour on subculture, all these lines retained their individual characteristics through a subsequent passage as callus. Fourteen of them were returned to suspension (friable - wet lines formed the most satisfactory suspensions) where they again showed different and stable characteristics over the first two passages in suspension. Comparison of some of these lines, in the first resuspended passage is shown in plate II.1.

These results suggest that substantial genetic variation is induced by the culture system and indicate that it may be possible to select physiological and biochemical variants without the use of mutagenic agents. The seven mixed lines (Table II.1) obtained are most likely due to two neighbouring cell aggregates on the plate growing to give rise to a single colony.

PLATE II.1. Suspensions initiated from callus lines derived by plating from a first passage suspension of N. sylvestris cell line H_7.

A. Highly aggregated suspension with frequent organogenesis.

B. Highly aggregated suspension with little organogenesis.

C. Moderately aggregated line with no organogenesis.

D. Finely dispersed line with few large aggregates.

PLATE II.1
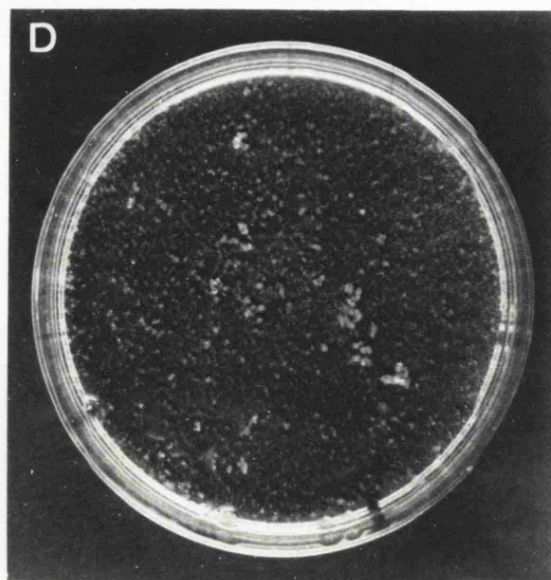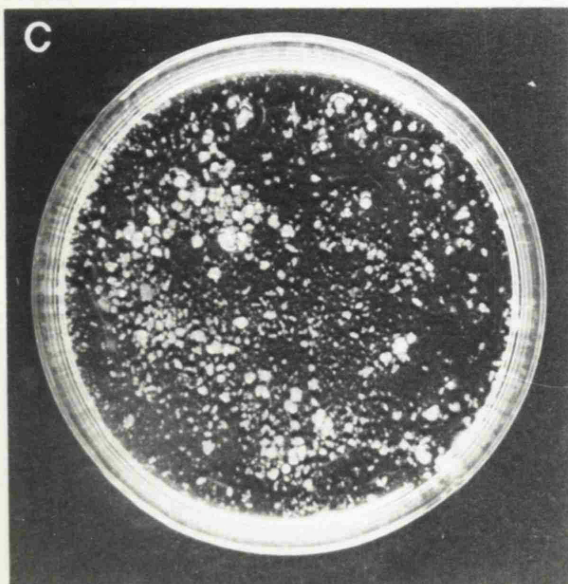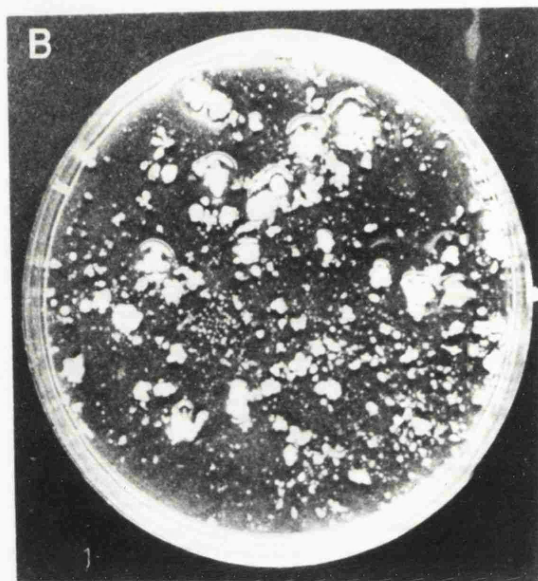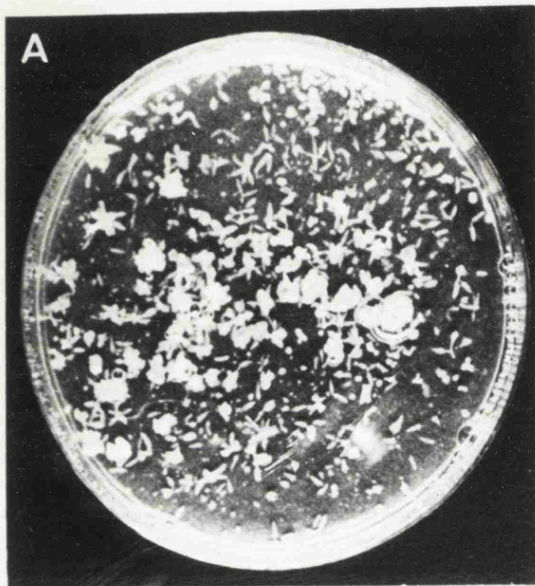
(B) <u>Selection of chilling tolerant variants</u>

(i) <u>Seedling test</u>

To establish the effect of chilling on survival of seedlings of
<u>N</u>. <u>sylvestris</u>, seeds were germinated on moist filter paper and seedlings
4 to 10 mm in length and 1.5 - 2.5 mm in width across the cotyledons were
placed in five rows of five on 10 ml Linsmaier and Skoog (1965) medium
lacking hormones and sucrose in 9 cm Petri dishes. These dishes were
placed at $5^{o}C$, $0^{o}C$ and $-3^{o}C$ in the dark and 4 were removed at weekly
intervals. They were then returned to the light and scored for survival
after 2 weeks. After this time, surviving seedlings were still green and
had increased in size; their root systems had developed and grown into the
agar. Dead seedlings had yellowed and showed no size increase or root
development. The percentage survival under the various conditions are shown
in Table II.2.

<u>TABLE II.2.</u>

<u>Percentage survival of seedlings exposed to chilling conditions</u>

Temperature (^{o}C)		Length of exposure (weeks)		
	1	2	3	4
5°	98	100	97	88
0°	98	2	0	0
-3°	0	0	0	0

Plates which had not been chilled but maintained at $25^{o}C$ throughout
showed 99% survival after 2 weeks. On the basis of these results, 3 weeks
at $0^{o}C$ or $-3^{o}C$ were chosen as the selective conditions likely to be suitable
for application to cultured cells.

(ii) Selection from callus cultures of N. sylvestris

Callus was initiated from leaf sections of a haploid N. sylvestris

plant. After two passages, callus pieces (0.5 cm^3) were placed individually

on 10 ml agar medium in Universal bottles. 40 of these were incubated at

between 0°C and -3°C for 21 days in the dark. After this time they were

removed from these conditions and incubated at 25°C under constant

illumination. The tissue rapidly browned and appeared dead, but after 4 to

6 weeks, small regions of pale callus growth appeared in 45% of the callus

pieces (Plate II.2). These rapidly proliferated and could be subcultured

after 8 weeks.

Three of these calli were maintained as separate lines and subjected to

the selection conditions a second time, again as callus pieces. After

this second exposure 80 - 90% of the callus pieces showed survival and in

many instances recovery was more rapid than after the first exposure. The

same pattern followed the chilling treatment, however, namely browning of

the bulk of the callus with small regions of the callus giving rise to

fresh growth. The inference from these results was that the callus lines

had been improved in their chilling tolerance, and this improvement was,

to some extent, persistent during callus culture at normal temperatures.

The tolerance was, however, incomplete and might be due to a physiological

acclimation to the chilling stress of the type that has been widely reported

(e.g. Wheaton and Morris, 1967; Kuraishi et al., 1968; Shpota and Bochkareva,

1974). The three "variant" lines had lost their ability to undergo morpho-

genesis on exposure to suitable conditions (Section V) and no plants were

obtained from them, which might have permitted examination of the persistence

of the tolerant phenotype.

Another consideration, when using such large pieces of callus for

selection of this type of variant, is that the bulk of the callus might

PLATE II.2.   Survival and regrowth of haploid callus of N. sylvestris

after chilling.

Chilling conditions: Between $0^\circ C$ and $-3^\circ C$ for 21 days.

A.   6 weeks after removal from chilling conditions. Fresh

callus growth from a surviving sector of the original

callus.

B.   8 weeks after removal from chilling conditions. Rapid

regrowth of surviving callus, compared with callus

pieces which showed no survival.

PLATE II.2

A

B

exert some sort of protective or shielding effect on a few cells, assisting in their survival.

For reasons such as this it was considered that selection might be more satisfactorily carried out on thin agar plates seeded with small aggregates from cell suspensions using the plating method devised in section I.

(iii) Selection from suspension cultures of Nicotiana sylvestris and Capsicum annuum.

A series of experiments were carried out in which cell suspensions were filtered and plated according to the method described in section I (p 44). After inoculation, the plates were sealed and incubated in the dark at $25^{\circ}C$ for 5 days before transfer to the "chilling" temperatures. At this time, control plates were left at $25^{\circ}C$ while remaining plates were transfered to $5^{\circ}C$, $0^{\circ}C$, or $-3^{\circ}C$, in continual darkness, and incubated for 21 days. After these chilling treatments they were returned to $25^{\circ}C$ and incubated for a further 6 weeks (C. annuum) or 8 - 10 weeks (N. sylvestris). Then the plates were photographed and scored for colony formation. Control plates were usually scored after 4 weeks (C. annuum) or 6 weeks (N. sylvestris) from their initiation. Scoring was as described in section I (p 45).

The results of some of these experiments are given in Table II.3. 7 to 10 day old suspensions were plated at cell densities ranging from 10,000 to 50,000 cells ml^{-1}. These results are fairly typical of a number of experiments with these lines and with other lines of N. sylvestris although there was some variation, within the same lines on separate occasions, and between different lines, in the control plating yield, and in the percentage reduction on chilling. These latter values tended to be higher at higher plating densities, as seen in the last column of Table II.3.

TABLE II.3

The effect of 21 day chilling treatments on the plating yield of cell lines of N. sylvestris and C. annuum.

Cell line	Plating density (cells ml^{-1})	Control maintained at 25°C	Mean colonies/plate for 10 plates				% Reduction in plating yield due to selection at 0°C
			Chilled at 5°C	Chilled at 0°C	Chilled at -3°C		
N. sylvestris	10,000	49	-	22	0		55
(H9)	20,000	164	-	68	2.4		59
	30,000	272	-	73	3.7		73
	50,000	351	-	102	4.5		71
N. sylvestris	10,000	88	-	64	0		28
(D)	20,000	208	-	84	0		60
	30,000	283	-	105	21		63
	50,000	387	-	139	20		64
C. annuum (C)	20,000	541	9.4	0	0		100

History of cell lines:
(H9): Haploid derived N. sylvestris cell line, plated during 2nd suspension passage
(D) : Diploid derived N. sylvestris cell line, plated during 2nd suspension passage
(C) : Diploid derived C. annuum cell line, plated during 10th suspension passage

This may merely reflect an idiosyncrasy of the scoring method and only appeared to be the case where there was a high degree of survival (i.e. after chilling at 0°C in N. sylvestris). The results of this experiment for one of the cell lines (D) are illustrated by photographs of representative plates on Plate II.3.

(C) The use of chemical mutagens

(i) Dosage response of cultures to chemical mutagens NTG and EMS

The length of exposure, and concentration of mutagen used were influenced by the reports of Mansfield, 1973, (NTG), and Carlson, 1970 (EMS), but in both cases a wide range of concentration was tried. The lethal effect of the mutagens was estimated both by vital staining with fluorescein diacetate (Widholm, 1974) 1 to 2 hours after exposure to the mutagen, and by the effect in reducing plating yield. In all short term exposures the suspensions were filtered before the application of the mutagen, and washed three times with fresh medium (as described in Materials & Methods) immediately afterwards. In the initial experiments the suspensions were plated immediately after the mutagen treatment, but it was found that in most cases a low plating yield was obtained even after mild mutagen treatments. The cells appeared to require a period of recovery between the mutagen treatment and plating. A large proportion of the cells may be impaired slightly by the mutagen treatment in such a way as to render them more sensitive to shock during incorporation into agar at 38°C. Incubation in shake flasks for 3 days gave less than 20% increase in cell number, yet gave more credible plating yields, and this recovery phase was incorporated into all further experiments. This step was more important in N. sylvestris suspensions than in C. annuum suspensions.

PLATE II.3. The effect of chilling on the plating yield of $\underline{N}$. sylvestris

cell line, D.

A. Control plate

B. Plate exposed to $0^\circ C$ for 21 days

C. Plate exposed to $-3^\circ C$ for 21 days

D. Plate exposed to $-3^\circ C$ for 21 days

Cells plated at 20,000 cell ml^{-1} (A,B and C) or 50,000 cell ml^{-1} (D).

PLATE II.3
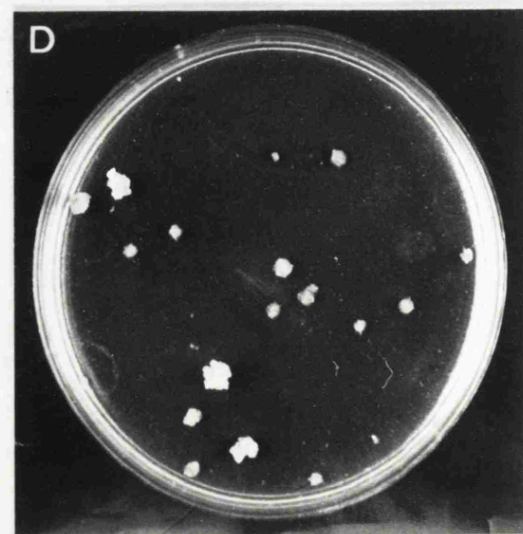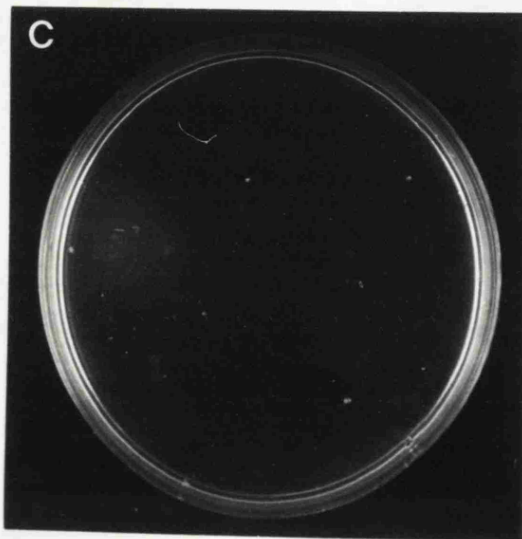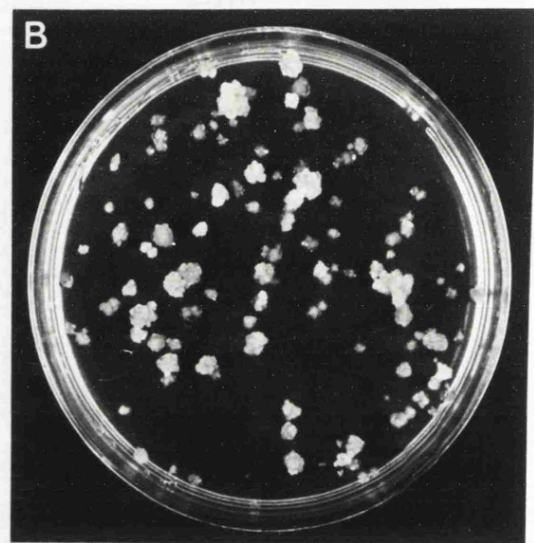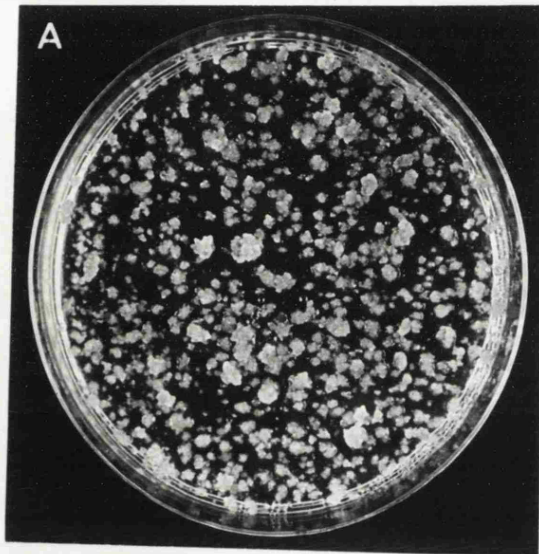
The results of one series of experiments with NTG are summarised

in Table II.4. In this case the cells were exposed to the mutagen for

10 minutes. Clearly the viability, as assessed by fluorescein diacetate

staining, does not closely reflect the ability to form colonies on agar

plates. The results using fluorescein diacetate agree favourably with

the work of Mansfield (M.Sc. thesis, 1973) in which incorporation of

radioactive sulphur (^{35}S) into protein was used as the index of viability.

However, in the two N. sylvestris lines all the treatments corresponding

to those used by Mansfield reduced the plating yield to zero, and it was

only at lower levels of NTG (50 μg/ml) that any colonies grew. C. annuum

cells were more tolerant of NTG and in this case the effect on plating

yield showed a greater resemblance to that on fluorescein diacetate staining.

A similar series of experiments was carried out using EMS as the mutagen.

In this case incubation for 60 minutes with various levels of EMS, was used

and the results are given in Table II.5.

Again there was not a close relationship between the results offered by

the two criteria for viability. In this instance certain treatments showed

100% killing according to fluorescein diacetate, yet still permitted some

colony formation.

With all three cell lines, treatments were established which gave a

partial reduction in colony formation, the conditions considered most likely

to induce mutation. An example of this is shown in Plate II.4. in which the

reduction in the plating yield of a C. annuum suspension due to a 60 minute

exposure to 0.3% EMS can be clearly seen.

For two cell lines, N. sylvestris line H_9 and C. annuum line C, the

effect of incorporation of the mutagen, at low concentrations, into the

growth medium was examined. In this case, 21 day old suspensions (2nd passage

H_9, 11th passage C) were inoculated at 10^5 cells ml^{-1} into fresh medium

TABLE II.4.

The effect of a 10' exposure to NTG on all lines of N. sylvestris and

C. annuum as indicated by fluorescein diacetate staining and plating yield

Cell line	NTG $\mu g/ml$	% viability as assessed by fluorescein diacetate staining	Mean colonies/ plate (5 plates)
H_9	0	87	149
	50	57	55
	100	53	0
	200	48	0
	400	19	0
D	0	92	193
	50	66	122
	100	35	0
	200	55	0
	400	31	0
C	0	97	349
	50	92	412
	100	32	33
	200	33	28
	400	12	10

History of cell lines: -

H_9:   Haploid derived N. sylvestris line; experiment performed on
           3rd passage suspension

D: Diploid derived N. sylvestris line; experiment performed on
       3rd passage suspension

C: Diploid derived C. annuum line; experiment performed on 12th
       passage suspension

All cell suspensions were plated at 20,000 cell ml^{-1}

TABLE II.5.

The effect of a 60' exposure to EMS on cell lines of N. sylvestris and

C. annuum as indicated by fluorescein diacetate staining and plating yield

Cell line	EMS % (v/v)	% viability as assessed by fluorescein diacetate staining	Mean colonies/ plate
H_9	0	89	49
	0.075	75	23
	0.3	42	9.6
	0.75	7	3.6
	1.5	0	5.8
	3.0	0	0
D	0	90	88
	0.075	78	65
	0.3	49	47
	0.75	28	27
	1.5	0	2.6
	3.0	1	20
C	0	95	620
	0.3	83	131
	0.75	49	128
	1.5	1	19
	3.0	3	17

History of cell lines: -   See Table II.4

Cell suspensions were plated at 10,000 cells ml^{-1} (H_9 and D)

or 20,000 cells ml^{-1} (C).

PLATE II.4. Reduction of plating yield of C. annuum cell line (C) after

treatment with EMS

A. Control plate

B. Plate from suspension incubated for 60 min. in medium

containing 0.3% (v/v) EMS

PLATE II.4
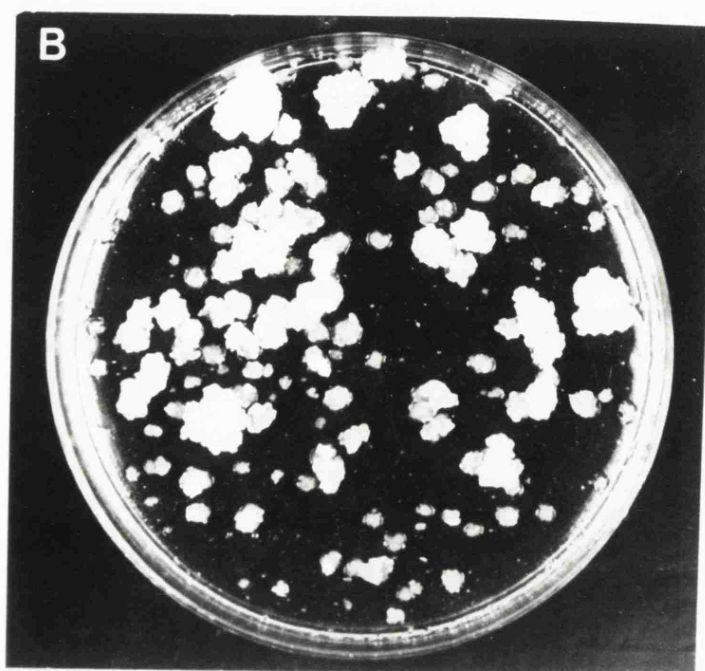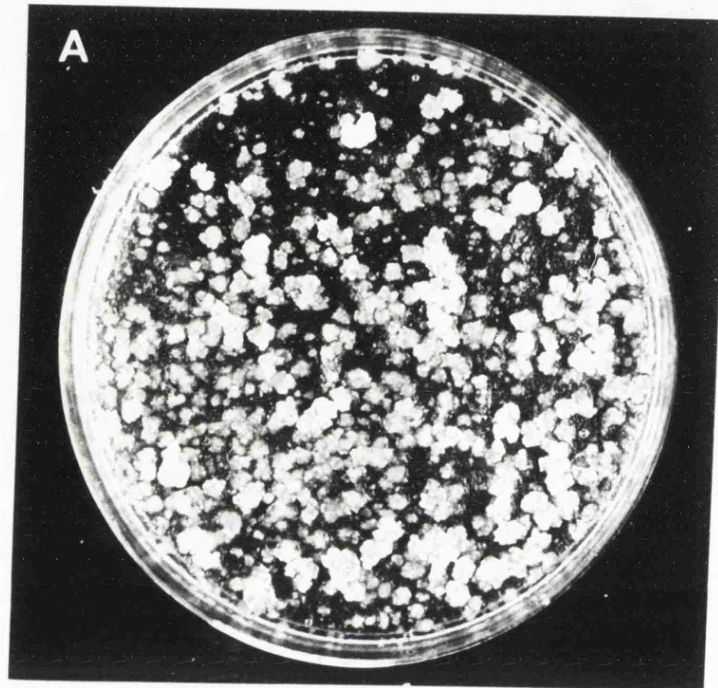
containing various levels of the mutagen. After 21 days incubation cell counts were performed and the percentage inhibition of cell number at that time, due to the presence of the mutagen, was calculated. The results are plotted in Fig. II.1. for NTG and expressed in tabular form (Table II.5(b)) for EMS. Again it is clear that C. annuum suspensions are susceptible to NTG only at higher levels than those required to inhibit the growth of N. sylvestris suspensions. The cell suspensions from these experiments were plated and gave good colony formation.

(ii) Effect of mutagens on the selection of chilling tolerant variants.

On the basis of the results obtained in the preliminary studies it was decided to concentrate on the use of short term exposures to EMS and longer term growth in the presence of lower concentrations of EMS and NTG in further work on the effects of the mutagens. In the former case the treatment with mutagen was terminated by washing with fresh medium (see Materials and Methods) followed by a three day incubation with fresh medium, prior to plating. In the case of growth in the presence of the mutagens the suspensions were filtered and plated, without a washing step, after 21 days. Plating provided at least a x10 dilution of the mutagen. In both cases the plates were incubated in the dark at 25°C before being submitted to the chilling procedure. Subsequent treatment and analysis of the plates was carried out as already described for the selection of spontaneous chilling tolerant variants from plates. The results of some of these experiments are summarised in Tables II.6 and II.7.

The analysis of variance of these results (Table II.6 (b) and Table II.7 (b) suggest significant interactions in some cases, but not in others. The implications of this are discussed further in the discussion that terminates this section.

Fig. II.1 Inhibition of final cell number by NTG incorporated into the

incubation medium of cell lines of N. sylvestris (H$_9$) and

C. annuum (C).

Fig II.1

21 days incubation with NTG
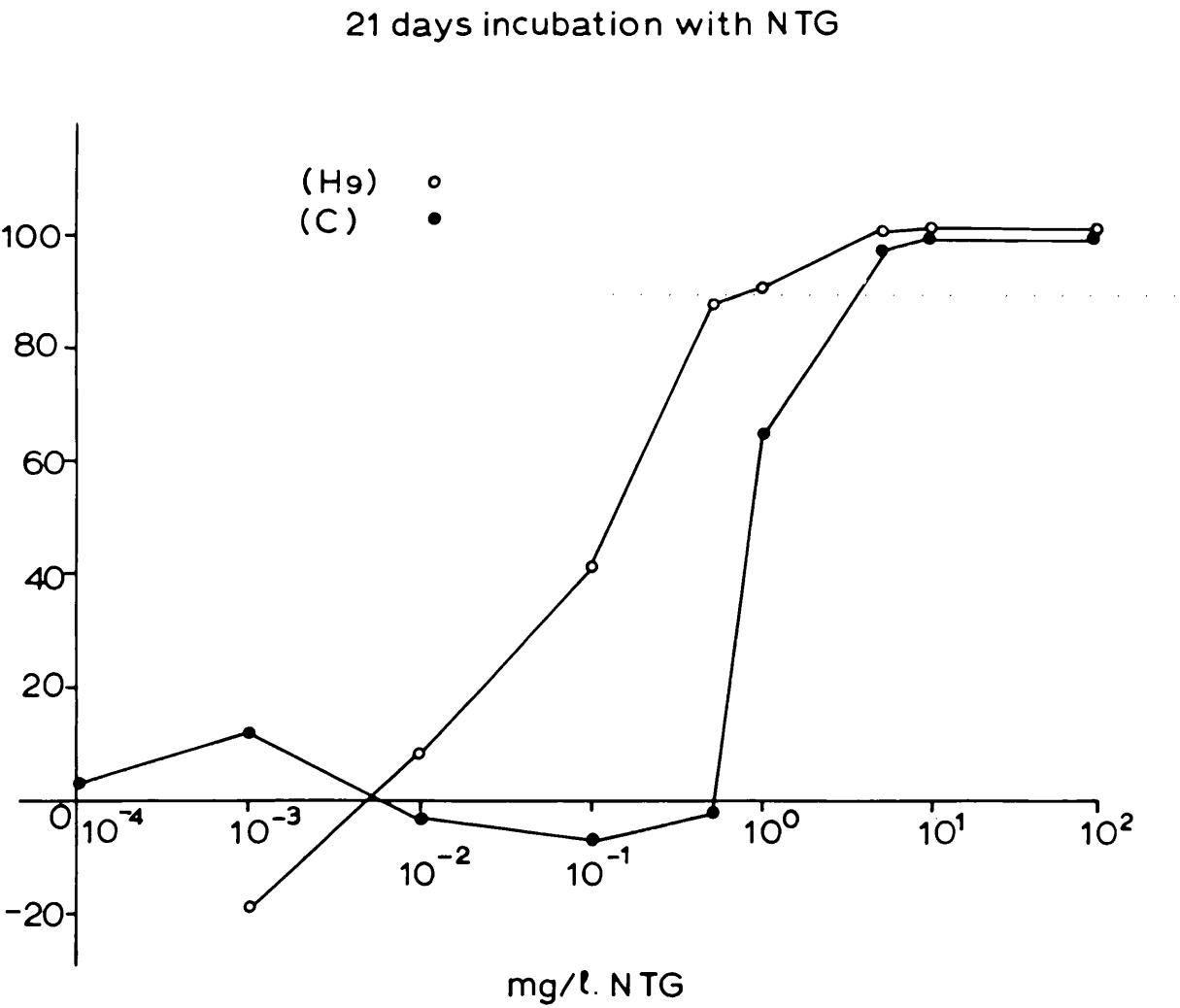
TABLE II.5 (b)

The effect of 21 days incubation in medium containing EMS on the cell number in suspensions of N. sylvestris and C. annuum

Cell line		EMS (%)			
		0	0.001	0.1	1.0
N. sylvestris (H9)	Cell ml^{-1} (x 10^{-6})	5.00	5.00	0.17	0.10
	% inhibition	-	0	99	100
C. annuum (C)	Cell ml^{-1} (x 10^{-6})	1.11	0.54	0.20	0.07
	% inhibition	-	57	90	103

TABLE II.6.

The effect of 60' exposure to EMS on the plating yields of cell lines of
N. sylvestris and C. annuum under non-chilling and chilling conditions

Cell line	EMS % (v/v)	Mean colonies/plate at 25°C	Mean colonies/plate after chilling	
			$0°C$ (H_9 and D) $5°C$ (C)	$-3°C$ (H_9 and D)
H_9	0	56	29	0.2
	0.075	27	4.0	0.3
	0.3	9.4	2.2	0
	1.5	6.2	1.4	0
D	0	95	69	0.4
	0.3	47	33	0.1
	1.5	20	14	0.5
	3.0	17	0.6	0
C	0	582	13	–
	0.3	146	17	–
	0.75	122	21	–
	1.5	13	0.8	–

For lines H_9 and D the experiment was performed on 3rd passage
suspensions which were plated at 10,000 cells ml^{-1}.
For line C the experiment was performed on 15th passage suspensions
which were plated at 20,000 cells ml^{-1}.

KEY TO COLUMN HEADINGS USED IN TABLES II.6 (b); II.7 (b) and II.12:

SS =   Sum of squares

^{o}F =   degrees of freedom

MS =   Mean square = $\dfrac{SS}{^{o}F}$

VR =   Variance ratio = $\dfrac{MS\ Item}{MS\ Error}$

P =   Probability of obtaining calculated variance ratios by chance

Interaction values of SS and ^{o}F are obtained by subtracting Chilling (or heat) and Mutagen values from Treatment values.

Error values of SS and ^{o}F are obtained by subtracting Treatment values from Total values.

TABLE II.6. (b)

Interaction analysis of variance of the data summarised in Table II.6.

Cell line	Item	SS	^o_F	MS	VR	P
H9	Total	13709.0	39			
	Treatment	12976.6	7	1853.8	80.95	<0.001
	Chilling	2418.0	1	2418.0	105.6	<0.001
	Mutagen	9621.3	3	3207.1	140.0	<0.001
	Inter-action	937.3	3	312.4	13.6	≈0.001
	Error	733.0	32	22.9		
D	Total	39941.6	39			
	Treatment	35113.6	7	5016.2	33.24	<0.001
	Chilling	2464.9	1	2464.9	16.33	<0.001
	Mutagen	32129.0	3	10709.7	70.97	<0.001
	Inter-action	519.7	3	173.2	1.15	0.25-0.50
	Error	4828.0	32	150.9		
C	Total	1358535	39			
	Treatment	1355342	7	193620.3	1940	<0.001
	Chilling	410062	1	410062	4108	<0.001
	Mutagen	479914	3	159971	1603	<0.001
	Inter-action	465365	3	155122	1554	<0.001
	Error	3193	32	99.8		

Summary: There are highly significant reductions in the plating yield of all
lines, brough about both by chilling and by the mutagen treatments.
There is no significant interaction between these effects in line D
but there is highly significant interaction in the other two lines.
In line H9 the interaction is in the opposite direction to that
expected.

TABLE II.7.

The effect of growth for 21 days in the presence of NTG or EMS on the plating yield of cell lines of N. sylvestris and C. annuum under non-chilling and chilling conditions.

Cell line	NTG (μg/ ml)	EMS (%)	Mean colonies/ plate at 25°C	Mean colonies/plate after chilling		
				5°C	0°C	-3°C
H₉	0	-	163	123	44	2.2
	0.001	-	169	130	93	2.8
	0.01	-	180	166	173	5.5
	0.1	-	150	117	60	4.9
	-	0	264	233	138	3.1
	-	0.001	215	191	158	3.0
	-	0.01	138	87	129	6.2
C	0	-	572	223	0	-
	0.0001	-	563	233	0	-
	0.001	-	587	196	0	-
	0.01	-	501	194	0	-
	0.1	-	449	212	0	-
	0.5	-	442	197	0	-
	1.0	-	426	337	0	-
	-	0	550	207	0	-
	-	0.01	462	200	0	-
	-	0.1	456	167	0	-

Mutagens were incorporated into the 3rd suspension passage of N. sylvestris line H₉ and the 15th suspension passage of C. annuum line C.

In all cases, cell suspensions were plated at 20,000 cells ml^{-1}.

TABLE II.7. (b)

Analysis of variance of the data summarised in Table II.7.

Cell line	Mutagen	Item	SS	^{o}F	MS	VR	P
H9	NTG	Total	405412	79			
		Treatment	303806	15	23587	29.3	<0.001
		Chilling	294790	3	98263	121.9	<0.001
		Mutagen	30850	3	10283	12.8	<0.001
		Inter-action	28186	9	3131	3.9	0.001–0.005
		Error	51606	64	806		
H9	EMS	Total	469753	59			
		Treatment	449155	11	40832	95.2	<0.001
		Chilling	350122	3	116707	272.0	<0.001
		Mutagen	52053	2	26026	60.7	<0.001
		Inter-action	46980	6	7830	18.3	<0.001
		Error	20598	48	429		
C	NTG	Total	1648524	69			
		Treatment	1579013	13	12232	9.9	<0.001
		Chilling	1360265	1	1360265	1096	<0.001
		Mutagen	67833	6	11305	9.1	<0.001
		Inter-action	150915	6	25152	20.3	<0.001
		Error	69511	56	1241		
C	EMS	Total	722083	29			
		Treatment	698287	5	139657	140.9	<0.001
		Chilling	666030	1	666030	672	<0.001
		Mutagen	23752	2	11876	12	<0.001
		Inter-action	8505	2	4252	4.3	≃0.025
		Error	23796	24	991		

Summary: There are highly significant reductions in the plating yield of both lines, brought about both by chilling and by the mutagen treatments. The interactions between the chilling and mutagen effects vary, but all are highly significant.

A large number of colonies were removed from plates exposed to chilling conditions both after no mutagenic treatment, and after exposure to EMS. The colonies were transferred to 10 ml agar medium in Universal bottles, and cultured independently for the subsequent study of the retention of the chilling tolerant phenotype.

(D) <u>Selection of high salt resistant variants</u>

Well established fast-growing suspensions were used for this work. In all cases, the cell lines had been in suspension for six passages or longer. After this period of time, most cell lines are far less aggregated than in earlier passages (see Section I), but it is also true that in lines initially isolated from haploid material few haploid cells remain (Section I, and Dix and Street, 1974).

A total of four different cell lines were used in this study and they are described as follows: -

<u>N. sylvestris</u>:　　D - Initial source, a diploid petiole. Culture predominantly diploid and tetraploid with a few octoploid and aneuploid cells.

H_9 - Initial source, a haploid petiole. Culture predominantly diploid and tetraploid with 10 - 15% haploid cells.

H_7:36 - Line clonally isolated from initially haploid line H_7. At time of cloning parent culture roughly 70% tetraploid, 30% diploid, with very few haploid cells. H_7:36 is almost entirely tetraploid.

<u>C. annuum</u>:　　C - Initial source a diploid petiole but ploidy state of culture not determined.

(i) <u>Sensitivity of parent cultures to NaCl.</u>

Various levels of NaCl were added to the culture medium, and each of the cell lines was incubated in the saline medium for 12 hours. After this time the cells were scored for viability (by floorescein diacetate staining) and plasmolysis. The results were similar for all of the lines and are summarised as follows: At 1% w/v NaCl there was little reduction in viability, over controls, and the cells were not plasmolysed. At 3% NaCl a high proportion of the cells were plasmolysed, but most of them remained viable. At 10% NaCl all the cells were killed. On the basis of these results cell lines were initially selected for growth in the presence of 0.7, 1 and 3% NaCl.

In contrast to the selection of chilling tolerant lines, most work with high salt resistance involved continuous selection, i.e. the variants were selected for their ability to grow in the continuous presence of the selection pressure. Selection was carried out from cell suspension cultures in two ways. The first involved the incorporation of suspensions into agar medium enriched with NaCl on Petri dishes, and the second involved the incorporation of NaCl into the liquid medium in which the cell lines were routinely cultured.

(ii) <u>Selection for NaCl resistance by Petri dish plating.</u>

Three cell lines, D, H_9 and C were used in these experiments. 6 to 9 day old suspensions were filtered (0.6 mm mesh) and plated at between 25,000 and 35,000 cells ml^{-1} in agar medium containing no Na Cl or containing 0.7, 1 or 3% NaCl. After 6 weeks incubation the plates were scored as already described. The results are summarised in Table II.8.

At 0.7% NaCl there is a dramatic reduction in the plating yield of <u>N. sylvestris</u> lines H_9 and D, but little reduction in the plating yield of <u>C. annuum</u> line C, suggesting that there might be a slight difference in NaCl

TABLE II.8.

Plating yields after 6 weeks incubation in agar medium containing NaCl.

Culture	% NaCl in medium	Plates scored	Mean colonies/plate
N.sylvestris	0	5	271
H$_9$	0.7	5	104
	1	7	29
	3	5	0
N.sylvestris	0	5	311
D	0.7	5	151
	1	8	29
	3	5	0
C.annuum	0	5	332
C	0.7	5	289
	1	8	38
	3	5	0

sensitivity between the two species. The effect of 1% NaCl, however, was of the same order for all three lines, reducing the plating yields to about 10% of those found on the control plates. No colonies were found growing on medium containing 3% NaCl. Sample plates from this experiment are illustrated for one of the cultures, line C, on Plate II.5.

For each of the three cultures used, 5 colonies from 0% NaCl plates and 20 from 1% NaCl plates were removed to fresh medium, containing no NaCl, in Universal bottles, where they were grown up as independent callus lines, for future experiments to study the retention of the ability to grow on NaCl containing medium.

(iii) Selection for NaCl resistance in suspension cultures.

Three cell lines, H_7:36, D and C were used in three experiments. 21 day old suspensions were subcultured at 10^5 cells ml^{-1} into fresh medium containing 0, 0.7, 1 or 3% NaCl. The cultures were incubated in the usual way, on a rotary shaker (120 r.p.m.) in the light at $25^{o}C$, for 21 days. Duplicate samples were removed for cell counts, after 7,14 and 21 days and the resulting growth curves are given on Fig. II.2., A, B and C. A direct comparison of the final cell numbers under all the treatments, together with standard errors is given in Fig. II.3. Clearly the addition of NaCl results in inhibition of growth, by cell division. At 0.7% NaCl this inhibition as expressed by reduction of cell number after 21 days was slight in the case of C. annuum but higher in both of the N. sylvestris lines. 1% NaCl had a more pronounced effect on all three cell lines and growth was completely inhibited by 3% NaCl. Aliquots were removed from these 1st passage (P1) cultures at day 21 of incubation in the presence of NaCl and treated in two ways.

(1) They were plated in the same way as described (Section II.D.(ii)) for the selection for NaCl tolerance on plates, either in the absence of NaCl, or in

PLATE II.5.   Colony formation by <u>C. annuum</u> cell line C, when plated in

medium containing various levels of NaCl.

A. Control plate (0% NaCl)

B. 0.7% NaCl

C. 1% NaCl

D. 3% NaCl

Cells were plated at between 25,000 and 35,000 cell ml^{-1}.

PLATE II.5
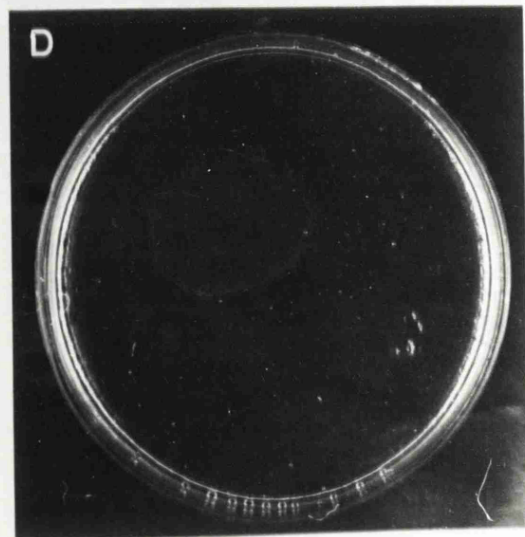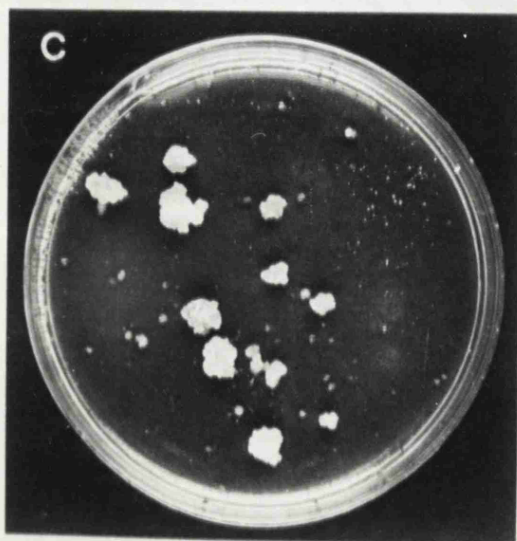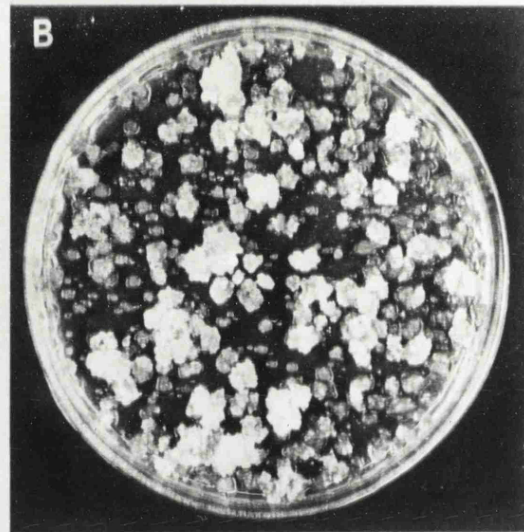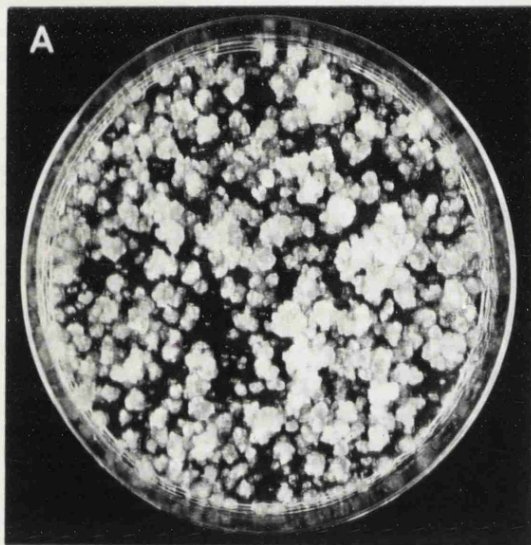
Fig. II.2 Growth of cell suspensions in medium containing NaCl.

A. $\underline{N}$. sylvestris cell line H_7:36

B. $\underline{N}$. sylvestris cell line D

C. $\underline{C}$. annuum cell line C

Abscissae: Time after inoculation (days)

Ordinates: Cell number $ml^{-1} \times 10^{-6}$

Medium contained no NaCl ($\triangle$) or 0.7% ($\blacktriangle$), 1% (O) or 3% ($\bullet$) NaCl (w/v).
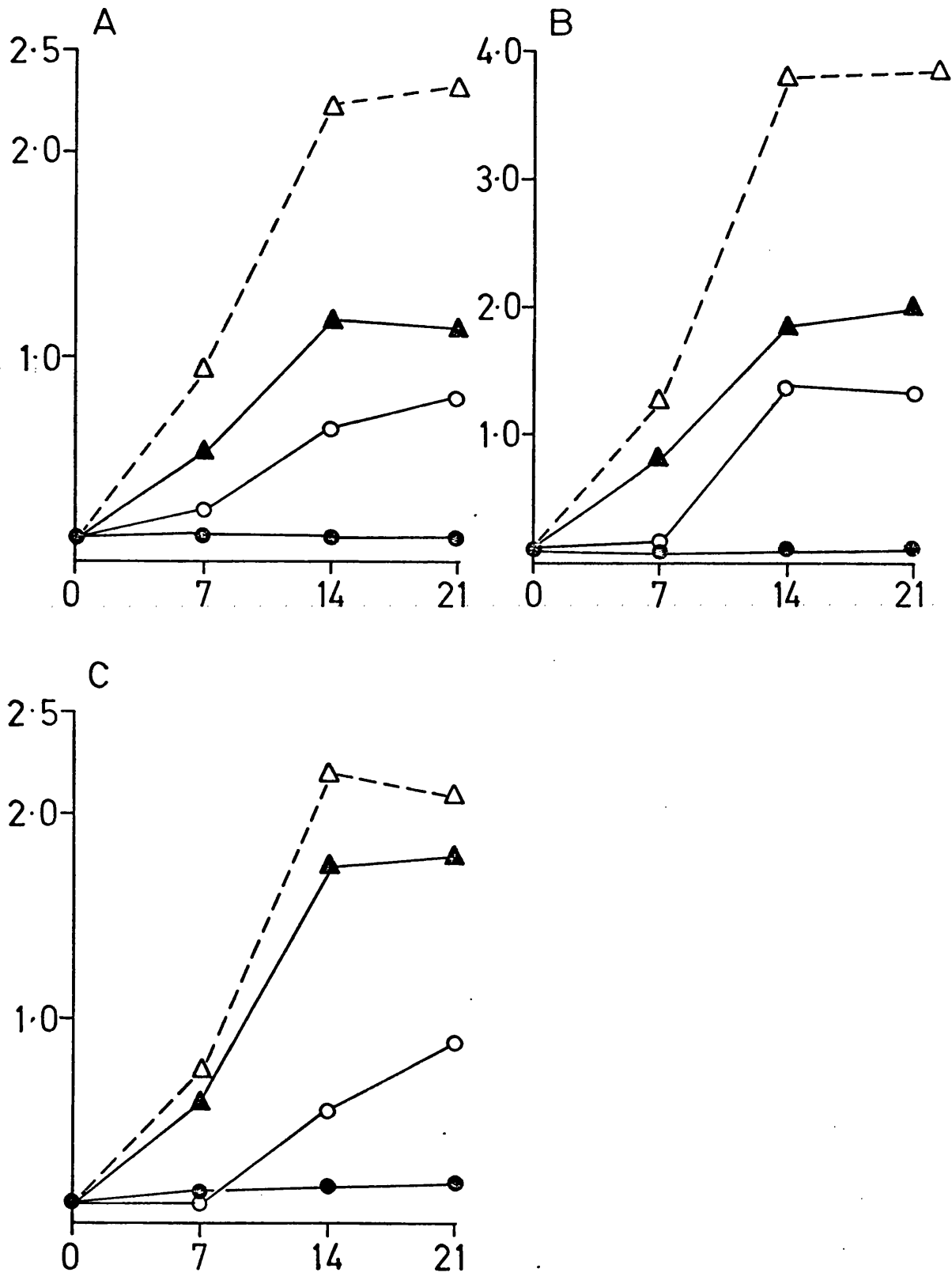
Fig. II. 2.

<u>Fig. II.3</u> <u>Effect of incorporation of NaCl into the culture medium, on the final</u>

<u>cell number in cell suspensions of N. sylvestris and C. annuum</u>

Cell numbers in suspension culture after 21 days incubation in the

presence of various levels of NaCl. Initial cell density 10^5 cell

ml^{-1}. The vertical lines equal twice the standard errors plotted

with the means as midpoints.

Fig. II. 3.
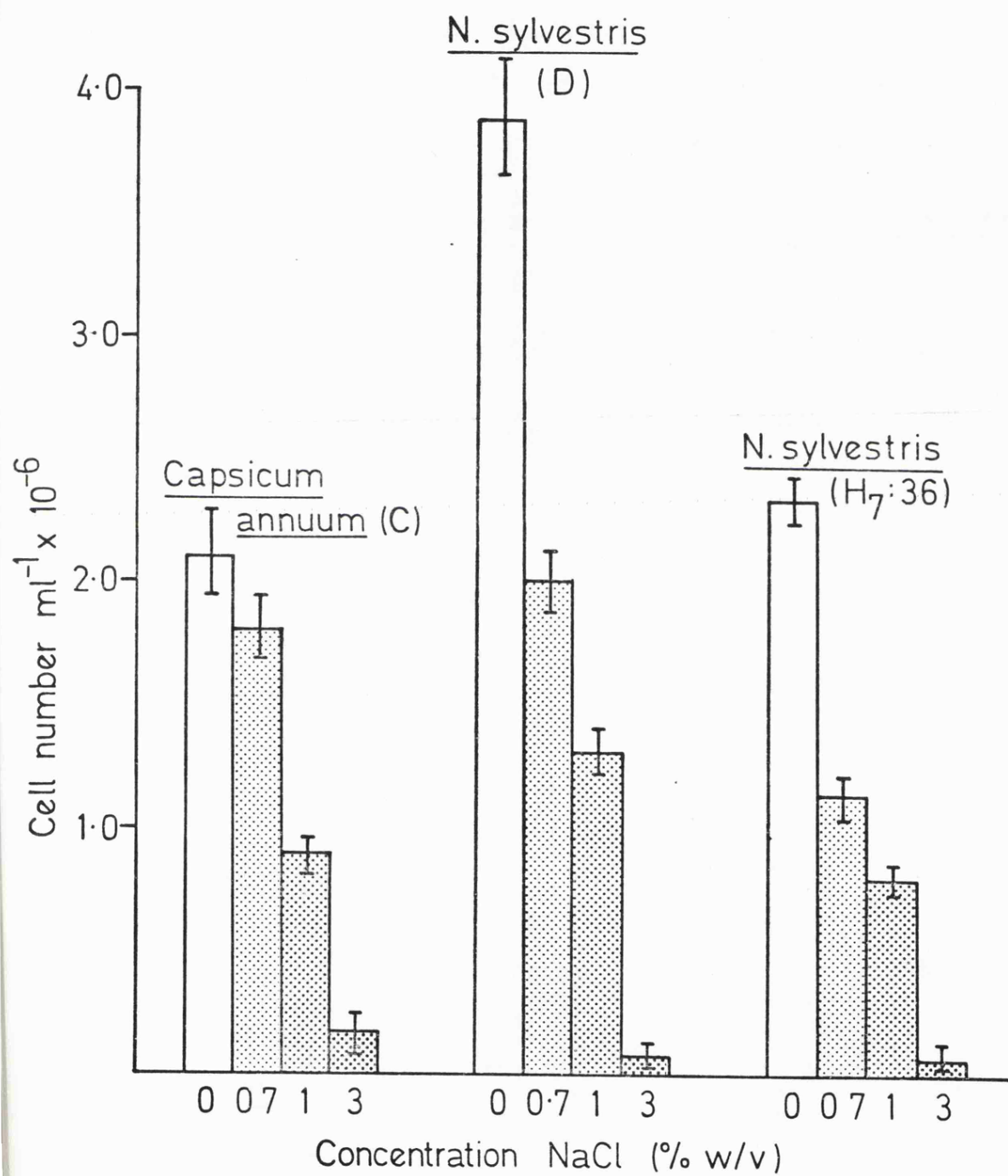
the presence of appropriate levels. Results from this plating experiment

are given in Table II.9. When compared with Table II.8, these data show

that the suspensions of D and C have been enriched in cells capable of

growth on agar plates containing NaCl. For example, there is a dramatic

improvement in the plating yield in 1% NaCl, brought about by pre-incubation

in medium containing 1% NaCl.

(2) Each suspension was subcultured on day 21 at 10^5 cell ml^{-1} into fresh

medium containing the same concentration of NaCl as in P1. The suspensions

have been maintained in this way to the present time. Over several

passages, growth in 1% NaCl improved markedly and in some cases the cell

number exceeded that in the absence of NaCl. After 3 to 4 passages in 1%

NaCl several of the resistant lines could also grow in medium containing

2% NaCl. A more detailed description of the growth of the resistant lines

after a number of passages is given in Section III.

In the case of suspensions incubated in 3% NaCl medium in P1 and showing

no growth, these were concentrated and inoculated into medium containing no

NaCl. In all cases a few large aggregates had formed after 3 to 5 weeks,

showing that a few cells were capable of surviving 3% NaCl for 21 days. This

point is also made by the appearance of a few colonies on the plates (0% NaCl)

taken from suspensions (3% NaCl) as shown in Table II.9.

(E) Selection of high temperature tolerant variants.

(i) Selection by Petri dish plating.

Preliminary investigation showed that, as compared with incubation at

25°C, callus of N. sylvestris could grow equally well, or even slightly more

rapidly at 30°C or 35°C but that at 40°C the callus gradually died. This

death on long-term exposure to 40°C could be due to chemical changes in the

medium, or dehydration of the medium, and not due to the sensitivity of the

TABLE II.9.

Plating yield after 6 weeks incubation following inoculation with cells
from first passage suspensions in the presence of various levels of NaCl.

% NaCl in medium		Mean colonies/plate		
Suspension passage	Agar plates	N.sylvestris H$_7$: 36	N.sylvestris D	C.annuum C
0	0	525	362	370
0.7	0	491	395	348
	0.7	551	204	362
1.0	0	431	332	325
	1.0	47	130	195
3.0	0	1.5	1	2
	3.0	0	0	0

callus to high temperature. Short term exposures to high temperature
were therefore used as the selective conditions to obtain high temperature
tolerant variants as this allows more rapid analysis of the temperature
effects and reduced the chance of an effect being caused by deleterious
changes in the medium.

A single line of $\underline{N}$. sylvestris, H_7:36 was used throughout this work
and selection was applied by holding the filtered suspensions in molten
agar medium at suitable temperatures, for a suitable period of time before
plating. After plating, they were incubated at $25^{\circ}C$ and plating yields
determined after 6 weeks.

Mansfield (1973) working with Acer pseudoplatanus showed that even a
very short exposure to $40^{\circ}C$ severely reduced the plating efficiency, and
10 or 20 minutes at $35^{\circ}C$ also had a substantial effect. This was not the
case for $\underline{N}$. sylvestris suspensions. 37 and $40^{\circ}C$ were found too low a
temperature for selection, as incubation of H_7:36 cells at these temperatures
for a range of times up to 27 hours gave no reduction in plating yield. In
fact the colonies appeared more rapidly after the 27 hour treatment suggest-
ing that the cells had been actively growing in the molten agar, so that
larger aggregates were being plated. Incubation at $45^{\circ}C$, however, for
periods of up to 60 minutes exerted a suitable level of selection. The
suspensions were filtered through 0.6 mm mesh and incorporated into 50 ml
agar in 100ml Erlenmeyer flasks at 20,000 cells ml^{-1} at $45^{\circ}C$ or $38^{\circ}C$. The
$38^{\circ}C$ flasks and one $45^{\circ}C$ flask were plated immediately. The remaining flasks
were incubated in a $45^{\circ}C$ water bath fitted with a shaker set at 60 strokes/min
and removed for plating at various intervals of time. The results are given
in Table II.10.

Incorporation into agar at $45^{\circ}C$ followed by immediate plating has only a
small effect on the plating yield, but the cells were susceptible to longer
exposures, plating yield falling off with time. The single colony growing

TABLE II.10.

The effect of short;term exposures to 45°C on the plating yield of
N. sylvestris cell line H_7:36.

Temperature ($^{\circ}$C)	Length of exposure (min)	Mean colonies/ plate for 5 plates	% reduction due to 45°C treat- ment
38	0	282	-
45	0	267	5.3
	15	50	82.3
	30	5	98.2
	60	0.2	99.9

after exposure to 45°C for 60 minutes, together with 10 colonies resistant

to exposure for 30 minutes and 5 control colonies (from 38°C plates) were

removed and grown independently in Universal bottles. The subsequent

examination of some of these cell lines is described in Section III.

(ii) The effect of EMS on the selection of high temperature tolerant
variants.

The aim of this work was to demonstrate a definite increase in the level

of variation found in a culture after exposure to a chemical mutagen. This

system is more suitable for such studies than the chilling tolerant selection

system already described, as exposure is short. This allows more rapid

analysis, and it is likely that a smaller number of metabolic events are

causing cell damage in the sensitive cells, i.e. gradual metabolic decay

is an unlikely cause of cell death. Also the treatment of 60 minutes at

45°C gives an almost complete reduction in plating yield. The growth

of a single colony after this treatment, as reported above, suggested that

this treatment is sufficiently close to the cells tolerance level to render

the selection of variants feasible. Filtered suspensions were treated with

EMS and washed as described in Materials and Methods (p 20) and plated at

a final cell density of 50,000 cells ml^{-1} after incubation for 0', 15' and

60' at 45°C. This experiment was carried out twice; the results are given

in Table II.11.

The results suggest there is a small increase in the survival after

exposure to 45°C of cells which have been treated with EMS and the analysis

of variance Table II.12 suggests that this is significant. The

implications of this are included in the following discussion,

TABLE II.11.

Effect of 30' treatment with EMS on the subsequent plating yield of a
suspension of N. sylvestris culture, H_7:36, after exposure to 45°C for
60 minutes.

EMS concentration (%)	Mean colonies/plate (5 plates counted)			
	Experiment 1		Experiment 2	
	0' exposure	60' exposure	0' exposure	60' exposure
0	537	10	502	7.4
0.075	477	16	455	15
0.3	342	1.6	223	2.0
1.0	90	0.2	88	0

TABLE II.12

The interaction analysis of variance of the pooled data from the two
experiments the results of which are summarised in Table II.11.

Item	SS	^{o}F	MS	VR	P
Total	3466239	79			
Treatment	3360040	7	480006	325	<0.001
Heat	2214784	1	2214784	1502	<0.001
Mutagen	606413	3	202104	137	<0.001
Interaction	538843	3	179614	122	<0.001
Error	106199	72	1475		

Summary: There are highly significant reductions in the plating yield

brought about both by the high temperature and mutagen

treatments and there are highly significant interactions

between these treatments.

DISCUSSION

A range of tolerance was found, to each of the three types of selection pressure applied, and in each case lines could be established from cells which had survived the selection pressure. The objective of the work, to be reported in later sections of this thesis (Sections III and V), is to establish whether all, or some, of the cell lines so obtained differ genetically, with respect to tolerance, from the remaining cells of the parent and non-selected lines, and so to establish and study such lines.

The shortcomings of the plating method used for the selection of variants has been discussed in Section I. The growth of plant cells both on plates and in suspensions is very much dependent on the inoculation density and this is shown for $\underline{N}$. sylvestris by the results on p 34 . This complicates analysis of survival (as established by growth) after subjection to a selection procedure which kills a large proportion of the cells, or impairs their ability to survive in some way. If most of the cells are killed then survivors may be at too low a density to grow, yet if the population remains, for the most part, metabolically active but unable to divide, the culture might be of a high enough effective inoculation density to allow the small proportion of truly tolerant cells to divide. This sort of complex balance, taken together with the fact that the colonies may arise from cellular units containing anything from 1 to 50 cells makes the system one where only pronounced differences are likely to be significant. In short, we are using an approach which is of intermediate sensitivity, compared on the one hand with the less satisfactory callus culture system as used in the initial chilling tolerant work and by Steponkus (1972), and Maliga et al. (1973) and on the other hand with any system where completely separated cells or protoplasts are plated with high efficiency (e.g. Takebe et al. 1971;

Cocking, 1973). A good comparison between these possible approaches is made by Binding (1974).

Given the limitations in the plating procedure, reduction in the plating yield was caused by the selection pressures, but in most cases "variant" lines could still be picked off at quite a high frequency, higher than one would expect to be due to genetic change in a culture that has previously been maintained in the absence of any selection pressure. This suggests that all, or a proportion, of the colonies formed, might be results of a rapid physiological adaption to unfavourable conditions by a small proportion of, perhaps, particularly healthy cells. For this reason a large number of isolates were taken from these initial selection plates in order that subsequent subjection to the selection pressures might reveal the proportion in which tolerance was highest and most stable, and therefore likely to be heritable, at least through mitosis.

Of the treatments with chemical mutagens the use of a short term exposure followed by washing is considered the more satisfactory. This method is more precise as well as more rapid, and the actual concentrations of the mutagens are known. Using long term incubation is likely to result in changes in the effective mutagen concentration, particularly in the case of NTG which is unstable in solution. Toxic degradation products may result in the killing effect observed in these treatments. The analysis of variance of the results obtained from the experiments using mutagens in most cases suggest that there are highly significant interactions between the reduction of the plating yield brought about by mutagen treatments and the selection pressures. This confirms the visual impression that the reduction in plating yield on selection, after using mutagens, is lower than would be expected from the results obtained without the use of mutagens; mutagen treatments are increasing the occurrence of survivors, and therefore probably giving rise to variants. These results are encouraging from the point of view of selecting genetically stable variants, but it should be

emphasised that the plating results are complicated by the fact that the plating yield is not directly proportional to the density of viable cells on the plates. Additional factors such as this might contribute to the statistical significance of the results found in these analyses. A big improvement in the yield of variants following a mutagenic treatment would have been better evidence that a genetic change was involved.

A number of lines were established from mutagen-treated and selected plates as it would be of interest to see whether lines which proved to be true genetic variants had been obtained with and without mutagen treatments, or only when a mutagen is used.

SECTION III

PERSISTENCE OF THE VARIANT PHENOTYPE IN CULTURE

INTRODUCTION

In the cases of the discontinuous selection used for chilling and high temperature tolerance all colonies growing on the plates (i.e. which survived the selection pressure) have been grown on in its absence. Thus, if they are again subjected to the selection pressure, and again show tolerance, this tolerance must have been maintained through a number of cell generations in the absence of the selection pressure, and therefore represents a stable adaption.

In the case of the continuous selection used for sodium chloride resistance, the resistant lines could be serially cultured in the presence of the selection pressure, and therefore must show continued resistance. In order to establish the stability of this resistance, it was clearly essential to study the retention of the resistant phenotype over a period of culture in the absence of the selection pressure.

The data presented here are the results of a number of experiments carried out on a proportion of the variant cell lines obtained as described in Section II. The object was to establish whether these lines do in fact differ from suitable control lines and, in cases where there is a difference, to find whether this can be amplified by repeated exposure to the selection pressure.

EXPERIMENTAL

(A) Persistence of chilling tolerance through a large number of cell generations.

The ideal comparison between variant lines would be drawn by comparing the effect of chilling on cell suspensions plated at the same density as

non-selected lines. Unfortunately the large number of lines under consideration makes it technically difficult to deal with them all as suspension cultures. For this reason, the second selection stage was applied to small callus pieces with the idea that any lines showing particularly dramatic recovery would then be resuspended and their resistance evaluated in suspension culture.

(i) Retention of chilling tolerance in callus culture.

This testing took place on callus which had been retained for a single 28 day passage after the subculture 6 to 8 weeks after the chilling treatment. Molten agar medium was transferred to Petri dishes (10 ml per dish) and allowed to cool. For each cell line the callus was cut into small pieces (0.025 to 0.050 g) which were placed in rows of four on the medium in a dish in the arrangement shown in Fig. III.1. Three (occasionally two or four) cell lines were placed on the same dish and two dishes set up for each treatment. Thus 8 or 4 (due to quite a high level of contamination in setting up these dishes) replicate callus pieces of similar size were scored in each treatment. The plates were sealed and immediately placed either under selective conditions (in the dark at $5^{\circ}C$, $0^{\circ}C$ or $-3^{\circ}C$) or under control conditions (illuminated at $25^{\circ}C$). After 21 days under chilling conditions the dishes were returned to $25^{\circ}C$ under constant illumination. The dishes were scored for survival, in terms of renewed growth, after 6 weeks at $25^{\circ}C$ (N. sylvestris) or 4 weeks at $25^{\circ}C$ (C. annuum) and checked for fresh growth 2 weeks later. The criterion for growth was increase in size by formation of fresh white, cream, or pale brown callus; in some of the N. sylvestris lines this was accompanied by shoot or root formation. In all cases where no fresh growth was observed, the callus pieces changed to dark brown

Fig. III.1 The arrangement of callus pieces on agar medium in Petri dishes,
for tests on chilling tolerance.

The arrangement is shown for callus pieces of three lines, x,
y and z.

Fig. III. 1.

line x

line y

line z
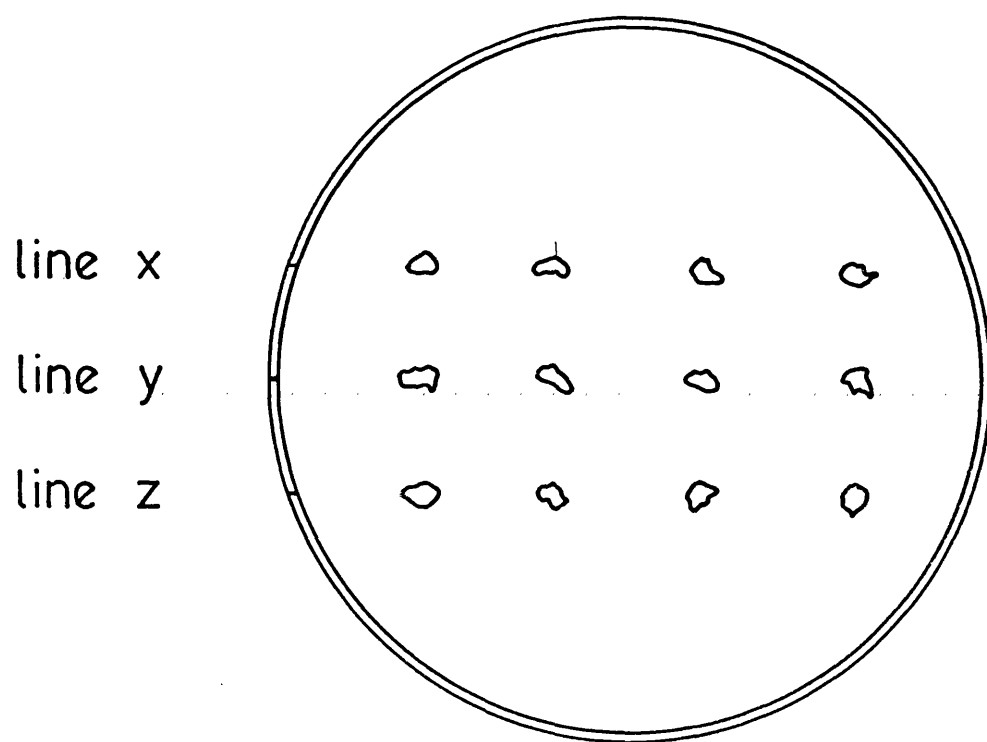
or black within a week of removal from the chilling conditions, and remained in this state until the time of scoring. The detailed results of these experiments for N. sylvestris are given in Table III.2. and summarised in Table III.3. Cell lines prefixed A are from the initial experiments in which mutagens were not used and cell lines prefixed B are from the experiments in which an EMS treatment was used, but include colonies from control plates. The history of all the cell lines used here are summarised in Table III.1.

There are too few cell lines in each individual treatment for any conclusive statistical deductions to be drawn. If, however, the lines are grouped into the three groups given at the bottom of Table III.3. it is found that 45% of the lines selected using EMS showed continued tolerance, 28% of lines selected in the absence of EMS and only 19% of lines not previously chilled were tolerant. These figures are thought to show a significant effect of the initial selection on subsequent resistance, indicating that a proportion of the variants were stable over 10 to 12 weeks in the absence of chilling conditions and this proportion was increased by the use of EMS.

The results of a similar experiment carried out with cell lines of C. annuum are given in Table III.4. and summarised in Table III.5. In this case, the selection pressure was 21 days at $5^{\circ}C$ and the cell lines used were derived as follows: -

CN1 - CN10 :	Non-selected lines from plates incubated at $25^{\circ}C$.
CV11 - CV30 :	Lines selected after $5^{\circ}C$ treatment without the use of a mutagen.
CV31 - CV60 :	Lines selected after $5^{\circ}C$ treatment from plates of a suspension exposed to 0.75% EMS.

TABLE III. 1.

The conditions of isolation for the callus lines of <u>N. sylvestris</u> used
for further examination of chilling tolerance.

Code for cell line	Parent cell line	EMS treatment (60')	Selection temp. ($^\circ$C)
A1 - A10	H_9	-	0°
A11 - A30	H_9	-	-3°
A31 - A50	D	-	0°
A51 - A70	D	-	-3°
B1 - B10	H_9	-	-3°
B11 - B13	H_9	0.075%	-3°
B14	H_9	1.5%	-3°
B15 - B24	D	-	-3°
B25 - B44	D	0.3%	-3°
B45 - B54	D	1.5%	-3°
AH1 - AH30	H_9	-	25°)non-)selected
AD31 - AD60	D	-	25°)controls

SH_{12}	Freshly initiated haploid callus.
H_9	)
D	) Parent cell lines)

TABLE III.2.

The survival and subsequent growth of cell lines of N. sylvestris exposed to chilling temperatures 0°C or -3°C for 21 days.

Cell line	0°C No. scored	No. showing growth	-3°C No. scored	No. showing growth	Vigorous growth
A1	4	4	8	1	
A4	4	4	8	0	
A5	4	4	8	0	
A6	8	8	8	1	
A7	4	4	8	0	
A13	4	4	8	0	
A14	4	4	8	0	
A15	4	4	8	0	
A16	4	3	8	2	+
A21	4	4	8	2	
A22	4	4	8	0	
A24	4	4	8	0	
A34	8	8	8	2	
A36	4	4	8	3	+
A37	8	8	8	1	
A39	4	4	8	0	
A41	4	4	8	3	
A42	4	4	8	0	
A44	8	8	8	0	
A45	8	8	8	0	
A46	4	4	8	0	
A51	8	8	8	0	
A52	4	4	8	0	
A53	4	4	8	0	
A54	4	4	8	0	
A56	4	4	8	1	
A57	4	4	8	0	
A58	4	4	8	0	
A59	4	3	8	2	
AH1	4	3	4	0	
AH2	4	4	4	0	
AH4	4	4	8	0	
AH7	4	2	8	0	
AH8	4	4	4	0	
AH9	4	4	8	0	
AH11	4	3	4	0	
AH13	4	3	4	0	
AH14	4	4	4	2	
AH17	4	4	8	0	
AH18	4	4	4	2	
AH19	4	4	8	0	
AH21	4	4	4	0	
AH24	4	3	4	0	
AH27	4	3	4	2	
AH29	4	4	4	0	
SH12	12	11	12	0	
H9	12	9	12	0	
D	12	8	12	0	

Cell line	0° No. scored	No. showing growth	No. scored	-3°C No. showing growth	Vigorous growth
A61	4	4	8	4	+
A63	4	4	8	3	+
B1	4	4	8	0	
B3	4	3	8	0	
B13	4	4	8	0	
B14	4	4	8	4	+
B15	8	3	8	0	
B18	8	8	8	0	
B19	0	0	4	0	
B20	0	0	4	0	
B21	0	0	4	0	
B23	4	4	8	3	
B25	4	4	8	2	
B26	4	4	8	1	
B29	4	4	8	0	
B30	4	4	8	3	
B31	4	4	8	1	
B33	4	4	8	1	
B34	4	4	8	2	
B35	4	4	8	7	+
B37	8	7	8	0	
B42	8	8	8	0	
B44	4	4	8	0	
B45	4	4	8	0	
B46	4	4	8	1	
B48	4	4	8	0	
B49	4	4	8	0	
B50	8	8	8	0	
B52	4	4	8	0	
AD31	4	4	8	5	+
AD32	4	4	8	0	
AD34	4	4	8	0	
AD35	4	3	8	4	
AD37	4	4	4	0	
AD39	4	4	8	0	
AD40	4	4	4	0	
AD41	4	1	8	0	
AD42	4	4	4	0	
AD43	4	3	4	0	
AD44	4	3	8	0	
AD48	4	4	8	0	
AD49	4	4	8	0	
AD50	4	2	4	0	
AD51	4	4	8	4	
AD52	4	3	8	0	
AD53	4	4	8	0	
AD54	4	4	8	0	
AD55	4	4	8	4	
AD58	4	4	8	0	

All lines showed complete survival when maintained at 25°C

TABLE III.3.

The survival and subsequent growth of cell lines of N. sylvestris exposed to -3°C for 21 days. (Summary of data from Table III.2.)

Parent cell lines	% EMS (60')	Initial selection temp (°C)	No. of cell lines	No. showing any recovery	% showing any recovery
H$_9$	0	0	5	2	40
	0	-3	9	2	22
	0.075	-3	1	0	0
	1.5	-3	1	1	100
	0	25(non-selected)	16	3	19
D	0	0	9	4	44
	0	-3	16	5	31
	0.3	-3	11	7	64
	1.5	-3	7	1	14
	0	25(non-selected)	20	4	20
Total selected after -3°C, no EMS			25	7	28
Total selected after -3°C, using EMS			20	9	45
Total non-selected			36	7	19

TABLE III.4.

The survival and subsequent growth of cell lines of C. annuum exposed to chilling temperature 5°C for 21 days

Cell line	Recovery after 3 weeks at 5°C			Cell line	Recovery after 3 weeks at 5°C		
	No. scored	No. showing growth	Vigorous growth		No. scored	No. showing growth	Vigorous growth
CN1	8	0		CV31	8	1	
CN2	8	0		CV32	8	0	
CN3	4	0		CV33	8	3	+
CN4	8	0		CV34	8	2	+
CN6	8	0		CV35	4	2	
CN7	8	0		CV36	4	4	+
CN9	8	0		CV40	8	0	
CV11	8	3	+	CV41	8	0	
CV12	8	0		CV42	8	0	
CV13	8	1		CV43	4	0	
CV14	4	1		CV44	8	1	
CV15	8	0		CV45	8	2	+
CV16	8	0		CV46	4	0	
CV17	8	0		CV47	8	0	
CV18	4	0		CV48	8	0	
CV19	4	0		CV49	8	0	
CV20	8	0		CV51	8	0	
CV21	8	1		CV52	8	5	
CV23	8	0		CV53	8	0	
CV25	8	0		CV54	4	0	
CV26	4	0		CV55	8	1	
CV28	8	0		CV58	8	0	
CV29	8	0		CV59	4	0	
CV30	8	1		C*	12	0	

*C = Parent cell line

TABLE III.5.

Survival and subsequent growth of cell lines of C. annuum exposed to 5°C

for 21 days. (Summary of data from Table III.4.)

Description of cell lines	No. of lines	No. showing any recovery	% showing any recovery
Selected after 5°C, no EMS	17	5	29.4
Selected after 5°C, using EMS	23	9	39.1
Non-selected lines, maintained at 25°C	7	0	0

The summary (Table III.5.) shows a similar effect to that obtained with the N. sylvestris lines, namely an increase in the proportion of lines showing resistance, from non-selected lines, to lines selected in the absence of EMS, to lines selected in the presence of EMS.

On the basis of the results described above the following cell lines of N. sylvestris and C. annuum were chosen for further study: -

N. sylvestris:	A16		C. annuum:	CV11	
	A36			CV14	
	A41	AH21)		CV33	CN2)
	A61	AD53) Controls		CV34	CN9) Controls
	A63	H9)		CV36	C)
	B14	D)		CV44	
	B23			CV45	
	B25				
	B35				

These were chosen for their rapid recovery after exposure to the chilling treatment ($-3°C$ in N. sylvestris, $5°C$ in C. annuum) followed by vigorous growth in the surviving callus pieces, with the intention of studying their survival after another (3rd) exposure to the chilling treatment. A number of other N. sylvestris cell lines, showing particularly vigorous growth after exposure to $0°C$ were also maintained as callus cultures with a view to attempting to obtain plants from a larger number of callus lines (Section V).

The variant cell lines listed above were each returned to liquid medium to provide suspension cultures which could be compared, in terms of chilling tolerance on plates, with the control lines and parent lines, as described in Section II.

In addition to this they were exposed to the chilling conditions once more as callus pieces in exactly the same way as described earlier in this section (p. 66). The results of this treatment are given in Table III.6. It is seen that most of the callus line surviving the second chilling treatment also gave some survival after the third, and in some cases all or most of the callus pieces survived. From now on all chilling tolerant lines will be expressed with a suffix Roman numeral indicating the number of times the line has been exposed to the chilling conditions; e.g. line CV34 taken from the above experiment would then be designated CV34 (iii).

A small experiment was carried out with some of these callus lines, in exactly the same way as above, to see if they were able to survive longer periods of chilling. In this experiment, the dishes were removed from the chilling conditions after 7, 14, 21, 28 or 35 days. The results are given in Table III.7. In the case of the N. sylvestris variant lines those already shown to have a high level of tolerance to 21 days at $-3^{o}C$ (B14 (iii) and B35 (iii)) continued to show a high level of tolerance after 21 days but still could not survive 28 or 35 days. Most lines surviving $0^{o}C$ for 21 days, however, (including some of the control lines) also showed some survival after 28 and 35 days. In the case of the C. annuum lines the picture is less clear. The most tolerant lines (CV34 (iii) and CV45 (iii)) showing survival after 21 days at $5^{o}C$, also showed some survival after 28 days but no results were obtained for plates chilled for 35 days.

(ii) Retention of chilling tolerance through suspension cultures.

Of the variant cell lines after two chilling treatments (listed on p.68) all the C. annuum lines and six of the N. sylvestris lines formed suspensions in the first passage, suitable for plating, and these were used to repeat the initial experiment on chilling tolerance, described in section II (p. 52).

TABLE III.6.

Recovery of callus pieces of cell lines of N. sylvestris and C. annuum after their 3rd exposure to a chilling treatment.

| | N. sylvestris | | | | C. annuum | | |
| | Recovery after 3 weeks at -3°C | | | | Recovery after 3 weeks at 5°C | | |
Cell line	No. scored	No.showing growth	Vigorous growth	Cell line	No. scored	No.showing growth	Vigorous growth
A16	8	4		CV11	12	0	
A36	8	4		CV14	12	4	
A41	8	8		CV33	12	2	
A61	8	5		CV34	12	10	+
A63	8	3		CV36	12	5	
B14	8	8	+	CV44	12	9	
B23	8	0		CV45	12	6 .	+
B25	8	3					
B35	8	6	+				
AH21*	8	1		C*	12	1	
AD53*	8	0		CN2*	12	0	
H9*	8	1		CN9*	12	0	
D*	8	1					

N.B. In many cases the recovery and growth was more rapid than after the previous exposure, but a '+' under "vigorous growth" in this table indicates only the best two lines for each species.

* Control lines, or parent lines, which have never been given a chilling treatment.

Photographs from some of the dishes in these treatments are shown in Plate III.1.

PLATE III.1. Survival and regrowth of callus pieces of chilling sensitive and tolerant lines after a chilling treatment.

A and B.   N. sylvestris line: -

A: A16   B: B14

     A41   H$_9$ (sensitive control)

     A61   B35

C and D.   C. annuum line: -

C :   CV14   D: CV44

      CV34   CV33

      C   (sensitive control)   CV45

      CV36

PLATE III.1
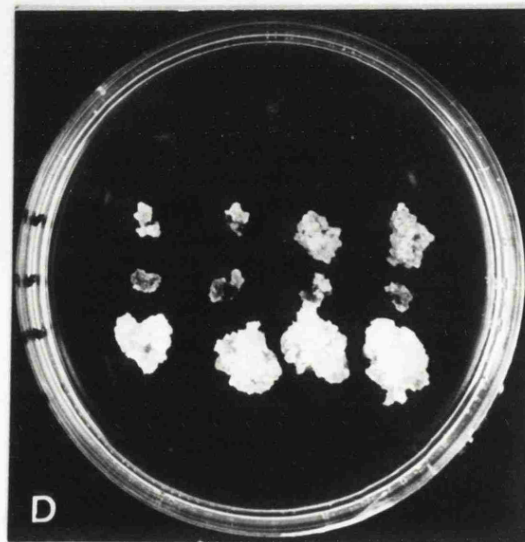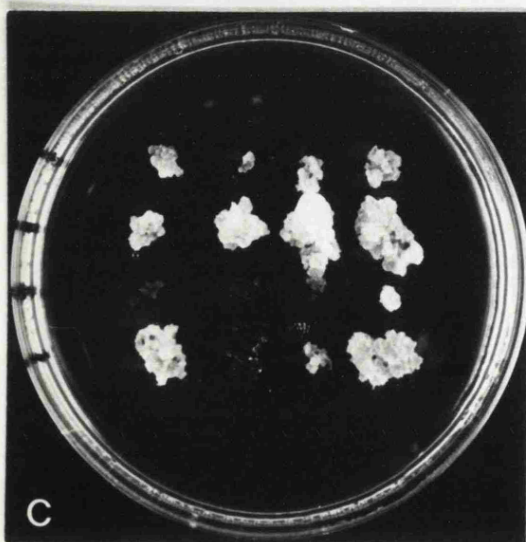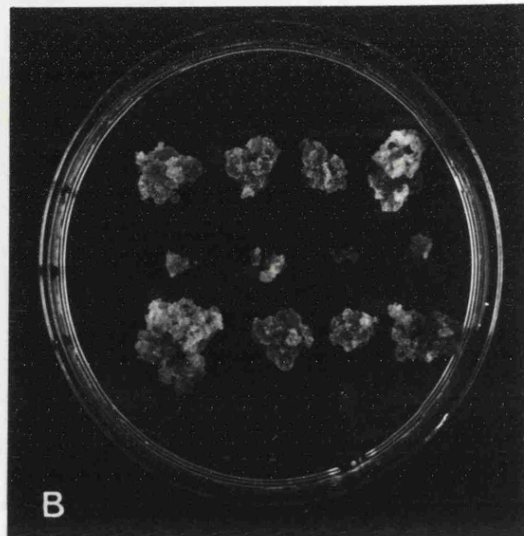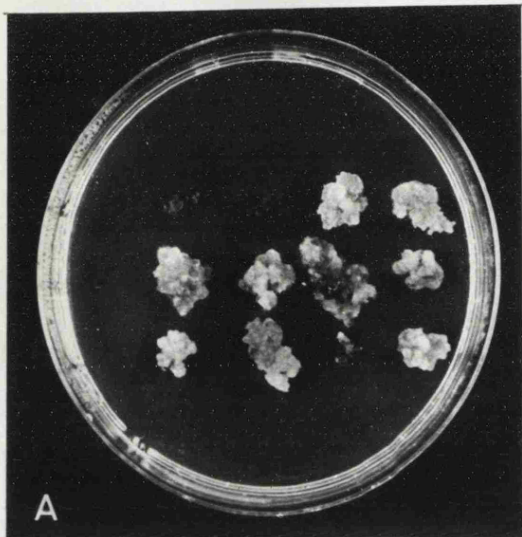
TABLE III.7.

Survival of some chilling tolerant and control callus lines after exposure
to chilling conditions for varying periods of times.

Species	Callus line (before expt.)	Chill-ing temp. ($^{\circ}$C)	Callus piece surviving (initially 4 per treat-ment)				
			Time of exposure (weeks)				
			1	2	3	4	5
N.sylv-estris	A36(iii)	0	4	4	4	4	2
		-3	4	3	0	0	0
	A21(ii)	0	2	3	2	1	1
		-3	1	0	0	0	0
	B14(iii)	0	4	4	4	3	4
		-3	4	3	3	0	0
	B35(iii)	0	4	4	4	4	2
		-3	4	4	4	0	0
	SH12	0	3	-	2	2	2
		-3	2	2	1	0	0
	AD48	0	4	4	4	4	3
		-3	1	2	0	0	0
	H9	0	3	3	1	0	1
		-3	1	0	0	0	0
C.ann-uum	CV14(iii)	5	-	1	2	1	-
	CV34(iii)	5	-	4	4	2	-
	CV44(iii)	5	-	0	1	0	-
	CV45(iii)	5	-	4	2	2	-
	CN9	5	-	0	0	0	-

Parent or control lines were also used. In this experiment all suspensions were filtered through 1 mm. mesh and plated at 20,000 cells ml.$^{-1}$. Plates were incubated at 25°C and at only one chilling temperature (-3°C for N. sylvestris, 5°C for C. annuum). No mutagenic treatment was applied. The results were recorded in the same way as described previously and are given in Table III.8.

There is clearly a wide range of tolerance to the chilling conditions and most of the variants are clearly more tolerant than the parent lines. There is no doubt about the significance of the high level of survival in the four lines marked *. These were also the most tolerant lines in terms of rapid recovery of callus pieces and were chosen as the lines most worthy of a serious attempt at characterisation as described in Section V.

(B) Persistence of high sodium chloride resistance through a large number of cell generations.

(i) Resistant lines selected from plates.

These lines were selected after incorporation into agar medium containing 1% NaCl as described in Section II. All the colonies were grown up on minus NaCl medium to form large callus masses which could be tested for resistance. These callus lines had therefore had a period of growth in the absence of the selective conditions. A few of the lines were then returned to liquid medium (minus NaCl) for subsequent examination of their resistance both in suspension and as small aggregates on plating. A larger number of the callus lines, together with appropriate control lines were examined for their continued resistance as callus cultures. This was carried out by placing small callus pieces on agar plates, as described for the chilling tolerant lines (Section III (A) (i) and Fig. III.1.) except that the plates contained 0, 1, 2 or 3% NaCl, incorporated into the medium. The plates

TABLE III.8.

The effect of a 21 day chilling treatment on the appearance of colonies on plates of N. sylvestris and C. annuum chilling tolerant variants, compared with the effect on parent cell lines.

Species	Cell line	Chilling temp (^{o}C)	Mean colonies/plate at 25°C	Mean colonies/plate after chilling
N.sylvestris	A16 (ii)	-3	68	4.2
	A41 (ii)	-3	188	29
	A61 (ii)	-3	152	16
	B14 (ii)	-3	167	54*
	B23 (ii)	-3	124	21
	B35 (ii)	-3	212	93*
	H9	-3	173	3.2
	D	-3	203	10.4
C.annuum	CV11 (ii)	5	370	4.3
	CV14 (ii)	5	482	27
	CV33 (ii)	5	324	0.2
	CV34 (ii)	5	528	124*
	CV36 (ii)	5	503	45
	CV44 (ii)	5	476	38
	CV45 (ii)	5	512	137*
	C	5	489	11
	CN9	5	425	21

were incubated in the dark at 25°C and scored for continued growth after 4 weeks (C. annuum) or 6 weeks (N. sylvestris). The growth on 0% NaCl medium was good from all callus pieces, and no growth was observed from any lines on 2 or 3% NaCl. A key to the history of the cell lines is given in Table III.9., and the results for growth on 1% NaCl medium are given in Table III.10.

A summary of the results is given in Table III.11. It is clear that many more of the lines selected from plates containing 1% NaCl showed some growth on this medium, than control lines removed from plates containing 0% NaCl. This suggests that many of these lines do possess increased resistance to the high salt concentration, and this has been retained through growth in the absence of the selection pressure. In most cases growth was slower on the selective medium than on medium with no added NaCl. A high proportion of the exceptions to this were lines selected from plates of suspensions of C. annuum which had previously been incubated for one passage in the presence of 1% NaCl.

Several lines selected from plates were resuspended in medium containing no NaCl and for two of them (derived from C. annuum line C) their growth in the presence of NaCl incorporated into the second passage was investigated. 250 ml Erlenmeyer flasks containing 60 ml medium were inoculated at a cell density of 5×10^4 to 10^5 cells ml^{-1} and duplicate flasks sampled for cell counts at intervals to give the growth curves shown in Fig. III.2. The growth curves are compared to that of a control line, CN2, which had never been exposed to 1% NaCl. The two resistant lines showed a long lag phase in the presence of 1% NaCl. This may be due, in part, to the rather lower inoculation densities used for these cultures, or to a phase of reconditioning to the high salt conditions. When they did commence growth, however, they grew more rapidly than the

TABLE III.9.

The history of callus lines used in investigations on high salt resistance

Code for cell lines	Parent cell line	NaCl in suspension passage prior to plating	NaCl incorporated into plates of suspensions
F1 - 10	H9	0	0
F11 - 30	H9	0	1%
F13 - 50	C	0	1%
F51 - 55	C	0	0
F56 - 75	D	0	1%
F76 - 80	D	0	0
G1- 30	H7	0	0
G31 - 40	C	1%	1%

TABLE III.10.

Growth of various callus lines on solid medium containing 1% NaCl.

Cell line	No. callus pieces	No. showing growth	Nature of growth (see key)	Cell line	No. callus pieces	No. showing growth	Nature of growth (see key)	Cell line	No. callus pieces	No. showing growth	Nature of growth (see key)	Cell line	No. callus pieces	No. showing growth	Nature of growth (see key)
F1	8	8	M	F38	4	3	M	F61	4	4	S	G20	8	0	
F3	4	0		F39	4	2	S	F63	8	8	M	G23	8	0	S
F4	8	8	M	F40	4	2	S	F67	8	8	M	G30	8	3	M
F6	8	0		F41	4	2	S	F69	8	8	M	G33	4	1	M
F7	4	4	S	F42	4	1	S	F70	8	8	M	G34	4	0	
F9	8	3	S	F43	4	4	M	F71	4	4	S	G35	4	2	M
F10	8	5	S	F44	4	1	S	F72	8	8	M	G36	4	4	G
F12	4	2	M	F45	4	2	S	F73	4	2	S	G37	4	3	G
F14	8	3	S	F46	4	3	S	F74	4	4	S	G38	4	4	G
F16	8	4	G	F47	4	3	S	F75	8	8	M	G39	4	0	
F19	8	4	S	F48	4	3	S	F76	4	2	S	G40	4	3	M
F20	8	4	G	F49	4	1	S	F77	4	0		C	4	1	S
F27	4	2	S	F50	4	0		F79	4	0		CN_2	4	0	
F31	4	4	M	F52	4	0		F80	4	0		D	4	0	
F32	4	0		F53	4	1	S	G1	4	1	S	H_9	4	1	S
F33	4	2	S	F54	4	0		G3	4	0		SH_{12}	4	1	S
F34	4	4	M	F55	4	0		G5	4	0					
F35	4	1	S	F57	4	2	S	G8	8	2	G				
F36	4	2	S	F59	4	4	S	G13	8	4	M				
F37	4	0		F60	4	1	S	G18	8	0					

KEY:

S = slow growth, calli 1 – 3 x original size, at time of scoring

M = moderate growth, calli 3 – 5 x original size, at time of scoring.

G = good growth, calli > 5 x original size, at time of scoring.

TABLE III.11.

Growth of various callus lines of N. sylvestris and C. annuum on medium
containing 1% NaCl (summary of data from Table III.10.)

Species	History of cell lines	No. of cell lines	No. of lines showing any growth
C. annuum	Selected from plates containing 1% NaCl. No previous exposure to high NaCl concentration.	20	17
	Selected from plates containing 1% NaCl after one suspension passage in medium containing 1% NaCl.	8	6
	Removed from plates containing no NaCl. No previous exposure to high NaCl concentration.	4	1
N. sylvestris	Selected from plates containing 1% NaCl. No previous exposure to high NaCl concentrations.	19	19
	Removed from plates containing no NaCl. No previous exposure to high NaCl concentration.	20	9

Fig. III.2   Growth of suspension cultures of two lines of C. annuum selected

for salt resistance, compared with a control line, in medium

containing 0%, 1% or 2% NaCl.

Lines F47 (●) and F48 (▲) had been selected from plates containing

1% NaCl and CN2 (○) removed from a plate containing no NaCl. Curves

represent growth in medium containing no NaCl (———), or 1% (— —)

or 2% (— — —) NaCl (w/v).

Fig. III 2.

Incubation (days)

Cell number ml^{-1} × 10^{-6}
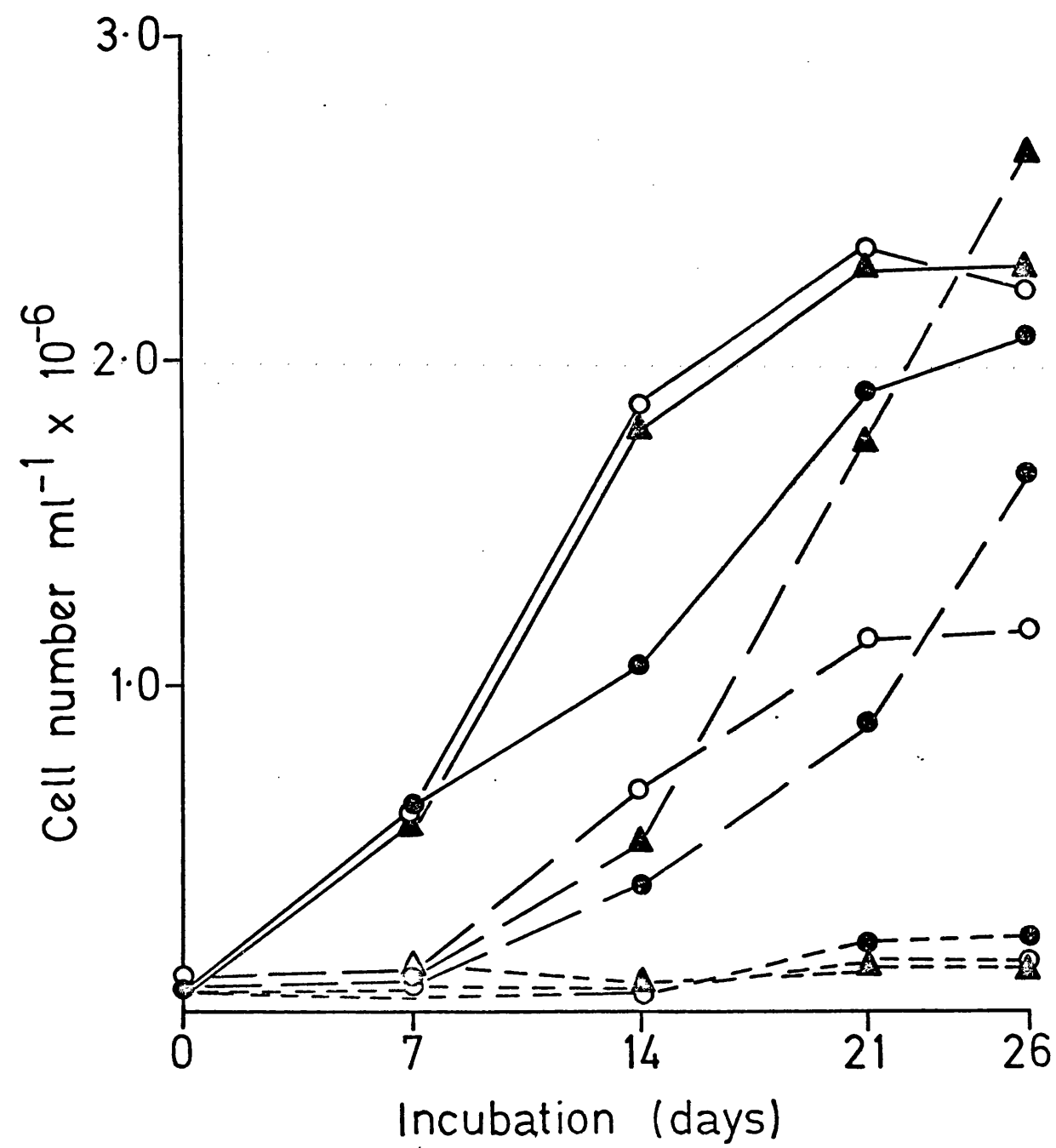
non-selected lines, were still dividing rapidly at the termination of the experiment, and appeared to be reaching a higher final cell density than any of the lines grown in 0% NaCl medium. There was little or no growth in medium containing 2% NaCl.

(ii) Resistant lines selected from cell suspensions.

As described in Section II.D.(iii). incubation of suspension cultures in the presence of NaCl for a number of passages gave rise to gradual increases in the growth rate and final cell density in medium containing 1% NaCl, and eventually gave growth in medium containing 2% NaCl. These cultures were being serially subcultured, continually in the presence of NaCl. The retention of the increased resistance of these cell lines in the absence of NaCl was examined in the following way. All three cultures used for the selection were examined (i.e. C, D, and H_7 : 36). If the first passage of selection in 1% NaCl is designated P1, the growth of each of the cell lines was examined in P7, each line having been subjected to the following three regimes: -

P1 - P6 in medium with 0% NaCl;

P1 - P6 in medium with 1% NaCl;

P1 - P3 in medium with 1% NaCl followed by

P4 - P6 in medium with 0% NaCl.

The detailed growth curves for these cell lines after all the above treatments are shown in Fig. III.3, Fig. III.4., and Fig. III.5. At certain points, packed cell volume data were determined, in addition to cell count data, and these values after 30 days are included with the growth curves.

Fig. III.3   Growth of a suspension culture of $\underline{N}$. sylvestris, line H_7:36, in

P7 (see text, p 72) in media containing 0% or 1% NaCl.

Previous regimes for P1 - P6: -

■ 0% NaCl throughout;

O 1% NaCl throughout;

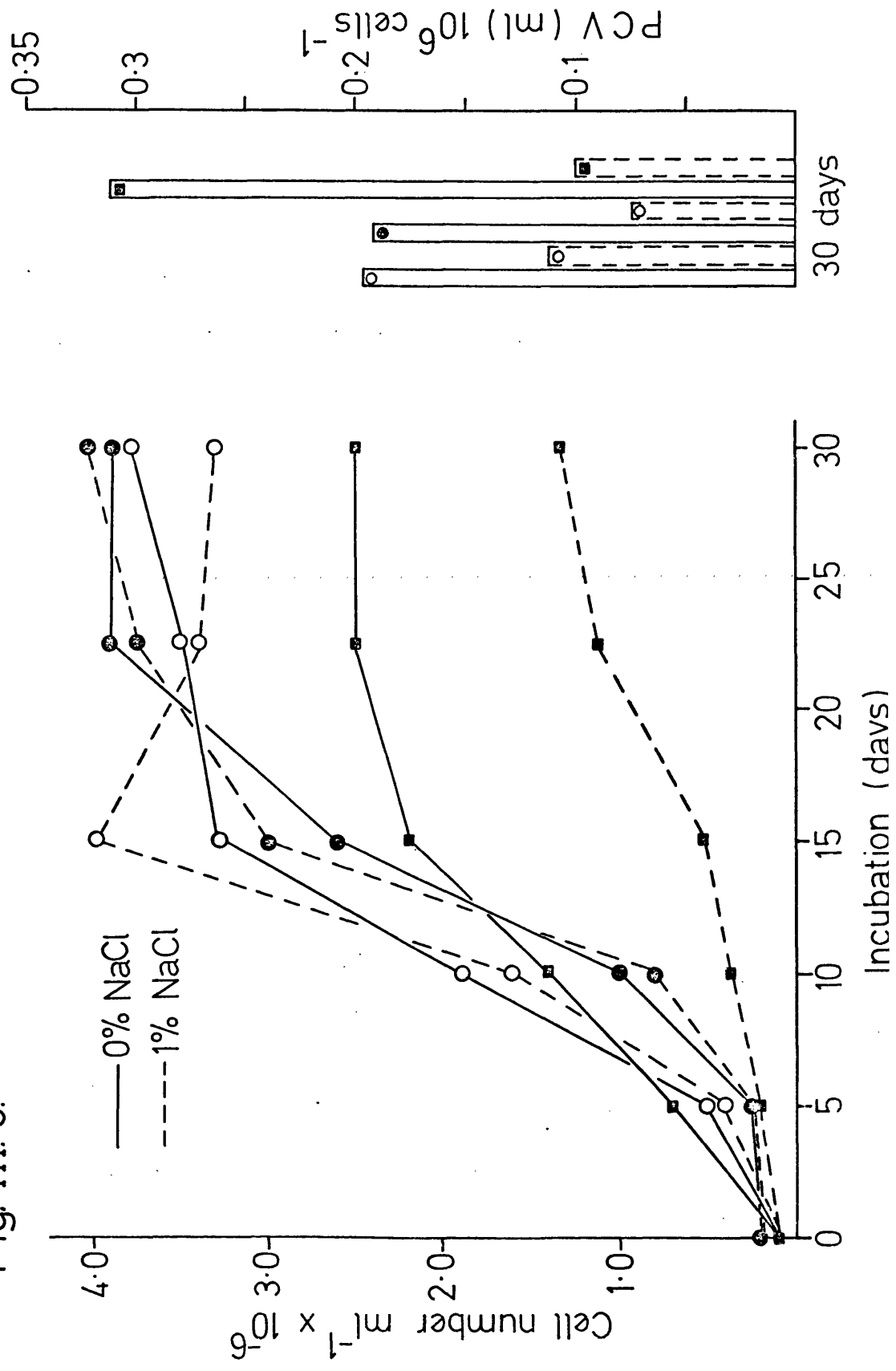● 1% NaCl, P1 - P3; 0% NaCl, P4 - P6.

Fig. III. 3.

Fig. III.4   Growth of a suspension culture of <u>N. sylvestris</u>, line D, in

media containing 0% or 1% NaCl.

For key to previous regimes, see Fig. III.3.

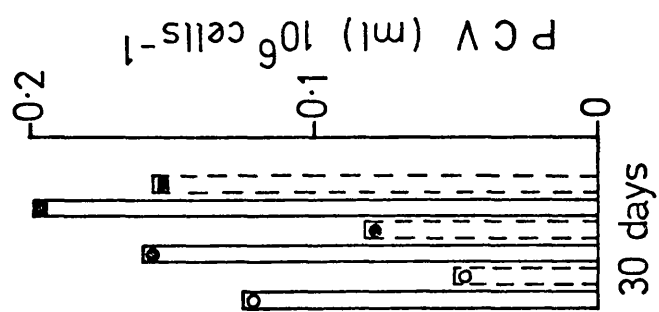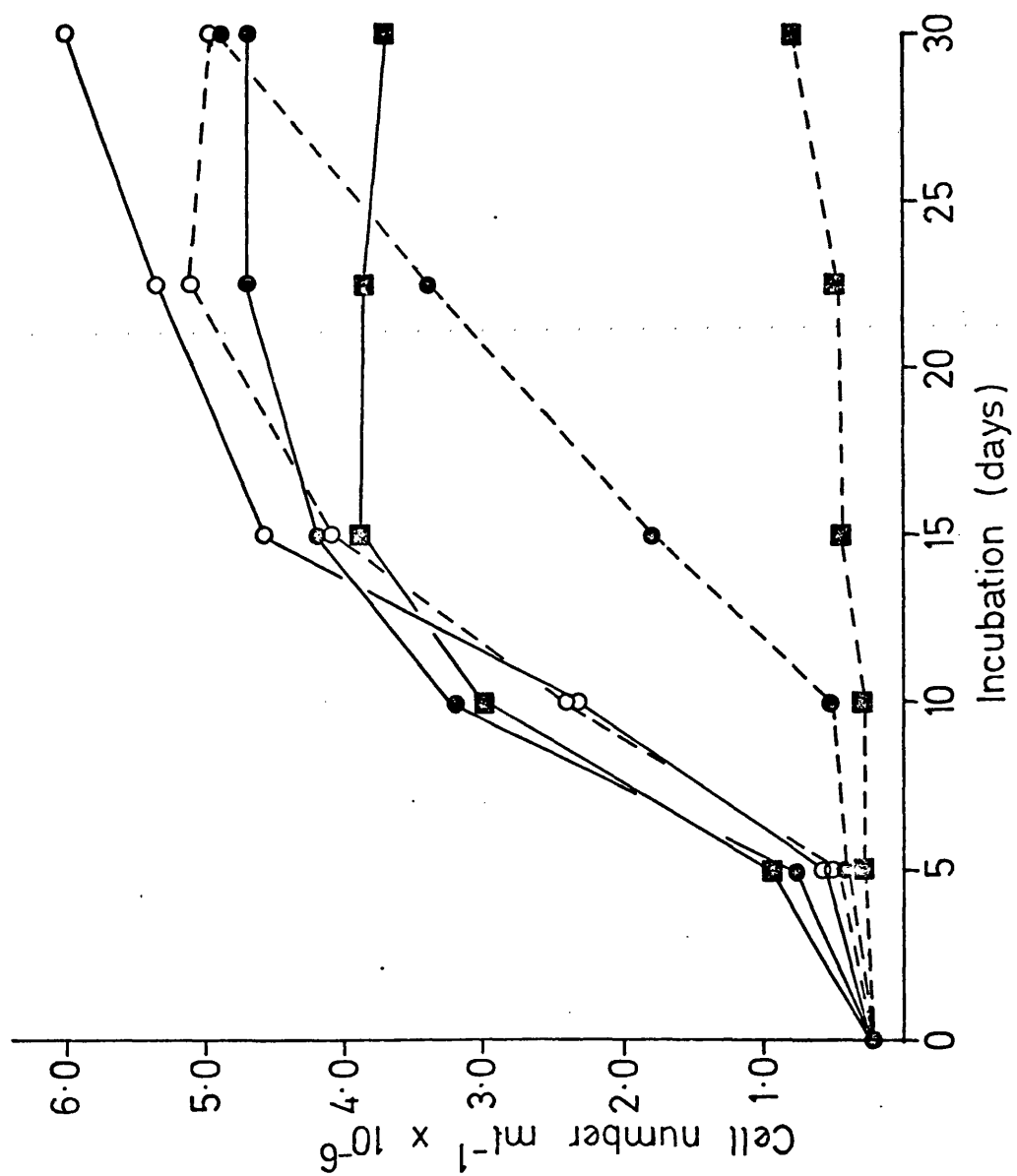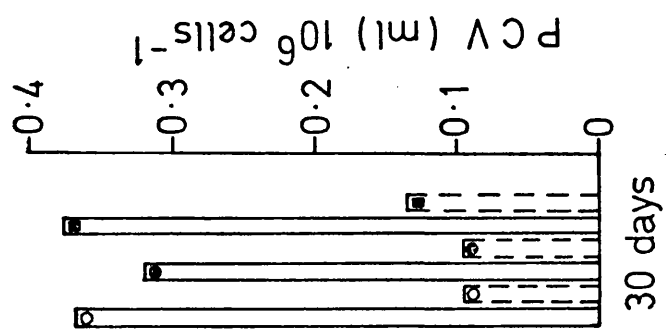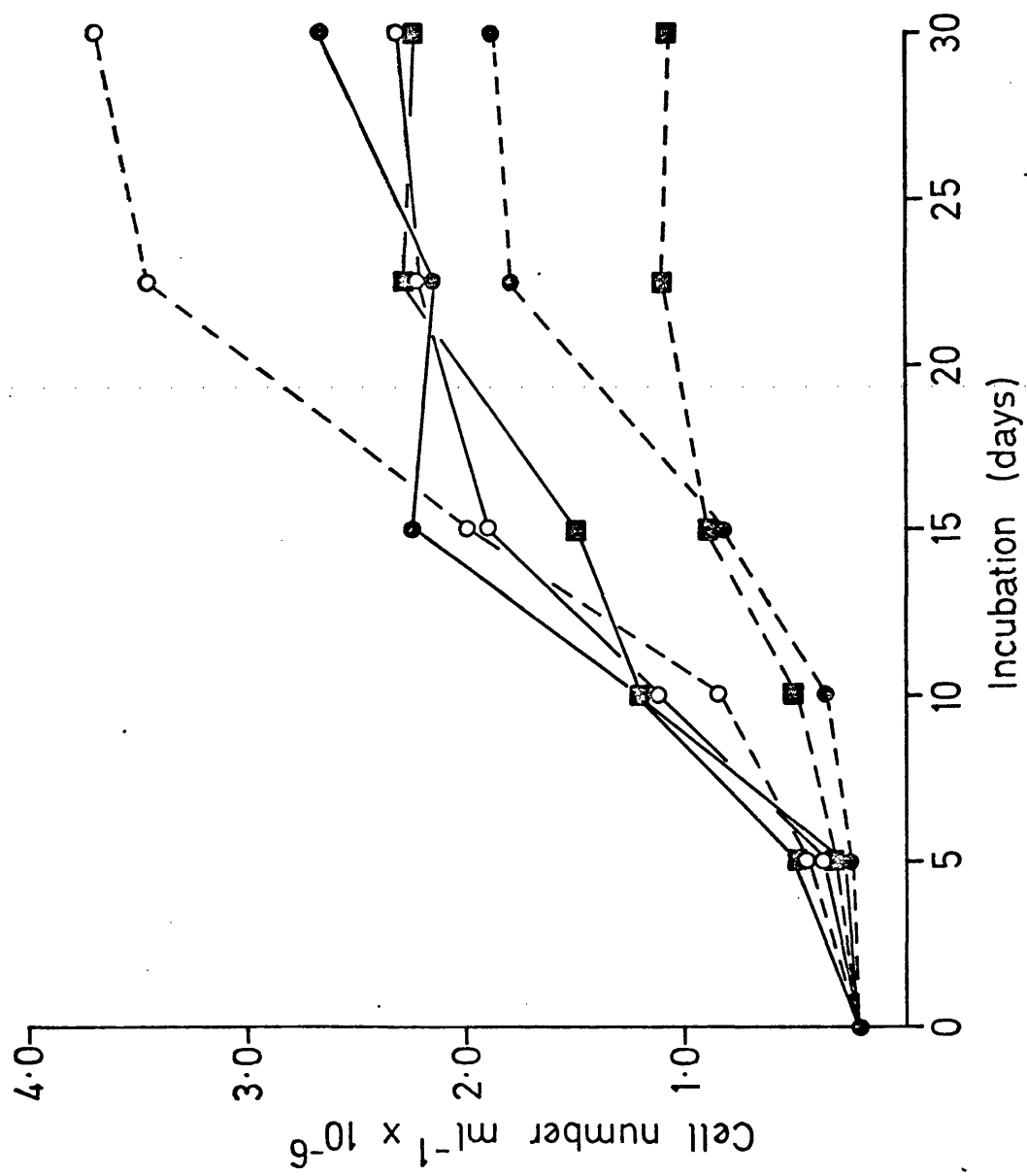Fig. III. 4.

Fig. III.5   Growth of a suspension culture of C. annuum , line C, in P7

(see text, p 72) in media containing 0% or 1% NaCl.

For key to previous regimes, see Fig. III.3.

Fig III. 5.

Cell number ml⁻¹ x 10⁻⁶ (vertical axis, values 1·0, 2·0, 3·0, 4·0)

Incubation (days) — 0, 5, 10, 15, 20, 25, 30

P C V (ml) 10⁶ cells⁻¹ — 0, 0·1, 0·2, 0·3, 0·4

30 days

The results clearly show that even after being grown in minus NaCl medium for three passages the tolerant lines were still able to grow successfully in medium containing 1% NaCl. In the case of lines C and D there was quite a long lag phase but the recovery was still far more rapid than in the same line which had never been subjected to 1% NaCl. The salt resistance selected line H_7:36 reached a higher cell number both in the presence and absence of NaCl than the control line in the absence of NaCl, and the selected line C, in the presence of 1% NaCl also reached a far higher cell density than the control line under any conditions. The packed cell volume values for cell lines in the presence of 1% NaCl were less than half those in the absence of NaCl, even where the final cell numbers were comparable with, or greater than, those in the absence of NaCl. This suggests a smaller mean cell size, a point which will be developed further in Section V.

(C) <u>Persistence of high temperature tolerance through a large number of cell generations.</u>

<u>N. sylvestris</u> cell lines derived from cells surviving exposure to $45°C$ (Section II (E)) were maintained as callus cultures. When enough callus had been obtained, four of these lines were returned to suspension culture. In their second passage after resuspension they were filtered and plated, after exposure to the same conditions as those under which they had been selected. For comparison, the parent cell line $(H_7$:36) was also exposed to the selection conditions and plated. The four cell lines under examination were designated as follows: -

E3, E4 and E9 : selected as resistant to 30' exposure to $45°C$.

E11 : selected as resistant to 60' exposure to $45°C$.

The plates were scored for colony formation after 6 weeks and
the results are presented in Table III.12., together with data from an
identical experiment carried out with line E11 one passage later (from P3).

The results show an extremely high level of retention of tolerance
in the line E11. It is of interest to note that the colonies of this
line, bother after 0' and after 60' exposure to 45°C were very uniform
in appearance and very tight and compact compared with those of the
parent line, H_7:36. Although compact, they are not hard, they grow
rapidly and form quite good suspensions. In contrast, the only other line
which seems to retain some measure of resistance, E3, gives rapidly growing,
loose, fluffy colonies. A comparison of plates showing the different
colony types is given in Plate III.2.

DISCUSSION

For examination of most of the variant lines a callus test was used.
The failings of this system are the same as those already mentioned for
the use of callus cultures for the selection of variants, primarily a
possible protective effect of the bulk of the callus on certain small
regions. It is also unsatisfactory from the point of view of comparing,
in a quantitative way, the effect of the selection pressure on different
lines. A large proportion of the lines showing what is termed "survival"
or "renewed growth", exhibit growth only from limited regions of the callus
which vary in size and number, and are difficult to measure. Thus, although
the results with callus indicate that some of the lines have acquired
resistance to stress, in very few cases is this resistance complete. This
applies to all of the different types of variant selected, but is particu-
larly true of the chilling tolerant variants.

TABLE III.12.

Colony formation by high temperature tolerant cell lines of N. sylvestris.

after a second exposure to the selective conditions.

Cell line	Mean colonies/plate (5 plates) Duration of exposure to 45°C		
	0'	30'	60'
E3	337	88	-
E4	285	2.0	-
E9	355	5.4	-
E11 (passage 2)	359	-	160
E11 (passage 3)	351	-	126
H_7:36 (parent line)	259	1.2	0

Initial cell density on plating was 50,000 cells ml^{-1} throughout.

Plating of high temperature tolerant and sensitive lines of

N. sylvestris, showing a diversity of colony type.

A and B. High temperature tolerant variant line E11 showing

the formation of tight compact colonies.

A. Control plate. B. Suspension plated after 60 min. exposure

to 45°C.

C. High temperature tolerant variant line E3 showing the

formation of loose, fluffy colonies after 30 min. exposure to

45°C. Colonies more closely resemble those of parent sensitive

line (H_7:36) plated without exposure to the high temperature (D)

Cells were plated at 50,000 cell ml^{-1}.

PLATE III.2
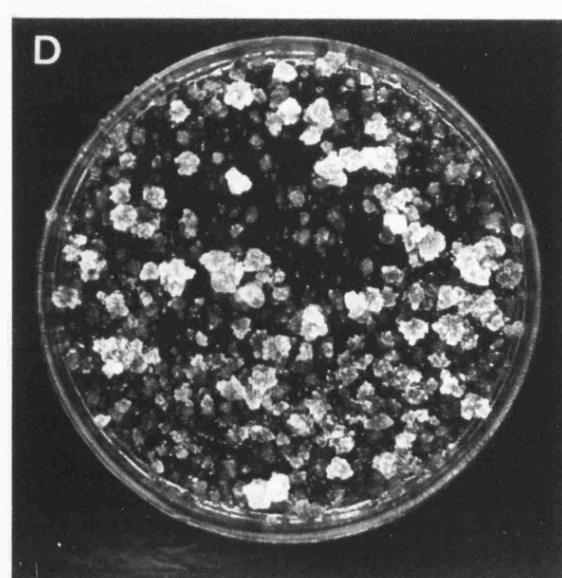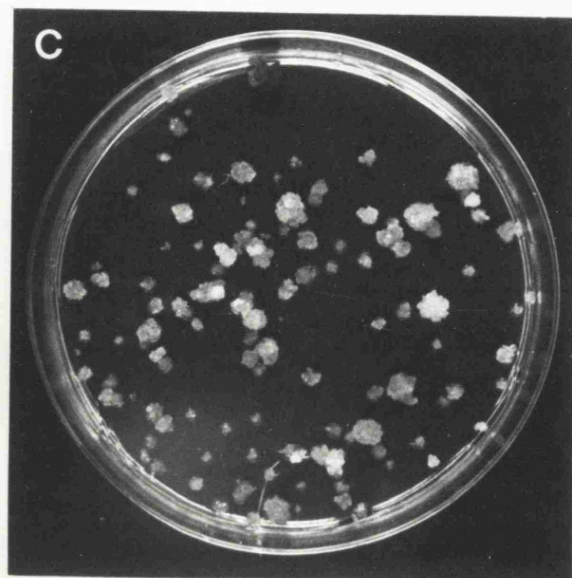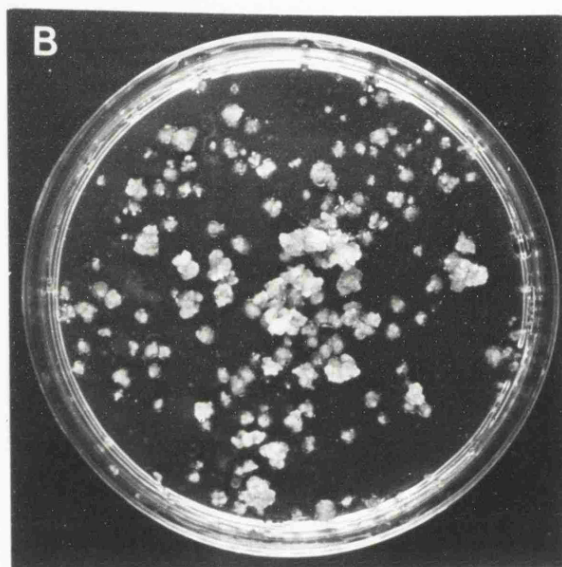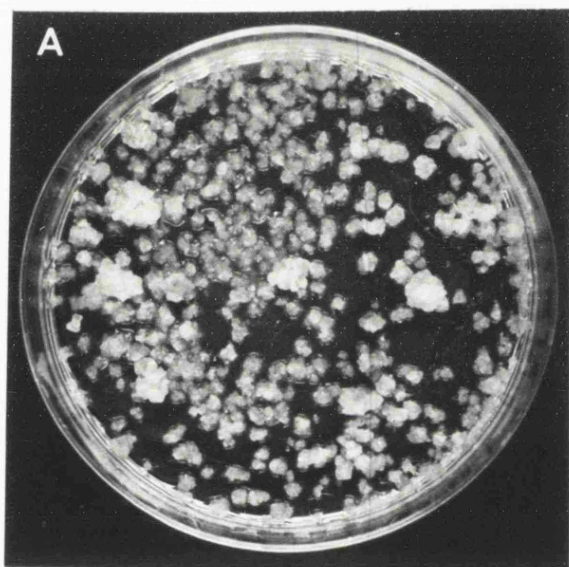
In the case of the chilling tolerant variants further exposures to the selective conditions resulted in a more predictable response, most callus pieces surviving and giving more evenly distributed growth and more rapid recovery. After three exposures to the chilling conditions the survival of several lines of both N. sylvestris and C. annuum, was very markedly better than that of control lines and such lines could be confidently described as improved in chilling tolerance.

The use of plated suspensions in the examination of continued resistance was a more satisfactory system as it allowed comparison with the parent and control lines. Although the plating technique is not a completely satisfactory quantitative method, it does allow examination of a far larger number of very much smaller units than the callus method. This was the only system used to study the high temperature tolerant variant lines and showed large and repeatable differences in tolerance between the lines originally selected after a high temperature treatment. One of these lines (E11) looks particularly promising but has not yet been studied further.

Sodium chloride resistance lends itself better to study in suspension culture, and resistance to 1% NaCl was selected with surprising ease, and largely retained over several passages in the absence of NaCl. These cultures showed just as much resistance to NaCl as the lines selected from plates, although presumably containing a wider range of genotypes.

One of the most fundamental objectives of this work is to establish whether variants resistant to environmental stresses and resulting from a genetic change, as opposed to a process of physiological adaption, can be selected using tissue culture methods. We therefore have to try to establish whether the variants we have obtained are due to mutation or adaption. This question will be discussed at length in the General Discussion, but will draw upon evidence provided by the data presented in other sections. To this end, it seems to important to emphasise certain points arising in this section. These are summarised in the following concluding paragraph.

Of the lines selected as tolerant, as described in Section II, only

a proportion proved tolerant on further examination. In the case of

chilling tolerance, these mostly showed incomplete tolerance which could

be improved gradually by repeated exposure to the selection pressure. Of

the chilling tolerant lines selected after exposure to a chemical mutagen

a higher proportion, in relation to those selected without use of a mutagen,

showed continued survival after a second chilling treatment.

SECTION IV

CHARACTERISATION OF VARIANT CELL LINES

INTRODUCTION

Most of the work described in this section relates to the chilling

tolerant variants, but some observations made on the high salt resistant

lines are also reported. No work was undertaken towards the characteris-

ation of the high temperature tolerant variants.

No single primary cause of chilling injury to plants has been

identified and the phenomenon of slow chilling injury (injury after an

extended period of exposure) under investigation here may be very complex.

Any one of a number of effects may result in injury when plants, or plant

material, are subjected to an extended period of stress under chilling

conditions. Most investigations into the causes of chilling injury have

involved comparison between a tolerant and a susceptible species or

variety, under both chilling and non-chilling conditions. In such studies

differences have been shown in membrane permeability (Lewis & Workman,1964),

photosynthesis (Sawada et al., 1974), protein breakdown (Razmaev, 1965),

cytoplasmic streaming (Lewis, 1956), respiration (Lewis & Workman, 1964;

Lyons & Raison, 1970 a & b; Tanaki & Uritani, 1974), and the rate of

particular enzymic reactions (Podin, 1966). It would thus appear that a

wide range of biochemical and physiological changes can occur when a

sensitive species is exposed to chilling conditions and that these changes

are different in different species.

Reviewing such studies, Levitt (1972) has suggested that the changes

observed can be grouped into two classes, (i) permeability changes and

(ii) metabolic changes. If the primary effect of chilling is an increase in

membrane permeability, effects such as starvation injury, inhibition of

protein synthesis, biochemical lesions, and the appearance of toxic products

could be secondary. A widely held theory is that the permeability changes

involve a phase change in membrane lipids. This was postulated by Lyons

et al. (1964) when they showed a higher level of polyunsaturated fatty acids

in the mitochondria of chilling tolerant plants than in those of chilling

sensitive plants. The inference was that membrane damage (and hence

increased permeability) did not occur because the increase in polyunsat-

uration resulted in a retention of flexibility of the membranes at low

temperatures. In support of this, Lyons & Asmundson (1965) showed a

marked depression in the freezing point of fatty acid mixtures when the

levels of unsaturated fatty acids were increased. Lyons & Raison (1970a)

isolated mitochondria from sensitive and tolerant plant tissues and assayed

their respiratory activity, in terms of succinate oxidation, at a range of

temperatures. They found discontinuities in the Arrhenius plots at between

9° and 12°C with mitochondira from the chilling sensitive tissues. Later,

similar discontinuities were established by electron-spin resonance spectros-

copy of various organelles (glyoxysomes, mitochondria and proplastids), of

their membranes, and of micelles of the phospholipids derived from these

membranes (Wade et al., 1974). These results all point to a phase change

in the membranes of the sensitive plants brought about by chilling. Further

evidence comes from study of chloroplast structural changes brought about by

chilling a chilling sensitive maize mutant (Millerd et al., 1969) and of

the damage caused by chilling or freezing biological membranes (e.g.

Santarius, 1973; Heber et al., 1973; and Steponkus & Wiest, 1973). A wide

range of ultrastructural changes were found in grass species exposed to

-5°C for 3 days (Kimball & Salisbury, 1973). Lyons (1972 and 1973) considers

the possibility that phase transitions might be involved with altering the

regulation of metabolism under chilling conditions.

Most evidence points to the lipid classes or component fatty acids as

the main factor involved with the membrane integrity associated with chilling

and freezing tolerance, but evidence has also been presented invoking changes

in protein (Shomer-Itan & Waisel, 1975) and nucleic acid (in black locust:

Siminovitch et al., 1968) components.

In the light of the work outlined above, an attempt was made in the present work to examine whether chilling injury resulted from a phase transition in the lipid components of cellular membranes, and whether tolerance was associated with either an inherent higher in the level of unsaturated fatty acids or from the ability to undergo such an increase upon chilling. Evidence in support of such a theory has previously always come from comparison between different species or varieties, but here we have the position where cell lines of the same origin but of differing levels of tolerance can be compared. Two approaches were used. The first of these was to record the response to chilling of the respiration of isolated mitochondria from chilling sensitive and chilling tolerant lines. A sharp discontinuity in the Arrhenius plot for mitochondria from a sensitive cell line, and the absence of such a discontinuity in the plot for mitochondria from a tolerant line would support the view that in the tolerant line as opposed to the sensitive line a phase change in the mitochondrial membranes did not occur over the temperature range tested. Tightly coupled mito-chondria have been isolated from cell suspensions of Acer pseudoplatanus (Wilson, 1971) and both this method and that of Raison and Lyons (1970) yielded tightly coupled mitochondria from actively growing suspensions of C. annuum. This approach was restricted to cell lines of C. annuum.

The second approach involved the isolation of lipids from the different cell lines, followed by an assessment of the proportions of the various component fatty acids. It was hoped that extractions from cultures maintained at $25^{\circ}C$ and from cultures subjected to chilling would yield differences which might be interpreted in the light of the theory of tolerance already discussed. Lipids and their component fatty acids have already been studied in plant tissue cultures (Tattrie & Veliky, 1973; Staba et al., 1971; Spener et al., 1974; Radwan et al., 1974; and Radwan et al., 1975). Tattrie and Veliky reported similar fatty acid compositions to those found in the leaves of the species under examination, but the other workers found some differences, for

example, a reduction in the level of sulfolipid in culture (Staba et al., 1971) and the occurrence of very long chain (up to 26 carbon atoms) fatty acids (Radwan et al., 1974). The most rigorous of these studies involved separation and identification of the lipids by 2-dimensional thin layer chromatography (Radwan et al., 1975) and indicated that the earlier conclusion that the lipid distribution of tissue cultures resembled that of plant organs was incorrect and based on insufficiently sensitive means of identification. They found very much higher levels of sterols and steryl derivatives, and lower levels of phospholipids and galactolipids than is typical of plant organs, and related this to the heterotrophic mode of nutrition in higher plants.

A number of possible mechanisms for salt resistance have been proposed (Levitt, 1972) including (i) avoidance mechanisms such as salt exclusion, salt excretion, and dilution within the cell, and (ii) tolerance due to ion secretion into the vacuole or to the cells becoming modified in order to be able to function despite the increased ion balance strain. In certain cases, secondary strains may be induced by high salt concentration such as osmotic strain or nutrient deficiency. Tolerance mechanisms against such strains might involve the accumulation of organic solutes (osmotic strain) and partial replacement of a deficient nutrient (e.g. K^+) by the abandonment ions in the salt solution (e.g. Na^+). In the present investigation, lack of time prevented examination of the salt resistant lines for the operation of these possible components of the observed resistance. However, differences in the growth form of the culture were examined both at the level of the culture and at the ultrastructural level in an attempt to throw some light on the possible nature of resistance in these cultures.

EXPERIMENTAL

(A) Respiratory activity of mitochondria from chilling tolerant and chilling sensitive cell lines of C. annuum.

Two cell lines of C. annuum were chosen for this work; line CN9 (a control line never exposed to chilling conditions and showing little tolerance when tested), and line CV34 (iii) (one of the most highly tolerant lines). For extractions of mitochondria the suspensions were grown up in 1 l. bottles containing 500 ml medium, incubated on a rotary shaker in the usual way. These bottles were inoculated by pouring one complete flask of suspension (60 ml) at stationary phase (21 days) into each bottle giving an inoculation density of about 2×10^5 cells ml^{-1}. Prior to extraction, all suspensions were washed and macerated as described by Wilson (1971). In a number of preliminary experiments two methods of extraction were tried; those of Raison & Lyons (1970) and Ikuma & Bonner (1967). Both these methods involved four centrifugations, but differed slightly in the speeds and times of centrifugation and in the natures of the maceration and wash medium.

Both the above methods of extraction were capable of giving coupled mitochondria but greater yields and tighter-coupling were usually obtained using the method of Raison & Lyons. Changes in some of the centrifuge speeds failed to yield more pure mitochondrial preparations and some of the activity was always lost during the second low-speed spin. The method, as described by Raison & Lyons, was used in all the isolations used in the experimental work reported below and details of the whole procedure, including the determination of mitochondrial protein, are described in Materials & Methods.

(i) Respiration of isolated mitochondria at $20^{\circ}C$

The apparatus is described in Materials & Methods. The final volume of the reaction medium was always 3 ml. Mitochondria were added at concentrations

above 2.0 mg mitochondrial protein (see Materials & Methods) per reaction

as a precaution against the anomalous response at low concentrations

reported by Raison & Lyons (1970). NADH at 1.6mM final concentration or

succinate at 3mM final concentration were used as substrates. From a

70mM stock solution of ADP (freshly prepared) suitable additions were made

to the electrode chamber. Using both culture lines mitochondria were

prepared from 6,12 and 21 day suspensions and their respiratory activity

was examined. For each time after subculture the experiment was repeated

several times. Differences were found between the mitochondria isolated

from cultures of different ages as shown by the polarigraphic traces in

Fig. IV.1. and the results of one experiment tabulated in Table IV.1. The

two cell lines had a similar respiratory activity using both substrates.

There was also very little difference in respiratory activity between

mitochondria isolated at 6 and 12 days after subculture. In these isolations

the rate of state 4 (ADP independent; Chance & Williams, 1955) respiration

was of the same order using both substrates, but the respiratory control

ratio was higher where NADH was used as the substrate, due largely to the

enhanced rate of state 3 (ADP dependent) respiration. Preparations from

21 day suspensions showed similar rates of state 3 and state 4 respiration,

regardless of substrate, and similar respiratory control ratios which were

lower than those of mitochondria from actively growing suspensions, due to

reduction in the state 3 rate. Fig. IV.1. compares the respiration of

mitochondria from 6 and 21 day suspensions of both cell lines, using NADH

as substrate.

During these studies there was considerable variation and occasional

preparations with low respiratory activity and little or no respiratory

control were encountered from cultures of all ages. For further experiments

mitochondria were therefore always extracted from suspensions 6 to 12 days

Fig. IV.1   Oxygen uptake by mitochondria isolated from C. annuum lines CN9

and CV34 after 6 and 21 days incubation, using NADH as substrate.

Line CN_9, mitochondria isolated, A, after 6 days and B, after

21 days incubation

Line CV34, mitochondria isolated, C, after 6 days and D, after

21 days incubation

Additions: -

NADH all additions 1.6 mM

ADP all additions 140 n mole

Fig. IV 1
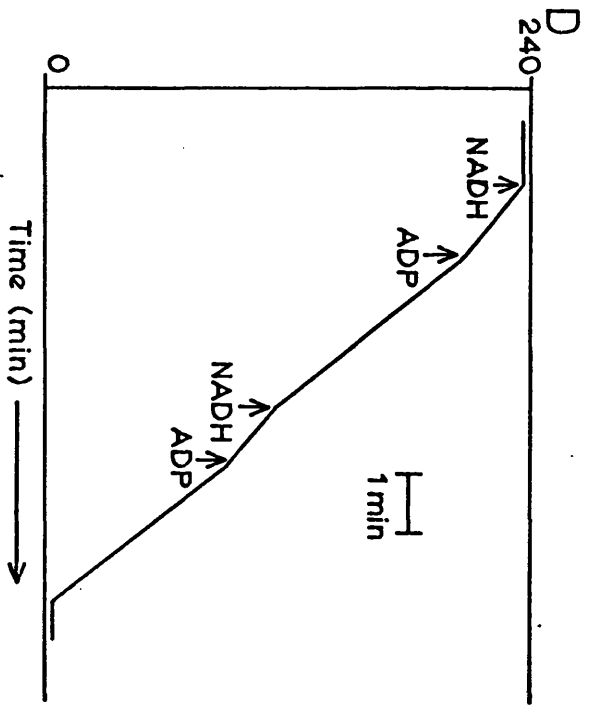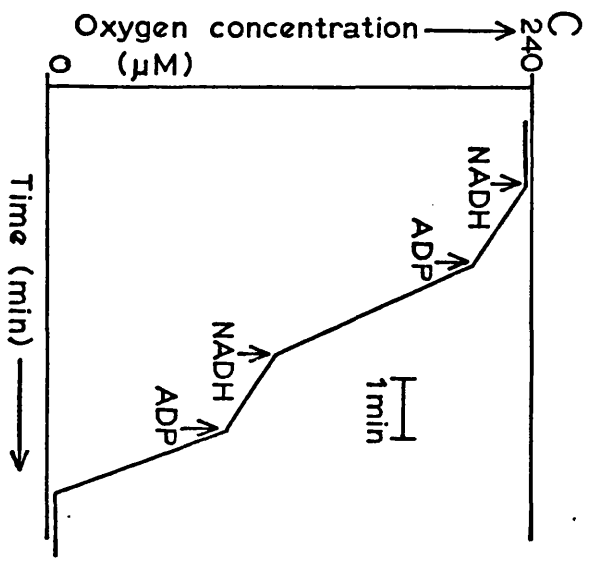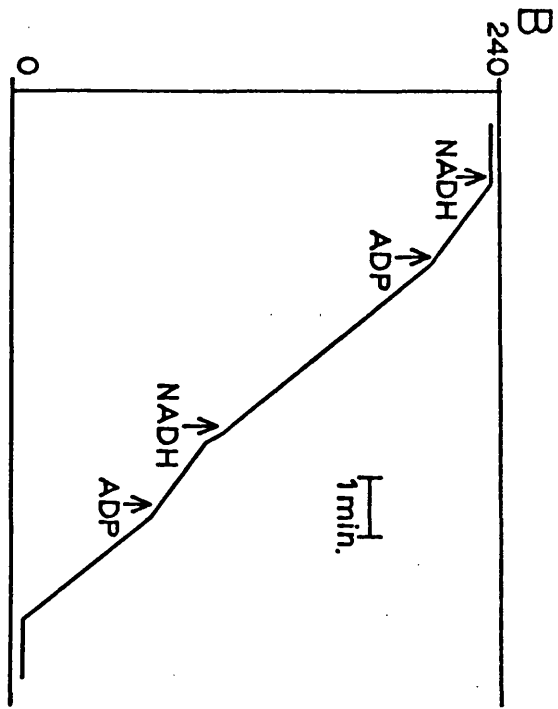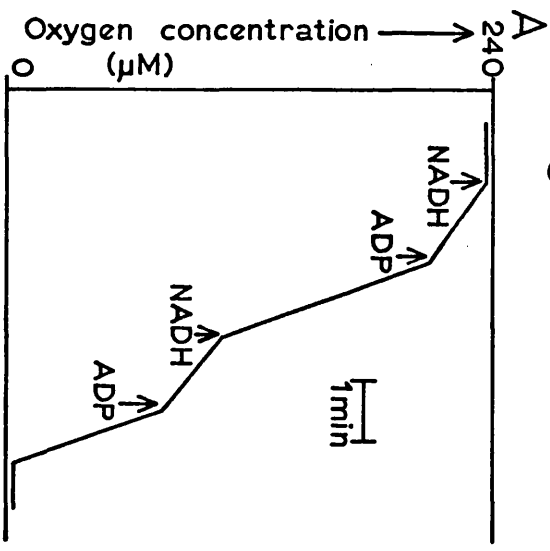
TABLE IV.1.

A comparison of the respiratory activities at $20^{\circ}C$ of mitochondria isolated from C. annuum cell lines, CN9 and CV34 at different times after subculture.

		Time from subculture (days)					
		CN9			CV34		
		6	12	21	6	12	21
Succinate oxidation (n mole O_2/min/mg protein)	State 4	56	59	49	62	55	58
	State 3	95	88	63	102	97	71
Respiratory control ratio:		1.7	1.5	1.3	1.6	1.8	1.2
NADH oxidation (n mole O_2/min/mg protein)	State 4	59	55	54	63	52	59
	State 3	125	114	72	117	99	76
Respiratory control ratio:		2.2	2.1	1.3	1.9	1.9	1.3

Values are taken from the second state 3 and second state 4.

after subculture and only preparations showing good respiration rates with NADH, and a high level of respiratory control were used in the chilling experiments. The effect of using levels of mitochondrial protein ranging from 2 to 10 mg per reaction was examined using isolations from 6 day suspensions of both lines and the results are plotted on Fig. IV.2. There is little change in the rate of respiration over this range of protein concentration. This is a useful observation as in subsequent experiments mitochondria were always used at concentrations within this range, but often the exact concentration was not determined until after the experiment. All experiments were performed within 2 hours of extraction of the mitochondria. During this time they were kept at room temperature and at the termination of the experiment their respiratory activity was checked at 20°C. Intact mitochondria generally maintained a high level of respiratory control over this period. Romani et al. (1974) have shown that plant mitochondria can be maintained intact for 4 days at 25°C but this was not found to be the case for the mitochondria from suspension cultures of C. annuum. Preparations kept overnight at 25°C exhibited only very slow oxygen uptake and no respiratory control.

Having established suitable conditions for the isolation of the mitochondria and the examination of their respiration, the effect of chilling on this respiration was studied. The details of the means by which the oxygen electrode was chilled are given in Materials & Methods, along with details of the calibration to counteract such effects as the temperature dependence of the solubility of oxygen and the sensitivity of the electrode. The experiments were performed in two ways. The first involved the examination of the polarigraphic trace as the mitochondria were slowly and continuously chilled and warmed, and the second involved maintaining the temperature constant at several key temperatures while measurements of respiration could be made. For both series of experiments NADH was used as the substrate throughout.

Fig. IV.2 The relationship between protein concentration and succinate
          oxidation in mitochondria isolated from 6 day suspensions of
          C. annuum line CN9.

          Values are succinate oxidation (n mole O_2/min/mg protein) during

          steady states 3 (●) and 4 (O) of respiration.

Fig. IV 2
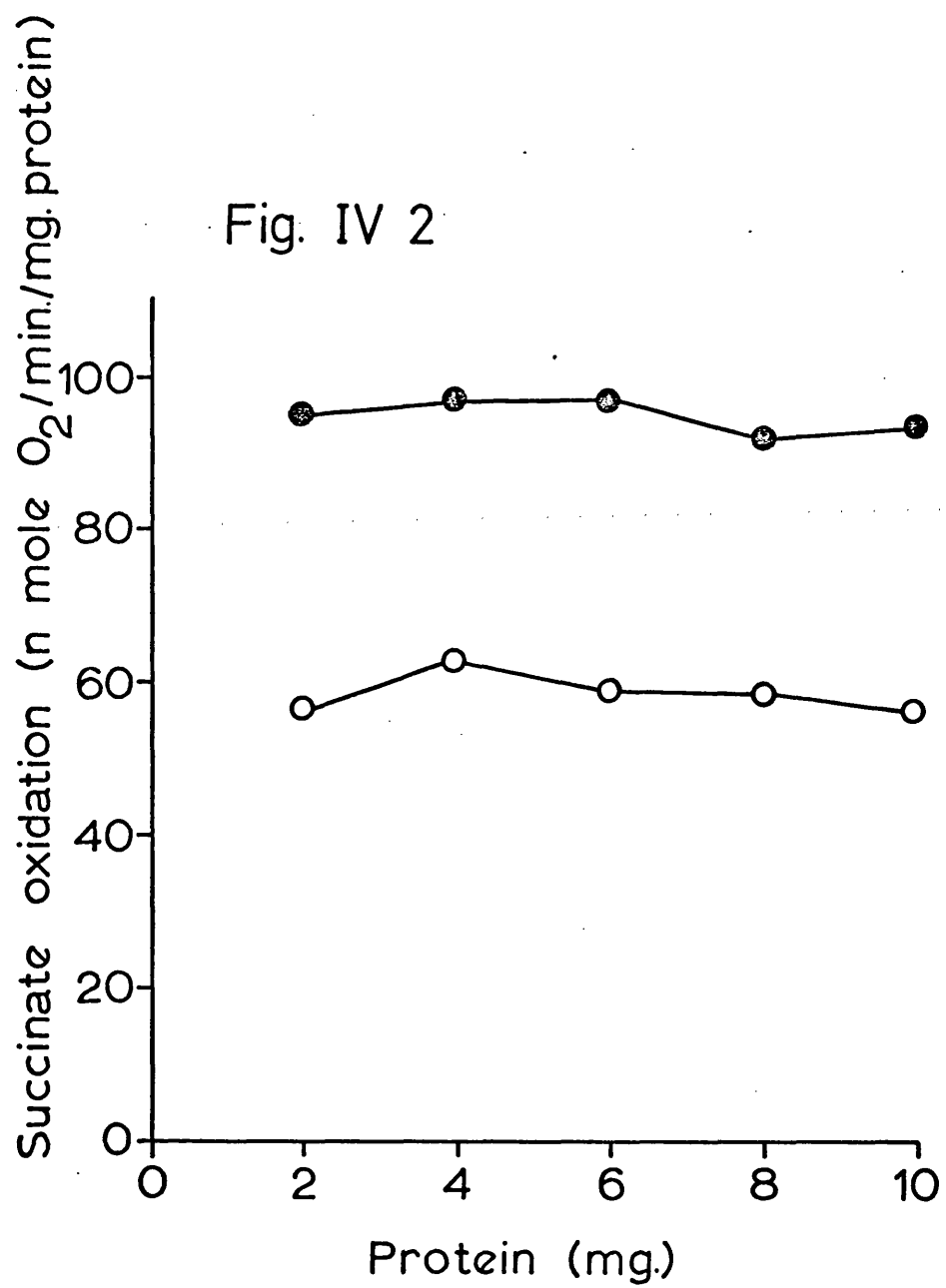
(ii) The effect of a continuous chilling regime on the rate of respiration
of isolated mitochondria

Only state 3 respiration was used in these experiments. 2 to 10 mg

mitochondria were added to the reaction medium followed by 0.16mM NADH.

To establish state 3 respiration an excess of ADP (700 n mole) was then .

added to ensure that state 3 would be maintained until all the dissolved

oxygen had been taken up. Once a steady rate of oxygen uptake had been

achieved at 20°C chilling was commenced. As the temperature reached 19.5°C

the chart event marker was depressed, and thereafter an "event" was noted

with every 1°C decrease in temperature in the electrode chamber (i.e. at

19.5°C, 18.5°C, etc.) down to the minimum which could be conveniently reached

with this apparatus (this varied between 4.5°C and 6.5°C). With each

mitochondrial isolation this experiment was repeated several times. Often

the complete temperature range could not be covered before the dissolved

oxygen became exhausted, but the repetitions covered portions of the

temperature range in such a way that at least one reading could be obtained

for each temperature. The curves obtained had a steep initial gradient

which gradually lessened as the temperature was reduced. The respiration at

individual temperatures was determined as shown on Fig. IV.3. The points at

which successive temperature events crossed the trace were joined with

straight lines, the gradients of which were taken as representing the

respiration rates at the midpoints between the successive temperature events

(i.e. at 19°C, 18°C, etc.). These gradients were converted to actual n mole

oxygen per unit time using the calibration curve given in Materials & Methods

(Fig. M.1.), and from these values state 3 respiration rates per mg mito-

chondrial protein could be determined for the complete range of temperatures.

These experiments were repeated several times with each of the two C. annuum

cell lines CN9 and CV34. In most cases increasing temperature regimes were

Fig. IV.3   Determination of respiration rates at individual temperatures

from the trace obtained from a continuous chilling experiment

This is a stylised trace, very much expanded and showing a larger

change in gradient than was normally found over this small temper-

ature range. Gradients were used to determine the real respiration

rate (n mole O_2/min/mg protein)

$a/_b$ = gradient for $19^{\circ}C$

$c/_d$ = gradient for $18^{\circ}C$

Fig. IV. 3.
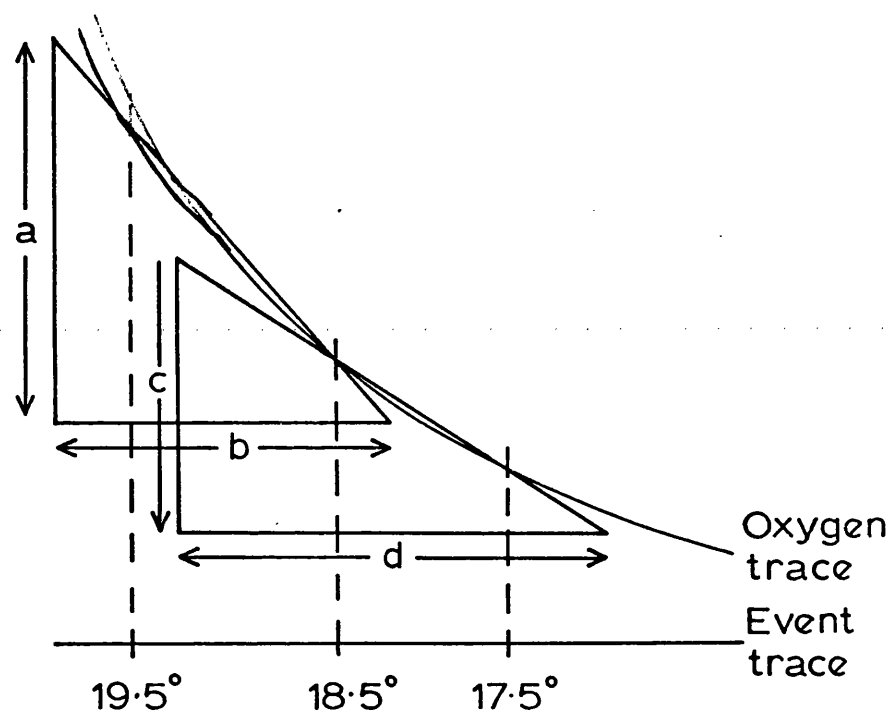
also tested but the curves obtained exhibited pronounced hysteresis effects, reacting only very slowly to the increasing temperatures until temperatures between 17 and 20°C were reached when there would be a burst of renewed respiratory activity. Only data accumulated from decreasing temperature regimes were used to compile Arrhenius plots ($\log_{10}$ respiration vs. reciprocal of absolute temperature). These plots are given on Fig.IV.4. (line CN9) and Fig.IV.5. (line CV34). The plots incorporate all the data from three or four experiments with each line, using batches of mitochondria isolated on separate occasions.

The plot for the sensitive line, CN9, shows a marked increase in gradient when the temperature gets down to 8°C, as expected from the results obtained by other workers and discussed in the introduction. The plot for the chilling tolerant line CV34 is more difficult to interpret. The data from two of the experiments confirm the theory that there is no sudden discontinuity in respiration at 8 to 10°C, but a third experiment, while giving comparable rates at higher temperatures gave lower and extremely erratic respiration rates below 12°C. Where the mean values are plotted there is no temperature at which the reduction of respiration rate with temperature can be seen to suddenly increase and the data is considered to support the view that this chilling tolerant line does not undergo the same changes, resulting in a sharp discontinuity in the respiration rate, as the sensitive line.

(iii) The use of a discontinuous chilling regime to study the effect of chilling on the respiration of isolated mitochondria.

The procedure in these experiments was the same as described above except that the heating and cooling elements of the apparatus (see Materials & Methods) were adjusted in order to maintain the temperature in the electrode chamber constant at each of several selected temperatures for long enough for

<u>Fig. IV.4</u> <u>Arrhenius plot for state 3 NADH oxidation by mitochondria isolated</u>

<u>from chilling the sensitive cell line, CN 9, of C. annuum.</u>

The different symbols represent data from independent experiments.
The curve drawn is a line connecting the means of the values
accumulated at each temperature.

The data were accumulated using continuous chilling regimes (p 84).

Fig. IV 4

Fig. IV.5 <u>Arrhenius plot for state 3 NADH oxidation by mitochondria isolated</u>

<u>from the chilling tolerant cell line, CV34, of C. annuum.</u>

The different symbols represent data from independent experiments.
The curve drawn is a line connecting the means of the values
accumulated at each temperature.

The data were accumulated using continuous chilling regimes (p 84).
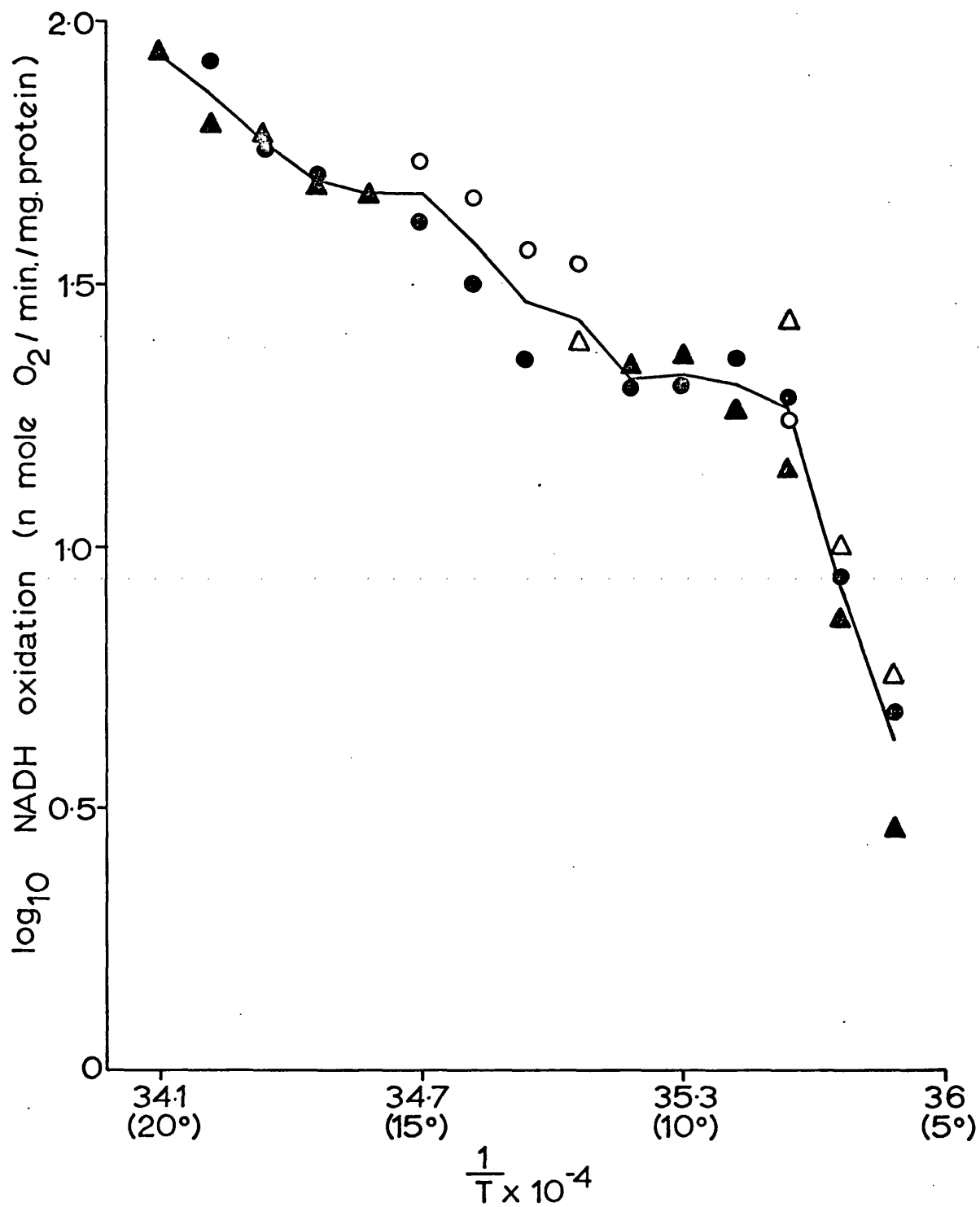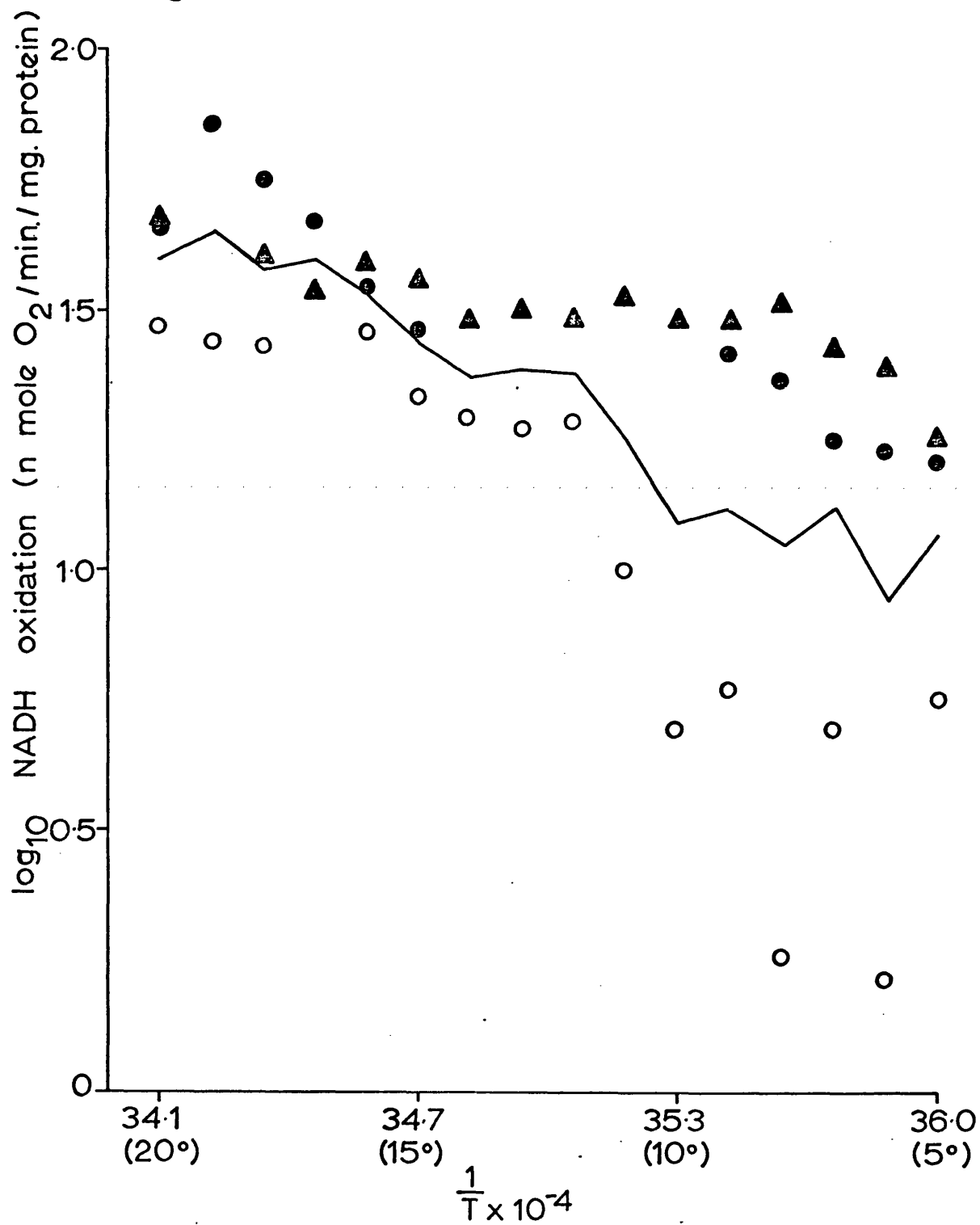
Fig. IV 5

steady respiration rates to be achieved (3 to 15 minutes). This compensates for any delay in the response of the mitochondria to changing temperatures, and allows greater lengths of trace to be used in calculating the rates. Arrhenius plots of data from a series of these experiments are shown on Fig. IV.6. (line CN9) and Fig.IV.7. (line CV34). In these cases, approximate lines of best fit are applied to the data in the form of one or two straight lines.

The data support that from the previous series of experiments. Again there are distinct differences between the two cell lines of the type reported by other works for mitochondria from chilling sensitive and chilling tolerant tissue. The main differences between these data and those from the experiments described in Section IV.A. (ii) are (i) the greater reproducibility of the results for experiments with the tolerant line, (ii) the difference in the point at which the discontinuity occurs in the respiration of mitochondria from the sensitive line ($10^{\circ}C$ instead of $8^{\circ}C$) and (iii) in that the gradient at temperatures below this critical temperature is less steep. These differences between the results of the two experimental procedures are considered in the discussion at the end of this section.

(B) Fatty acid compositions of lipids extracted from chilling sensitive and chilling tolerant cell lines of C. annuum and N. sylvestris.

The methods used in the extraction of total lipids are described in Materials & Methods. All the data presented in this section are based on extracts prepared using method A, i.e. a chloroform/methanol extraction followed by a saline wash, but with no pre-extraction with iso-propanol. A number of key samples were also prepared using the pre-extraction with iso-propanol (method B) and their fatty acid compositions compared with those prepared by method A. The purpose of the iso-propanol step was to inactivate lipases which might otherwise be able to function in the chloroform/

Fig. IV.6 <u>Arrhenius plots for NADH oxidation by mitochondria isolated from the</u>

<u>chilling sensitive cell line, CN9, of C. annuum.</u>

The different shaped symbols represent the results of three different

experiments.

Closed symbols: state 3 respiration

Open symbols: state 4 respiration

Approximate lines of best fit were drawn through the points, in the

form of two straight lines.

The data were accumulated using discontinuous chilling regimes (p 85)

Fig. IV 6
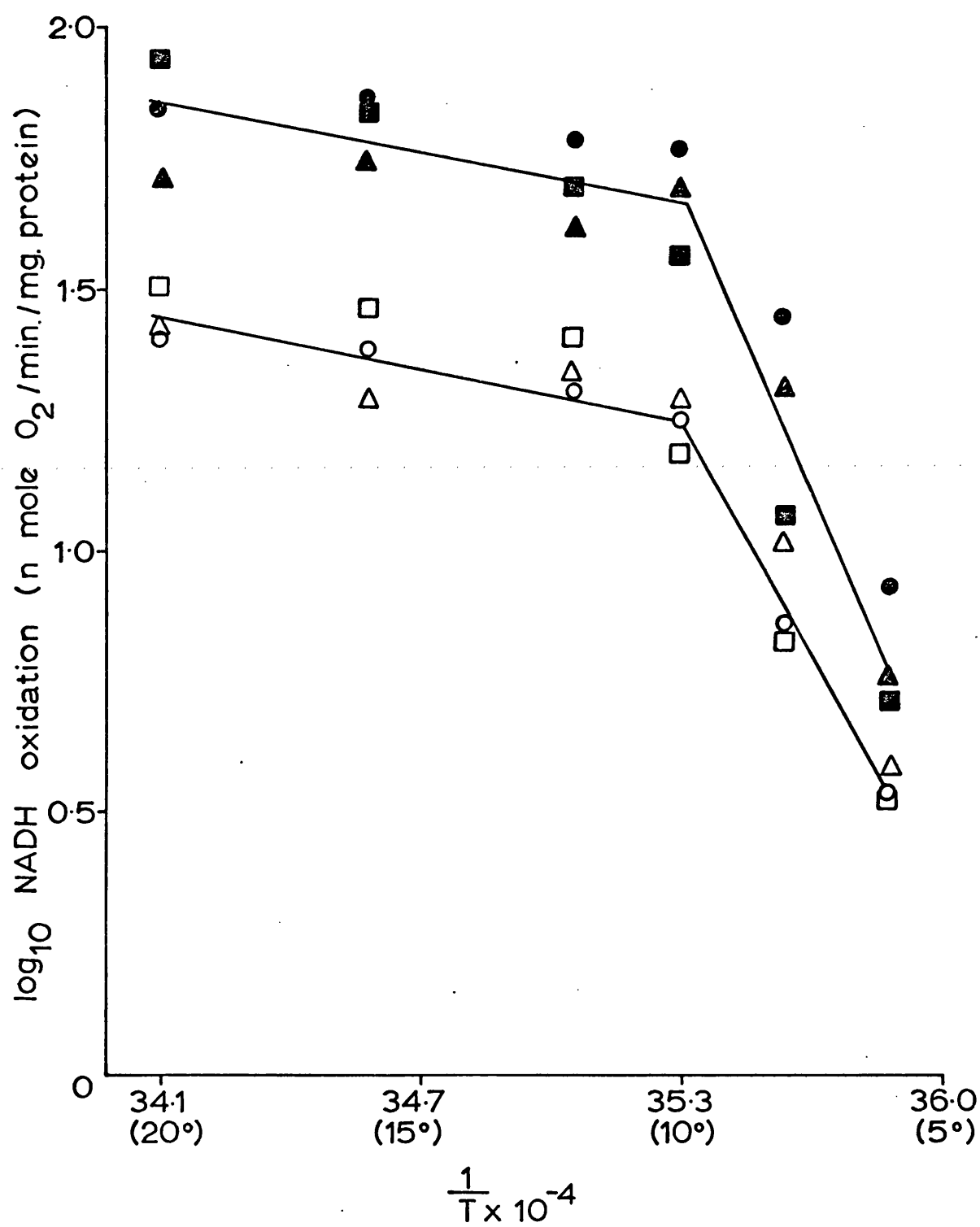
Fig. IV.7 Arrhenius plots for NADH oxidation by mitochondria isolated from the chilling tolerant cell line, CV34, of C. annuum.

The different shaped symbols represent the results of three different experiments.

Closed symbols : state 3 respiration

Open symbols : state 4 respiration

Approximate lines of best fit were drawn through the points, in the form of single straight lines.

The data were accumulated using discontinuous chilling regimes (p 85).

Fig. IV 7
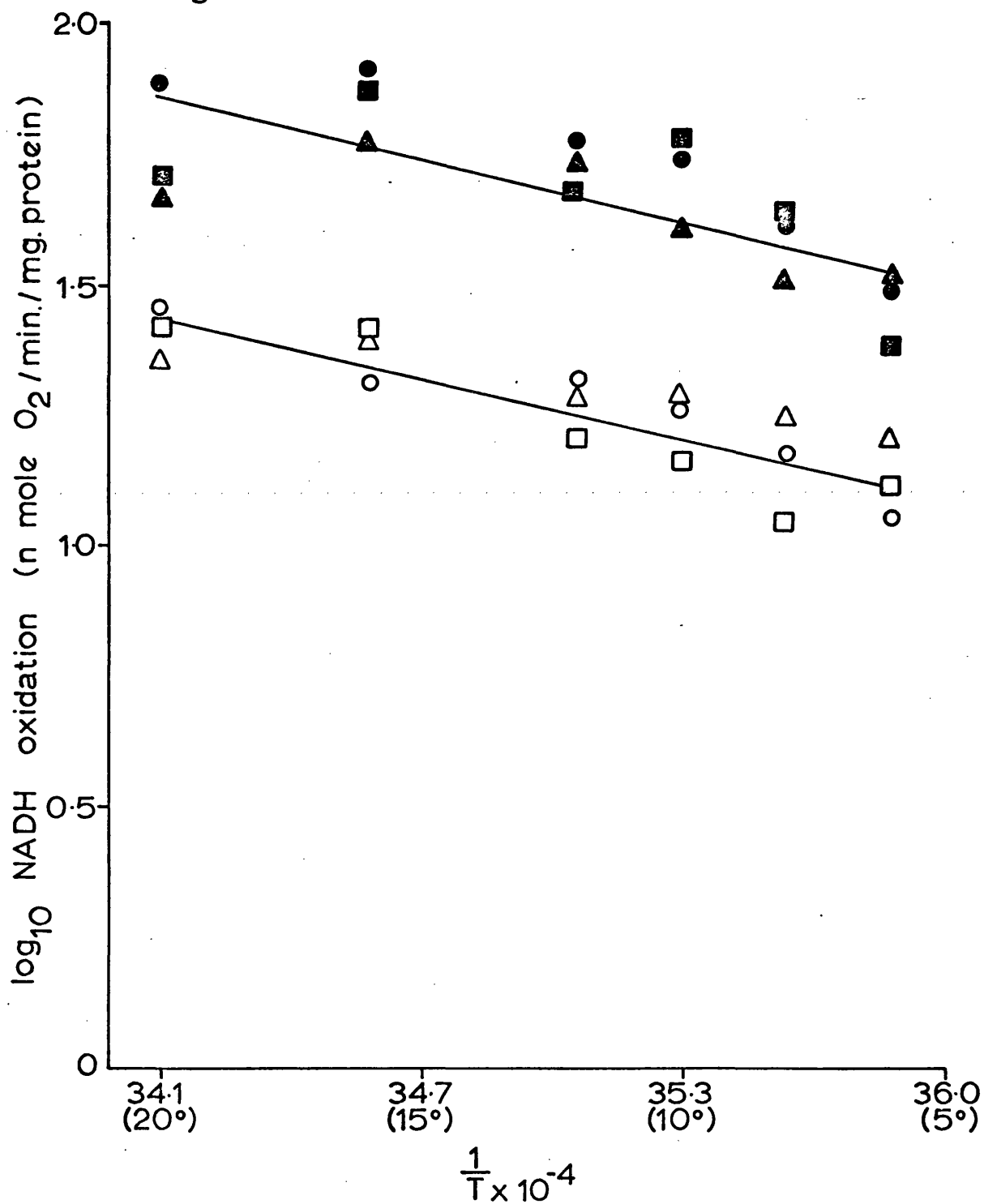
<u>Fig. IV.8</u> <u>GLC traces of methyl esters of fatty acids from the total lipid</u>

extracts of <u>N</u>. <u>sylvestris</u> cell lines, incubated at 25°C.

A. Chilling sensitive line, H_9.

B. Chilling tolerant line, B14.

C. Chilling tolerant line, B35.

D. Chilling sensitive line, H_{13}.

The positions of peaks corresponding to palmitate (16:0), stearate (18:0), linoleate (18:2) and αlinolenate (18:3) are indicated.

The loadings varied between the traces.

Fig. IV. 8.
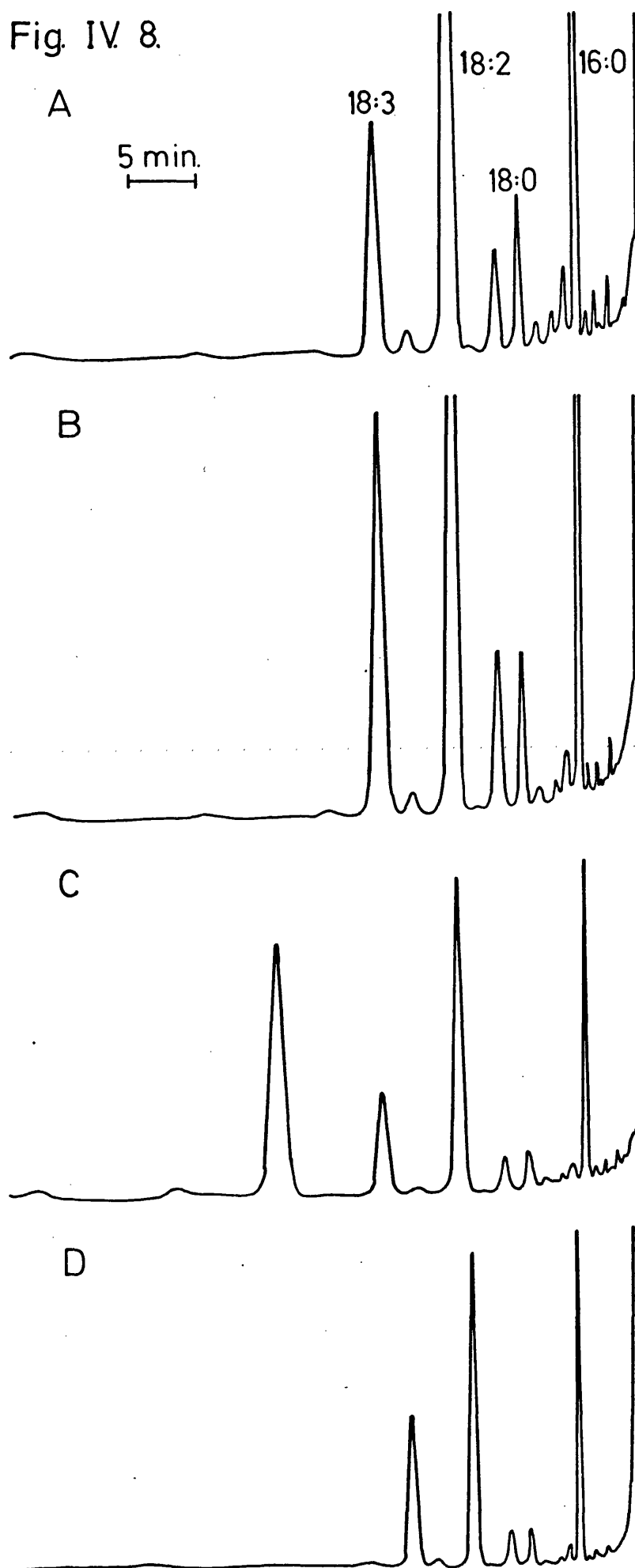
Fig. IV.9 GLC traces of methyl esters of fatty acids from the total lipid

extracts of $\underline{C}$. annuum, incubated at 25°C.

A. Chilling sensitive line, CN2.

B. Chilling sensitive line, CN9.

C. Chilling tolerant line, CV34.

D. Chilling tolerant line, CV45.

The positions of peaks corresponding to palmitate (16:0), stearate
(18:0), and linoleate (18:2) are indicated.

The loadings varied between the traces.

Fig. IV. 9.

A

5 min.

18:2

16:0

18:0

B

C

D
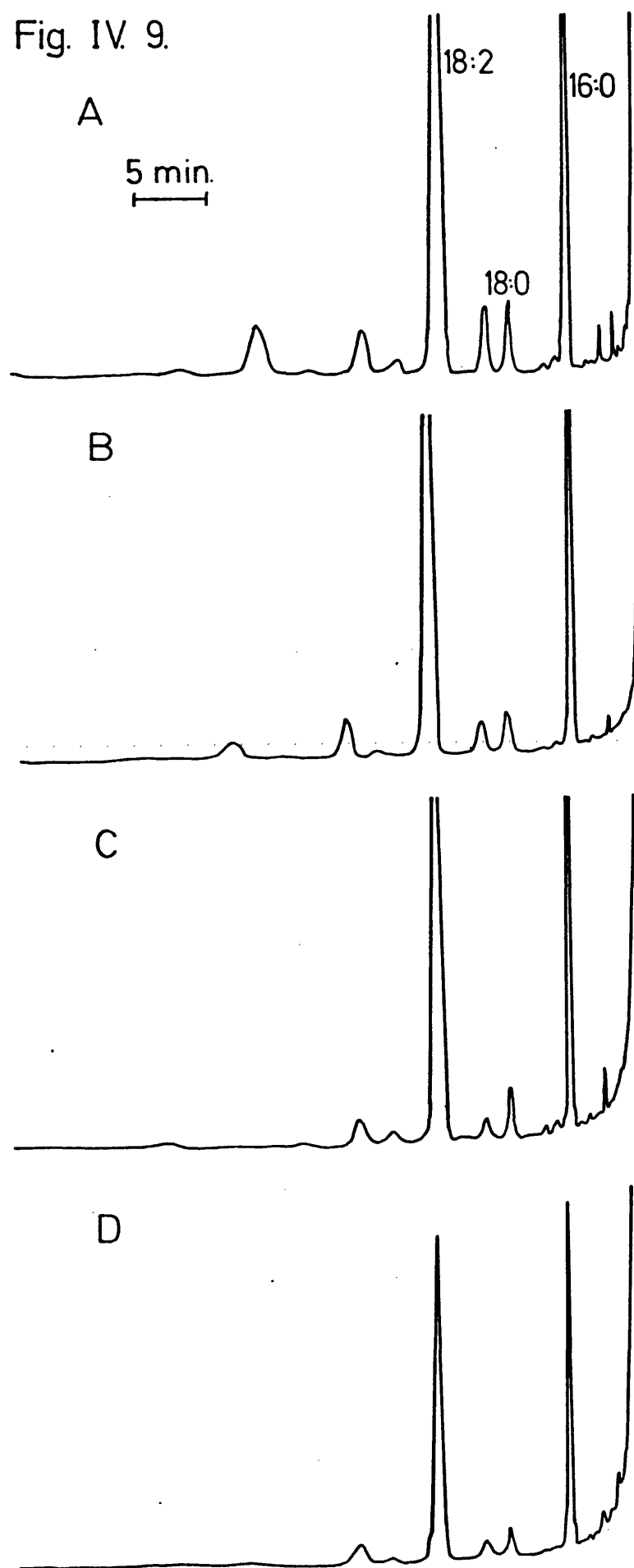
methanol mixture and affect the eventual lipid composition. However, in no case did the extracts obtained by the two methods differ substantially in their fatty acid compositions and the iso-propanol step was subsequently discarded. The transmethylation step was carried out in the same way throughout and the methyl esters of the fatty acids subjected to analysis by gas-liquid chromatography as described. No attempt was made to determine the absolute amounts of lipid and different lipid classes in the extracts, this study being confined to the proportions of different fatty acids in the total lipid fraction.

Two chilling tolerant cell lines of each species were chosen, together with chilling sensitive control lines, for comparison in this way, in addition to a freshly initiated and highly morphogenic line of N. sylvestris. The cell lines studied were as follows: -

N. sylvestris: B14) Chilling tolerant lines
       B35)

       H9 Chilling sensitive control
       H13 Freshly initiated haploid line (highly morpho-
           genic)

C. annuum: CV34) Chilling tolerant lines
      CV45)

      CN2) Chilling sensitive controls
      CN9)

These lines were studied both under routine culture at 25°C, and when exposed to a chilling and warming regime.

(i) <u>Fatty acid compositions of lipids from chilling tolerant and chilling sensitive lines during culture at 25°C.</u>

Cell suspensions incubated in 1 l. bottles were harvested at 6 to 12 days after subculture.

Approximately 20 g. fresh weight of tissue were used in each extraction. The GLC loading of the samples was varied to give about a full scale deflection of the peak corresponding to palmitate (16:0), but the exact level of loading

was unimportant, the computer analyses of peak areas being independent

of the actual GLC trace. Representative traces are shown in Figs. IV.8.

(for N. sylvestris lines) and IV.9. (C. annuum lines) and the appropriate

fatty acid compositions are given in Tables IV.2. and IV.3. respectively.

In these tables all fatty acids comprising less than 1% of the total are

classed together under "others" unless the individual percentage is

important for the comparisms. The nomenclature used for the different

fatty acids is described under Table IV.2. Peaks not corresponding to

acids in the mixture of standards on which the programme was based are

indicated with a letter (e.g. a,b). Where possible a tentative identific-

ation of the peaks was made using argentation TLC (see Materials & Methods).

The distributions for all the C. annuum cell lines (Table IV.3.) are

very similar, the bulk of the fatty acids comprising palmitate and an

18 carbon diene (probably linoleate). There are only slight differences

between the chilling sensitive and chilling tolerant lines, for example,

the latter show reduced levels of the unidentified long chain fatty acid, b,

and correspondingly higher proportions of 18:2. Chilling sensitive lines

H9 and H13 and chilling tolerant line B14 of N. sylvestris have similar

fatty acid compositions and they differ from those of C. annuum only in

having a far higher proportion of 18:3 (α - linolenate). This fatty acid is

largely associated with chloroplasts and may relate to the higher proportion

of differentiation in the N. sylvestris lines. Line H13 was highly morpho-

genic, and line B14 showed more frequent signs of shoot formation than line

H9, so the proportions of 18:3 in these three lines do appear to reflect these

differences. Chilling tolerant line B35 had a very different pattern of

fatty acid composition, the most abundant being the long chain acid b (thought

to be 22:0). The other major peaks were all reduced by about the same

proportion. This distribution was based on a single extract as the line

was lost through bacterial contamination shortly afterwards. In view of the

TABLE IV.2.

Percentages of component fatty acids of the total lipids of cell lines
of N. sylvestris

Fatty acids*	H9 (sensitive control)	B14 (tolerant line)	B35 (tolerant line)	H13 (freshly initiated line)
16:0	19.5	22.3	12.2	25.2
a	2.0	1.2	0.6	1.2
18:0	5.7	4.2	2.3	3.4
18:1	5.1	6.0	2.3	4.3
18:2	44.5	40.4	25.3	37.7
18:3	16.0	20.7	11.3	23.4
b	0	0	38.0	0
Others	8.0	5.2	8.0	4.8

Figures are the relative quantities of individual fatty acids expressed as
percentages of the total.

* First figure indicates number of carbon atoms. Second figure

  indicates number of ethylenic bonds.

  Examples: 16:0 is 16 carbon saturated acid (palmitate)

               18:0 „ 18   „   „   „ (stearate)

               18:1 „ 18   „   monounsaturated acid (e.g. oleate)

               18:2 „ 18   „   diunsaturated acid (e.g. linoleate)

               18:3 „ 18   „   triunsaturated acid (e.g. α-linolenate)

Peak identification:

Using argentation thin layer chromatography (see Materials & Methods)
unidentified peaks were tentatively assigned as follows:

      a   16 carbon monounsaturated acid (16:1)

      b   22 carbon saturated acid (22:0)

TABLE IV.3.

Percentages of component fatty acids of the total lipids of cell lines
of C. annuum.

Fatty acids*	CN2 (sensitive control)	CN9 (sensitive control)	CV34 (tolerant line)	CV45 (tolerant line)
16:0	28.9	27.0	26.9	27.5
18:0	2.9	3.2	3.3	4.0
18:1	2.7	2.8	2.4	2.5
18:2	52.0	52.7	56.5	56.2
18:3	4.6	5.4	4.1	5.2
b	5.1	4.4	0.2	0
Others	3.8	4.5	6.6	4.6

Figures are the relative quantities of individual fatty acids expressed
as percentage of the total of the mixture.

* For explanation of nomenclature and tentative identification of
component b see Table IV.2.

great difference between this extract and any others examined, it would have been desirable to repeat it. There are no marked differences between the fatty acid compositions of the other chilling tolerant line and the chilling sensitive lines.

(ii) Fatty acid compositions of lipids from chilling tolerant and chilling sensitive cell lines extracted during chilling and after re-warming.

Lipids were extracted after chilling for 21 days (at -3°C for N. sylvestris lines, 5°C for C. annuum lines) and 6 hours after the return to 25°C following the chilling treatment. The aim was to examine the possibility that changes in the overall fatty acid composition might be induced in the tolerant lines by the chilling process, and that such changes might be readily reversible. For exposure to the chilling conditions the medium was removed from suspensions (6 to 12 days after subculture) by filtration through four layers of muslin and the cells thinly spread on the surface of 200 ml. agar medium in 500 ml Erlenmeyer flasks. Two flasks for each cell line were placed under chilling conditions. After 21 days one flask was returned to 25°C while lipids were extracted from the cells in the second flask as described in Materials & Methods, except that the first two hours of the extraction were performed in ice. After 6 hours at 25°C the lipids were extracted from the cells in the other flask at room temperature. The fatty acid compositions of lipids extracted from these treatments are given in Tables IV.4. (N. sylvestris lines)and IV.5. (C. annuum lines). The results for only one sensitive line are given in each case since the other sensitive lines gave very similar distributions.

In neither species do the results show a clear characteristic difference between the chilling tolerant and control lines but in each species one chilling tolerant line gives distributions closely related to the control line, and the other responds very differently. The differences

are of the same type for both these lines (B35, N. sylvestris and C45,

C. annuum) and are best seen by comparison of Tables IV.4. and IV.5.

with Tables IV.2. and IV.3. The main changes apparently induced in

these lines by chilling are increases in the proportions of 18:0 (stearate)

and, to a lesser extent, several shorter chain acids (e.g. 17:0, a and c)

and decreases in the proportion of 18:2 (linoleate) in CV45 and component

b, which completely disappears from B35. The same changes occurred, but

only to a very small extent in chilling sensitive control lines CN2 and

CN9. In all these cases the changes appear to be partly reversible on

warming and maintenance at 25°C for 6 hours. The peaks corresponding to

18:0, 17:0, a and c decrease and the peak corresponding to 18:2 increases

so the distributions approach those of the extractions from cells maintained

at 25°C. The exception is that component b has not reappeared after this

period, in B35 and the increase is in the proportion of 18:2 as in the

other lines.

(C) A comparison of the morphology and ultrastructure of cell lines
sensitive and resistant to sodium chloride.

As indicated by cell number and packed cell volume data (Figs. III.3.,

III.4., and III.5.) for the cell suspensions, the NaCl resistant lines

differ in some aspects of their growth (particularly mean cell size) when

grown in the presence and absence of NaCl. On the basis of this the

growth forms of these suspensions were compared with each other and with

sensitive lines in the absence of NaCl. In addition to this, some of the

C. annuum lines were examined for ultrastructural changes which might

relate to the increased NaCl resistance.

TABLE IV.4.

Fatty acid compositions of lipids extracted from N. sylvestris cell lines
after 21 days chilling, during the chilling regime (-3°C) and after
re-warming for 6 hours (-3°C $-$ 25°C)

Fatty acid	-3°C			-3°C-25°C		
	H9 (sensitive)	B14 (tolerant)	B35 (tolerant)	H9 (sensitive)	B14 (tolerant)	B35 (tolerant)
16:0	23.4	27.5	28.2	24.9	26.9	22.8
a	1.4	0.8	1.7	1.3	1.0	2.7
17:0	0.2	0.3	1.6	0.3	0.3	1.0
c	0.3	0.6	3.9	0.4	0.6	0
18:0	3.2	4.4	14.8	3.6	6.7	4.3
18:1	4.4	5.4	5.8	4.2	7.1	6.3
18:2	36.2	39.2	17.6	36.8	35.0	37.5
18:3	22.7	17.5	5.5	23.6	13.8	17.8
b	0.2	0	0	0.8	2.7	0
d	2.5	0.4	10.7	0	0.4	0
Others	5.5	3.9	10.2	4.1	5.5	7.6

Figures are the relative quantities of individual fatty acids expressed as
percentage of the total.

Peak identification: -

    For a and b see Table IV.2.

    c is thought to be an unsaturated 16 carbon acid (possibly 16:2)

    d is thought to be a long chain unsaturated acid (possibly 22:1)

TABLE IV.5.

Fatty acid compositions of lipids extracted from C. annuum cell lines after 21 days chilling, during the chilling regime (5°C) and after re-warming for 6 hours (5°C - 25°C)

Fatty acids	5°C			5°C-25°C		
	CN2 (sensitive)	CV34 (tolerant)	CV45 (tolerant)	CN2 (sensitive)	CV34 (tolerant)	CV45 (tolerant)
16:0	32.0	25.3	35.4	28.9	27.9	32.9
a	1.3	0.8	1.7	0.2	0.5	1.5
17:0	0.3	0.4	1.9	0.1	0.1	0
c	0	0	3.5	0	0	0
18:0	3.7	4.4	18.1	2.9	3.7	6.4
18:1	3.3	3.2	5.0	2.7	3.6	5.7
18:2	41.8	52.1	18.3	52.0	55.1	41.6
18:3	4.5	4.3	3.1	4.6	4.2	4.5
Others	13.1	9.5	13.0	8.6	4.9	7.4

Figures are the relative quantities of individual fatty acids expressed as percentages of the total.

Peak identification: -

    For a see Table IV.2.

    For c see Table IV.4.

(i) <u>The appearance of the suspensions</u>

Samples of various suspensions were removed at 9 days after subculture
and examined on cell counting slides. They were allowed to settle on the
slides and photographed. Photographs of <u>N</u>. <u>sylvestris</u> line D, and
<u>C</u>. <u>annuum</u> line C, of various cultural histories are shown on Plate IV, 1.
and Plate IV.2. respectively.

The morphology of the resistant lines growing in the presence of
1% or 2% NaCl is clearly very different to that of the same lines growing in
its absence. In the presence of NaCl they grow predominantly as tight
aggregates containing very small cells. There are some larger cells present
either as single cells or units of several cells, but many of these are
plasmolysed and very few stain with the vital stain flourescein diacetate,
while the large aggregates of very small cells flouresce brightly. In the
absence of NaCl the resistant lines vary in the degree of aggregation but in
all cases most of the aggregates comprise fewer, larger cells and the
suspension resembles far more closely the parent line than the resistant line
grown in the presence of NaCl.

(ii) <u>Ultrastructural changes associated with NaCl resistance.</u>

This comparison was restricted to a resistant cell line of <u>C</u>. <u>annuum</u>
derived by culturing line C for a number of passages in 1% NaCl. To avoid
confusion the resistant line is called CR and the parent, sensitive line is
called CS. CR and CS are in fact the same resistant and sensitive cell lines
of <u>C</u>. <u>annuum</u> as those discussed in section (i) above and samples were removed
at the same time (9 days after subculture) and processed for electron
microscopy as described in Materials & Methods. Sections were cut from
2 to 3 blocks of each sample and photographs taken, and electron micrographs
prepared of as wide a diversity of cell type as could be found within a given
sample, emphasis being placed on the distinctive features of cells of the

PLATE IV.1. Appearance of cell suspensions of N.sylvestris (line D) after

selection for NaCl resistance in suspension.

A. Suspension never exposed to NaCl

B. Suspension selected for resistance by growth for 9 passages

in the presence of 1% NaCl but here growing (10th passage)

in the absence of NaCl

C. Suspension serially subcultured in the presence of NaCl

for 10 passages

D. Suspension selected for resistance by culture for 7 passages

in the presence of 1%, and 4 passages in the presence of 2%

NaCl. Here growing in medium containing 2% NaCl.

x 50
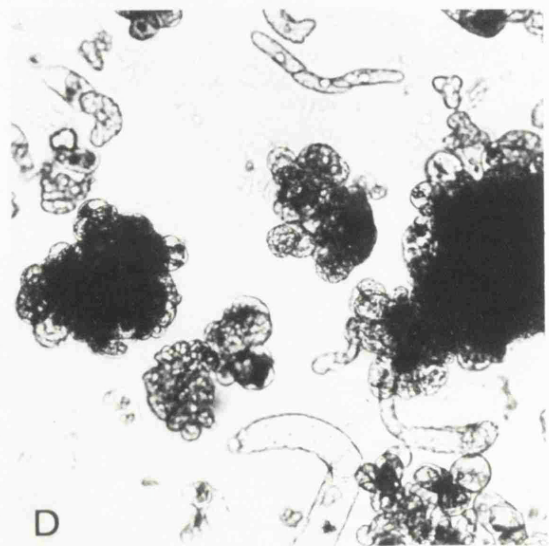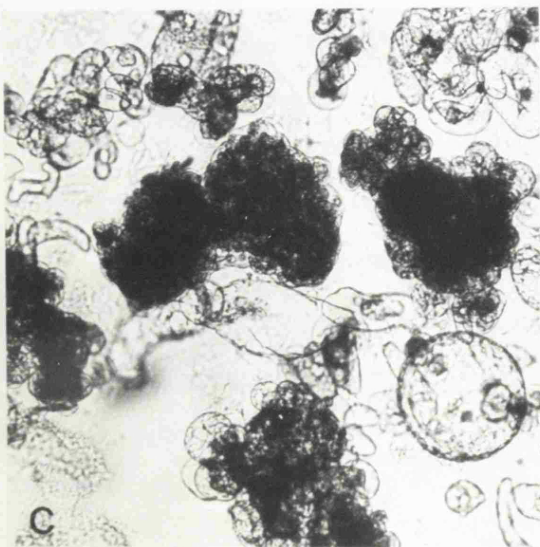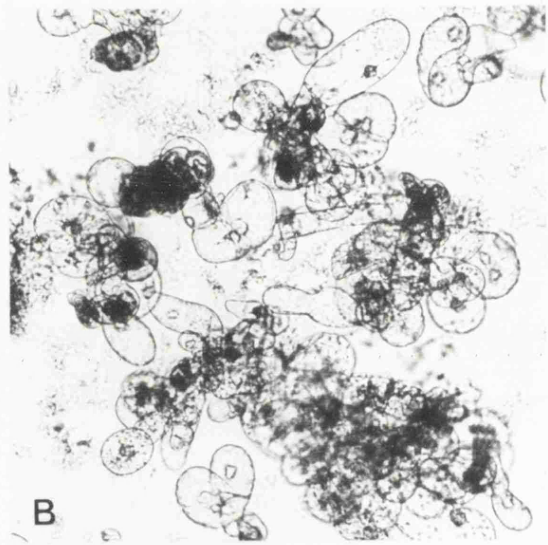
PLATE IV.1

PLATE IV.2. Appearance of cell suspensions of C. annuum (line C) after
selection for NaCl resistance in suspension

A. Suspension never exposed to NaCl

B. Suspension selected for resistance by growth for 5 passages
in the presence of 1% NaCl but here growing (6th passage)
in the absence of NaCl

C. Suspension serially subcultured in the presence of NaCl
for 6 passages

D. Suspension selected for resistance by culture for 4 passages
in the presence of 2% NaCl. Here growing in medium
containing 2% NaCl.

x 50

PLATE IV.2

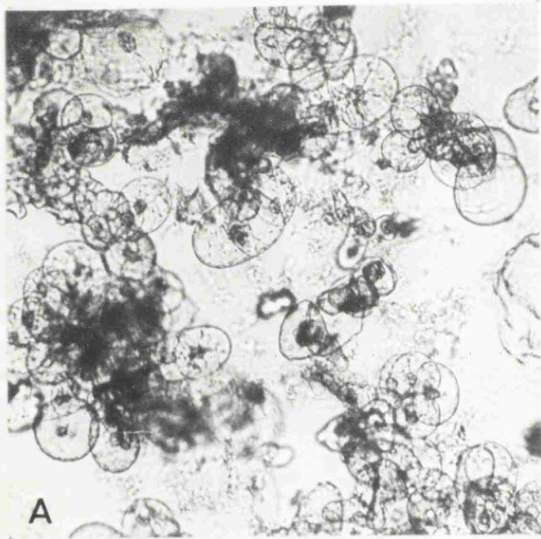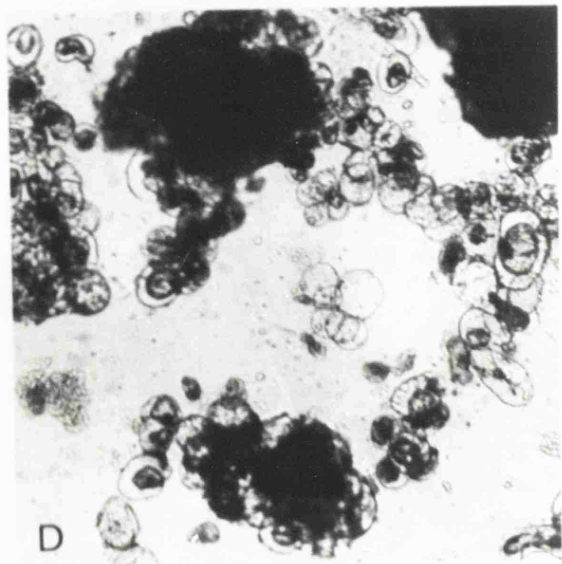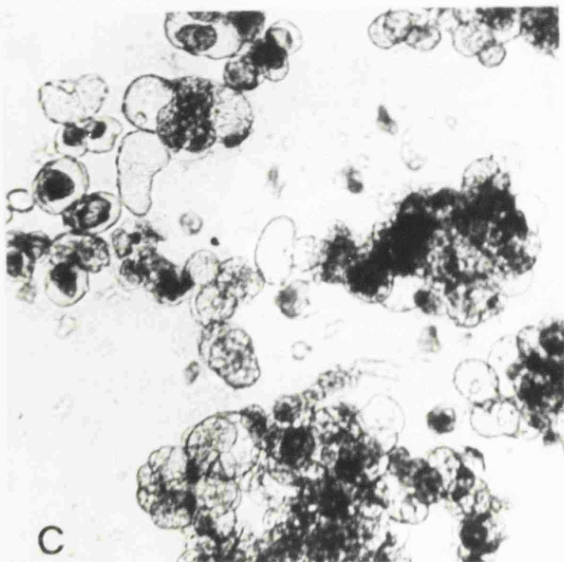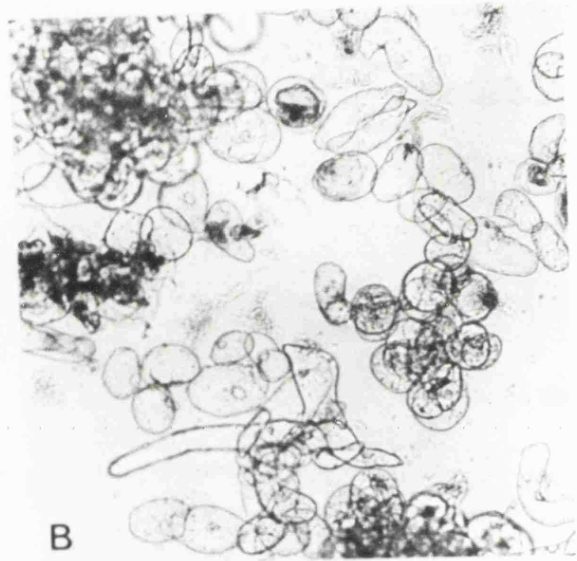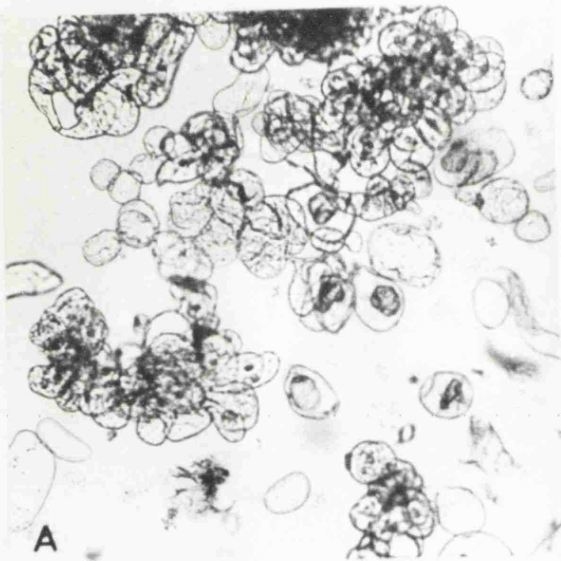different cultural histories. Some of these electron micrographs are shown on the following plates.

There are marked differences in the appearance in thin sections of the cells from the three samples. The cells of the sensitive line, CS, never grown in the presence of NaCl. (Plate IV.3.) are large, highly vacuolated and contain only a thin layer of cytoplasm adpressed to the cell wall, except where the plasmolemma is invaginated into secondary vacuoles (Mahlberg et al., 1974) (A and B). These vacuoles comprise a variety of membranous structures resembling lomasomes (Sutton-Jones & Street, 1968). The cytoplasm contains many osmophilic bodies (A and D), compact mitochondria (C), and amyloplasts (B), but not many dictyosomes or visible plasmodesmatal connections. There is also some electron dense vacuolar material (E). The nuclei tend to be elongate and flattened against the cell wall (D).

The resistant line CR, growing in the presence of NaCl (Plate IV.4), on the other hand, comprises mostly smaller cells which are highly cyto-plasmic containing few small vacuoles (A and B). There are very few amyloplasts but many large plastids (C) containing well developed lamellae. The mitochondria exhibit a wide diversity of form (D) in comparison with the parent line, many of them being highly elongated. There is a great deal of dictyosome activity, many dictyosomes comprising up to eight lamellae (F). There are comparatively few secondary vacuoles, very few osmophilic bodies and little electron dense vacuolar material but more frequent plasmodesmatal connections between cells. There is some randomly distributed rough endo-plasmic reticulum (B and C). The nuclei tend to be compact (A and B) and, being situated more centrally within the cells, are more apparent than in the parent line. Cells of the same line growing in the absence of NaCl show similarities to and differences from the above (Plate IV.5.). They tend to be slightly less cytoplasmic (A) with rather more vacuolar material including

PLATE IV.3. Ultrastructure of C. annuum cell line C, never exposed to NaCl.

A and B. Largely vacuolar (v) cells with cytoplasm (c) adpressed to cell wall (cw). Frequent secondary vacuoles (sv) and amyloplasts (a) are present.

       A x 16,000   B x 30,000

C. Mitochondria (m) in the form usually found in cell cultures.

                      x 120,000

D. A cell junction showing a thin layer of cytoplasm on one side and nucleus (n) close to the cell wall on the other. Lipid bodies (lb) are present.

                      x 50,000

E. Highly vacuolar cells showing electron dense vacuolar material (vm).

                      x 20,000

PLATE IV.3
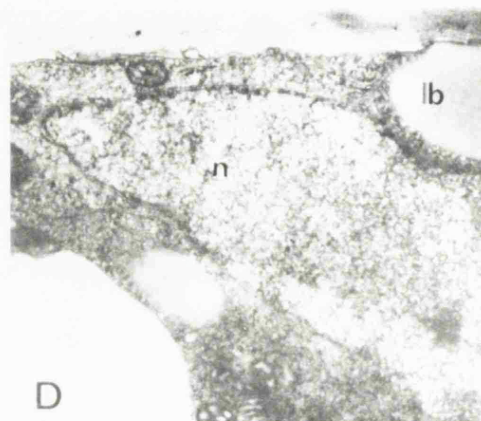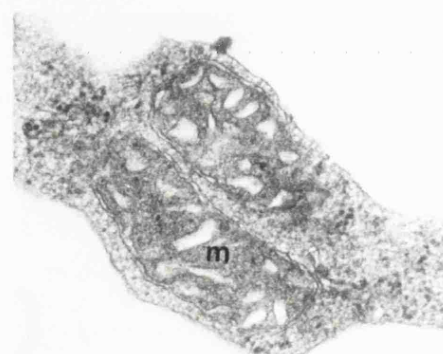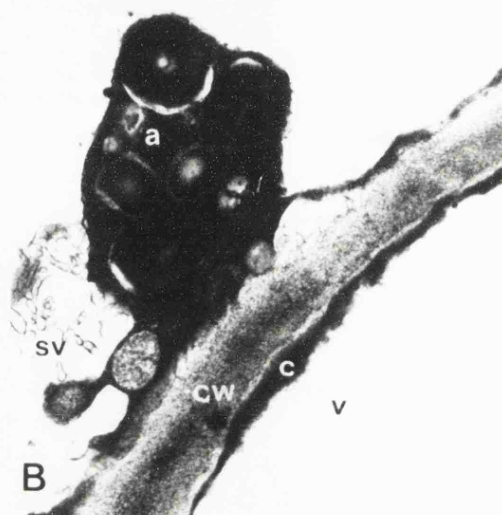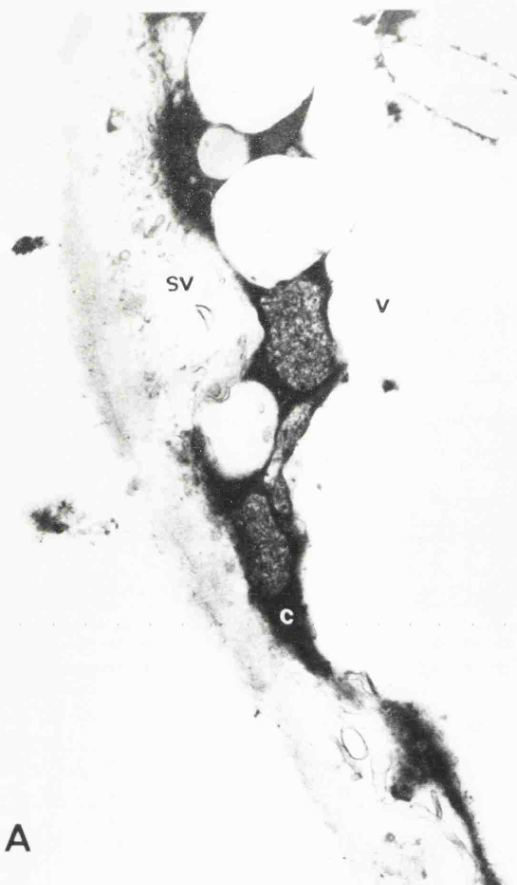
PLATE IV.4. Ultrastructure of C. annuum cell line C, serially subcultured in the presence of 1% NaCl for 6 passages.

A and B. Highly cytoplasmic cells with central nuclei (n), small vacuoles (v) and many organelles including proplastids (pp). B shows a cell junction with cell wall (cw).

A x 10,000   B x 5,000

C. Plastid (p) with well-developed lamellae (l).

x 100,000

D and E. Elongated mitochondria (m) and microtubules (mt) adjacent to cell wall.

D x 50,000   E x 70,000

F. Well developed dictyosome (d) with 8 lamellae.

x 125,000

PLATE IV.4
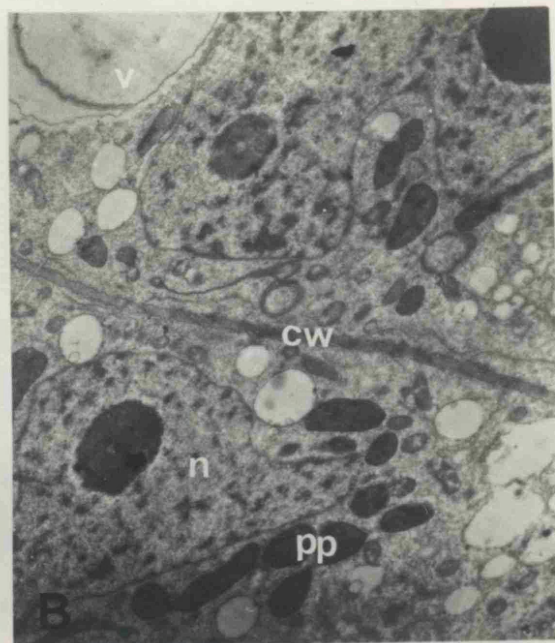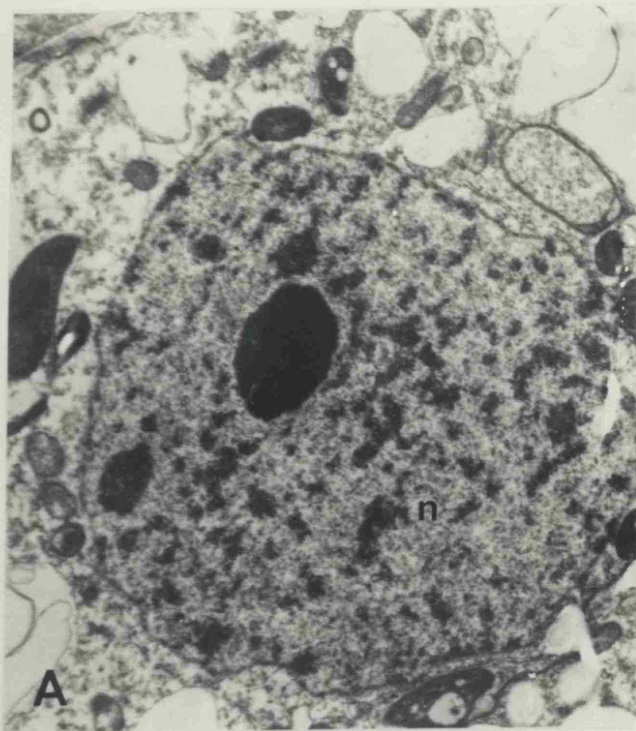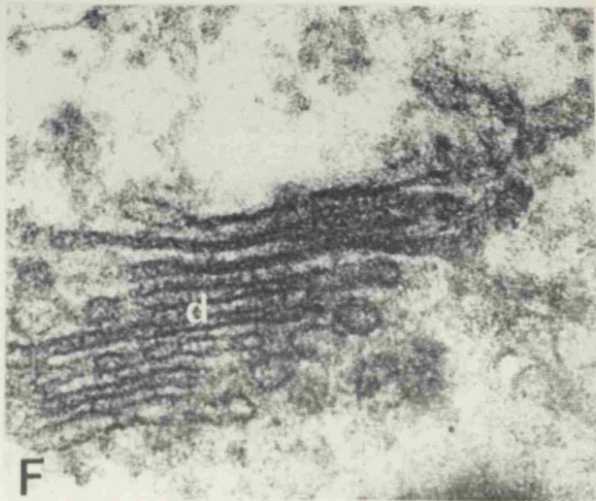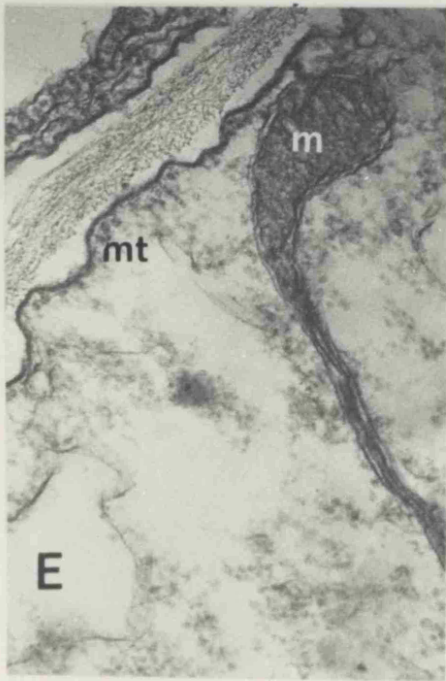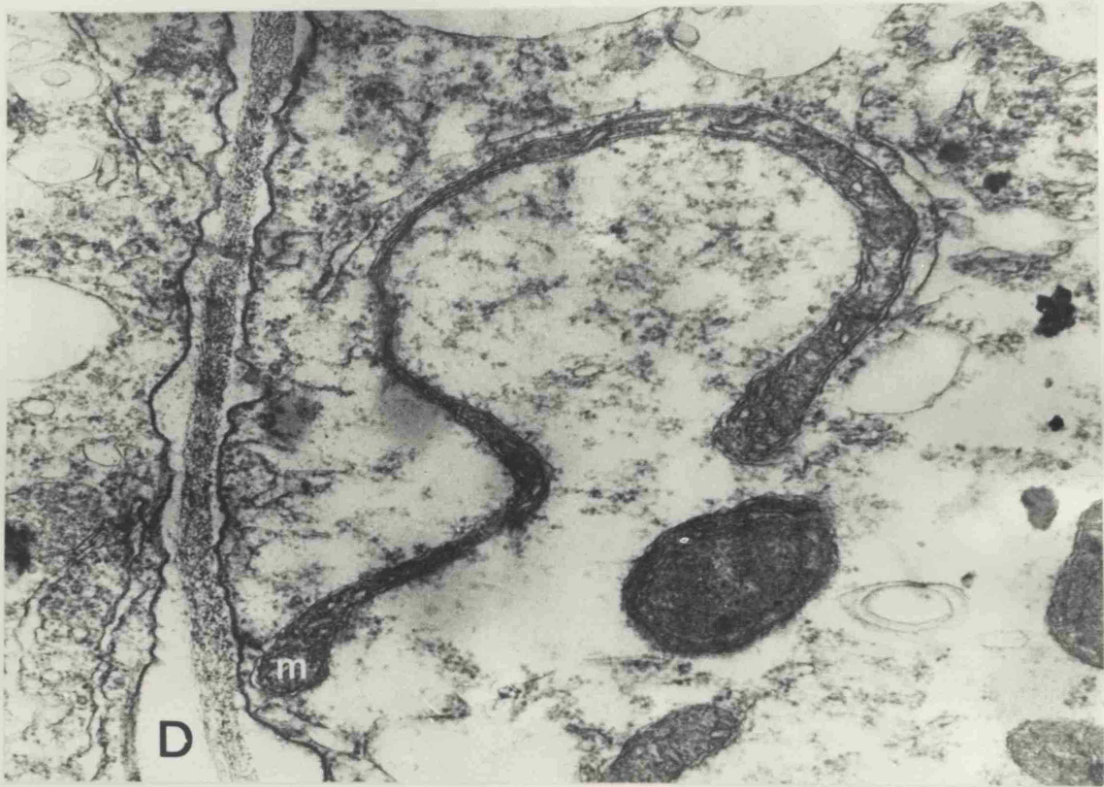
PLATE IV.5. Ultrastructure of C. annuum cell line C, selected for resistance by culture for 5 passages in the presence of 1% NaCl but here growing (6th passage) in the absence of NaCl.

A. Tissue plan showing nucleus (n), vacuole (v), cell wall (cw) and many organelles.

x 5,000

B. Vacuole with secondary vacuole (sv) and electron dense vacuolar material (vm).

x 100,000

C. Amyloplasts (a) and vacuole containing "tanin-like" vacuolar material (t).

x 25,000

D and E. Plastids (p) with well-developed lamellar systems (l).

x 100,000

F. Some cells were highly vacuolate with very large vacuoles (v) and thin layersof cytoplasm (c) adpressed to the cell wall.

x 50,000

D

E

F
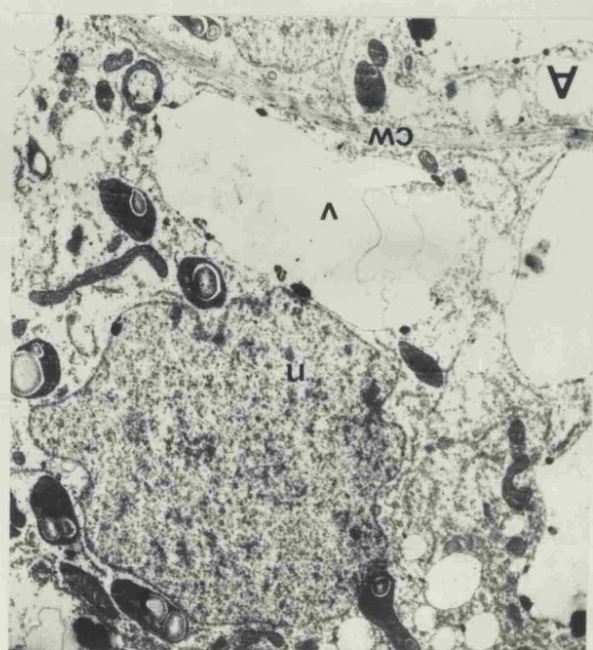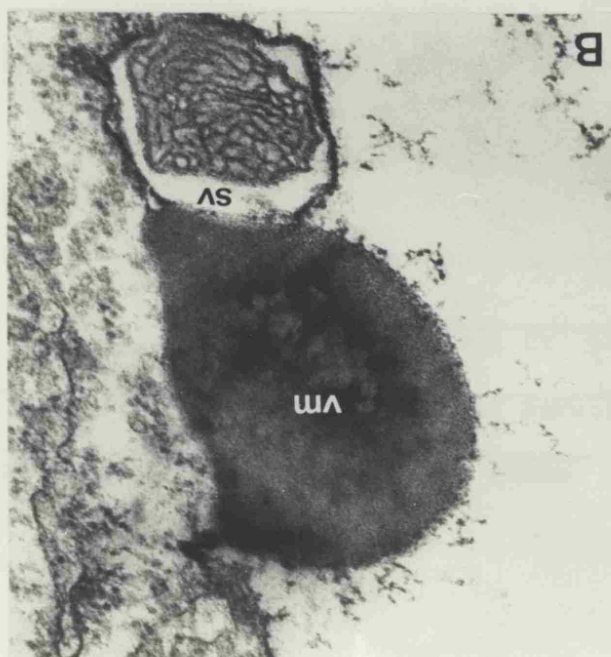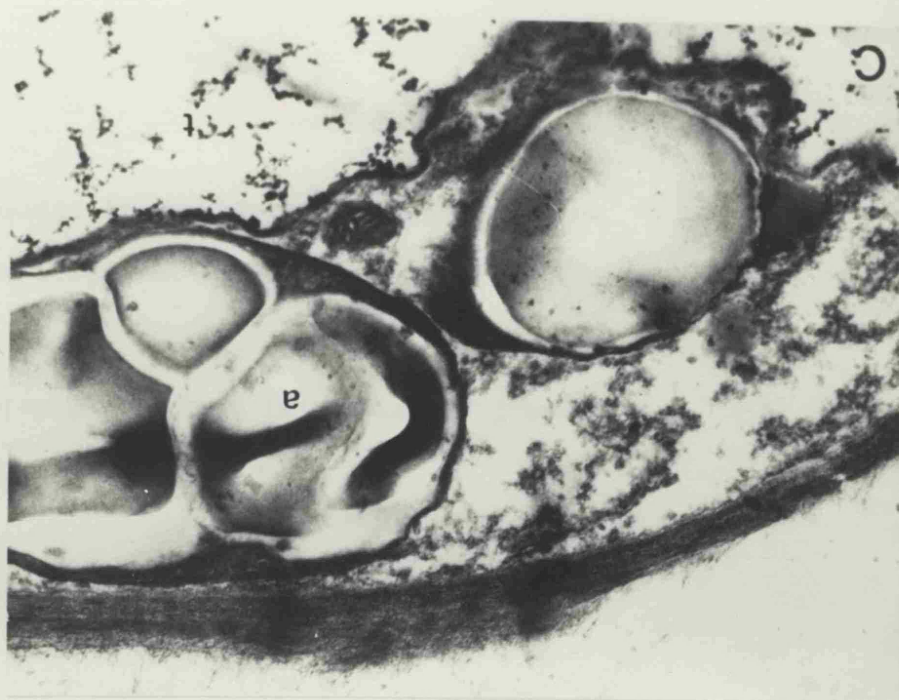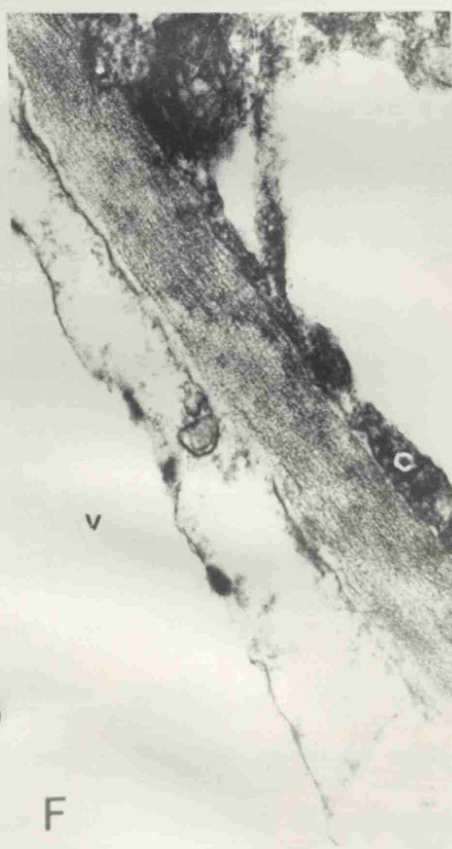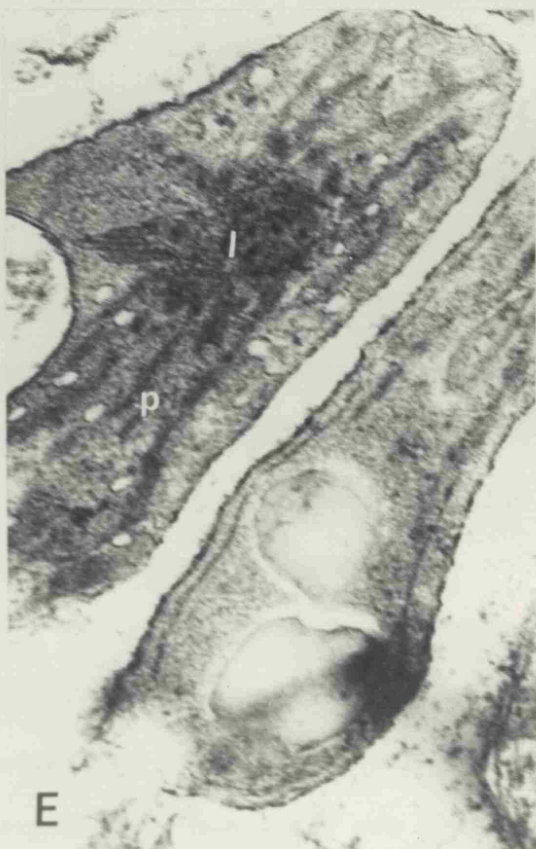
considerable quantities of "tannin-like" material (C) and there are

some secondary vacuoles (B). There is a wide diversity of plastids

including small, undifferentiated proplastids (A), amyloplasts (C) and

large plastids containing lamellar structures (D and E) some resembling

pro-lamellar bodies (E). These plastids have the appearance of developing

chloroplasts. Most of the mitochondria are small and compact (B) the

highly elongated ones having disappeared. They also appear to be less

abundant than in the presence of NaCl. Again there are very few osmophilic

bodies and fewer plasmodesmatal connections than in the presence of NaCl.

Some cells are more highly vacuolated (F) but none as large and vacuolate

as the cells of line CS.

DISCUSSION

In the time available only very limited characterisation of some of

the variant cell lines obtained in Sections II and III could be achieved.

Despite this some of the work did reveal differences between the lines,

which may shed some light on the nature of the mechanisms associated with

tolerance. In this discussion these differences are emphasised and the

techniques used in their study are critically examined with consideration,

where relevant, of how the work could be improved and extended.

The studies on the respiration of isolated mitochondria revealed

convincing differences between one chilling tolerant and one chilling

sensitive cell line of C. annuum. Despite the variable quality of the

preparations of isolated mitochondria, sufficient good preparations were

obtained for the experiments to be carried out, and in these the yields

were similar for the two cell lines and they were capable of similar rates

of respiration and levels of respiratory control. The two methods used

in these experiments had their own particular advantages and disadvantages.

The initial aim was to use the continuous chilling regime throughout this

work as it allows determinations from a large number of temperatures, so
accumulative data from several experiments would allow an accurate
estimation of the line of best fit on an Arrhenius plot. There are,
however, two major disadvantages to this method. Measuring gradients
at a large number of points means that only very short regions of the trace
can be used to determine each gradient, resulting in a loss of accuracy
and a wider scatter of the points than might otherwise be the case. The
second disadvantage is related to the long delay in the recovery of mito-
chondria exposed to a continuous warming regime commencing at about $5^{o}C$.
This type of hysteresis effect is less pronounced using a cooling regime
but it may still result in the change in respiration rate with temperature
lagging 2 to $3^{o}C$ behind the actual change in temperature. Evidence in
support of this comes from comparison of the traces and plots obtained
using the continuous and discontinous methods. The traces for the discontin-
uous experiments often took 1 to 2 minutes at a given steady temperature in
the cooling regime for the respiration to slow down and reach a steady rate,
In addition to this, where the Arrhenius plot of respiration for the
chilling sensitive line exhibit a sharp discontinuity this appears to occur
at a lower temperature ($8^{o}C$ against $10^{o}C$) in the plots obtained using the
continuous regime. The discontinuous method, therefore, yields more accurate
determinations of respiration rate and can pinpoint more accurately the
temperatures at which changes occur. Its disadvantages are that measurements
can only be made at a restricted number of temperatures with any one
preparation and that it is not always easy to hold the temperature steady
for the time required. This difficulty was overcome as experience was gained
in adjusting the balance between the heating and cooling elements.

Results obtained using both the methods discussed above showed differences
between the lines comparable with those shown by Lyons & Raison (1970) between
chilling tolerant and chilling sensitive plant organs, and suggest that a
similar method might be involved in the chilling tolerance of C. annuum cell
line CV34.

The comparison of the fatty acid distributions of the total lipids

extracted from chilling tolerant and chilling sensitive lines relies upon

all the lipid being extracted to ensure that the resulting mixture of

fatty acids is truly representative. The good repeatability that was

obtained in lipid extractions performed on suspensions growing at 25°C

using both methods of extraction described in Materials & Methods suggests

that these procedures are efficient and the fatty acid distribution observed

is the real distribution within the lipids of the culture. Comparison

between the fatty acid compositions of different cell lines grown at 25°C

showed, with one exception, only small differences between sensitive and

tolerant lines. In the case of the C. annuum lines these are of the

type predicted as involved with increased chilling tolerance, namely an

increase in the level of unsaturated fatty acids (particularly 18:2) but

it seems unlikely that the magnitude of this difference is sufficiently

great to result in lipids with very different physical properties. In

the case of the N. sylvestris cell lines the position is complicated by the

fact that differences in the proportions of certain fatty acids (e.g.18:3)

might relate to differences in the morphogenic nature of the individual

lines.

The extracts from cells which have been chilled, and rewarmed differ

in some cases (e.g. CV45 and B13) from the extracts of the same lines

maintained at 25°C, but these differences vary between individual extracts

and although they have not been found to be substantial in any chilling

sensitive lines, neither are they common to all tolerant lines. The

response is erratic and unpredictable. Where changes do occur on chilling

the main changes (a decrease in the proportion of 18:2, and an increase in

the proportion of 18:0) are opposite in type to those which might be expected

to result in a lower rigidity of the membranes, and hence improved chilling

tolerance.

A criticism of the method used in these experiments is that the cells were spread in a thin layer on agar medium for exposure to the chilling conditions. Being placed on the interface between a solid and gaseous environment might result in a gradient which could bring about a variation in the metabolism of the cells due to some cells having better access to nutrients or oxygen than others. Oxygen supply could be particularly relevant in dictating the level of unsaturation of the component fatty acids of lipids. By comparison the cell suspension system provides a uniform source of metabolites and oxygen to all the cells. Re-warming, for only 6 hours, of the chilled tissue resulted in a reversion of the fatty acid compositions of the lines which had shown changes on chilling, until they resembled those of the same lines incubated as suspensions at $25^{o}C$. This suggests a rapid turnover of fatty acids or desaturation of certain saturated acids in-situ, but does not preclude the possibility that such changes might occur due to the temperature affecting the availability of certain metabolites to a proportion of the cells. In retrospect the effect of chilling on the fatty acid composition of the lipids would be best examined after the incubation of the appropriate cell suspensions in agitated liquid medium under chilling conditions. This should be feasible in the case of C. annuum where the temperature in question is $5^{o}C$, but maintaining N. sylvestris suspension cultures at $-3^{o}C$ might give rise to technical problems.

The study could be further improved by: (i) quantitative extraction and estimation of the components, rather than determination of the relative proportions, (ii) estimation of the proportions of different classes of the lipids themselves and (iii) estimation of the lipids and fatty acids of isolated membrane or mitochondrial preparations. This last would preclude the possibility that a variable proportion of the lipid under study might arise from lipid storage bodies which are unlikely to be involved with chilling tolerance. Work along these lines was commenced but not satisfactorily completed.

A purely qualitative study was made of the morphology and ultra-structure of the sodium chloride resistant lines. There were pronounced differences between the resistant lines growing in the presence and absence of NaCl, but smaller differences between the resistant and parent lines growing in the absence of NaCl. The latter might not be related to NaCl resistance at all, but due to divergence in the morphology and anatomy of cells in culture, of the type reported, within a culture, for

N. sylvestris (Section II.A.). For the same reasons, distinctive features of the resistant line, apparent in both the presence and the absence of NaCl, such as the large plastids with lamellae, might be quite independent of the phenomenon of improved NaCl resistance. A large number of resistant lines need to be examined to establish whether features of this type are commonly associated with resistance. Factors exhibited by the resistant line only in the presence of NaCl, however, are likely either to be associated with the mechanism by which the cells escape damage by salt stress and secondary strains, or to be a result of the cells adaption to a hostile environment. This applies to such factors as the high level of aggregation in the suspensions, the highly cytoplasmic nature of the cells, the low level of storage starch, and the large numbers of mitochondria, many of which are highly elongated. These observations do not throw any certain light on the mechanism of salt resistance in cell suspensions but perhaps they do suggest that some possible mechanisms (Levitt, 1972) are unlikely. For example, salt dilution within the cell would probably result in expansion of the cells aided by increased extensibility of the walls, and we would not expect the small compact cells observed. Also, secretion of excessions into the vacuole would require, or result in, a high level of vacuolation. The changes in the subcellular organisation (e.g. the unusual properties of mitochondria) might relate to a mechanism which allows growth

despite an elevated intracellular ion concentration; a true tolerance

mechanism. It is also possible that an ion exclusion mechanism is

operating and preventing the accumulation of high intracellular

salt concentrations.

RETENTION OF CHILLING TOLERANCE THROUGH A PLANT STAGE

INTRODUCTION

In section III, variant cell lines were shown to maintain their tolerance through a period in culture in the absence of the selective conditions, and in section IV, certain lines were shown to have physiological or structural differences which may be associated with increased tolerance. Tests of this kind can only confirm that cultured cells of variant lines do possess different characteristics to the parent lines. They do not prove that a genetic change, as opposed to a physiological adaption to the hostile environment, has taken place. If plants can be obtained from variant cell lines, fresh callus initiated from these, and tested for tolerance, this would establish whether such tolerance at the cellular level is retained through a period when the cells are constituents of the highly organised structure of the plant. Further, if the plants are fertile, they can be selfed and the tolerance of the progeny examined. Continued tolerance would require the tolerant trait to be stable through meiosis and point to it having a genotypic basis. A more rigorous genetic analysis might also be possible if a seed stock could be built up for the variant plants. The aim of the work described in this section was to regenerate plants from as many of the chilling tolerant variant lines of N. sylvestris as possible and to examine them in the ways outlined above. Culture conditions suitable for shoot regeneration in cultures of C. annuum were not found, thereby preventing the use of variant lines of this species in this aspect of the research.

In view of the high level of instability of chromosome number found in the suspension cultures (Section I) it was considered likely that the variant cell lines would be of mixed ploidy and plants obtained unlikely to be haploid. For this reason, no chromosome doubling stage (Jensen, 1974) was applied to suspensions; a colchicine treatment was planned for application to the buds of any regenerated plants which might prove haploid.

EXPERIMENTAL

(A) Regeneration of plants

Shoot initiation was induced in a proportion of the variant cell lines
by subculture into medium lacking 2,4-D. 20 variant cell lines were sub-
cultured at 10^5 cells ml^{-1} into this medium, both in 250 ml Erlenmeyer
flasks, to be maintained as shake cultures, and in 100 ml tumble tubes
(10 ml per tube) or 250 ml nipple flasks (100 ml per flask) for culture as
described by Steward et al. (1952) and Steward & Shantz (1956). Growth in
2,4-D omitted medium was very slow and in all cases the suspensions became
highly aggregated. In some lines shoot, or shoot and root initiation occurred
and after 5 to 12 weeks shoots were excised and rooted as described for
cuttings of N. sylvestris (Appendix 1). When an extensive root system had
formed, Feulgen stained preparations of the root tips were made, and the
plants potted in Levingtons potting compost. No plants were shown to be
haploid, so the intended chromosome doubling step was omitted. After 3 months
growth in soil, some of the plants had flowered. Of the 20 variant culture
lines to which this procedure was applied, only 3 gave rise to fertile plants.
The fates of the individual lines are given in Table V.1. The variant lines
used were those which showed good recovery after their second exposure to
$-3°C$, and some which showed particularly rapid recovery after exposure to
a second chilling treatment at $0°C$ after the selection had been at 0 or $-3°C$.
For the detailed histories of the individual lines Tables III.1 and III.2
can be examined.

The ploidy estimates were based on very few chromosome counts and for
some plantlets no metaphase nuclei could be counted. Of the seven plants from
which estimates could be made, five were approximately tetraploid, one diploid
and one of intermediate ploidy. Two of the plants obtained from line B13 had
different ploidy levels. One was the only truly diploid plant and was also

TABLE V.1.

Shoot initiation and plant regeneration from chilling tolerant variant lines of N. sylvestris

Variant cell line	Temp. of last exposure °C	Shoot initiation	Root initiation	Estimated ploidy	Successful transfer to soil	Flowering	Setting seed
A16(ii)	-3						
A36(ii)	-3						
A41(ii)	-3	+					
A61(ii)	-3	+	+				
A63(ii)	-3						
B14(ii)	-3	+	+	-	+	+	
B23(ii)	-3	+	+	4n	+	+	+
B25(ii)	-3						
B35(ii)	-3						
A1(ii)	0	+	+	4n	+		
A6(ii)	0	+	+	4n	+	+	+
A13(ii)	0	+	+	-	+	+	
A21(ii)	0	+	+	4n	+		
B13(ii)a	0	+	+	2n	+	+	+
B13(ii)b	0	+	+	4n	+		
B29(ii)	0	+	+	-	+		
B31(ii)	0						
B33(ii)	0	+					
B42(ii)	0						
B46(ii)	0						
AD31(i)	-3	+	+	$2n^+$	+		

one of the three plants which flowered and set seed. The plants exhibited

a range of morphological differences in factors such as leaf and flower

structure. Some of these are illustrated on Plate V.1. The older leaves

of several of the plants were very large and in two lines the midrib appeared

to have extended farther than allowed by the expansion of the lamina, causing

a "bowing" effect (E.). In another plant (the B13 tetraploid plant) the

leaves were small, dark green, and turned up around the edge of the lamina.

The flowers of two plants (one fertile and one sterile) had anthers which

extended 2 to 3 mm beyond the corolla. Two of the flowering plants were

sterile and one of these (B14) had highly distorted early flowers (C.). The

flowers of the three fertile plants were selfed and the seed collected.

(B) <u>Chilling tolerance of callus derived from variant plants</u>

Callus cultures were initiated in the usual way (Materials & Methods,

p.9) from 5 variant plants: -

   B14

   B23

   A6

   B13

   AD31

and also from a diploid plant of the parent seed stock and a haploid plant (H9).

Second passage callus was cut into pieces which were subjected to chilling

conditions for 3 weeks as described previously (Section III, p.66, and

Fig. III.1) and survival noted 8 weeks after removal from the chilling conditions.

The results are given in Table V.2. In terms of the number of callus pieces

showing regrowth, the results show only minimal differences between the lines,

but there are more pronounced differences in the rapidity of regrowth and the

proportion of callus contributing to it. Lines A6 and AD31 show markedly better

recovery than controls (Plate V.2), and there are smaller differences between

lines B13 and B23 and controls.

PLATE V.1. Morphology of plants regenerated from chilling tolerant lines of

N. sylvestris.

A. Fertile flowering plant regenerated from chilling tolerant cell

line, B13.

B. Fertile flowering plant regenerated from chilling tolerant cell

line, B23.

C. Distorted and sterile flower on plant derived from chilling

tolerant cell line, B14.

D. Comparison of leaves of plants derived from different variant

cell lines.

L to R: B23, B13a, B14, B13b, AD31 and A6.

E. Side view of leaves of B14 (left) and AD31 (right) plants

showing "bowing" of the midrib.

PLATE V.1
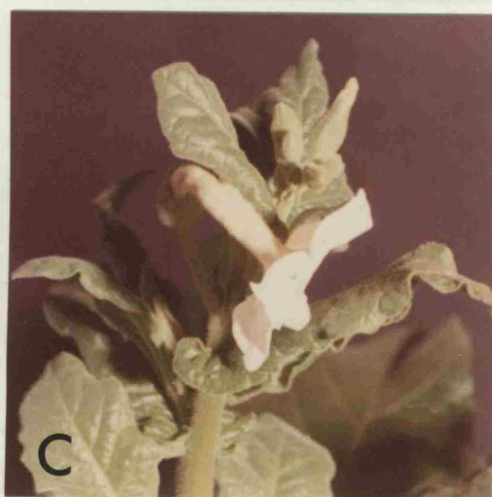
TABLE V.2

Survival and regrowth of callus pieces 8 weeks after exposure to a 3 week chilling treatment

Callus line	Chilling temp. ^{o}C	Callus pieces showing regrowth	Extent of regrowth
B14	0	16	++
	-3	11	+
B23	0	20	+++
	-3	9	+
A6	0	20	+++
	-3	12	++
B13	0	20	+++
	-3	7	+
AD31	0	20	+++
	-3	12	++
Diploid	0	18	+++
	-3	8	+
H9	0	17	++
	-3	10	+

20 callus pieces were initially used in all treatments.

+   Average regrowth less than 0.5 x initial callus volume

++   Average regrowth 0.5 to 2.0 x initial callus volume

+++ Average regrowth greater than 2.0 x initial callus volume

PLATE V.2. Survival and regrowth of callus derived from plants regenerated from chilling tolerant lines of N. sylvestris.

A. Line A6

B. Line AD31

C. Diploid control

Callus pieces were photographed 8 weeks after removal from the chilling conditions.

PLATE V.2
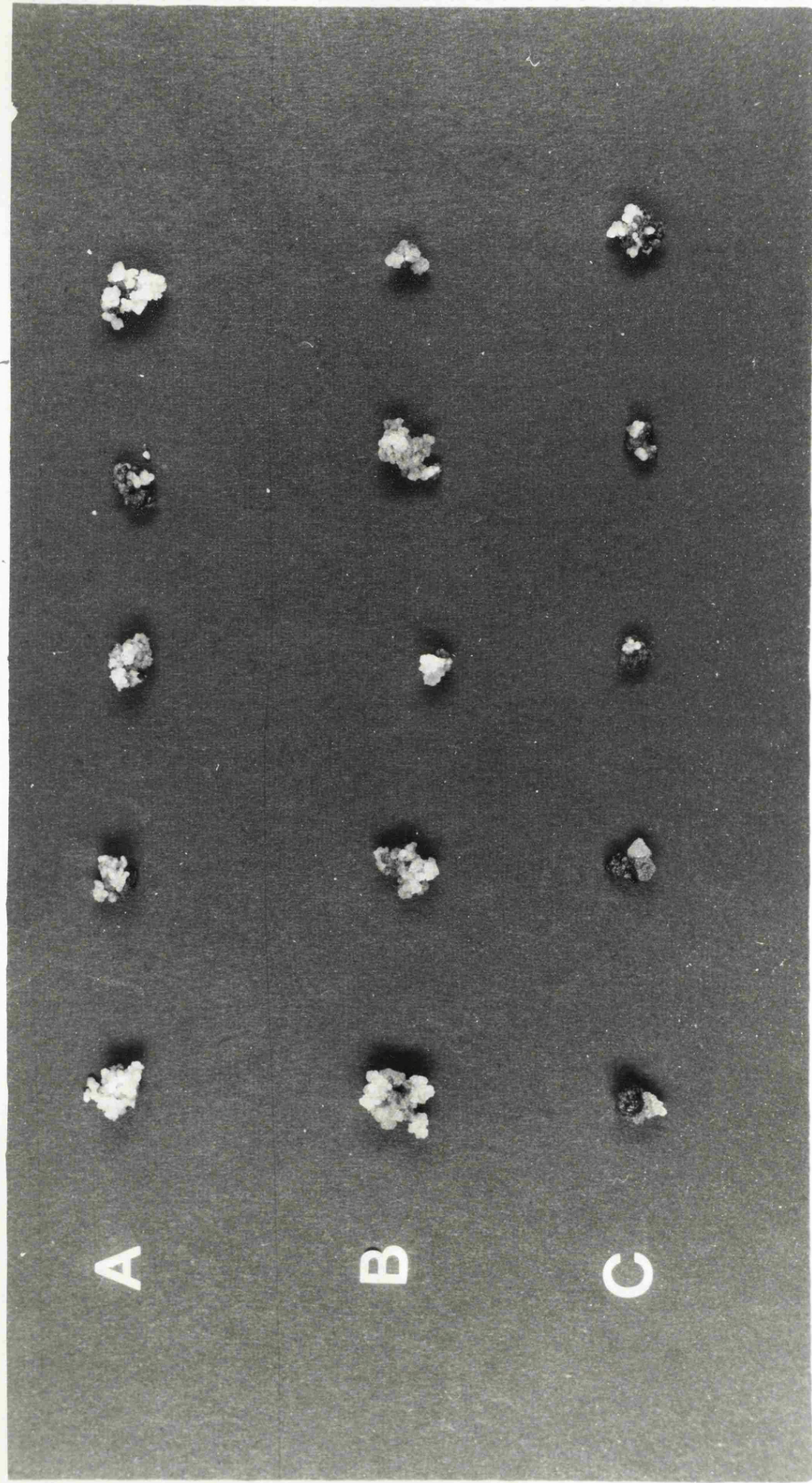
(C) <u>Chilling tolerance of seedlings derived from variant plants</u>

Seeds from the three fertile plants from variant cell lines, and from the parent seed stock were germinated on moist filter paper and the seedlings placed on agar medium in Petri dishes as described in Section II (p.50). Dishes were placed at $0^{o}C$ or $-3^{o}C$ for 1,2 or 3 weeks and survival, in terms of retention of chlorophyll by the cotyledons, estimated 7 days after removal from the chilling conditions. The results are given in Table V.3.

Seedlings of line B13 show no improved chilling tolerance and in fact appear to be killed more rapidly than the seedlings of the normal seed stock. All the lines show a high level of survival after chilling at $0^{o}C$ and it is the results for $-3^{o}C$ that indicate differences between the lines. Seedlings of lines A6 and B23 show a high level of survival after exposure to $-3^{o}C$ for one week, after which time only 20% of the control seedlings survive. In line A6 many seedlings survive chilling for a second week, by which time all the control seedlings have been killed. Only one seedling survived the full 3 week treatment and this was of line B23.

<div align="center">DISCUSSION</div>

The use of a callus system for assay of chilling tolerance again provided problems due to the large size of the callus pieces under examination but suggested that at least two lines, A6 and B23 possessed an increase in chilling tolerance which had been retained through a plant stage. This does not preclude the possibility that tolerance is due to a stable physiological adaption. The tolerance remains incomplete and is less emphatic than in the same lines prior to plant regeneration. Time did not permit the more thorough investigation of these cultures that would have been provided by resuspension of the callus cultures and plating of the resulting suspension in agar medium.

TABLE V.3

Survival of seedlings 7 days after removal from chilling conditions

Length of chilling treat- ment (weeks)	Chilling temp. °C	Plants from which seedlings derived			
		Normal seed stock	A6	B13	B23
1	0	84	100	66	100
	-3	20	100	0	78
2	0	96	100	52	82
	-3	0	64	0	0
3	0	96	100	73	88
	-3	0	0	0	1

Values are percentages of seedlings showing survival and are based on a total of 50 (1 week and 2 week treatments) or 100 (3 week treatment) seedlings.

The seedling test gave a strong indication that at least one of the
lines (A6) had an improved level of chilling tolerance over the normal seed
time, and suggested that this trait was inheritable through meiosis. This is
the best evidence obtained so far that chilling tolerant mutants can be
selected at the cellular level and exhibit tolerance in the form of the
entire plant. Some caution must be maintained, however, until the results of
this test prove repeatable. Reference back to Table II.2 shows that on an
earlier occasion control seedlings showed different (lower) levels of survival
than in the present case. In the earlier experiment there was a wider
fluctuation of temperature within the incubators and this could account for
the higher apparent level of sensitivity, but until normal seedlings can be
shown to respond to temperature in a predictable way, the results obtained
using this assay cannot be considered conclusive. However, in the current
series of experiments seedlings of all the lines were exposed to exactly the
same conditions and the differences do appear to be significant. Tracing the
history of line A6 reveals that it was selected, without a mutagenic treat-
ment, from an initially haploid cell line after twice showing rapid recovery
from exposure to the milder selection temperature ($0^{o}C$). The plant recovered
from this line was approximately tetraploid. A second line (B23) showing a
clear but lower level of improved tolerance over the control line was again
selected without use of a mutagen, this time from an initially diploid cell
line, and a plant regenerated after two exposures to $-3^{o}C$. Again the plant
was approximately tetraploid.

If the seedling test is regarded as valid, the diversity of response
shown between the lines (two showing different levels of tolerance, one showing
no tolerance) is difficult to explain except on the basis that some of the
clonal variant lines do, and others do not represent resistant genotypes.
This confirms evidence obtained in Section III in which it was found that the
retention of the variant phenotype in culture varied. However, a high level of

tolerance in culture did not necessarily give rise to a high level of tolerance in seedlings from regenerated plants. The reasons for this may lie in the complexity of the genotypes of cultured cells which may involve selection for the very unusual conditions of culture; polygenic mutations might occur which would have very different effects on the growth of seedlings. These arguments are developed further in the following General Discussion, together with an appraisal, based on the data presented here, of the potential of plant cell cultures for the selection of variants resistant to environmental stresses.

GENERAL DISCUSSION

The general scheme for this project, outlined in the General introduction, encountered two major technical difficulties which were not resolved. No haploid material of C. annuum was obtained, and no successful method was found for maintaining N. sylvestris cultures as stable haploid suspensions. In this discussion these two problems will be considered, and the extent to which the initial objectives were achieved despite these setbacks assessed. Suggestions for the course of future work are made in the light of the difficulties encountered in the present study.

In order to apply C. annuum to the problem in the same way as envisaged for N. sylvestris it was essential to obtain haploid material and to establish conditions for plant regeneration. The range of media used in anther culture studies failed to show any involvement of the microspores in callus formation from the anthers of any of the five varieties of C. annuum. Callus cultures of C. annuum showed no sign of differentiation into shoots or roots and microscopic examination of the derived suspension cultures showed they were quite free from vascular tissue (e.g. tracheal elements) of the type frequently found in cultures of N. sylvestris. During the course of this work a number of workers have reported limited success in obtaining haploid material with cultivars of C. annuum (Wang, Y-Y et al., 1973; Kuo, J-S et al., 1973; George and Narayanaswamy, 1973; Sukhanov et al., 1974; and Novák, 1974). In most of these cases varieties of "chilli" pepper were used and the success rate was extremely low. This usually took the form of pollen derived callus with very occasional embryo formation. The most detailed reported on "sweet" pepper is that of Novák (1974) in which five different cultivars were tested on a wide range of media. Haploid callus was found on several variations of the medium of Nitsch (1969) and could be distinguished from the diploid callus on the same anthers by its different growth form. No embryos were obtained and organogenesis could not be induced. The five cultivars reacted differently across the range of media and the results are regarded as encouraging evidence

that, while it is a far less amenable species, C. annuum may eventually
prove usable in the same way as Nicotiana species and hence its advantages
be fully exploited. Two of the media used by Novák closely resembled media
used in the present study and if a greater number of anthers had been used
success might have been achieved. Conditions for stabilising the haploid
cultures and for the induction of organogenesis would still have to be
established which would further delay the objective of obtaining variant
plants via cell cultures of this species.

The rapid growth rate of diploid derived suspensions of C. annuum
and the success obtained in plating it in agar medium made the cultures at
least as convenient for the application of selection pressures as those of
N. sylvestris. For this reason it was used in parallel studies on the
selection of variants, with a view to its used in the characterisation at the
cellular level of any variants obtained. This proved worthwhile as the
diploid origin of the suspensions did not prevent the selection of variants
and their fast growth rate and lack of differentiation, together with their
susceptibility to chilling at higher temperatures made them more amenable to
biochemical and physiological studies than those of N. sylvestris.

The other major problem, the tendency towards chromosome duplication in
cell cultures, may be more difficult to overcome in a way which allows the
suspensions to remain in a suitable form for mutation and selection. A
detailed study of PFP as an agent for the selection of haploid cells demonstrated
that it did not promote the preferential growth of haploid cells in a mixed
population. This work did however show that cultures of different origins
may respond differentially to this compound and that this may have a genetic
basis. Studies on the rate of chromosome duplication in freshly initiated
cultures of N. sylvestris also revealed great differences between culture lines,
suggesting that it should be possible to select more highly stable cultures. The

cloning method used in the present work did not in fact yield any stable haploid lines but the establishment of a stable diploid line suggests that haploid lines might be found if a sufficiently large number of lines was examined. The relative stability of haploid cultures of Lycopersicon esculentum (Gresshoff and Doy, 1972a) and Atropa belladonna (Rashid and Street, 1973b) can be interpreted in one of two ways   Either certain species have a genetic basis for chromosome stability in culture, in which case this character should be inducable in other species, or the culture conditions, or the components of the culture medium, adopted by these workers particularly favoured chromosome stability in these species. If this is the case it should be possible to achieve enhancement of the stability of the ploidy level by modification of the culture conditions for other species. Despite the range of media tested here for N. sylvestris, without success, this approach could be extended.

An alternative approach to the problem is to bypass it. In this work this was attempted by the use of freshly initiated suspensions still containing a high proportion of haploid cells. The difficulties associated with this approach are the highly aggregated nature of freshly initiated suspensions, and the extended period of time in culture required for the application of repeated selection pressures. By the time the variant lines had been established in culture few haploid cells remained. However, if mutations are induced in haploid cells during the early stages of culture, chromosome doubling would provide homozygous diploids or tetraploids which would continue to exhibit recessive variations in the phenotype. The nature of mutations obtained in cell cultures containing mixed ploidy levels must remain obscure, unless genetic analysis can be performed on regenerated plants. Only in this way can it be determined whether a mutation occurred in a haploid cell which gave rise to a homozygous diploid or in a diploid or tetraploid cell,

providing a dominant heterozygous mutant line. For elucidation of the

phenomenon of mutation in cultures a genetically uniform source of material

is required.

One method of stabilising a favourable cell line is the freeze

preservation of cultured cells successfully performed with carrot cultures

by Latta (1971) and Nag and Street (1973). If haploid cultures of

N. sylvestris could be stored indefinitely in liquid nitrogen, while

maintaining a high level of viability, they could be used to provide a

standard inoculum for a series of experiments. This might be applicable

to freshly initiated suspensions of haploid origin or to lines selected as

having a greater (but not complete) inherent genetic stability than the parent

cultures. The procedures involved with freeeze preservation have been

evaluated and studied in some depth by Nag and Street (1975 a and b) for

carrot cultures and they have also proved the method applicable to suspension

cultures of other species (Atropa belladonna and Acer pseudoplatanus). As

yet very few such experiments have been performed with the species used in the

present study and no success has been achieved with suspension cultures of

N. sylvestris. C. annuum however has yielded a very low level of viable cells

after freezing and thawing (Withers, unpublished observations). Extension of

this work should reveal suitable conditions for the freeze preservation of

both of these species. Freeze preservation provides a means for the mainten-

ance of a uniform population of cells in a non-dividing state. As such it is

a parallel in plant cell suspension culture, to the leaf as a uniform source

of cells for protoplast culture.

The potential of the culture of isolated protoplasts as a system for

the selection of variants has already been considered in the General Introduction

but its value should be stressed here, in the light of the difficulties

encountered due to the genetic instability of suspension cultures. A great

advantage of the protoplast system is that in addition to providing a

genetically fairly uniform inoculum, this inoculum comprises unicellular units,

rendering it more amenable for quantitative studies of the type that can
be so readily performed with bacteria. This requires a high plating
efficiency of viable protoplasts. The main difficulty associated with the
use of protoplasts is one which also haunts other "in-vitro" culture methods,
namely the individuality of different plant species concerning their
particular cultural requirements. Protoplast culture should provide a
worthwhile system for the isolation of mutant lines of any species for
which the conditions for isolation and culture, at a high level of efficiency,
of haploid protoplasts from vegetatively propagated haploid plants, can be
elucidated. This system, despite being an improvement on the plating of cell
aggregates, is still imperfect as protoplasts, like cell cultures, require a
minimum density for growth. A single protoplast, surviving selection, will
not give rise to a colony on a plate. Future work on the selection of
variants would be expected to involve the use of either haploid protoplasts
or haploid cell cultures stabilised by freeze preservation.

The genetic instability of the cultures was accepted as a severe
disadvantage but not as prohibiting their use in the isolation of variants.
Such cultures were subjected to the chosen selection pressures using the
plating method devised for the purpose (Section I). This method proved
suitable for the isolation of variants resistant to environmental stresses,
where a highly quantitative estimation of the rate of occurrence of such
variants was not required. It also allowed a preliminary evaluation of the
importance of mutagen treatments in the isolation of variants.

The three types of environmental stress chosen for application to the
cultures each elicited a differential response with a small proportion of
the cells surviving and giving rise to colonies on plates. The selection of
chilling tolerant and high temperature tolerant variants requires survival
of cells and subsequent regrowth from the survivors. In these cases the
range of metabolic and physiological states of the cultured cells could result

in some being more "fit" to survive extreme conditions than others, and these may result in colonies with no genetic advantage and a similar range of cell types. Cell lines of this type might be expected to show a decay of resistance during growth in the absence of the selection pressure. This would account for the proportion of lines initially selected as variants, which subsequently behaved in the same way as controls. Other lines exhibited a persistent improved tolerance which could result either from a rapid physiological adaption to the environmental stress or from a genetically altered physiology enabling the modified cells to survive the stress as they are, or rapidly change to a form in which they can survive. The selection procedure for high salt tolerance differs in that it requires the selected cells to undergo persistent cell division in the presence of the selection pressure. Under these circumstances it is unlikely that cells will be selected purely on the basis of their physiological state at the time of application of the selection pressure, but physiological adaption and mutation still remain as alternative possibilities. Whatever the basis of the tolerance, the selective systems adopted proved successful in the isolation of variant cell lines stable in culture, even in the absence of the selection pressure, tolerant of all three of the stresses applied.

Another key step in the original research plan was the regeneration of plants from variant lines. The chilling tolerant lines were chosen for this aspect of the work and some success was achieved. In not all cases could organogenesis be induced in the variant cell lines. This could in part be due to a general decline in morphogenic and embryogenic potential associated with an extended period in culture (e.g. Smith and Street, 1974), a phenomenon probably connected with the genetic instability of cultures, or to the genetic changes selected being coupled to others associated with the loss of morphogenic potential. The latter situation may be more frequent where chemical

mutagens are used and could account for the higher proportion of lines selected without use of a mutagenic treatment giving rise to shoot, and eventually plant regeneration. The regeneration of fertile plants, albeit from only three variant lines, brought about the completion of the cycle of events envisaged at the outset, and allowed an assessment of the maintenance of the chilling tolerant phenotype through a plant stage. If the seedling test is regarded as a valid assay of tolerance the different lines again showed a spectrum of tolerance, two of them possessing a higher level than controls. The importance of regenerating plants from variant lines cannot be overemphasised as it allows a real assessment of the genetic change and breeding studies to examine its inheritance. Future work should include attempts to regenerate plants from a larger number of variant lines, a goal which may be more feasible if selection is from stable cultures or protoplasts, in which point mutations have been induced in a controlled way.

Consideration of evidence from the different phases of the project suggests that at least a proportion of the variant lines resulted from selection of genetic variants from the population of cultured cells. Analyses of variance showed that mutagen treatments did significantly increase the incidence of colony formation by cells surviving selection pressures although variant lines could be selected without the use of mutagens. Furthermore a greater proportion of the lines selected after a mutagen treatment maintained an elevated level of tolerance through subsequent exposures to the selection pressures. The implications of this are that genetic instability of the culture system brings about many chromosome mutations affecting varying numbers of genes and some of these may result in changes at the structural level, or in physiology, which could directly or indirectly confer tolerance of certain stresses. The incidence of such changes could be increased by mutagen treatments which would induce scattered point mutations providing further genetic variation in the culture. If changes do take place in a large number of genes, the resulting modifications at the cellular level may

be so great that tolerance is just one of a number of side effects of a modified anatomy or physiology. This might be particularly true where chilling tolerance is selected by using a long term exposure during which various metabolic factors might influence tolerance. The multiplicity of gene changes would also account for the variation in the morphogenic potential of the culture, the diversity of form of the regenerated plants and the infertility of some of them. Diploid cultures could be used for selection, although no comparison could be drawn between their effectiveness and that of haploid cultures, which is probably indicative of the occurrence of numerous genetic changes in the cultured cells. The results of the experiments with isolated mitochondria suggested a basic membrane change might be associated with the tolerance of one particular variant line, but this may not represent the situation in all the lines.

In summary, there are three possible viewpoints which can be adopted to account for the appearance of variant phenotypes tolerant through repeated stresses and persistent in their absence. (i) They in no way relate to mutations on loci affecting tolerance, but are merely due to a rapid and stable physiological acclimation to the stress conditions. (ii) The variant lines may all be the results of mutation, but the genetic changes themselves may be complex, not directly linked to, and certainly not confined to aspects of metabolism involved in naturally occurring chilling and salt resistance. That is to say multigenic changes are giving rise to cells with a radically altered anatomy and physiology which coincidently confers a measure of tolerance. (iii) Some lines may result from mutation in one or several genes controlling aspects of metabolism primarily concerned with increased tolerance. Variants resulting from genetic changes are probably being selected along with colonies arising due to stable physiological acclimation. These can only be differentiated with confidence after retention of tolerance through to the progeny of a regenerated plant.

The data presented in this thesis are regarded as supporting the view that (ii) or (iii) above are operating, and that mutant lines resistance to environmental stresses can be selected using cell cultures. The regeneration of plants from some lines and the retention of tolerance in their progeny suggest that, although the system is far from straightforward, plant cell cultures do have a potential both for crop improvement and for basic studies on somatic cell genetics. For application to agriculture, careful attention should be paid to the development of a suitable selection technique. For example, in the cases of chilling or freezing tolerance, selection must occur at a level of tolerance valuable to commercial application. we should be prepared to raise the intensity of the selection pressure, even at the expense of having to screen very large cell populations for surviving lines. In cases of environmental stresses which must have been operating naturally throughout the evolution of the species under study, the selection of variants at the cellular level is of interest, but the inheritance of tolerance must be studied carefully in field experiments over a number of generations. It may be that the plants have,associated with the changed physiology conferring tolerance, other attributes which place them at a selective disadvantage. In addition to the potentials of selection in cell cultures for cell improvement and studies on cell genetics, the establishment of mutant cell lines with differing levels of tolerance, from the same stock, could provide a valuable tool for the study of the physiological basis of tolerance.

APPENDIX

APPENDIX 1

Haploid material of <u>Nicotiana</u> <u>sylvestris</u>

Haploid plantlets were obtained by anther culture using the medium
and methods described by Nitsch and Nitsch (1969). Several variations in
this medium were tried but none yielded plantlets from a substantially greater
proportion of the cultured anthers than the unmodified medium. It was found
that the inclusion of plant hormones such as 1AA, 2,4-D and kinetin, did
not give a greater yield of plantlets and hence these were subsequently
omitted from the medium. Removal of the filament from the anther did enhance
the proportion of anthers yielding plantlets, probably because the attached
filaments extend during culture and often push the anthers away from the
surface of the agar medium.

Plantlets wereremoved shortly after the bursting of the anther wall
and placed individually in test tubes on Nitsch and Nitsch (1969) medium,
again with no hormones, and with the sucrose level lowered to 1%. On this
medium good root formation occurred, and root tips could soon be removed for
chromosome counts (Materials & Methods) to verify the haploid nature of the
plantlets. A small number of plants were selected and maintained as a
continual supply of haploid material. Every 28 to 35 days the selected plants
were clonally maintained by removing them from the tubes and cutting them
laterally into two or three pieces each containing at least one node with
axillary buds. These pieces were placed on fresh medium; lateral buds sprouted
and rooting quickly occurred. Callus could be routinely initiated from the
leaves or petioles of these plants (see Materials & Methods) or, where larger
plants were required these could be obtained by plating out rooted cuttings
in Levington's potting compost.

APPENDIX 2

Anther culture using Capsicum annuum

A wide range of bud stages were examined for each of the five varieties. In each case the bud was dissected open, the anther wall split and the contents stained with acetocarmine or propionic orcein to examine the stage of pollen development. In all cases the uninucleate microspore stage was seen to correspond to the point in bud development where the calyx has just withdrawn to reveal the white corolla. Anthers from buds close to this stage were used for anther cultures.

Twelve different media, mostly modifications of the medium of Nitsch and Nitsch (1969) and of Murashige and Skoog (1962). Details of the different media are given in Table A.2.1. Medium XII is similar to that used by Wang et al. (1973) for the induction of haploid callus from anthers of a variety of C. annuum but lacks their RNA-nucleotide mixture. Anthers were dissected from the buds of each of the five varieties of C. annuum and placed on the complete range of media in Universal bottles which were incubated at 25°C under constant illumination. In many cases callus initiation occurred after 2 weeks in culture. After 5 to 6 weeks the numbers of anthers showing callus formation for each variety in each medium were counted and the extent of callus formation estimated. The results are given in Table A2.2.

The five varieties differed in their response over the range of media. For example, New Ace was the only variety showing callus formation on medium I, while Canape and Westlandia were the only varieties showing callus formation on medium VI. These two varieties in fact showed similar responses to each other across the range of media. The type of callus formed appeared to be more dependent on the medium than on the variety. Media II, IV, VI and XII (all containing high auxin levels) giving rise to firm, white callus, while the remainder gave rise to brown or cream friable callus.

TABLE A2.1.

Compositions of media used for anther culture of C. annuum

Component	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Basal medium	H	H	H	MS	MS	MS	H	H	H	H	H (NH$_4$NO$_3$ 500mg/1)	MS
Iron and chelate	FeSO$_4$.7H$_2$O 28 NaEDTA 37.5	FeSO$_4$.7H$_2$O 280 NaEDTA 37.5	FeSO$_4$.7H$_2$O 28 NaEDTA 75	as I	as I	as I	as I	as III	FeEDDHA 15	FeEDDHA 15	as I	as I
Vitamins	H	H	H	Myoinositol 100 Thiamine 0.1 Pyridoxine HCl 0.5 Nicotinic acid 0.5 Glycine 2	as IV	as IV	H	H	H	H	H	H No myoinositol
Hormones	IAA 0.1 kinetin 0.2	NAA 1 kinetin 0.2	IAA 0.1 kinetin 0.2	IAA 6 2,4-D 2 kinetin 1.5	NAA 0.5 kinetin 10	NAA 2 kinetin 0.03	IAA 0.1	IAA 0.4 kinetin 0.1	IAA 0.1 kinetin 0.2	IAA 0.4 kinetin 0.1	IAA 0.1 kinetin 0.2	2,4-D 1 kinetin 1
Other additions							5% coconut milk					15% coconut milk

H = Medium of Nitsch & Nitsch (1969)
MS = Medium of Murashige & Skoog (1962)

Concentrations are given in mg/l.

TABLE A2.2

Callus formation from anthers of five varieties of *C. annuum* after 6 weeks incubation on a range of media.

Medium	Bell Boy			Canape			New Ace			Prona			Westlandia		
	No. of anthers	No. with callus	Amount of callus	No. of anthers	No. with callus	Amount of callus	No. of anthers	No. with callus	Amount of callus	No. of anthers	No. with callus	Amount of callus	No. of anthers	No. with callus	Amount of callus
I	10	0		23	0	0	27	10	+++	28	0		28	0	
II	18	2	+	24	0		27	7	++	21	3	+	29	0	
III	23	12	++	45	25	++	39	25	++	41	41	+++	47	28	+++
IV	12	6	+++	22	11	+++	21	5	+++	26	7	+++	28	9	+++
V	16	10	+++	21	5	++	25	13	+++	23	0		26	5	+++
VI	16	0		20	6	+++	11	0		25	0		28	4	++
VII	7	0		31	15	++	28	12	++	28	12	++	33	13	++
VIII	13	2	+	12	12	+++	16	12	+++	16	5	++	18	6	++
IX	5	0		10	10	+++	10	10	+++	27	3	+	24	12	++
X	10	5	+	11	6	++	15	5	++	16	5	++	16	5	++
XI	11	11	+++	15	5	++	15	10	++	14	10	++	18	5	++
XII	15	7	+	15	8	++	15	8	++	15	4	+	15	6	++

+ = Callus not enveloping the anther   ++ = Callus completely enveloping anther   +++ = Callus more than 4x volume of the anther.

Anthers from all the above treatments (including those in which callus formation had not occurred) were cut open, their contents squashed and stained in acetocarmine and examined for multicellular pollen grains. No signs of divisions within microspores were observed in any of the treatments, but without examination of every anther the possibility of divisions occurring at a low rate cannot be precluded. Pieces of some of the fast growing calli were pretreated with colchicine, stained with Feulgen and squashed for chromosome counts. They all had low mitotic indices and only diploid cells were found suggesting that in these cases, at least, the callus was of sporophyte origin.

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ABSTRACT

Cell cultures initiated from Nicotiana sylvestris and Capsicum annuum
were used in studies on mutation and the selection of variants. Both
diploid and haploid derived cultures of N. sylvestris were used, but the
genetic instability of these cultures invariably resulted in the application
of selection pressures to cultures of mixed ploidy. No haploid material of
C. annuum could be obtained, so diploid derived cultures alone were used for
this species. A plating method was devised in which small aggregates from
the cell suspensions were incorporated into a thin layer of agar medium in
Petri dishes, and exposed to the selection pressures in this form. Using
these methods, lines of both species were selected with improved chilling
tolerance, high temperature tolerance and high salt resistance. In some
cases chemical mutagens were shown to increase the yield of lines selected as
tolerant.

Many tolerant lines were maintained in culture for a number of passages
and a proportion of them maintained their tolerance through extended periods
in the absence of the selection pressures. For three chilling tolerant lines
of N. sylvestris, fertile plants were regenerated and two of these exhibited
the maintenance of an improved tolerant phenotype in the seedlings.

Metabolic and anatomical aspects of the suspension cultures of variant
lines were examined and certain characteristics were found which could relate
to the improved tolerance. These included changes in the temperature depend-
ance of respiration, associated with chilling tolerance and ultrastructural
changes associated with high salt resistance.