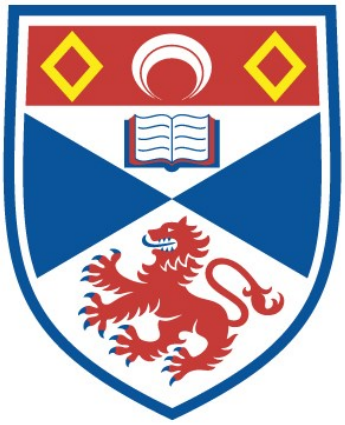
ISOLATION AND CHARACTERISATION OF NITRATE REDUCTASE-MINUS CELL-LINES OF NICOTIANA TABACUM

Roger J. Buchanan

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1983

ISOLATION AND CHARACTERISATION OF NITRATE REDUCTASE-MINUS CELL-LINES OF *NICOTIANA TABACUM*.

by

Roger J. Buchanan

A thesis submitted to the University of St. Andrews in application for the degree of Doctor of Philosophy.

University of St. Andrews, Feb. 1983.
Department of Biochemistry and Microbiology,
North Street,
St. Andrews,
Fife.

CONTENTS

CERTIFICATE

I hereby declare that Roger James Buchanan has spent nine terms in research work under my direction and that he has fulfilled the conditions of Ordinance General No 12 (St. Andrews) and that he is qualified to submit this thesis for the Degree of Doctor of Philosophy.

DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that this thesis is my own composition and that no part of it has been presented previously for a higher degree.

The research work was carried out in the Department of Biochemistry and Microbiology of the University of St. Andrews under the supervision of Dr. J. L. Wray.

ACADEMIC RECORD

I matriculated at the University of St. Andrews in October 1973 and graduated with the Degree of Bachelor of Science, Upper Second Class Honours in Biochemistry.
In October 1977 I matriculated as a research student at the University of St. Andrews.

PUBLICATIONS

"Isolation of Molybdenum co-factor defective cell-lines of Nicotiana tabacum," R. J. Buchanan and J. L. Wray. Molec. Gen. Genet. 188 : 228 - 234 (1982).

"Isolation and partial characterisation of a chlorate-resistant cell-line of Nicotiana tabacum possessing nitrate reductase activity, " J. A. Qureshi, R. J. Buchanan and J. L. Wray.
Proc. Int. Symp. on Nitrate Assimilation - Molecular and Genetic Aspects, Gatersleben, DDR, P22 (1982)

ACKNOWLEDGEMENTS

This research was supported by a grant from the Agricultural Research Council.

I am greatly indebted to Dr. J. L. Wray for his guidance and assistance throughout the course of this work.

I would also like to thank my wife for her patience and support and Mrs J. Binnie for typing this manuscript.

ABBREVIATIONS

NR	-	nitrate reductase
NAA	-	naphthalene acetic acid
2,4-D	-	2,4-dinitrophenoxy-acetic acid
EMS	-	ethyl methane sulphonate
Mo-co	-	molybdenum co-factor
DCPIP	-	dichlorophenolindophenol
BSA	-	bovine serum albumin
XDH	-	xanthine dehydrogenase

All other abbreviations and nomenclature are accepted terms as given in "Instructions to Authors". Biochemical Journal 193 pp 4-27 (1981)

SUMMARY

The object of this reseach was to isolate nitrate reductase-minus cell-lines of a higher plant species in order to gain information on the genetic control of nitrate reductase production in higher plants. This demanded a culture system consisting of isolated cells or protoplasts, ideally carrying single-copy genetic information. To this end, haploid Nicotiana plants were raised by anther culture and abortive attempts were made to culture haploid N. sylvestris protoplasts. Callus and cell suspension cultures of haploid N. sylvestris also proved unsuitable but eventually dihaploid N. tabacum suspension cultures were obtained which, it was decided, would be suitable for this work.

After treatment of these cultures with EMS, 39 cell-lines were selected for resistance to chlorate. Four of these were unable to grow on nitrate medium and were later shown to possess an assembled but inactive nitrate reductase. None of the four possessed xanthine dehydrogenase activity indicating that they were molybdenum co-factor (Mo-co) defective lines (cnx variants). These cnx lines are different from other Mo-co defective N. tabacum lines thus far described and are therefore new types, providing evidence that more than one gene locus is involved in the formation of functional Mo-co in N. tabacum nitrate reductase.

Introduction

For many years now there has been a great deal of interest in the improvement, both quantitatively and qualitatively, of the protein produced by food crops. Conventional breeding programmes have achieved remarkable successes in this field, for example, the development of high lysine grains and high yield cereals. Our knowledge of the biochemical basis behind protein production in higher plants is, however, not all that might be desired.

Synthesis of cell protein and nucleic acids is dependent on a source of reduced nitrogen. For those plants not in symbiotic association with nitrogen-fixing microbes, the majority of this nitrogen is derived from nitrate in the soil. Although ammonium is commonly used as a fertiliser and is freely taken up by higher plants, it is rapidly oxidised to nitrate in the soil by Nitrobacter and Nitrosomonas species. As nitrogen fixation is an energy-intensive process, it is carried out maximally in leguminous plants only when combined nitrogen is limiting.

The nitrate assimilation pathway is responsible for the uptake of nitrate from the plant's external environment and its reduction to ammonium for use in protein synthesis and other quantitatively less important biosyntheses. The pathway consists of a nitrate uptake system and two intracellular enzymes, nitrate reductase (NADH: nitrate oxidoreductase, EC. 1.6.6.1) and nitrite reductase (ferredoxin: nitrite oxidoreductase, EC. 1.7.7.1).

Since this pathway is the major port of entry of combined nitrogen into the plant cell, a greater knowledge of the regulation of this pathway and the structure and assembly of its component parts might lead to ways of improving its efficiency and manipulating it to improve protein production.

Nitrate reductase is perhaps the most important enzyme in this pathway (Beevers and Hageman, 1969). This assimilatory enzyme is found in higher plants, fungi and algae and should not be confused with the respiratory nitrate reductase found in some micro-organisms eg. E. Coli.

Although nitrate reductase is an ubiquitous enzyme activity of higher plants (Beevers and Hageman, 1969), the enzyme has been studied more widely in micro-organisms and it is in fungi, particularly Aspergillus nidulans and Neurospora crassa, but now also Penicillium chrysogenum, that we have the most complete knowledge of its structure and the genetic control of its assembly and regulation. Nitrate reductase in micro-organisms accepts elections from NADPH rather than NADH (NADPH: nitrate oxidoreductase, EC 1.6.6.3.).

Nitrate reductase in fungi.

Cove and Coddington (1965) were the first to purify Aspergillus nidulans nitrate reductase and later produced evidence indicating that it contained a flavin and a b or c-type cytochrome (McDonald and Coddington, 1974). Downey (1971; 1973), however, was unable to detect a cytochrome, but verified the presence of molybdenum, reported as early as 1939 by Steinberg, and a flavin. It is still not clear whether A. nidulans nitrate reductase contains a b_{557} cytochrome similar to that in N. crassa.

Downey (1971) reported a molecular weight of about 200,000, a sedimentation coefficient of 7.8S and a Stokes radius of 64nm. In SDS polyacrylamide gels, the enzyme yielded a single 49,000 mol. wt. band (Downey and Focht, 1974). Subsequently, Downey and Steiner (1979) reported four distinct protein bands upon disc gel electrophoresis. Two bands of about 360,000 and

and 300,000 mol. wt. respectively, possessed haem iron and gave rise to sub-units of about 49,000 mol. wt., appearing to correspond to those of Downey and Focht (1974). The other two bands of about 240,000 and 118,000 mol, wt. respectively, dissociated into sub-units of 50,000 and 70,000 mol. wt. None of these bands possessed nitrate reductase activity.

Extensive genetic work has been carried out with mutants of A. nidulans lacking nitrate reductase. At least seven separate loci are known to be involved in the production of nitrate reductase activity (Cove, 1979). Pateman et al (1964) found five loci involved in the production of a molybdenum co-factor (Mo-Co) and named these cnx ABC, E, F, G and H. Mutants defective at any of these five loci lacked not only nitrate reductase but also xanthine dehydrogenase, a molybdenum-containing enzyme presumed to have a similar molybdenum co-factor. Mutants defective at the nia D locus were unable to produce the major structural component of nitrate reductase (McDonald, Cove and Coddington, 1974) but produced an active enzyme when incubated with cell-free extracts from any of the five cnx mutants (Garrett and Cove, 1976). This situation thus appears analogous to that in N. crassa in that both nit - 1 and cnx mutants produced an active nitrate reductase when incubated in vitro with a source of the molybdenum co-factor.

The seventh locus, nir, seems to produce a regulatory element required for full expression of not only nitrate reductase but also nitrite reductase. Mutants defective at the nir locus produce non-inducible nitrate reductase (Cove and Pateman, 1969). Cove and Pateman (1969) proposed that the nitrate reductase molecule was also involved in the regulation of its own synthesis.

Cove (1979) presented a model for the assembly of A. nidulans nitrate reductase (Figure 1). This model does not, however, adequately account for the observations of Downey and Steiner (1979) (above).

Nitrate reductase in Neurospora crassa has also been the subject of intensive study. The molecular weight of the Neurospora enzyme is reported to be 228,000 with a sedimentation coefficient of 8.0S (Garrett and Nason, 1969) and a Stokes radius of 7.0nm. It is composed of two identical 115,000 mol. wt. sub-units, each possessing cytochrome b_{557} and probably FAD, with an associated molybdenum co-factor (Mo-Co) (Nason et al, 1970; Pan and Nason, 1978).

Much genetic work has been carried out with this fungus. Sorger (1965; 1966) isolated and identified mutant strains of N. crassa lacking nitrate reductase. These mutations mapped at five different loci which he named nit - 1 to 5. More loci have subsequently been identified and there are now at least seven different loci known to be directly involved in the production of nitrate reductase activity (Tomsett and Garrett, 1980).

Sorger demonstrated that nit - 1 mutants possessed cytochrome c reductase activity and that nit - 3 mutants possessed reduced methyl viologen - nitrate reductase activity and he postulated that these activities were associated with different parts of the NADPH - nitrate reductase molecule. Subsequently, Ketchum et al (1970) and Nason et al (1971) showed that, by incubating the cytochrome c reductase species produced by nit - 1 mutants with acid-treated cell-free extracts from not only nit - 3 mutants but from a wide range of

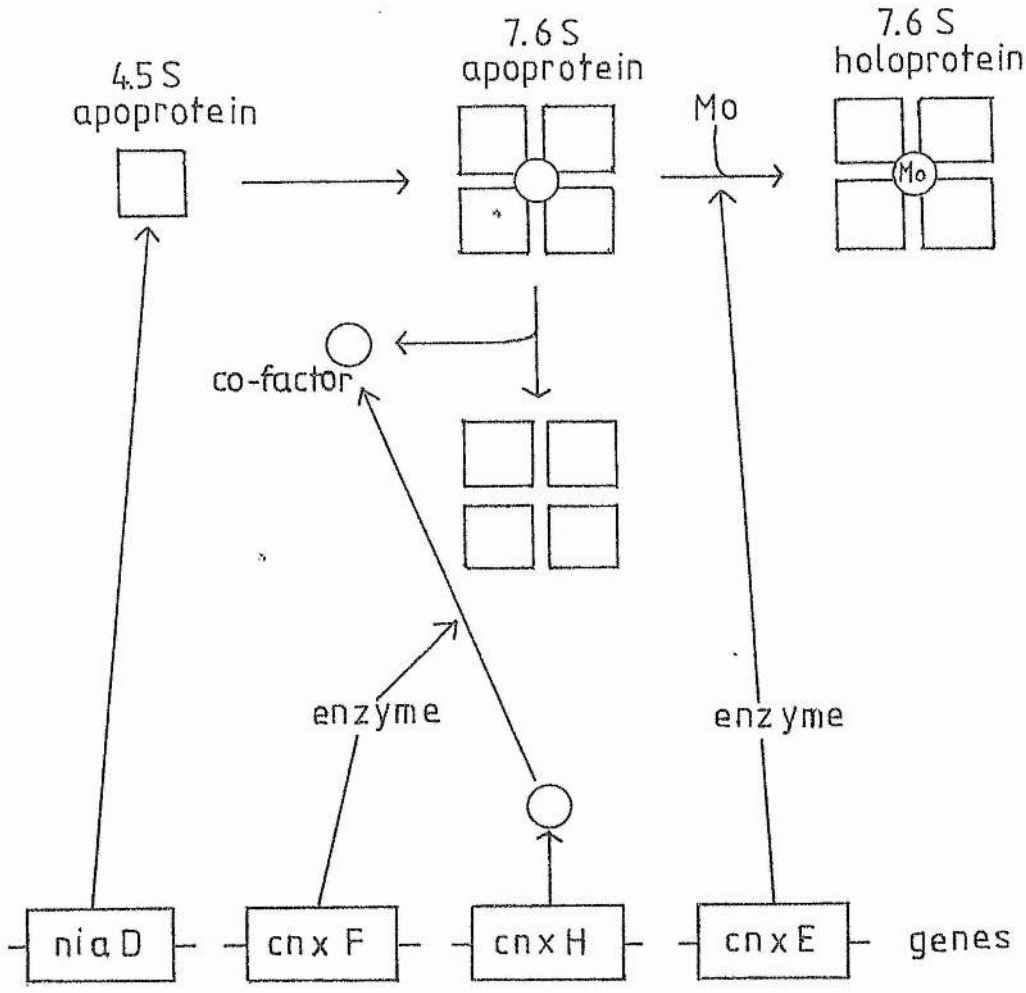


Figure 1 Model for the genetic control of synthesis and assembly of Aspergillus nidulans nitrate reductase (Cove, 1979).

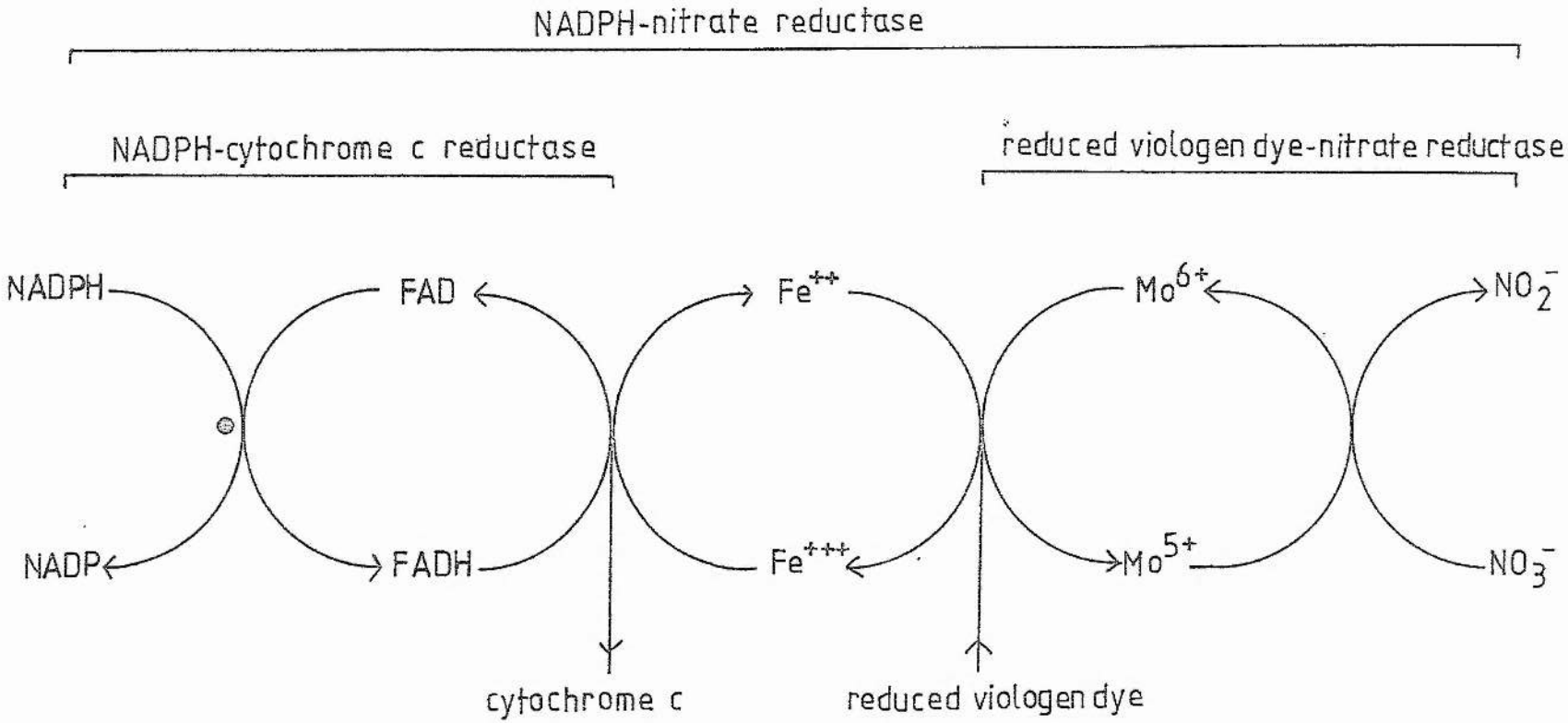
phylogenetic sources and even other molybdenum-containing enzymes, they could recover NADPH - nitrate reductase activity. Lee et al (1974) suggested that the factor responsible for the restoration of this activity was a small dialysable molybdenum-containing moiety (Mo-Co) of about 1000 mol. wt. The nit - 1 locus then, is apparently involved in the synthesis of the molybdenum co-factor and the nit - 3 locus therefore, codes for the enzyme apoprotein. nit - 7,8 and 9 are now also thought to be involved in the Mo-Co production. nit - 2 and nit - 4/5 are involved in the regulation of nitrate reductase production (Tomsett and Garrett, 1980).

Neurospora nitrate reductase is subject to induction by nitrate and/or nitrite and repression by nitrogen metabolites (Garrett and Amy, 1978). Until recently, ammonium was thought to be the repressor (Coddington, 1976: Facklam and Marzluf, 1978) but recent work suggests that ammonium must be assimilated into glutamine before it can exert a repressive effect (Dunn-Coleman et al, 1979: Premakumar et al, 1979). It is proposed that glutamine prevents the action of a positive regulatory molecule encoded by the nit - 2 locus, either directly (Grove and Marzluf, 1981) or via glutamine synthetase (Dunn -Coleman and Garrett, 1980).

Tomsett and Garrett (1981) have now also produced evidence which suggests that Neurospora nitrate reductase can autogenically regulate its own synthesis.

As a result of Sorger's work (above), Garrett and Nason (1967;1969) were able to draw up a scheme for electron transport through N. crassa nitrate reductase (Figure 2). This scheme remains substantially unchanged in the face of later discoveries (above) and indeed it now seems possible that all higher plant, algal and other fungal nitrate reductases studied to date have

Figure 2 Scheme for electron transport through N. crassa NADPH-nitrate reductase, EC.1.6.6.3. (Garrett and Nason, 1967 and 1969).



⊙ A sulphydryl function is now thought to be an intermediary in the transfer of reducing equivalents from NADPH to FAD (Garrett and Amy, 1978).

a similar or identical election transport scheme to this one.

Renosto et al (1981) have recently investigated Penicillium chrysogenum nitrate reductase. They reported the molecular weight to be 199,000, with a sedimentation coefficient of 7.4S and a Stokes radius of 6.3nm. SDS gel electrophoresis of the purified enzyme revealed two diffuse bands of about 97,000 and 98,000 molecular weight respectively and as such gave a similar pattern to that obtained in N. crassa by Pan and Nason (1978).

Nitrate reductase in algae.

Solomonson et al (1975) reported that Chlorella vulgaris nitrate reductase has a molecular weight of 356,000, a sedimentation coefficient of 9.7S and a Stokes radius of 8.9nm. Giri and Ramadoss (1979) have suggested that the true molecular weight is 280,000 and that the enzyme is composed of three identical sub-units of 90,000 mol. wt., each possessing flavin, haem and a molybdenum co-factor. Evidence has now been obtained, however, which indicates that the Chlorella enzyme is a homotetramer with dihedral symmetry (Solomonson, 1982).

Chlamydomonas reinhardii nitrate reductase was also thought to have a trimeric structure (Sosa, Ortega and Barea, 1978), but in view of the recent work on Chlorella (Solomonson, 1982), this may be open to some doubt. Chlamydomonas mutants, similar to the cnx and nia mutants of Aspergillus and the nit mutants of Neurospora, have been reported (Nichols and Syrett, 1978; Sosa et al, 1978).

Nitrate reductase in yeast.

Guerrero and Gutierrez (1977) reported a molecular weight of 230,000 for nitrate reductase of the "false" yeast, Rhodotorula glutinis. The enzyme had a sedimentation coefficient

of 7.9S and a Stokes radius of 7.05nm and possessed flavin and a b_{557} cytochrome. The number of moles of haem per molecule was only 0.7x that in Chlorella vulgaris (Guerrero and Gutierrez, 1977). SDS gel electrophoresis of the enzyme produced a protein band of 118,000 mol. wt., suggesting a dimeric structure (Guerrero and Gutierrez, 1977). R. glutinis nitrate reductase thus has a similar structure to that in N. crassa.

Nitrate reductase in higher plants.

Our knowledge of higher plant nitrate reductase is considerably less complete that for that in micro-organisms. In general, the plant enzyme is specific for NADH, but in soybean and in corn scutellum and roots, a bi-specific NAD(P)H: nitrate oxidoreductase (EC. 1.6.6.2), apparently distinct from NADH: nitrate oxidoreductase is also present (Redinbaugh and Campbell, 1981). The NADH-specific enzyme with which we are concerned has been studied most widely in spinach (Spinacea oleracea) and in barley (Hordeum vulgare).

Spinach nitrate reductase has been shown to contain FAD (Hewitt, 1975), cytochrome b_{557} (Notton, Fido and Hewitt, 1977) and molybdenum (Notton and Hewitt, 1971). Flavin stoichiometry is not known but the haem to molybdenum ratio is about 2:1 (Notton and Hewitt, 1979). The location of these prosthetic groups on the polypeptide chain(s) of the enzyme is not known. Notton, Fido and Hewitt (1977) measured a sedimentation coefficient of 8.1S and a Stokes radius of 6.0nm, and from these parameters calculated a molecular weight of 197,000 by the method of Siegel and Monty (1966). The frictional ratio (f/fo) was reported to be 1.55 and the axial ratio (r_1/r_2) was 10:1.

Kuo, Kleinhofs and Warner (1980) reported a molecular weight of 221,000 for the barley (cv. Steptoe) enzyme. Small and Wray (1980) calculated the molecular weight of the barley (cv. Golden Promise) enzyme to be 203,000 from a sedimentation coefficient of 7.7S and a Stokes radius of 6.4nm. The frictional ratio was reported to be 1.65 and the axial ratio to be 11:1. Information on the prosthetic groups is less complete than in spinach but the enzyme is certainly stabilised by FAD and the effect of tungsten on the activity of the enzyme suggests that it too contains molybdenum (Wray and Filner, 1970).

Higher plant nitrate reductase can reduce nitrate using electrons from not only NADH but also FMNH and reduced viologen dye (Wray and Filner, 1970). As FAD is unnecessary for reduced viologen dye-nitrate reductase activity (Wray and Filner, 1970), the dye must feed electrons directly to the haem or molybdenum. The enzyme also possesses a dehydrogenase function and can transfer electrons from NADH to electron acceptors such as cytochrome c, nitroblue tetrazolium and DCPIP (Wray and Filner, 1970; Small and Wray, 1980).

However, the enzyme cannot reduce cytochrome c using electrons from reduced viologen dye, nor is molybdenum necessary for NADH-cytochrome c reductase activity. Cytochrome c must then receive electrons directly from the haem or FAD.

The electron flow in the enzyme is thus considered to be the same as that in Neurospora crassa nitrate reductase (Figure 2), which is

$$NADH \longrightarrow FAD \longrightarrow Haem \longrightarrow Mo \longrightarrow NO_3^-$$

Table 1 Comparism of the physical parameters of assimilatory nitrate reductase from various sources.

Organism	Molecular weight	Sedimentation coefficient(S)	Stokes radius (nm)	Reference
Rhodotorula glutinis	230,000	7.9	7.05	Guerrero and Gutierrez(1977)
Penicillium chrysogenum	199,000	7.4	6.3	Renosto et al(1981)
Aspergillus nidulans	a,200,000	7.8	6.4	Downey(1971)
"	b,197,000	7.6	6.3	MacDonald and Coddington(1974)
Neurospora crassa	228,000	7.9	7.0	Pan and Nason(1978)
Chlorella vulgaris	a,356,000	9.7	8.9	Solomonson et al(1975)
"	b,280,000	-	-	Giri and Ramadoss(1979)
Spinach	197,000	8.1	6.0	Notton,Fido and Hewitt(1977)
Barley cv.Golden Promise	203,000	7.7	6.4	Small and Wray(1980)
Barley cv.Steptoe	221,000	-	-	Kuo,Kleinhofs and Warner(1980)

Plant nitrate reductase is unstable *in vitro* and turnover *in vivo* is high (Beevers and Hageman, 1969). This has made purification difficult and it is only recently that the enzyme has been purified to electrophoretic homogeneity (Campbell and Wray, 1982).

Small and Wray (1980) and Brown, Small and Wray (1981) showed that proteolytic degredation of the barley enzyme was accompanied by the appearance of small (40,000 and 60,000 mol. wt.) cytochrome c species. BSA and leupeptin (a barley cysteine endoprotenase inhibitor were independently shown to both stabilize nitrate reductase in cell-free extracts and prevent the appearance of the small cytochrome c reductase species (Brown, Small and Wray, 1981: Wray and Kirk, 1981). This suggests that these species are probably proteolytic degredation products of nitrate reductase.

SDS gel electrophoresis of the purified barley enzyme revealed a major protein species of 59,000 mol. wt., minor protein species of 38,000 and 20,000 mol. wt. and a trace protein species of about 103,000 mol. wt. (Campbell and Wray, 1982). Under conditions where proteolysis during extraction is inhibited, the 59,000 mol. wt. species is much less prominent and the major protein component is the 103,000 mol. wt. species (Campbell and Wray, 1982.)

Campbell and Wray (1982) interpret these results as showing that barley nitrate reductase is a dimer of 103,000 mol. wt. sub-units, which are susceptible to proteolytic attack at specific sites. Kuo, Kleinhofs and Warner (1980) also favour a dimeric structure for the barley enzyme but Notton and Hewitt (1979) have interpreted data from SDS gel electrophoresis of the spinach enzyme to show that it is composed of several sub-units

of about 40,000 mol. wt. each. Wray (1982) has speculated that the complex pattern of sub-units seen after SDS gel electrophoresis of the spinach enzyme may result from proteolytic cleavage of what is also a dimeric enzyme of about 100,000 mol. wt. sub-units.

Use of auxotrophic cell-lines in the investigation of higher plant nitrate reductase.

Whilst our knowledge of the structure of higher plant nitrate reductase is advancing steadily, it is lagging well behind that in micro-organisms. We still know little of the genetic control of the higher plant enzyme's production. One of the major reasons for this has been the non-availability of defined auxotrophic cell-lines, such as have been used in Neurospora, Aspergillus and Chlamydomonas to study their respective nitrate reductases. In a wider context, defined auxotrophic cell-lines of micro-organisms have been immensely useful in developing biochemistry in the past thirty years. Were it possible to obtain auxotrophic cell-lines lacking nitrate reductase from a higher plant species, then these would be a useful tool with which to investigate the genetic control of the higher plant enzyme.

Auxotrophic mutant cell-lines are characterized essentially by the loss or disfunction of a single protein which is due to a mono-genetic mutation (Wagner and Mitchell, 1964). The isolation of such cell-lines requires a unicellular culture system in order to avoid metabolic co-operation and allow clones of mutant cells to be raised (Zryd, 1978). Expression of a mono-genetic mutation also requires that the cells be haploid at the time of mutagenesis. These conditions are much harder

to fulfil with higher plant systems than with micro-organisms. The reasons for this are basically due to the differences in biochemical organisation between them.

Bacteria, for example, have extended haploid phases and express phenotypic variation quickly due to their very limited reserves of nutrients. Homogeneous populations with a short generation time can be cultivated on a fully-defined medium, thus allowing efficient screening for variants of mutational origin or otherwise (Chaleff and Carlson, 1975). In general, higher plants have none of these advantages. In addition, chromosomes in higher plants are both smaller and more numerous than those in micro-organisms. This makes detailed genetic analysis more difficult.

A further complication is the lack of suitable procedures for selection of auxotrophic cell-lines of higher plant species, replica plating not being possible because of cell adhesion.

Recent advances in higher plant tissue culture, however, now offer the prospect of overcoming some of these difficulties. Haploid sporophyte plants can now be raised from pollen of many plant species (Sunderland, 1980). Cells and protoplasts from a wide range of higher plant species can be cultured in fully-defined media under aseptic conditions (Yeoman and Macleod, 1977; Evans and Cocking, 1977). Techniques are now available for raising clones from single cells (Street, 1977) and sporophytes can be regenerated from cell and protoplast cultures (Vasil and Vasil, 1972; Reinert et al, 1977), allowing the use of conventional plant genetic techniques to determine whether or not variant cell-lines are of mutational origin.

In 1977, Warner, Lin and Kleinhofs isolated nitrate reductase-minus mutants of barley without recourse to tissue culture

techniques. However, all of these cell-lines were "leaky", possessing low levels of nitrate reductase and growing normally in the field. The object of this project was to take advantage of recent advances in higher plant tissue culture techniques (discussed above) to isolate non-leaky nitrate reductase-minus cell-lines from a higher plant species, to characterize such mutants, and by doing so, further investigation into the structure, assembly and regulation of the higher plant enzyme.

Choice of species for nitrate reductase-minus cell-line isolation experiments.

The first step was to find the most suitable species with which to carry out this work. Ideally, the species should be a "true" diploid and not allopolyploid (Many common higher plant species are allopolyploid (Allard, 1960) and carry duplicated genetic information even in the gametic chromosome complement). The species should be one which gives rise to vigorous haploid plants by anther culture and from which stable haploid cell-lines can be initiated. These cell-lines should grow as dispersed cell suspensions or in protoplast culture, and finally, it should be possible to regenerate whole plants from single cultured cells.

Unfortunately, a search through the literature shows that there is no such "ideal" species. However, Nicotiana species are popular for mutant selection work due to their favourable cultural and regeneration capabilities (Collins and Legg, 1980) and the Nicotiana species which most nearly met the above requirements was N. sylvestris. This is generally regarded as a true diploid species (Zenk, 1974), and is one which gives rise to vigorous haploid plants by anther culture (Nitsch, 1969). N. sylvestris has also been grown successfully in protoplast

culture and whole plants have been regenerated from such cultures (Nagy and Maliga, 1976). Were it possible to culture stable haploid cell-lines in a similar manner then this species would fulfil all of the above requirements.

Choice of culture system.

The initial objective then, after raising haploid plants by anther culture, was to obtain cytologically stable haploid protoplast or dispersed cell suspension cultures of N. sylvestris. Of the two, protoplast cultures would be preferable as they offer the prospect of a culture system consisting of isolated protoplasts embedded in agar on a fully-defined medium, in which the progress of single protoplasts can be followed, clones of variant protoplasts can be obtained, and whole plants regenerated therefrom.

As in other forms of plant tissue culture, there is still a strong empirical aspect to protoplast culture. Although diploid N. sylvestris protoplasts had been successfully cultured with a high plating effeciency by Nagy and Maliga (1976), and both haploid and diploid protoplasts cultured by Bourgin, Missonier and Chupeau (1976), albeit with a low plating efficiency, it was likely that successful culture in this laboratory would involve a degree of trial and error. This proved to be something of an understatement. Despite repeated attempts, a satisfactory protoplast culture system was not achieved. Limitations of time dictated that these efforts, described in Chapter two, be abandoned.

The alternative to protoplast culture was cell suspension culture. As this does not involve exposing the cells to possibly toxic cell-wall degrading enzymes (p. 51), and as the

cell-wall protects the cells from the osmotic stresses to which protoplasts are subject, such culture methods are better established and more dependable. However, cell suspension cultures have certain disadvantages as a culture system from which to isolate auxotrophic cell-lines. Firstly, cells grow in suspension not only as isolated cells but also in aggregates of variable size (Street, Henshaw and Buiatti, 1965). Many of the cells in a treated culture might therefore be protected from the effect of mutagenic or selective agents. There is also the possibility that metabolic co-operation might mask the existence of auxotrophic cells.

Secondly, cells in suspension culture are typically cytologically unstable and stable haploid cell-lines are difficult to obtain (Sunderland, 1971).

Nevertheless, it was felt that these problems could be overcome. The revised objective was therefore to obtain dispersed, cytologically stable haploid suspension cultures of N. sylvestris.

Dispersed, haploid cell cultures of N. sylvestris were rapidly raised on the B_5 medium of Gamborg et al (1968). Unfortunately, attempts to grow N. sylvestris suspensions on a nitrogen source other than nitrate were unsuccessful (p. 57). As it would be impossible to maintain nitrate reductase-minus cell-lines on a medium containing nitrate as a sole nitrogen source, this was clearly an obstacle to the use of this species for the work envisaged.

Attention was therefore transferred to an alternative species, N. tabacum. N. tabacum is considered a less suitable species from which to isolate auxotrophic mutants due to its

amphidiploid nature (Gray et al, 1974.) However, as discussed in Chapter three of this thesis, it is likely that much of the N. tabacum genome has become functionally diploid, and so in dihaploid cell cultures, much of the genetic information will be single-copy. It should therefore be possible to isolate non-leaky auxotrophic cell-lines from this species as long as the character(s) involved are amongst those for which only single-copy genetic information is carried in the dihaploid cell.

In its favour, N. tabacum as a species is amongst the most amenable to anther culture (Nitsch, 1969), has a long history of use in mutant selection work, and so, in the short term, was perhaps the most suitable species to use in this work also.

Procedures for isolation of nitrate reductase-minus cell-lines.

Most procedures for the isolation of auxotrophic plant cell-lines involve the killing of prototrophic cells in minimal medium containing nucleic acid analogues, followed by development of auxotrophic cells on supplemented medium (Zryd, 1978). Fortunately, in the particular case of nitrate reductase, a positive selective agent is available for isolation of nitrate reductase-minus cell-lines. This agent is the herbicide, chlorate. Both respiratory and assimilatory nitrate reductase are thought to be able to reduce chlorate to chlorite, which is toxic to most plants and micro-organisms (Pichinoty, 1966; Lewis and Fincham, 1970; Solomonson and Vennesland, 1972; Hofstra, 1977). Cell-lines lacking nitrate reductase would therefore be resistant to chlorate toxicity. However, in some organisms at least, chlorate resistance can arise by mechanisms independent of nitrate reductase (Cove, 1976a) and as yet the mechanism of chlorate toxicity is not fully understood (see

discussion to Chapter four), although the success of Piechaud et al (1967) and Oostindier-Braaksma and Feenstra (1973) has shown that this need not be an obstacle to the use of chlorate for isolating nitrate reductase-minus cell-lines.

The type of selection procedure required to isolate nitrate reductase-minus cell-lines is therefore essentially that for selection of resistance rather than auxotrophic mutants, as initial selection is for chlorate-resistance only. Many workers have successfully isolated resistance mutants from N. tabacum cell cultures. Selected traits include methionine sulphoxinine resistance (Carlson, 1973), base analogue resistance (Lescure, 1975), salt tolerance (Nabors et al, 1975) antibiotic resistance (Maliga et al, 1973), and herbicide tolerance (Barz and Umiel, 1977; Aviv and Galun, 1977). Müller and Grafe (1978) isolated chlorate-resistant cell-lines, all of which were also nitrate reductase-minus. The work of Müller and co-workers is discussed in detail in the general discussion at the end of this thesis.

The procedures used by the aforementioned groups of workers generally involve the use of a chemical mutagen to increase the frequency of variation in the wild-type cultures, followed by a one-two week incubation to allow expression of variant phenotypes prior to application of selection pressure. It was therefore anticipated that a similar procedure would be suitable for isolation of chlorate-resistant cell-lines in this laboratory.

Growing cells in suspension culture is an efficient way of obtaining a large cell mass in a short time. Such cultures can also be easily and efficiently treated with mutagenic agents. However, since we wish to clone resistant cell-lines from individual variant cells, it will be necessary to immobilise

the mutagenised cells by plating them at a low density in petri-dishes containing agar medium. In this way individual variant cells can develop into discrete calli without risk of contamination from other variant cells (Street, 1977).

This then was the approach to be used in this work. Dihaploid N. tabacum cell suspensions would be treated with a chemical mutagen from which the cells would then be washed free. The treated cells would be given one week in suspension culture to express themselves phenotypically before being plated in petri-dishes containing agar medium and the selective agent chlorate. Cell-lines established from chlorate-resistant calli would be screened for nitrate reductase activity and nitrate reductase-minus cell-lines obtained would be studied biochemically. Attempts would also be made to regenerate nitrate reductase-minus plants from these cell-lines.

Isolation and characterisation of nitrate reductase-minus cell-lines of N. tabacum - Summary of objectives.

The project can be said to consist of three main parts.

1) Development of a tissue culture system consisting of isolated cells growing on a fully-defined medium containing a nitrogen source other than nitrate, and possessing single-copy genetic information. (Chapters one to three.)

2) Treatment of cultured cells with a mutagenic agent and selection for resistance to chlorate toxicity. Screening of chlorate-resistant cell-lines for nitrate reductase activity. Culture of nitrate reductase-minus cell-lines. (Chapter four).

3) Study of nitrate reductase-associated enzyme activities in nitrate reductase-minus and wild-type cell-lines, in order to determine what genetic defects are responsible for their lack of nitrate reductase activity (Chapter five). Such studies should further investigation of the genetic control of nitrate reductase production and nitrate assimilation in higher plants.

Materials and methods

MATERIALS

Alcohol dehydrogenase (yeast), bovine serum albumin (Fraction V), cytochrome c (horse heart type III), dithiothreitol FAD (grade III), NAD^+ (yeast, grade V), NADH (yeast, grade III), myoglobin (whale skeletal muscle, type II), and ethyl methane sulphonate were all obtained from Sigma London Chemical Company Ltd., Poole, England.

Colchicine and phenosafranine were obtained from BDH Chemicals, Poole, England.

Szechrome NAS was supplied by Yedatek Ltd., Rimon 10, Omer, Israel.

Millex-GS filters were from Millipore SA, 67120, Molsheim, France.

Miracloth was obtained from Chicopee Mills Inc., Broadway, New York, U.S.A.

Plastic petri-dishes and universal bottles were obtained from Sterilin Ltd., Teddington, England.

Euparal came from GBI (Labs) Ltd., Heaton Mills, Heaton Street, Denton, Manchester, England.

Macerozyme R-10 and Cellulase R-10 (Kinki Yakult Manuf. Co. Ltd., Nishinomiya, Japan) were obtained from R.W. Unwin, Prospect Place, Welwyn, Herts.

Meicelase was obtained from Meiji Seika Kaisha Ltd., Chuo-Ku, Tokyo, Japan.

All other chemicals were of the highest grade available from the usual commercial sources.

CULTURE METHODS.

Composition and preparation of media.

1) Anther culture medium.

Anthers were cultured on Medium A of Kasperbauer and Collins (1972) (Table 2) which is a modification of the medium of Murashige and Skoog (1962).

Anther culture medium was prepared from stock solutions of vitamins, hormones and micronutrients with the other components added as solids before autoclaving.

2) Rooting medium.

Young anther-derived plantlets were raised aseptically on the R medium of Kasperbauer and Collins (1972) (Table 3) which is based on that of White (1943). Rooting medium was prepared from a stock solution of micronutrients, all other components being added as solids before autoclaving.

3) Cell culture media.

Callus initiation and routine maintenance of callus and cell suspension cultures were carried out on a medium based on the B_5 medium of Gamborg, Miller and Ojima (1968) (Table 4).

A number of variations of this medium were used (Table 5). These differ essentially only in the nitrogen supplements. Components A,B,C,D and G (Table 4) were prepared as a ten-fold concentrated solution and stored frozen for up to one month. The complete medium was prepared by addition of components E, F and H, followed by volume and pH adjustment. The medium was autoclaved when complete except in the case of glutamine medium, where glutamine was added from a filter-sterilised stock solution to the autoclaved incomplete medium.

Table 2 Composition of anther culture medium (A medium of Kasperbauer and Collins, 1972).

Component	$mg.litre^{-1}$ final concentration	
Macronutrients (A)		
KNO_3	1900	
NH_4NO_3	1650	
$CaCl_2.6H_2O$	660	
Micronutrients (B)		
Na_2EDTA	22.4	
$Fe_2(SO_4)_3.9H_2O$	15	
KI	0.83	from a 100x stock solution
$MgSO_4.7H_2O$	370	
KH_2PO_4	170	
Micronutrients (C)		
H_3BO_3	6.2	
$Na_2MoO_4.2H_2O$	0.25	
$CoCl_2.6H_2O$	0.025	from a 100x stock solution
$MnSO_4.5H_2O$	22.3	
$ZnSO_4.7H_2O$	8.6	
$CuSO_4.5H_2O$	0.025	
Vitamins (D)		
Thiamine HCl	0.1	
Nicotinic acid	0.5	from a 10x stock solution
Pyridoxine HCl	0.5	
Glycine	2	
Inositol	100	
Hormones (E)		
NAA	0.1	from a 100x stock solution
Kinetin	0.2	
Sucrose (F)	20,000	
Agar (G)	6,000	

Final pH adjusted to 6.0

Table 3 Composition of rooting medium (R medium of Kasperbauer and Collins, 1972).

Component	$mg.litre^{-1}$ final concentration	
Macronutrients (A).		
$Ca(NO_3)_2.4H_2O$	300	
Na_2SO_4	200	
KNO_3	80	
KCl	65	
$NaH_2PO_4.H_2O$	19	
$MgSO_4.7H_2O$	750	
Micronutrients (B).		
$MnSO_4.5H_2O$	7	from a 100x stock solution
$ZnSO_4.7H_2O$	3	
H_2BO_3	1.5	
KI	0.75	
$CuSO_4.5H_2O$	0.01	
$NaMoO_4.2H_2O$	0.25	
$Fe_2(SO_4)_3$	2.5	
Sucrose (C).	20,000	
Agar (D).	6,000	

Final pH adjusted to 5.8.

Table 4 Composition of cell culture medium (B_5 medium of Gamborg, Miller and Ojima, 1968).

Component	$mg.litre^{-1}$ final concentration
Macronutrients (A)	
$CaCl_2.6H_2O$	223
$MgSO_4.7H_2O$	250
$NaH_2PO_4.2H_2O$	167
Micronutrients (B)	
H_2BO_3	3
$MnSO_4.5H_2O$	14
$ZnSO_4.7H_2O$	2
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Micronutrients (C)	
KI	0.75
FeEDTA	40
Vitamins (D)	
Nicotinic Acid	1
Thiamine HCl	10
Pyridoxine HCl	1
m - Inositol	100
Hormones (E)	
2,4 - D	2
Nitrogen supplements (F)	
see Table 5	
Sucrose (G)	20,000
Agar (H) (if present)	8,000

Final pH adjusted to 5.6

Table 5 Cell culture media showing various nitrogen supplements (see also Table 4).

	aspartate	arginine	glycine	$KClO_3$	KNO_3	KCL	succinate	glutamine
amino-acid medium	2	1	0.1	-	-	25	6	6
amino-acid/20mM-chlorate medium	2	1	0.1	20	-	-	6	6
amino-acid/40mM-chlorate medium	2	1	0.1	40	-	-	6	6
nitrate medium	-	-	-	-	25	-	-	-
glutamine medium	-	-	-	-	-	25	6	8

All values are given in $mmol.l^{-1}$ final concentration.

4) Protoplast culture media.

Protoplasts of N. sylvestris were normally cultured on the K_3 medium used by Nagy and Maliga (1976) (Table 6). This medium, protoplast culture medium I, is based on those of Kao, Constabel, Michalyuk and Gamborg (1974), Nagata and Takebe (1971), and Gamborg, Miller and Ojima (1968).

The T_2 medium of Bourgin, Missonier and Chupeau (1976) was also used (Table 7). This medium, protoplast culture medium II, is based on that of Murashige and Skoog (1962). Both media were prepared from stock solutions of vitamins, hormones and micronutrients with other components added as solids before autoclaving.

5) Washing medium.

When required cells were washed in cell culture medium without any nitrogen supplement (Table 4).

6) Regeneration medium.

Cell culture medium with differing hormone complements was used in attempts to raise plantlets from callus or cell suspension cultures of both N. sylvestris and N. tabacum.

Sterilisation of equipment and media.

Forceps and scalpels, wrapped in metal foil, plugged pipettes in metal canisters and suitably wrapped glassware were sterilised in an Electro Helios oven at 180°C for 3h.

Media and Washing solutions were autoclaved at 15 pounds per square inch for 20 min. Where components of the media were filter-sterilised, this was carried out on concentrated pH-adjusted solutions of the component. Millex - GS 0.22 µm filter units were used and aliquots of the filtered component were added to the already autoclaved incomplete media.

Table 6 Composition of protoplast culture medium I (K_3 medium of Nagy and Maliga, 1976).

Component	$mg.litre^{-1}$ final concentration	
Macronutrients (A)		
$NaH_2PO_4.2H_2O$	228	
$CaCl_2.6H_2O$	1400	
$MgSO_4.7H_2O$	250	
$(NH_4)_2SO_4$	134	
KNO_3	2500	
$NH_4.NO_3$	250	
$FeSO_4.7H_2O$	27.8	
Na_2EDTA	37.3	
Micronutrients (B)		
$MnSO_4.5H_2O$	14.2	
H_3BO_3	3	
$ZnSO_4.7H_2O$	2	from a 100x stock solution
$Na_2MoO_4.2H_2O$	0.25	
$CuSO_4.5H_2O$	0.025	
$CoCl_2.6H_2O$	0.025	
KI	0.75	
Vitamins (C)		
Nicotinic acid	1	from a 10x stock solution
Thiamine HCl	10	
Pyridoxine HCl	1	
m - Inositol	100	
Hormones (D)		
NAA	1	from a 100x stock solution
2,4 - D	0.1	
6 - benzylaminopurine	0.2	
Xylose (E)	250	
Sucrose (F)	13,700	
Agar (G) (if present)	8,000	

Final pH adjusted to 5.6

Table 7 Composition of protoplast culture medium II (T_2 medium of Bourgin, Missonier and Chupeau, 1976).

Component	$mg.litre^{-1}$ final concentration	
Macronutrients (A)		
NH_4NO_3	825	
KNO_3	950	
$CaCl_2.2H_2O$	220	
$MgSO_4.7H_2O$	185	
KH_2PO_4	85	
Micronutrients (B)		
$FeSO_4.7H_2O$	28	
Na_2EDTA	37	
$ZnSO_4$	1	from a 100x stock solution
H_2BO_3	1	
$MnSO_4.4H_2O$	0.1	
$CuSO_4.5H_2O$	0.03	
$AlCl_3$	0.03	
$NiCl_2.6H_2O$	0.03	
KI	0.01	
Vitamins (C)		
Inositol	100	
Calcium pantothenate	1	
Nicotinic acid	1	from a 10x stock solution
Thiamine HCl	1	
Pyridoxine HCl	1	
Biotin	0.01	
Hormones (D)		
NAA	3	from a 100x stock solution
6 - benzyladenine	1	
Mannitol (E)	80,000	

Final pH adjusted to 5.5

Pre-sterilised plastic 9 cm petri-dishes and universal bottles were used in all cases. All aseptic operations were carried out in a laminar flow bench equipped with UV light, supplied by SLEE Ltd., London, England.

Plant Material.

Seeds of Nicotiana sylvestris were supplied by N. Sunderland, John Innes Institute, Norwich. Nicotiana tabacum cv. Xanthi seeds (15th generation inbred) were supplied by J.B. Power, Department of Botany, University of Nottingham, as were seeds of Nicotiana affinis (alata), Nicotiana otophora, Petunia parodii and Petunia axillaris. Plants were raised under glass without a light supplement at the University Botanic Gardens, St. Andrews.

Protoplast Isolation and Culture.

Haploid and diploid N. sylvestris protoplasts were initially isolated exactly as described by Nagy and Maliga (1976), but during the course of this work many alterations were made. The final protocol is given in full below.

Fully expanded leaves from both mature haploid and diploid N. sylvestris plants were surface-sterilised by immersion in a solution of 5% (v/v) Domestos for 15 min in a 20 cm oval Pyrex dish. The sterilising solution was poured off, and after washing three times with sterile distilled water the leaves were left for 30 min to become flaccid. The leaves were placed on a glass plate and as much of the lower epidermis as possible was peeled off, using jewellers' forceps. The leaf tissue was then placed, peeled face down, on protoplast culture medium I (16% sucrose) (Table 6) in 9 cm plastic petri-dishes (15 ml

medium per dish) for one hour to allow plasmolysis to take place. The medium was removed from underneath the leaf tissue with a pasteur pipette and a filter-sterilised solution of protoplast culture medium I (16% sucrose), containing 4% (w/v) Meicelase and 0.3% (w/v) Macerozyme R-10, pH adjusted to 5.8, was added to the petri-dishes (15 ml medium per dish), causing the tissue to be refloated. After about four hours (depending on the type of tissue) the medium containing the enzymes was removed with a pasteur pipette and replaced with a similar volume of the same medium without the enzymes. The tissue was then teased to release the protoplasts into the medium. The petri-dishes were tilted to about 5^{o} and, using a mounted needle, the debris was pulled to the upper end of the dishes. The solution containing the protoplasts was transferred to conical 15 ml glass bench centrifuge tubes which were stoppered and centrifuged at 100 g for 5 min in an MSE bench centrifuge. The resultant layer of intact protoplasts was removed by pasteur pipette and resuspended in fresh medium. After a further centrifugation the protoplasts were suspended in a known volume of fresh medium, and after counting on a Hawksley modified Fuchs-Rosenthal haemocytometer, were adjusted to the desired number per unit volume.

Culture conditions were varied widely and are outlined in Chapter two.

Attempts were also made to culture protoplasts from several other species using media and culture conditions determined by other workers. The species were N. tabacum (Power, Frearson and Hayward, 1974), N. affinis (alata)(Bourgin and Missonier, 1978) P. parodii (Frearson, Power and Cocking, 1973) and P. axillaris (Frearson, Power and Cocking, 1973), and in each case the

isolation procedure used was essentially that outlined above.

Anther culture of Nicotiana and Petunia species.

Seedlings of Nicotiana and Petunia species were raised to maturity under glass at the University Botanic Gardens, St. Andrews, and when suitably developed (pp. 40, 41) buds were removed and anthers cultured essentially as described by Nitsch (1969).

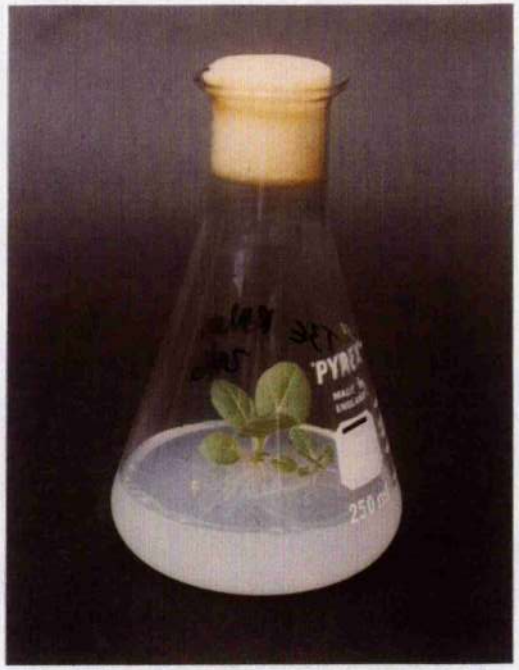
The buds were chilled for five days at 4°C in sealed polythene bags (Nitsch, 1974). The sepals were removed and the buds sterilised by dipping in 70% (v/v) ethanol for 10s and then immersion in 10% (v/v) Domestos for 10 min. After washing the buds three times with sterile distilled water, anthers were excised and transferred by their filaments to the agar surface of anther culture medium (Nitsch, 1969) (Table 2) in plastic universal bottles. The bottles were incubated at 25°C at a low light intensity in a Fisons Growth Cabinet. Plantlets which developed were removed and grown aseptically on the R medium of Kasperbauer and Collins (1972) (Table 3) in 250 ml conical flasks. When plantlets were 2-3 cm high they were removed, potted in Levingtons No 1 compost and grown under glass (Figure 3).

All aseptic operations were carried out in a laminar flow bench equipped with UV light, supplied by SLEE Ltd., London, England.

Initiation and maintenance of callus cultures of N. sylvestris and N. tabacum.

Sections of stem, taken from between 3.5 cm and 10.5 cm

Figure 3 Development of haploid Nicotiana plantlets from cultured anthers.



from the apex of anther-derived, cytologically verified haploid N. sylvestris and dihaploid N. tabacum plants were excised, stripped down to the woody layer and sterilised by immersion, firstly in 70% (v/v) ethanol for 30s and then in 20% (v/v) Domestos for 30 min. The stem sections were washed thoroughly with sterile distilled water. Pith explants were dissected out and placed on the agar surface of nitrate or amino-acid B_5 medium (Tables 4,5). Explants were incubated in plastic universal bottles or 250 ml conical flasks at 25°C in a low light intensity. Callus generally developed within two or three weeks (Figure 4).

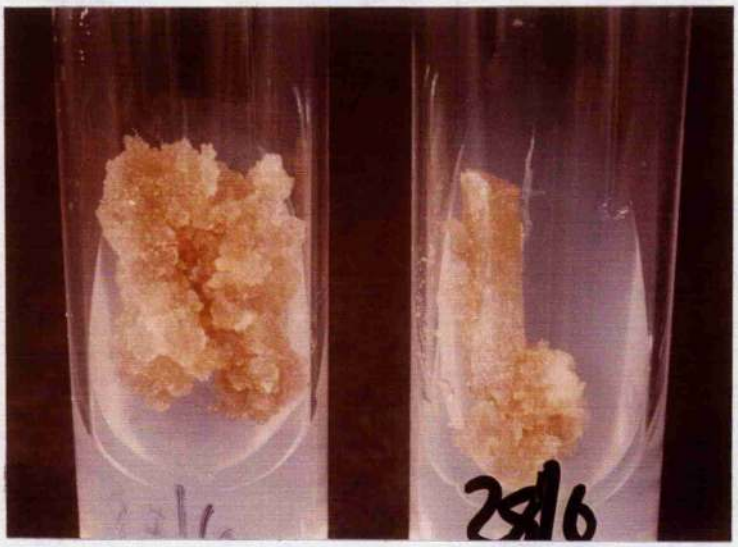
Callus cultures were routinely subcultured every four weeks by removing portions of callus and placing on fresh medium.

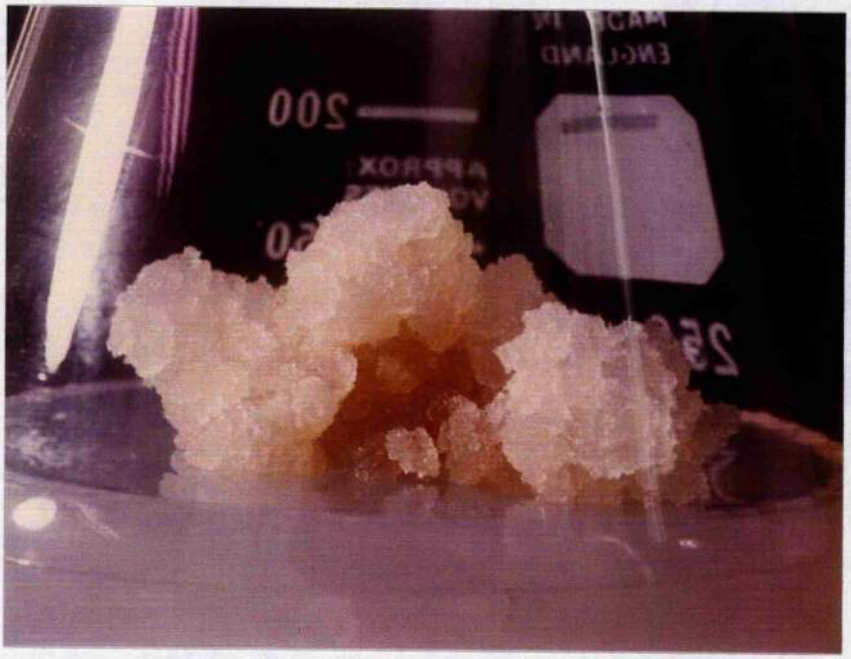
Initiation and maintenance of cell suspension cultures of N. sylvestris and N. tabacum.

Cell suspension cultures were initiated by squashing several grams of callus with a spatula, and suspending the cells in 100 ml liquid nitrate or amino-acid B_5 media (Tables 4,5). 250 ml conical flasks containing the cell suspension were placed on an LHE orbital shaker, operating at 120 cycles/min in the dark at 28°C. Generally, callus would quickly break up to form a suspension of single cells and small cell aggregates, as determined by examination under the microscope. Cultures reached stationary phase within two to four weeks.

Cell suspension cultures were routinely subcultured approximately every fourteen days with wide-bore (0.75 cm) pipettes. 5 or 10 ml aliquots of cell suspensions in stationary phase were transferred to 250 ml conical flasks each containing 100 ml fresh medium. As aliquots were withdrawn, the pipettes

Figure 4 Development of callus cultures from haploid *Nicotiana* pith explants.


2816


200

were rotated and the flasks were shaken to ensure an even distribution of cells. All subculturing was carried out in a laminar flow bench.

Treatment of N. tabacum cell suspensions with the chemical mutagen, ethyl methane sulphonate (EMS).

The protocol for selection of chlorate-resistant cell-lines from EMS-treated cell suspensions is given in full in Chapter four.

The following procedure was developed for the safe handling of the chemical mutagen EMS during the treatment of plant cells.

Using a 2 ml disposable syringe and disposable Millex-GS 0.22 µm filter unit, 0.25 ml or 0.4 ml EMS was transferred aseptically to a 250 ml conical flask containing 100 ml cell suspension in logarithmic phase. Excess EMS in the syringe was returned to the stock bottle and the syringe and filter were placed in a bucket of 2N-sodium hydroxide. The flask containing EMS was incubated on the orbital shaker for the requisite period, then cells were harvested on a 5 cm Buchner funnel containing a Miracloth filter. The cells were washed free of EMS with 300 ml washing medium (Table 4), weighed and transferred to one or more 250 ml conical flasks each containing 100 ml fresh B_5 medium (Table 4), normally containing a selection of amino-acids as nitrogen supplement (Table 5). These flasks were incubated on an orbital shaker for one week before application of selection pressure.

Operations involving EMS were carried out with disposable gloves in a SLEE laminar flow cabinet with the fan off. The washes, which were collected in a one litre Buchner flask, contained most of the EMS and were added to an equal volume of

4 M-sodium hydroxide in a waste bottle, left for two days and then poured down a sink with the tap running. The Buchner flask and funnel were placed in 2 M-sodium hydroxide for 2 days prior to washing. High pH causes decomposition of methane sulphonic esters such as EMS (Osterman-Golkar, Ehrenberg and Wachtmeister, 1970).

Plating techniques.

Prior to plating, cells were harvested on a Buchner funnel containing a Miracloth filter and washed with washing medium (Table 4). The cells were weighed aseptically and 1g of cells was suspended in 8 ml liquid B_5 medium (Tables 4,5), and spread evenly on 12 ml agar B_5 medium in each of several 9 cm disposable petri-dishes. The petri-dishes were sealed with Nescofilm and incubated at 25°C in a low light intensity.

CYTOLOGICAL TECHNIQUES.

Determination of ploidy of anther-derived N. sylvestris and N. tabacum plants.

The ploidy of anther-derived plants was determined by chromosome analysis of stained squash preparations from root-tips.

Root-tips were excised around midday and placed in a 0.05% (w/v) colchicine solution. After two hours, the colchicine was siphoned off using a pasteur pipette and replaced with Farmers fluid (absolute ethanol/glacial acetic acid 3:1). After 18h in this fixative the root-tips were removed with tweezers and placed in 1 M-HCl for 8 min (Kept at 60°C in a hot water bath). Hydrolysis time is critical: if too short squashing will be impossible, if too long staining will be poor. After this time the HCl was siphoned off and replaced with leuco-basic Fuchsin stain (Purvis, Collier and Walls, 1966). Staining took about two hours.

Slides of each stained root-tip were prepared by firstly placing each root-tip in a drop of 45% (v/v) acetic acid on a microscope slide, applying a cover slip, tapping the area of the root-tip to spread the tissue and carefully squashing to spread the cells. Temporary preparations were made by sealing the slide with Euparal. Photographs were taken on a Zeiss photomicroscope.

In the case of N. sylvestris, good preparations allowed accurate determinations of the ploidy of the plants to be made by counting the number of chromosomes per cell when in metaphase. Due, however, to the large number of chromosomes in N. tabacum it was not often possible to determine the exact number per

cell, and good photographs were not obtained.

Determination of ploidy of cultured plant cells.

Chromosome analysis of callus and cell suspension cultures was much more difficult than with root-tip samples, since actively dividing cells in metaphase were seldom found in reasonable numbers. Furthermore callus and suspensions all contained cells of differing ploidies.

The procedure was similar to that for root-tip samples with the exception that all treatments, up to and including staining, were carried out in 15 ml glass conical centrifuge tubes, each treatment being terminated by spinning down the cells at 100 g for 3 min in an MSE bench centrifuge and discarding the supernatant. The cells were then resuspended for the next treatment. The numbers of chromosomes in as many intact cells as possible were determined in order to give an indication of the range of ploidy.

Determination of Cell Viability.

Viability of cultured plant cells was determined with phenosafranine dye, using a method developed from that of Widholm (1972). The dye does not stain live cells but stains intact dead cells. Dead cells which have lost their contents are not stained. The dye is probably excluded from live cells by the intact plasma membrane (Widholm, 1972).

One drop of phenosafranine solution (0.02% (w/v) in 2 M-sucrose) was mixed with one drop of cell suspension on a Hawksley modified Fuchs-Rosenthal haemocytometer. A cover-slip was applied and the numbers of stained and non-stained intact cells were counted under low power on a light microscope between 5 and 20 min after mixing.

PREPARATION OF CELL-FREE EXTRACTS FROM N. TABACUM CELL CULTURES.

Preparation of cell-free extracts.

N. tabacum cells were harvested by filtration of suspensions on 7 cm Buchner funnels with Miracloth discs and washed with washing medium. The cells were weighed and then homogenised in 0.05 M-KH_2PO_4, pH 7.5 containing 0.1 mM-Na_2EDTA, 10 mM-FAD, 1 mM-cysteine and 3% (w/v) bovine serum albumin or in 0.1 M-KH_2PO_4, pH 7.5 containing 1 mM-Na_2EDTA, 12 mM-β mercaptoethanol and 3% (w/v) bovine serum albumin (both 5 ml per gram fresh weight of cells) by twenty strokes in a motor-driven Potter homogeniser. The homogenate was centrifuged at 38,000 g for 20 min at 3°C in an MSE High Speed 18 refrigerated centrifuge. The resultant supernatant was used as the source of enzyme.

Concentration of enzyme extracts.

If required, enzyme extracts were concentrated by ammonium sulphate precipitation (Dawson, Elliot and Jones, 1969). The required amount of saturated ammonium sulphate solution, pH 7.5 was added to the enzyme extract which was stirred throughout in an ice water bath. After 30 min the precipitated protein was collected by centrifugation at 20,000 g for 15 min at 3°C in an MSE High Speed 18 centrifuge. The precipitate was then redissolved in a small amount of the appropriate buffer.

ENZYME AND OTHER ASSAYS.

NADH - nitrate reductase.

NADH - nitrate reductase was assayed in cell-free extracts (p.30) by the method of Wray and Filner (1970). The reaction mixture contained

0.5 ml 0.1 M-potassium phosphate buffer, pH 7.5.

0.1 ml 0.1 M-potassium nitrate.

0.1 ml 0.1 mM-NADH.

0.1 ml distilled water.

This was added to 0.2 ml enzyme extract to start the reaction. The reaction was carried out at 25°C for a suitable time period (generally 30 min). Nitrite formation was measured by adding 1 ml of 1% (w/v) sulphanilamide in 3 M-HCl followed by 1 ml of 0.02% (w/v) N - (1-naphthyl)ethylenediamine dihydrochloride (Snell and Snell, 1949). After mixing and allowing 15 min for colour to develop, the precipitated protein was removed by centrifugation at top speed for 5 min in an MSE bench-top centrifuge.

Nitrite produced was estimated by measurement of absorbance at 540 nm of this solution, compared with a control where the reaction mixture was added to the enzyme extract immediately prior to the addition of the sulphanilamide solution.

A calibration plot of 0-100 nmol potassium nitrite was prepared (Figure 5) and absorbance values were converted to nmol nitrite formed by reference to this plot.

NADH - cytochrome c reductase.

This was assayed using the method of Wray and Filner (1970). A reaction mixture was prepared containing

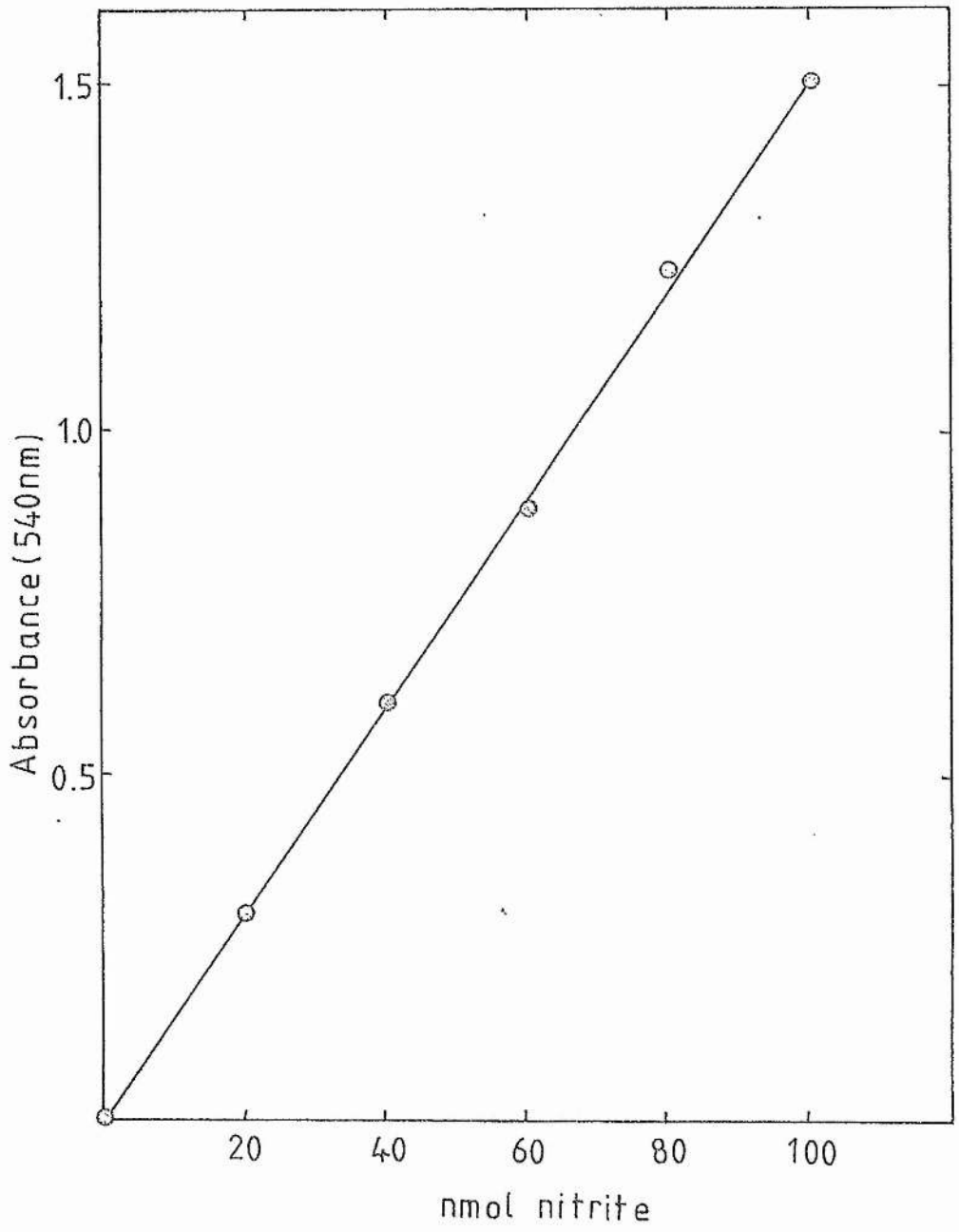


Figure 5 Nitrite standard curve showing relationship between nitrite concentration and absorbance at 540nm.

10 ml 0.1 M-potassium phosphate buffer, pH 7.5

5 ml distilled water.

20 mg cytochrome c.

3.6 mg NADH.

To 0.3 ml of this mixture was added 0.1 ml enzyme extract. The change in absorbance at 550 nm due to reduction of cytochrome c was followed on a Pye Unicam SP500 Series II UV/visible spectrophotometer linked to a Pye Unicam AR25 chart recorder or a CECIL CE 272 UV/visible spectrophotometer linked to a Bryans 28000 chart recorder (double assay volumes required).

Nitrite reductase.

This was assayed by the method of Wray and Filner (1970). A reaction mixture was prepared containing

25 ml 0.1 M-potassium phosphate buffer, pH 7.5.

5 ml 0.5 mM-potassium nitrite.

5 ml 1 mM-methyl viologen.

To 0.7 of the above mixture was added 0.2 ml enzyme extract. The tubes were flushed with nitrogen and the reaction was started by the addition of 0.1 ml of 15 mg/ml sodium dithionite in 95 mM-sodium bicarbonate. The tubes were again flushed with nitrogen and sealed with Nescofilm. The methyl viologen was reduced to its blue form by the dithionite. After a suitable incubation period at 25°C, the reaction was stopped by removal of the Nescofilm followed by vigorous agitation of the assay tube on a Whirlimix. This re-oxidises the methyl viologen to its colourless form. The change in absorbance at 540 nm due to reduction of nitrite was converted to nitrite reductase activity by reference to a standard plot of absorbance at 540 nm against nitrite (Figure 5).

Xanthine Dehydrogenase.

Attempts were made to measure xanthine dehydrogenase activity in concentrated cell-free extracts from N. tabacum cells. It was not found possible to measure xanthine dehydrogenase activity by the method of Scazzocchio, Holl and Foguelman (1973), due to a high background level of cytochrome c reduction above which no activity could be detected. This assay was also very expensive in reagents. No xanthine dehydrogenase activity could be detected using the method of Mendel and Müller (1976) either, but a modification of this method was derived which also allowed qualitative though not quantitative determination of xanthine dehydrogenase. This procedure is outlined below.

Enzyme extracts from N. tabacum cells (p. 60) were concentrated by precipitation of protein with an equal volume of saturated ammonium sulphate solution, pH 7.5. The precipitate was suspended in as small a volume of 0.1 M-sodium pyrophosphate buffer, pH 8.0 as possible.

0.2 ml aliquots of these concentrated extracts were incubated at room temperature in the dark for 3h with 0.2 ml of the staining mixture. The staining mixture contained

0.1 M-sodium pyrophosphate buffer, pH 8.0

2 mM-hypoxanthine.

1 mM-nitroblue tetrazolium.

0.1 mM-phenazine methosulphate.

The presence of xanthine dehydrogenase was shown by the development of a red-brown colour, not present in blanks which contained no hypoxanthine. The colour is not stable to light and alters with time.

Catalase.

Catalase was assayed using a modified form of the Beers

and Sizer (1952) method. A reaction mixture was prepared containing

0.4 ml 30-volume hydrogen peroxide.

100 ml 0.1 M-potassium phosphate buffer, pH 7.5.

30 µl of enzyme extract was added to 3 ml of this mixture and the decrease in absorbance at 240 nm, corresponding to the breakdown of hydrogen peroxide by catalase, was followed on a recording spectrophotometer.

Alcohol Dehydrogenase.

This was assayed by using a modification of the method of Vallée and Hoch (1955). A reaction mixture was prepared containing

30 ml 0.1 M-Tris/HCl buffer, pH 8.5.

75 ml distilled water.

90 mg NAD^+

30 mg dithiothreitol.

0.3 ml absolute ethanol.

30 µl of enzyme extract was added to 3 ml of this mixture and the increase in absorbance at 340 nm due to production of NADH was followed on a recording spectrophotometer.

Myoglobin.

The distribution of myoglobin in sucrose gradients was determined by measuring the absorbance at 415 nm of fractions.

Nitrate.

Nitrate was estimated using the method of Aviram (1975). This method uses Szechrome NAS reagent, prepared for use by dissolving 5 g Szechrome NAS in a mixture of 500 ml concentrated

phosphoric acid (85-86%). The acid mixture was prepared at least one week prior to addition of the reagent.

0.5 ml of a diluted extract containing up to 100 nmol nitrate was added to 5 ml of the reagent mixture. After mixing of contents by inversion, a violet colour was produced by the action of nitrate on the diphenylamine sulphonic acid in the reagent.

Absorbance at 570 nm was converted to nmol nitrate by means of a previously constructed calibration plot (Figure 6).

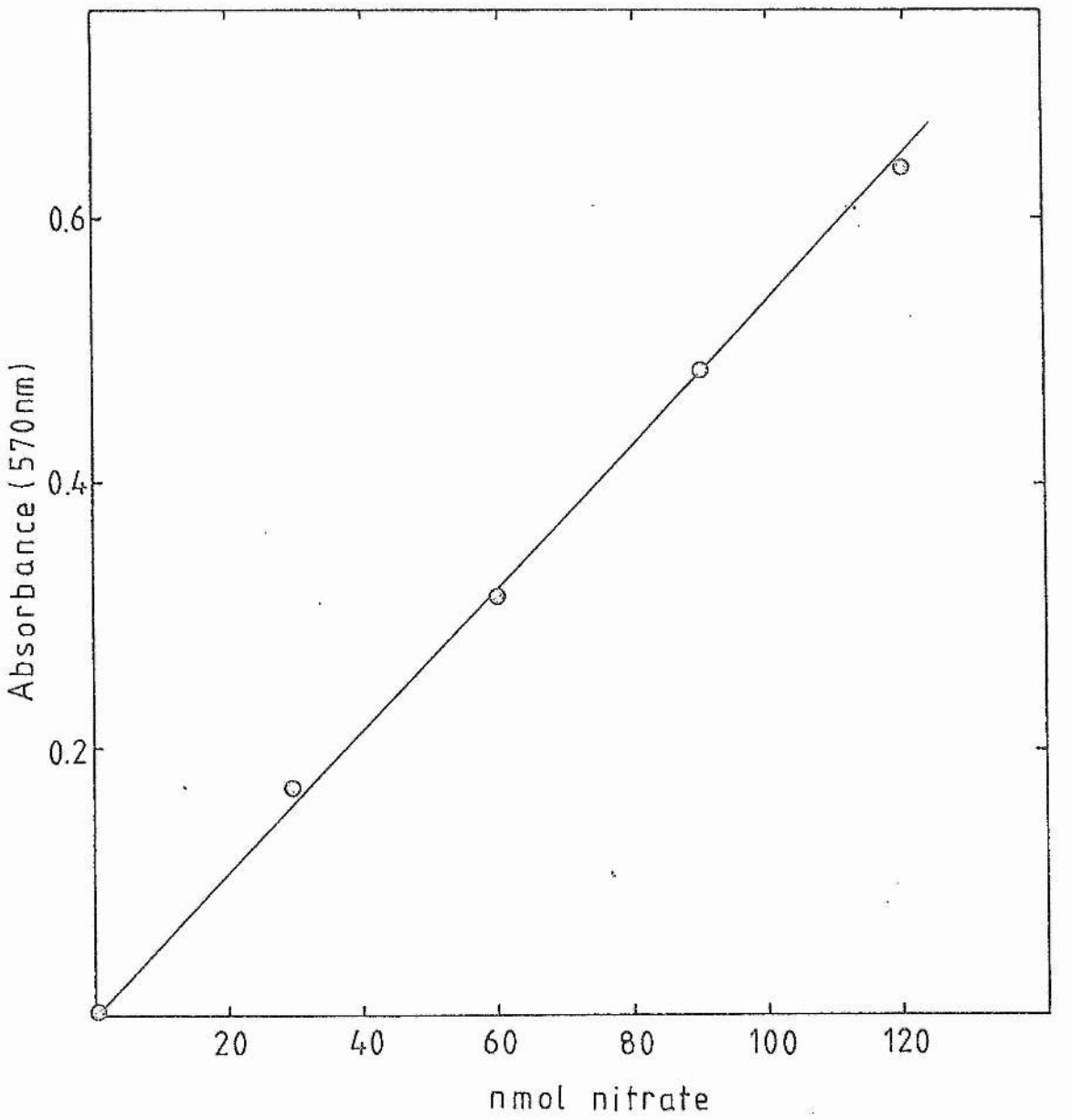


Figure 6 Nitrate standard curve showing relationship between nitrate concentration and absorbance at 570nm.

SUCROSE DENSITY GRADIENT CENTRIFUGATION

In order to determine the sedimentation coefficients of cytochrome c reductase and nitrate reductase species in cell-free extracts from N. tabacum cell cultures (p.99), sucrose density gradient centrifugation was carried out, essentially as described by Small and Wray (1980).

Preparation of gradients.

Solutions of 4,6,8,10,12,14,16,18 and 20% (w/v) sucrose were prepared from stock solutions of 0% and 20% sucrose in either 0.1 M-KH_2PO_4 buffer, pH 7.5 containing 0.1 mM-EDTA and 10 µM-FAD or with the addition of 1 mM-β-mercaptoethanol.

Gradients were prepared by layering 2 ml of each successively less concentrated sucrose solution into 25 ml polypropylene centrifuge tubes (MSE-34411-138). The gradients were then sealed with Nescofilm and left to equilibrate at 4°C for 18h.

Centrifugation of gradients.

Samples for analysis were mixed with aliquots of each of three reference proteins, namely catalase (11.3S), alcohol dehydrogenase (7.4S) and myoglobin (2.04S). 0.4 ml of this mixture was carefully layered on top of each of the gradients which were then centrifuged at 84,000 g for 24h at 0-2°C in an MSE Superspeed 65 preparative ultracentrifuge (Mark I).

Fractionation of Gradients.

After centrifugation, the gradients were fractionated into approximately 40 fractions of 15 drops each, using an MSE tube

piercer. The linearity of the gradients was confirmed using a Bellingham and Stanley Abbe-type refractometer.

Calculation of Sedimentation Coefficients.

These were determined by reference to the sedimentation characteristics of the three reference proteins included in each gradient. These proteins were estimated in each fraction and the peak fractions of each were subsequently plotted against their known sedimentation coefficients to yield a straight line. From this plot the sedimentation coefficients of the proteins under study could be determined, knowing the peak fraction in which they sedimented (Small and Wray, 1980).

Chapter 1. Isolation of haploid plants by anther culture.

INTRODUCTION

Pollen from diploid plants is haploid and the techniques of pollen and anther culture exploit this situation to produce whole plants carrying single copy genetic information. Subsequently, haploid cell-lines can be produced which, because genetic variations would be expressed in them without interference from duplicated genetic material, are potentially very useful in the isolation of autotrophic cell-lines (Carlson, 1970; Sadisivaiah, 1974; Nitsch, 1972).

Pollen and anther culture.

The first successful pollen culture was reported by Tulecke (1953, 1957), who raised callus from mature pollen grains of Ginkgo. Attempts to culture angiosperm pollen by similar means were unsuccessful until Guha and Maheshwari (1964) discovered that Datura innoxia pollen could be stimulated to form callus by culturing intact anthers on an appropriate medium. More significantly, cultured anthers produced organised haploid plants. Their procedures were rapidly adopted by other workers and applied to other species, initially only to other members of the Solanaceae family (Bourgin and Nitsch 1967; Nitsch and Nitsch, 1969; Sharp, Dougall and Paddock, 1971) but eventually to cereals (Clapham 1973; Picard and de Buyser, 1973; Wang, Sun, Wang and Chien, 1973). The developmental pathway along which pollen is diverted from its normal role to form haploid plants is described well by Sunderland and Dunwell (1977).

Vigorous plants are now readily obtained by anther culture from a number of plant species, especially Datura innoxia (Guha and Maheshwari, 1964), Nicotiana tabacum (Bourgin and

Nitsch, 1967) and Nicotiana sylvestris (Nitsch, 1969), but some species are more suitable than others for genetic work requiring single copy genetic material. Datura innoxia has the disadvantage that most plants produced by anther culture are in fact polyploid due to a high rate of meiotic instability (Collins, Dunwell and Sunderland, 1974), and the large number of chromosomes present in anther-derived N. tabacum plants (n=24) makes cytological studies difficult. There is also the possibility of there being duplicated genetic material in N. tabacum "haploids" (p.69). N. sylvestris, however, is particularly suitable as it gives rise easily to vigorous haploid plants by anther culture and is a "true" diploid species (2n=24).

The raising by anther culture in this laboratory of haploid N. sylvestris plants is described below, followed by an outline of efforts to raise haploid plants from several other species, namely N. tabacum, N. affinis (alata), N. otophora, Petunia parodii and Petunia axillaris.

RESULTS

Anther culture of N. sylvestris.

Buds were removed from maturing N. sylvestris plants during 1976, 77 and 78, and anthers cultured as described on p. 24. A range of bud sizes was used but, whenever possible, buds were picked when their petals had just grown beyond their sepals (Stage 3 in Figure 7). Nitsch (1969), found that, in N. sylvestris, maximal embryo formation was obtained from anthers removed at this stage of bud growth, which corresponds to the occurrence of mitosis in the microspore, before starch accumulation.

Only a small percentage of the cultured anthers gave rise to plantlets (Table 8) but most of these androgenic anthers would produce more than one. Anther-derived plants were of two distinct morphological types (Figure 8); those which were identical to seed-grown plants, possessing broad leaves and large flowers and producing seed, and those which were smaller than seed-grown plants, possessing narrow leaves and small flowers and producing no seed. The numbers of these two types of plant obtained during each season are given in Table 9.

Root-tip chromosome analysis (p.28) showed that all plants of the first morphological type were diploid, but, although most plants of the second morphological type were found to have a haploid chromosome complement, two of these plants were diploid, indicating that haploid plants cannot be distinguished from diploid plants by morphology alone.

Haploid N. sylvestris plants were maintained vegetatively and have retained their haploidy for up to five years. The chromosome complement of root-tip cell, prepared as on p. 28

Figure 7 N. sylvestris buds used for anther culture. Stage 3 is most suitable.

Figure 8 Stature and flower size of Types 1 and 2 anther-derived *N. sylvestris* plants.

Type 1 Type 2 Type 1

Type 2

Type 1

Type 2

Table 8 Androgenic anthers as a percentage of the total number of N. sylvestris anthers plated during 1976, 1977 and 1978.

Year	Anthers plated	Androgenic anthers (no.)	Androgenic anthers (%)
1976	320	7	2.2
1977	590	23	3.0
1978	210	5	2.4

Table 9 Morphology of anther-derived *N. sylvestris* plants.

Year	Total no. of plants.	Morphology of plants. Type 1	Type 2	Unknown.
1976	16	6	8	2
1977	33	9	15	9
1978	18	1	12	5

Type 1: similar to seed-grown plants, possessing broad leaves and large flowers and producing seed.

Type 2: Smaller than seed-grown plants, possessing narrow leaves and small flowers and producing no seed.

Unknown: These plants died before maturity.

from a haploid anther-derived plant, is shown (Figure 9), but the large number of chromosomes present made it difficult to obtain good photographs from diploid plants.

Anther culture of other Nicotiana and Petunia species.

When it became apparent that N. sylvestris might not be a suitable species from which to obtain nitrate reductase-minus cell-lines (p.64), plants of several other Nicotiana species and two Petunia species were raised from seed for anther culture.

N. tabacum.

Buds were removed from maturing N. tabacum plants in 1978 and anthers were cultured as described on p.24. Wherever possible buds were picked when their petals reached to the tips of their sepals (Stage 2 in Figure 10) because Nitsch (1969) had found that this stage of bud growth corresponded to the occurrence of mitosis in the microspore, when maximal embryo formation could be obtained from cultured anthers.

Only four anthers out of seventy plated gave rise to plantlets but seventeen plants were finally raised from these. All of these plants were considerably smaller than seed-grown plants (Figure 11). They did not produce seed, and were dihaploid as determined by root-tip chromosome analysis (p.28). These plants were maintained vegetatively and have retained their dihaploidy for three years.

N. affinis (alata), N. otophora, P. parodii and P. axillaris.

Buds of various sizes were removed from maturing N. affinis (alata) (2 varieties), N. otophora, P. parodii and P. axillaris plants and anthers cultured as on p.24 . No mature haploid plants were produced from any of these anthers

although some N. affinis (white flowered) anthers did produce weak, sickly plants, which all died when only a few inches high.

Figure 9 Chromosome complement of a haploid (n=12) *N. sylvestris* root-tip cell.

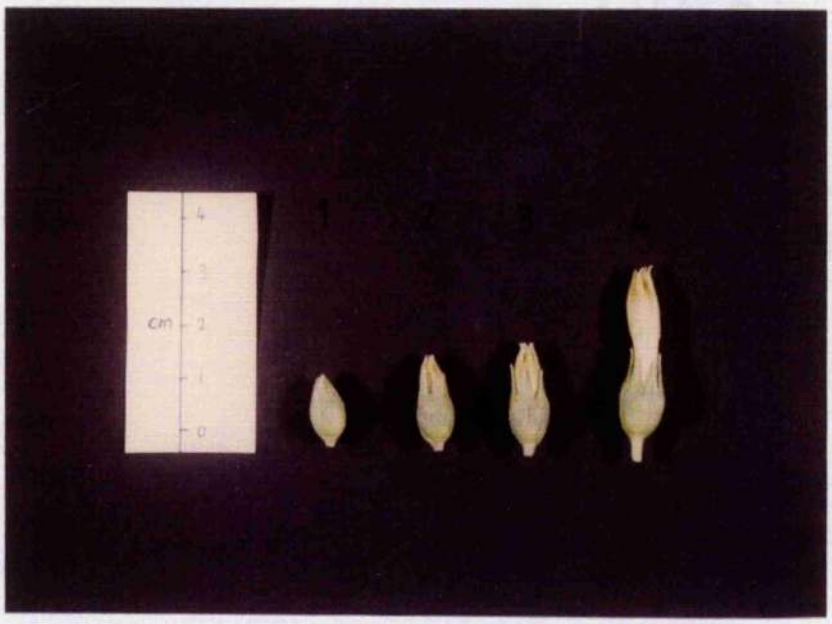
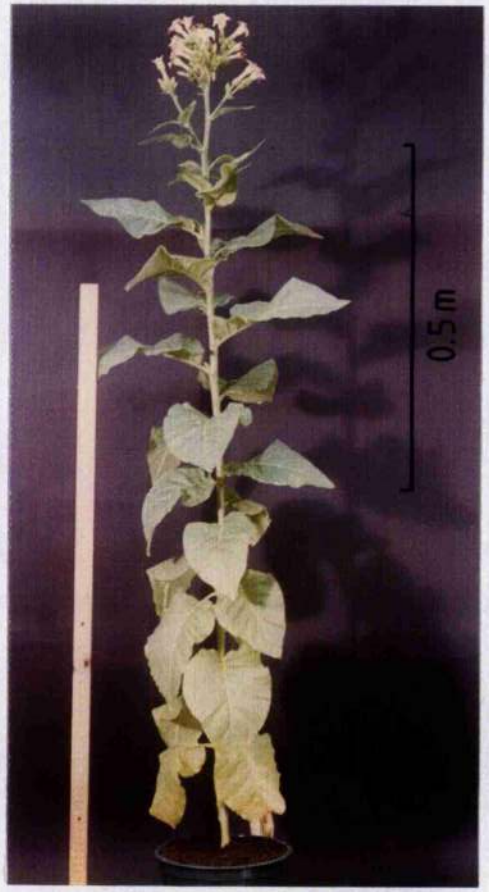


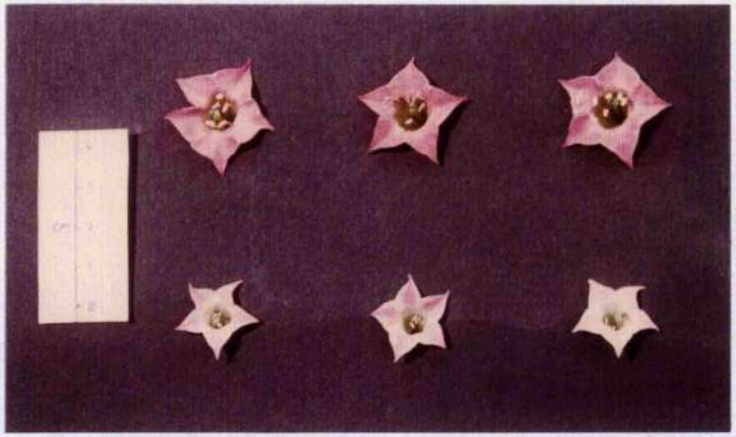
Figure 10 N. tabacum buds used for anther culture. Stage 2 is most suitable.

Figure 11 Stature and flower size of seed-grown (amphidiploid) and anther-derived (dihaploid) N. tabacum plants.



Amphidiploid

Amphidiploid



Dihaploid

Dihaploid

Table 10 Androgenic anthers as a percentage of the total number of anthers plated for different species.

Species	Anthers plated	Androgenic anthers (no.)	Androgenic anthers (%)
N. affinis (white flowered)	570	2	0.3
N. affinis (crimson bedder)	1055	0	0
N. otophora	264	0	0
P. parodii	65	0	0
P. axillaris	60	0	0
N. tabacum	70	4	5.7

DISCUSSION

Anther culture of N. sylvestris.

Although it proved relatively easy to raise N. sylvestris plants by anther culture, many of these were not haploid but diploid (p. 40). This was not anticipated as Nicotiana species generally give rise exclusively to haploid plants by anther culture (Sunderland 1970; Collins and Sunderland, 1974). However non-haploids have been noted by Collins, Legg and Kasperbauer (1972) who suggested that they might have arisen from diploid tapetal cells in the anther, or from an early failure of telekinesis in a haploid cell-line.

A number of haploid plants were produced though, and in general these could be distinguished from diploid plants by their morphology, haploid plants being smaller in stature and possessing narrower leaves. Unfortunately, however, some of these small narrow-leaved plants proved subsequently to be diploid, and it was apparent from this that all anther-derived plants must be karyotyped in order to determine whether they are haploid or not.

Anther culture of N. tabacum, N. affinis (alata), N. otophora, P. parodii and P. axillaris.

Seventeen plants were produced by anther culture of N. tabacum and in this case all carried the gametic chromosome number. Healthy plants could not be obtained from any of the other species, although a few weak plantlets were produced from N. affinis (white flowered) anthers.

It is not yet totally clear why some species produce strong haploid plants by anther culture and why some produce only weak

plants or none at all (de Fossard, 1974). N. tabacum is recognised as perhaps the easiest species to obtain "haploid" plants from by anther culture (Nitsch, 1969). Nitsch suggested that this may be due to the amphidiploid nature of N. tabacum (2n=48), such species yielding plantlets carrying the gametic chromosome number more easily than diploid species. The percentage of anthers which produced plantlets in this laboratory was certainly higher in N. tabacum than in any of the other species tested, none of which were amphidiploid.

Of the diploid Nicotiana species, anther culture of N. affinis (alata) and N. otophora were not sucessful (p.41) but anther culture of N. sylvestris was (p.40). This may have been because N. sylvestris is a self-compatible species, whereas N. affinis (alata) and N. otophora are both self-incompatible (Nitsch 1972). The N. sylvestris plants used in this work were from an inbred line and would exhibit a lower degree of heterozygosity, and thus contain fewer deleterious recessive genes, unmasked and expressed in the haploid, than more heterozygos self-incompatible species such as N. affinis (alata) and N. otophora.

Unfortunately, time was not available for attempts to raise haploid plants from other possibly suitable species such as N. plumbaginifolia (Novak and Vyskot, 1975) or N. knightiana (Collins and Sunderland, 1974).

Chapter 2. Protoplast culture of *N. sylvestris*.

INTRODUCTION

The successful culture of Nicotiana protoplasts was first reported by Nagata and Takebe (1970). Since then the culture of N. tabacum protoplasts has become a routine procedure in many laboratories but the culture of other Nicotiana species has proved much more difficult (Banks and Evans, 1976).

However, adequate plating efficiencies (i.e. the percentage of plated protoplasts which formed calli) were eventually achieved by Nagy and Maliga (1976) with protoplasts from N. sylvestris diploid leaf tissue, and, albeit less successfully, by Bourgin, Missonier and Chupeau (1976) with both haploid and diploid N. sylvestris protoplasts. It was hoped that haploid and diploid N. sylvestris protoplasts could be cultured by similar means in this laboratory.

There is often a lot of trial and error involved in protoplast culture as, quite apart from the culture conditions, the condition of the starting material and the method of protoplast isolation used, can have a considerable bearing on the subsequent behaviour of the protoplasts once cultured. N. sylvestris, however, is an apparently homogeneous species with no notable variants, so previously successful procedures offered a good starting point.

RESULTS AND DISCUSSION

Isolation and culture of N. sylvestris protoplasts as described by Nagy and Maliga (1976).

Fully expanded leaves of haploid and diploid N. sylvestris plants were surface-sterilised by immersion in 70% (v/v) ethanol for 15s and then 5% (v/v) Domestos for 20 min, followed by four washes in sterile distilled water. After removal of the lower epidermis, the leaves were placed, face down, on protoplast culture medium I (Table 6) for 3h in the dark at 28°C, in order to plasmolyse the exposed cells. After this time, the plasmolysing solution was replaced with protoplast culture medium I, containing 2% (w/v) Cellulase R-10 and 0.5% (w/v) Macerozyme. After a further 3-4h in the dark at 28°C, the tissue was teased and the resultant protoplast suspension was passed through a 64μ nylon filter, in order to remove debris. The protoplast suspension was then centrifuged at 100 g for 3 min, resulting in the accumulation of protoplasts at the surface. Protoplasts were then washed three times with culture medium and plated in 5 cm petri-dishes at a density of $1\text{-}4 \times 10^4$ protoplasts per ml. Dishes were maintained at 28°C, firstly at a low light intensity (about 300 lux) for three days, then subsequently at a high density (about 1500 lux).

Unfortunately, using the above isolation procedure, yields of intact protoplasts were never more than 10^4 per gram of peeled tissue. Furthermore, although they remained intact for up to two weeks, cultured protoplasts did not divide more than once under these culture conditions. Some aspect(s) of the isolation procedure and culture conditions of Nagy and Maliga (1976) obviously required adaptation to suit the

particular tissue available in this laboratory.

Adaptation of the isolation procedure and culture conditions.

When assessing the isolation procedures and culture conditions used successfully for protoplast culture of a given species in other laboratories, it is often difficult to determine which aspects are important. A particular aspect of the method may be critical to its success or there may be considerable flexibility. Often it is simply not known which, because methods of protoplast isolation and culture are usually derived from other established methods, which, if successful, are retained. Possible improvements are not investigated with the result that less than optimal conditions may be employed.

In order to obtain a workable system in this laboratory it was therefore necessary to systematically investigate and possibly vary every aspect of the method, applying improvements where possible. Any improvement in the isolation procedure would be represented by increased yields of intact protoplasts, and, since the yields achieved above were so low, there was much scope for this. The effect of alterations to the culture conditions, however, can only be judged by whether the protoplasts divide or not.

A summary of the different conditions used, and alterations made to the method in effort to stimulate the protoplasts to form calli, together with a short explanation of the rationale behind them, is given below.

Source of plant material.

Protoplasts were isolated and cultured from leaf tissue of

plants raised under the following conditions:

a) Diploid plants grown from seed in a greenhouse without light supplement.

b) Diploid plants grown under artificial light supplied by Gro-lux lamps at 25°C.

c) Haploid plants derived by anther culture (p.40) and grown to maturity in a greenhouse without light supplement.

In all three cases, leaves were normally used when fully expanded; about 80-100 days after planting in the case of seed-grown diploid plants, and about four months after potting or re-potting in the anther-derived plants. In practice, however, it was not possible to ensure standardised supplies of haploid leaf tissue throughout the year, as the plants were all derived by anther culture at the same time of year. Immature leaves from both haploid and diploid plants were also used in order to determine whether they might be more suitable for protoplast culture.

Leaves of a similar development age from the three groups, a, b and c, gave approximately similar yields of intact protoplasts, all other conditions being equal, but much poorer yields were invariably obtained from immature leaves. This was probably due, in part at least, to the extreme difficulty experienced in peeling the lower epidermis off younger leaves. In many cases it was not possible to peel any of the epidermis off.

Unfortunately, viable cultures were not obtained with any of the above tissue types so it was not possible to say what effect the differences in source of plant material had on the subsequent behaviour of the protoplasts in culture.

Isolation of Protoplasts.

a) Preparation of tissue

Leaf tissue was initially sterilised by immersion of the intact leaves in 70% (v/v) ethanol for 15s and then 5% (v/v) Domestos for 20 min, but this procedure caused pitting of the leaf tissue which doubtless contributed to the low yields of protoplasts originally obtained. Omission of the ethanol treatment and the reduction of the time immersed in Domestos to 15 min was found to greatly reduce the extent of this pitting, whilst retaining asepsis.

Peeling the lower epidermis from leaves was always very difficult, particularly with immature leaves (see above). This is an inherent problem in this species (Banks and Evans, 1976), and it was never fully overcome, but with practice a sufficiently large area of leaf tissue from fully-expanded leaves could be peeled within a reasonable time period. The difficulties experienced, however, were responsible for considerable mechanical damage to the fragile tissue and the risk of microbial contamination was also increased.

b) Plasmolysis

After removing the lower epidermis and before enzyme treatment it is necessary to plasmolyse the exposed cells. Plasmolysis minimises the deleterious effects of the enzymes (Tribe, 1955) and the choice of plasmolyticum is important. Mannitol, which penetrates cells slowly and is effectively inert, is a possible plasmolyticum although sucrose can also be used. Metabolism of sucrose by the plant cell may be a problem, but may also be useful since continued culture of protoplasts requires that the osmolarity of the culture medium be reduced.

This would occur as sucrose was metabolised (Michalyuk and Kao, 1975). The concentration of the plasmolyticum is also important since low concentrations can cause spontaneous protoplast fusion (Evans and Cocking, 1977).

In the studies reported here, two media were used. These were protoplast culture medium I (PCM I), based on the K_3 medium of Kao, Constabel, Michalyuk and Gamborg (1974) and containing 13.7%(w/v) sucrose, and protoplast culture medium II (PCM II) which contains 8% (w/v) mannitol and was used by Bourgin, Missonier and Chupeau (1976).

Protoplasts did not float on PCM II and subsequent separation of protoplasts from debris was difficult. In most of the experiments PCM I was used. Increasing the sucrose concentration to 16% gave a much higher yield of protoplasts since many protoplasts did not float on 13.7% sucrose and were lost during the washing procedure. It is unlikely that an increase of this order would be sufficient to damage the protoplasts. Nagy and Maliga (1976) carried out plasmolysis for three hours, but a one hour period of plasmolysis was adopted here since microscopic examination showed that plasmolysis was complete by this time.

c) Enzyme treatment.

Albersheim (1974) described the cell wall of dicotyledons as being composed of cellulose fibrils bound to each other by pectic polymers which bind covealently to a xyloglucan monolayer coating. Associated with this is a glycosylated hydroxyproline-rich protein of uncertain position. For the degredation of such a structure a number of enzymes are required and crude cellulases have proved suitable. Macerozyme, a crude pectinase

for separation of cells and Cellulase, a crude cellulase, were used in succession (Takebe, Otsuki and Aoki, 1968), and later concurrently (Power and Cocking, 1969) to isolate N. tabacum mesophyll protoplasts. The crudity of these preparations undoubtedly assisted their degredative properties but they also contained potentially damaging low molecular weight material, so, for this reason, commercially desalted enzymes were used in this work, viz. Cellulase R-10 and Macerozyme R-10. Lower concentrations of degredative enzymes caused less damage to the protoplasts than shorter incubations at higher concentrations. In this laboratory Macerozyme R-10 and Cellulase R-10, at one-tenth of their original concentrations (p.46) were fully effective at releasing protoplasts within a 16h incubation but the cultured protoplasts still did not divide.

Finally, different batches of the three enzymes, Macerozyme R-10, Meicelase and Cellulase R-10, were also used in case the originally used batches were exceptionally toxic to the protoplasts, but no differences could be detected in their subsequent behaviour in culture.

d) Separation from debris and washing of intact protoplasts.

Separation of intact protoplasts from debris after enzyme treatment and their subsequent washing were initially carried out as described on p. 46. Protoplasts were teased into the medium containing the enzymes and passed through a 64μ nylon filter to remove large debris. The filtrate was then centrifuged gently and intact protoplasts which floated to the top were washed three times with culture medium.

It was immediately apparent, in my hands at least, that most of the protoplasts were held back by the filter and very few

passed through intact. The filtration step was therefore omitted and instead large debris was pulled to the upper end of the culture dish (tilted to 5^{o}) with a mounted needle. The protoplast suspension was then washed as before. Omission of the filtration step caused the yield of intact protoplasts to be increased at least ten-fold.

e) Yields of intact protoplasts.

As a result of all the work described above, yields of up to $4x10^{6}$ protoplasts per gram of peeled tissue could be obtained, representing a hundred-fold improvement over the method originally chosen. The protoplast isolation procedure, as finally used, is given in the Materials and Methods section.

Culture of protoplasts.

Despite the alterations made to the isolation procedure, protoplasts still did not divide when cultured as on p. 46. Attempts were thus made to vary the culture conditions in order to find those most suitable for protoplast division.

a) Culture media.

Two different media were used for the culture of the isolated protoplasts, PCM I (Table 6) and PCM II (Table 7), both of these having previously been used successfully in the culture of N. sylvestris protoplasts (Nagy and Maliga, 1976); Bourgin, Missonier and Chupeau, 1976).

In general, cultured protoplasts appeared healthier in PCM I, those in PCM II bursting within a week.

b) Plating density.

Protoplasts were plated at a range of densities for 10^{4}

to $5x10^5$ per ml, but there was little difference in their appearance under the microscope after one week incubation.

c) Culture support.

Protoplasts were plated in plastic petri-dishes either embedded in agar, in liquid suspension, or in liquid suspension on an agar layer, but again there was little difference in their appearance after a period in culture.

d) Incubation conditions.

Temperatures of from 20-30°C were used for incubation of the protoplasts and lighting regimes were varied systematically from constant darkness to a constant high light intensity.

Unfortunately protoplast division and calli formation was not achieved under any of these culture conditions.

Although protoplasts would retain their freshly-isolated appearance for up to two weeks, they did not divide and would eventually burst.

Conclusion

All attempts at protoplast culture from both diploid and haploid N. sylvestris leaves were ultimately unsuccessful despite ten months' effort, and the successful culture of diploid protoplasts by Nagy and Maliga (1976) could not be repeated.

It would appear that there was something fundamentally unsuitable in either the condition of the plant material, the methods used for protoplast isolation, or the culture conditions. It has been suggested that N. sylvestris protoplasts are lethally sensitive to Macerozyme (Dix 1975). However, Nagy and Maliga (1976) also used Macerozyme, so this could only explain the problems experienced if the toxicity were batch

dependent. As Macerozyme is a fairly crude mixture of enzymes this is certainly a possibility, but three different batches of Macerozyme R-10 were used in this work and in each case subsequent culture was unsuccessful.

One thing which had become abundantly clear during this work was that protoplast culture was much more difficult than was first anticipated. As time was limited, it was decided not to continue with further efforts to culture protoplasts, and attention was diverted to cell culture techniques (Chapter three).

Much useful experience was gained however, in the techniques of protoplast culture and a greater understanding of the problems involved resulted.

Chapter 3. Callus and cell suspension cultures of haploid *Nicotiana* species.

INTRODUCTION

To be suitable for the isolation of auxotrophic cell-lines, a cell suspension culture system must possess certain features (pp. 11 - 13). Essentially, the cultured cells should possess single-copy DNA and grow in suspension as discrete cells or as very small aggregates. For the isolation of nitrate reductase-minus cell-lines, it is additionally necessary that the cells should be capable of growth on a sole N source other than nitrate, as by definition such cells will be unable to utilise nitrate.

Attempts to establish such dispersed cell suspensions growing on N sources other than nitrate are thus described, firstly from haploid N. sylvestris and then from dihaploid N. tabacum plants. The nitrate reductase activity of the cultured cells is determined, and their ploidy is checked to see whether haploidy is retained in culture. It should not, however, be a hindrance to have cells of higher ploidies present in the cultures so long as a sufficient percentage of haploid cells remain by the time of variant cell-line selection.

RESULTS

Initiation of N. sylvestris callus cultures.

Callus cultures were initiated from a haploid N. sylvestris plant as described in Materials and Methods, on four different media (Table 11), containing 25 mM nitrate as sole N source and 2 mg/l 2,4-D and 0.1 mg/l kinetin as a source of growth hormone. Callus developed in all cases within two-three weeks from excision, but callus maintained in medium B_5 (Gamborg et al, 1968) was faster-growing and more friable than that grown on the other three media (Figure 12). B_5 was therefore selected as the standard medium for use in subsequent work.

Growth of N. sylvestris callus cultures on different nitrogen sources.

Since an N source other than nitrate would be needed to rescue nitrate reductase-minus cells from selection plates (p. 14) the ability of N. sylvestris callus to grow on other N sources was examined. 0.5 g of N. sylvestris callus, previously grown on B_5 medium containing nitrate as sole N source (nitrate medium) was subcultured onto agar B_5 medium containing various N sources (Table 12).

Callus did not grow on media containing either casein hydrolysate, urea or γ-aminobutyric acid as sole N source, and turned brown (Figure 13). Growth on glutamine and on selected amino-acids (Müller and Grafe, 1978) was poor and callus appeared yellowish and nodular, suggesting differentiation. On transfer to the same agar medium lacking hormones, shoots formed within one to two weeks. Even callus from cultures maintained on nitrate medium for twelve months did not form friable undifferentiated callus.

Table 11 Culture media used in callus culture of N. sylvestris and N. tabacum.

In the work described here, all media contained 25mM-nitrate as sole N source and 2mg/l 2,4-D and 0.1mg/l kinetin as hormones.

References: B_5 - Gamborg et al (1968).

K_3 - Kao et al (1974).

UM - Uchimiya and Murashige (1974).

MS - Murashige and Skoog (1962).

	B_5	K_3	UM	MS
$CaCl_2.2H_2O$	150	950	440	440
$MgSO_4.7H_2O$	250	250	370	370
$NaH_2PO_4.2H_2O$	167	228	-	-
KH_2PO_4	-	-	170	170
Na_2EDTA	-	37.3	37.3	37.3
$FeSO_4.7H_2O$	-	27.8	27.8	27.8
FeEDTA	40	-	-	-
H_3BO_3	3	3	6.2	6.2
$MnSO_4.4H_2O$	14	14	22.3	22.3
$ZnSO_4.7H_2O$	2	2	8.6	8.6
KI	0.75	0.75	0.83	0.83
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025	0.025	0.025
Sucrose	20,000	13,700	30,000	30,000
Xylose	-	250	-	-
Glycine	-	-	2	2
m-Inositol	100	100	100	100
Nicotinic acid	1	1	5	0.5
Pyridoxine HCl	1	1	10	0.5
Thiamine HCl	10	10	10	0.1
Agar	8,000	8,000	6,000	6,000
pH	5.6	5.6	5.7	5.8

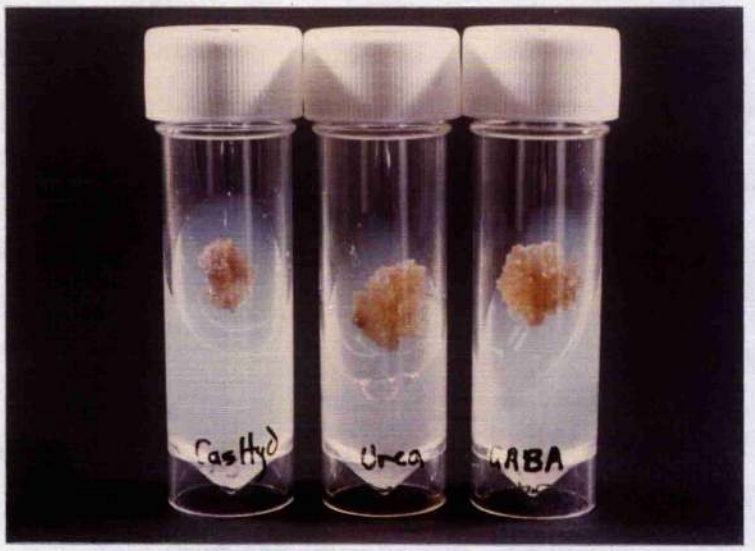
All values in mg/l final concentration.

Figure 12 Growth of N. sylvestris callus in various culture media.

Table 12 Alternative nitrogen sources used with B_5 medium in callus culture of *N. sylvestris* and *N. tabacum*.

casein hydrolysate (2 g/l).
urea (6 mM).
glutamine (8 mM).
glutamine (6 mM), aspartate (2 mM), arginine (1 mM) and glycine (0.1 mM).
γ - amino butyric acid (6 mM).

In all cases the hormones were 2 mg/l 2,4-D and 0.1 mg/l kinetin.



casein hydrolysate | urea | γ-amino butyric acid

amino-acids | nitrate | nitrogen-free

Figure 13 Growth of N. sylvestris callus on B_5 medium with various nitrogen sources.

Cell suspension cultures of N. sylvestris.

Cell suspension cultures were initiated in nitrate medium by macerating callus and placing it in a small volume (10 or 20 ml) of liquid medium on an orbital shaker (120 cycles/s) and were maintained as described in Materials and Methods. At first cells grew as aggregates which tended to differentiate but, after two - three subcultures, a friable suspension was obtained.

Attempts to raise a dispersed cell suspension by subculture of newly formed callus into medium containing any of the alternative N sources (Table 12) were unsuccessful. In all cases aggregates differentiated into knobbly, yellowish callus covered in shoot primordia (Fig.14). Cultures which had undergone fifteen passages in liquid nitrate medium were no more capable of forming friable suspensions in these media.

In certain species increased auxin to cytokinin ratios were found to decrease the extent of differentiation in cell cultures (Kamada and Harada, 1979). However, increasing the 2,4-D level to 5 mg/l and decreasing the kinetin level to 0.05 mg/l did not decrease the tendency of suspensions of N. sylvestris cells to differentiate.

Regeneration of plants from N. sylvestris cell cultures.

After four passages in liquid nitrate medium, 10 ml from each of two suspension cultures, derived from haploid N. sylvestris plants A28 and A31, was pipetted onto rooting medium (Table 3). Within two weeks roots had formed from many cell aggregates, followed by green shoot formation (Figure 15). Two mature plants were eventually raised from suspension A28,

Figure 14 Shoot primordia developed in N. sylvestris callus on amino-acid medium.

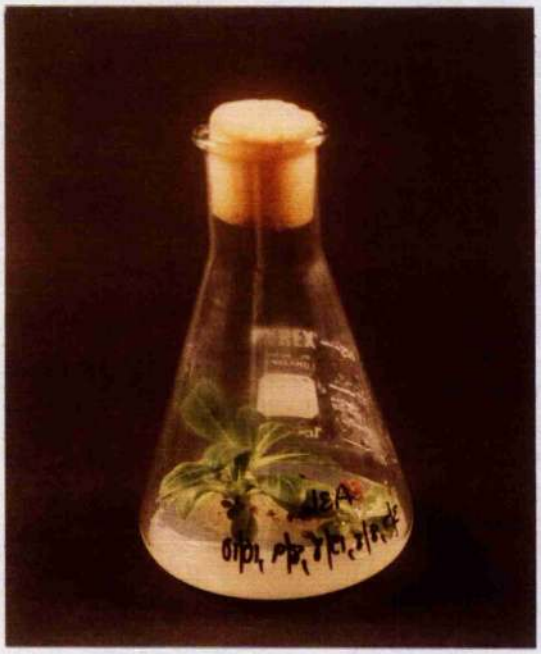


Figure 15 Regeneration of plantlets from suspension cultures of N. sylvestris.

Figure 16a-f Haploid (b and e) and diploid (a,c,d and f) plants regenerated from N. sylvestris.

a, Diploid

b, Haploid

C, Diploid

d, Diploid

e, Haploid

f, Diploid

and four from suspension A31. Of these six plants, four were diploid and two were haploid (Figure 16) as determined by root-tip chromosome analysis (p. 28).

Initiation of N. tabacum callus cultures.

Callus cultures were initiated from an dihaploid N. tabacum plant on four different media (Table 11), all containing 25 mM-nitrate as sole N source and 2 mg/l 2,4-D and 0.1 mg/l kinetin as a source of growth hormone. Pith explants were taken from the upper stem, as in N. tabacum the incidence of mixed ploidies amongst cells is least in the region just below the apex of the plant (Murashige and Nakano, 1967). Callus developed in all cases within two-three weeks from excision. Light friable callus was obtained on media B_5 MS and K_3 but growth was poor on UM medium (Figure 17). The most friable and fastest-growing callus was grown on medium B_5 which was therefore selected as the standard medium for subsequent work.

Growth of N. tabacum callus cultures on different nitrogen sources.

The ability of N. tabacum callus to grow on other N sources was examined as for N. sylvestris (p. 56). 0.5 g of N. tabacum callus, previously grown on B_5 medium containing nitrate as sole N. source, was subcultured onto agar medium containing the alternative N sources shown in Table 12 and a medium containing no N source.

Friable callus developed on media containing either glutamine, selected amino-acids (Müller and Grafe, 1978) or γ-aminobutyric acid, but there was no growth on media containing casein hydrolysate, urea or with no N source (Figure 18). Callus was most friable on the medium containing

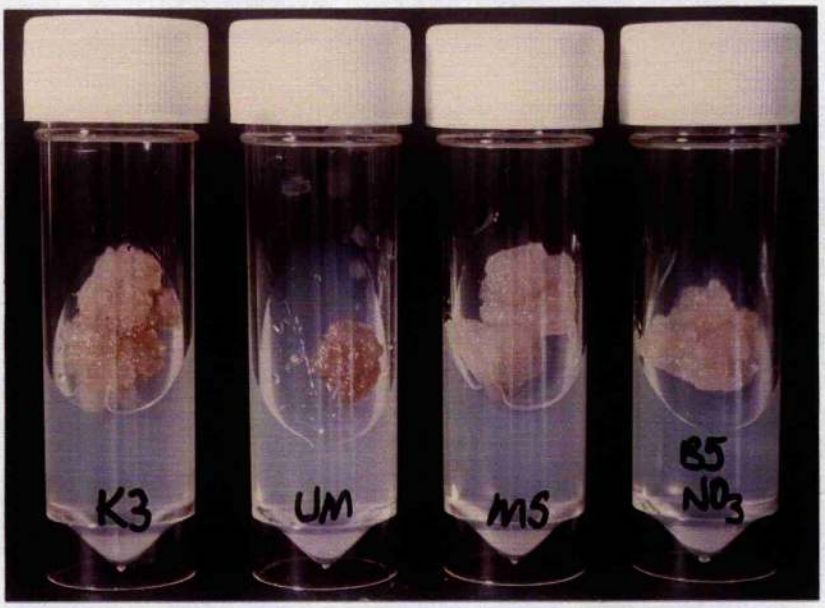
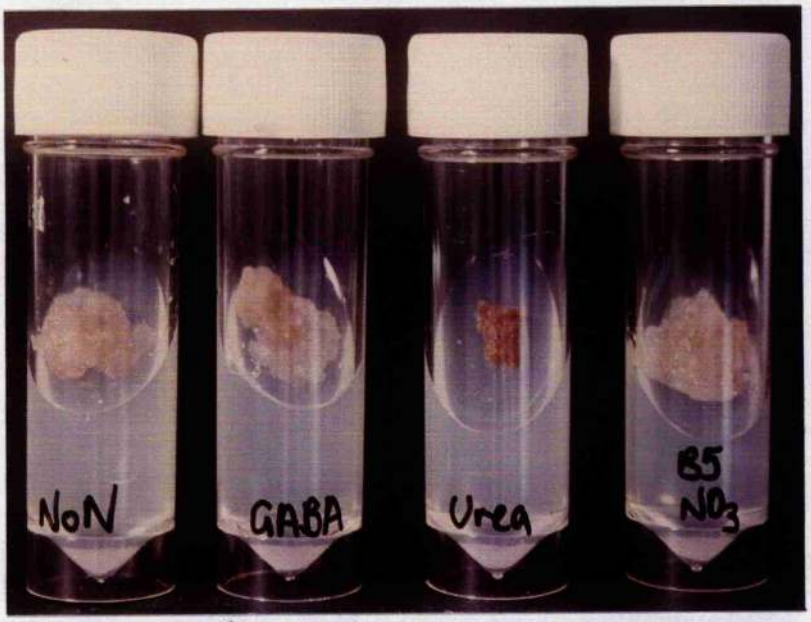


Figure 17 Growth of N. tabacum callus in various culture media.



nitrogen-free | γ-amino butyric acid | urea | nitrate

casein hydrolysate | glutamine | amino-acids

Figure 18 Growth of N. tabacum callus on B_5 medium with various nitrogen sources.

a mixture of selected amino-acids which was thus chosen as standard medium (amino-acid medium). This medium contains a large amount of glutamine (Table 5), and as Fukunaga, King and Child, (1978) have noted, glutamine as an N source provides cells with two equivalents of amino groups and one of an organic acid. It is reasonable to expect that the cells growing on this medium may have been able to assimilate some but not all of the amino groups and that the addition of an organic acid e.g. citrate or succinate, might stimulate growth. This idea is supported by Behrend and Mateles (1976) who found that the addition of 6 mM-succinate to ammonium-based media stimulated growth of N. tabacum cells.

6 mM-succinate was thus added subsequently to amino-acid medium and was found to enhance cell growth.

Cell suspension culture of N. tabacum.

Cell suspension cultures were initiated from callus growth on nitrate or amino-acid medium, by macerating callus and suspending it in a small volume (10 - 20 ml) of the same liquid medium on an orbital shaker (120 cycles/s). Cells grew rapidly as a dispersed suspension in both media, and were maintained as described in Materials and Methods. The mean fresh weight of 100 ml stationary phase suspension was 33.5 g in nitrate medium but only 14.1 g in amino-acid medium. However, addition of 6 mM-succinate to amino-acid medium allowed an increased stationary phase fresh weight of 25.1 g and 6 mM α-ketoglutarate allowed a fresh weight of 19.5 g. This supports the observations of Fukunaga et al (1978) and 6 mM-succinate was subsequently added routinely to liquid amino-acid and glutamine media.

Extraction and assay of nitrate reductase activity from *N. tabacum* cell cultures.

Before investigating the growth characteristics of these *N. tabacum* cell lines, it was necessary to develop a reliable method for extraction and assay of nitrate reductase from them.

Initially, cell-free extracts were prepared as described on p.30 from cultures growing in nitrate medium, using the buffer of Small and Wray (1980), containing 0.05 M-KH_2PO_4, pH 7.5 with 0.1 mM-Na_2EDTA, 1 mM-cysteine and 10 µM-FAD. Nitrate reductase activity in these extracts, assayed as described in Materials and Methods, was in the order of 0 to 0.4 µmol nitrite produced/h per gram fresh weight of cells, but activity was lost rapidly with storage at 0°C making quantitative assay difficult.

For a reliable and reproducible assay, a more efficient extraction and increased *in vitro* stability of the enzyme was required. In an attempt to achieve this, 3% (w/v) bovine serum albumin (BSA) was added to the extraction buffer and 0.1 M-Tris was substituted for 0.05 M-phosphate. The extraction and stability of the enzyme in these modified buffers was compared with that in the original buffer by assay of nitrate reductase in cell-free extracts from a homogeneous cell suspension after storage at 0°C (Table 13).

No significant difference was found between extraction and stability in phosphate and Tris buffers, and as this phosphate buffer was used routinely by other workers in this laboratory, it was retained as standard extraction buffer. However, the addition of 3% (w/v) BSA to either extraction buffer caused a marked increase in total nitrate reductase activity extracted and its subsequent stability *in vitro* (Table 13). 3% (w/v) BSA

was thus added to the extraction buffer of Small and Wray (1980) which, thus modified, was used routinely in the remainder of this thesis for preparation of cell-free extracts.

Table 13 Nitrate reductase activity of cell-free extracts from N. tabacum cells after extraction in various Buffers and storage at 0°C.

Buffer	0 h	2 h	4 h
0.1 M-Tris.	0.313	0.185 (59%)	0.126 (40%)
0.1 M-Tris, 3%(w/v)BSA.	0.590	0.521 (88%)	0.459 (78%)
0.05 M-Phosphate.	0.344	0.198 (58%)	0.152 (44%)
0.05 M-Phosphate, 3%(w/v)BSA.	0.561	0.521 (93%)	0.436 (78%)

All buffers were pH. 7.5 and also contained 0.1mM-Na_2EDTA, 10mM-FAD and 1mM-cysteine.

Nitrate reductase activities are given in µmol nitrite produced/h per gram of tissue and are the mean from two assays of each of three separate extractions. Figures in parentheses are percentages of the activities at 0 h.

Growth characteristics of N. tabacum cell suspensions.

Cell suspensions, each initiated from callus derived from pith of a dihaploid N. tabacum plant were maintained routinely on nitrate, amino-acid and glutamine media (Tables 4 and 5). The increase in fresh weight with time and the nitrate reductase level was followed for several of these cell-lines in these three media. Growth profiles were similar for different cell-lines in a given media.

Nitrate medium

In cultures grown on nitrate medium, nitrate reductase activity was highest one day after subculture, fell immediately after, and rose to a second maximum five days after subculture. This corresponded to the mid-logarithmic phase of culture growth (Figure 19). This cell-line was also subcultured into similar media with lower levels of nitrate (10 and 2.5 mM). Growth is shown to be restricted by availability of nitrate (Figure 20).

Amino-acid medium.

Cultures grown in amino-acid medium reached stationary phase within ten days (Figure 21) but the fresh weight of cells per stationary phase culture was lower than that in similar cultures grown on nitrate medium. There was a single peak of nitrate reductase activity when the level was 40% of that in nitrate medium (Figure 19), but in stationary phase the activity dropped to only 10% of the corresponding level in nitrate medium.

Glutamine medium

Culture growth in glutamine medium was similar to that in amino-acid medium, but the nitrate reductase levels were much lower. The maximum level was only 10 - 15% of that in similar cultures grown in nitrate medium (Figure 22).

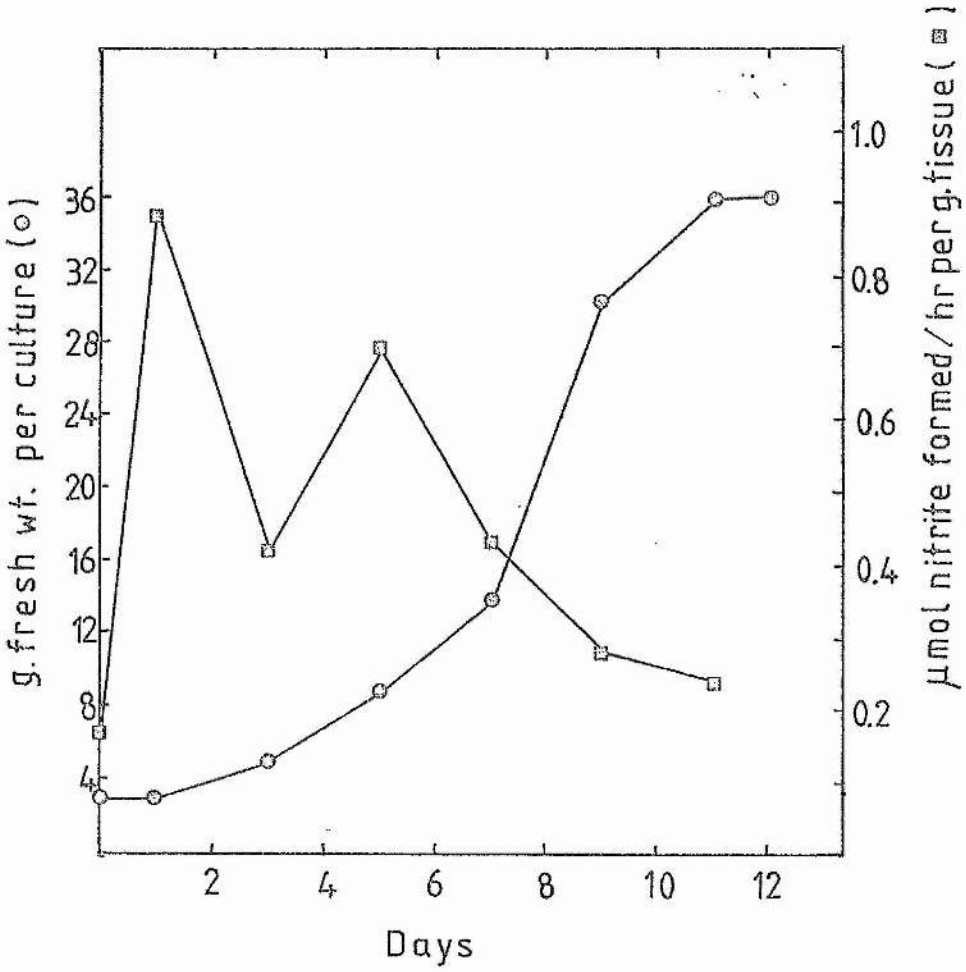


Figure 19 Growth (◎) and nitrate reductase activity (■) of wild-type cells subcultured into nitrate medium.

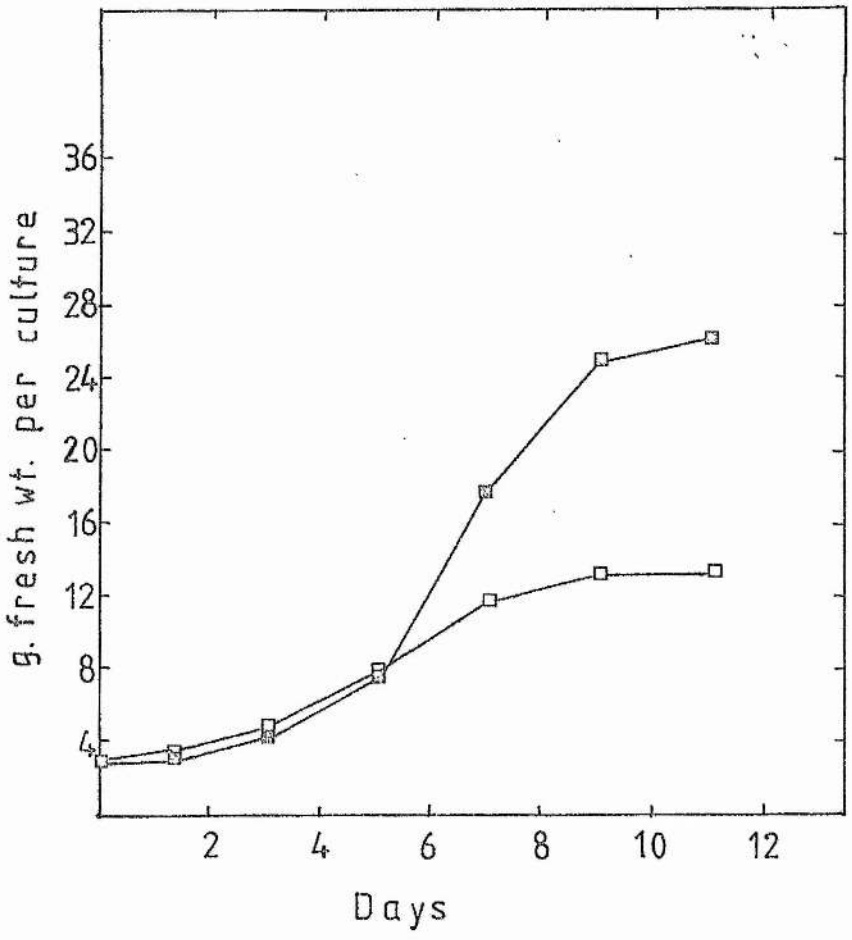


Figure 20 Growth of wild-type cells subcultured into B_5 medium with 2.5mM (□) or 10mM (▨) nitrate as a sole nitrogen source.

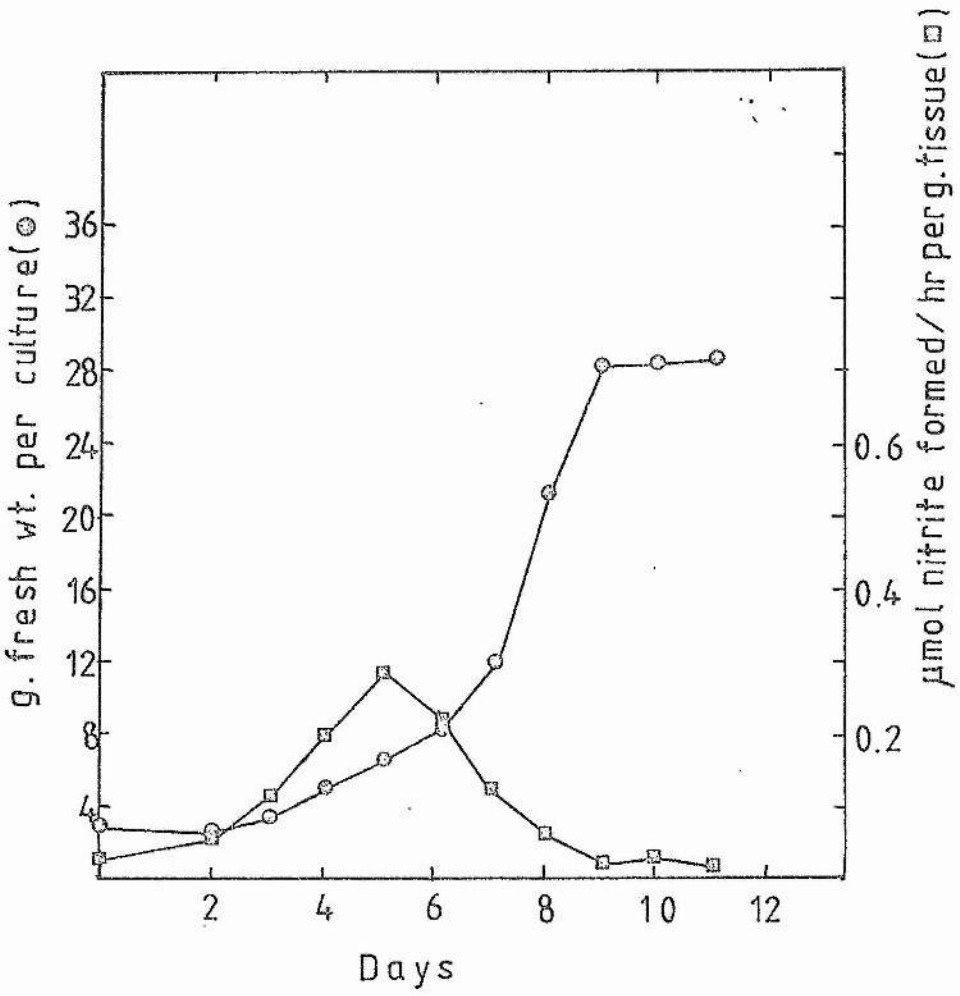


Figure 21 Growth (⊙) and nitrate reductase activity (▣) of wild-type cells subcultured into amino-acid medium.

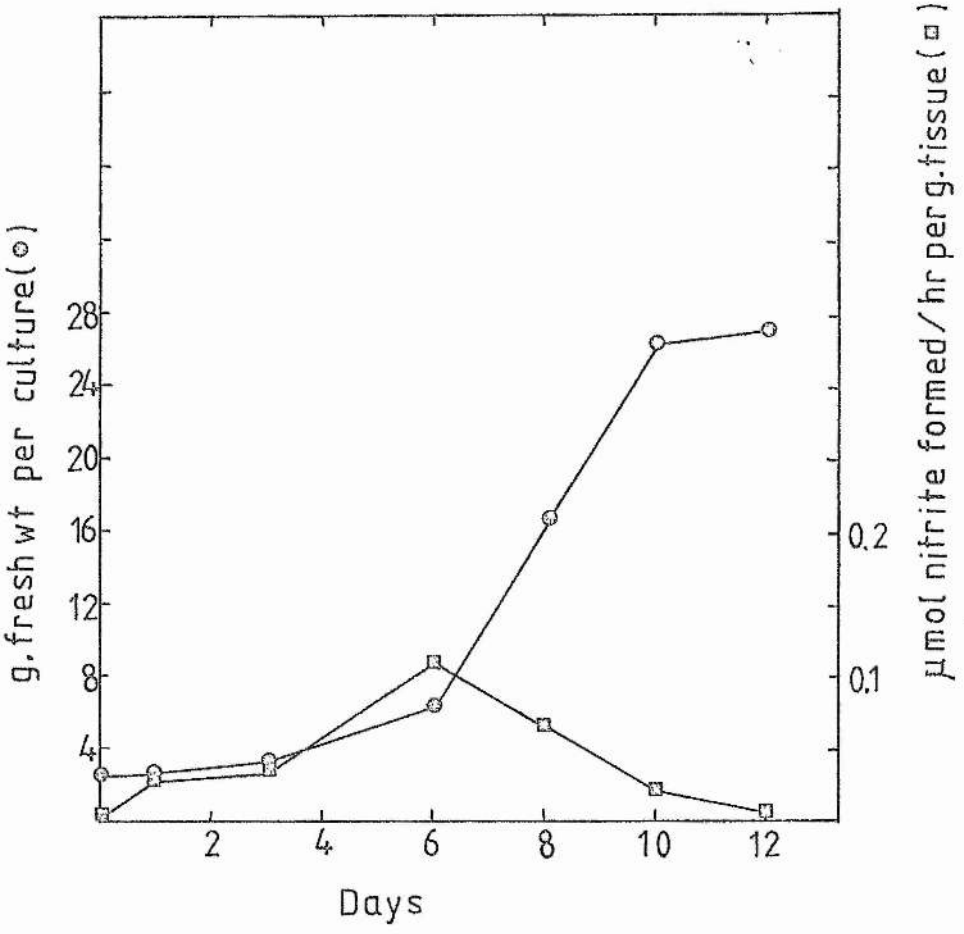


Figure 22 Growth (○) and nitrate reductase activity (▣) of wild-type cells subcultured into glutamine medium.

Ploidy in N. tabacum cell cultures.

Callus cultures were initiated on amino-acid medium from pith explants of the upper stem of a cytologically-verified dihaploid N. tabacum plant, and suspension cultures were initiated from these after four weeks, both as described in Materials and Methods. The ploidy of these cultures was determined at intervals as described on p.29 but, since pith explants contain few dividing cells, it was not possible to determine the ploidy of fresh explants (Table 14). In addition, the large number of chromosomes per cell made it difficult to determine the exact number of chromosomes in cultured cells as suitably spread metaphases in squash preparations were few. For this reason Table 14 shows only ranges of chromosome number.

Sample numbers were low, but it appears that older cultures contain fewer dihaploid cells (Table 14). This may be due to a progressive loss of dihaploid cells with increasing time in culture, but it is also possible that this merely demonstrates varying levels of polyploidy amongst cell-lines, due to differing levels of somatic polyploidy amongst pith explants. The fact that cell-line B retained 45% dihaploid cells after five months and cell-line C had only 14% dihaploid cells after one month longer suggests that this latter explanation must be taken into account. To obtain a clearer picture of the loss of dihaploidy in N. tabacum cell suspensions, the ploidy of the cells in a single cell-line, cultured as above, was determined two, six and eleven weeks after pith excision and culture initiation (Table 15). It is apparent that in this cell-line the percentage of dihaploid cells declined rapidly with increasing time in culture.

Table 14 Ploidy of cells in wild-type *N. tabacum* cell-lines after culture in amino-acid medium.

Cell-line	Weeks since pith excision	dihaploid range (%)	amphidiploid range (%)	polyploid range (%)
A	2	70	20	10
B	22	45	55	0
C	26	14	57	29

Table 15 Ploidy of cells in a single wild-type *N. tabacum* cell-line after 2, 6 and 11 weeks in culture.

Weeks since pith excision	dihaploid range (%)	amphidiploid range (%)	polyploid range (%)
2	90	10	0
6	44	29	27
11	26	36	38

DISCUSSION

N. sylvestris cell culture.

Attempts to raise dispersed cell suspension cultures from haploid N. sylvestris plants on a medium containing a nitrogen source other than nitrate were not, in the end, successful. Cells either did not grow at all on the alternative nitrogen sources or grew poorly as differentiated aggregates. However, the regeneration of two haploid plants out of six plants regenerated from N. sylvestris cell-lines after fourteen weeks in culture was interesting, as it shows that cell-lines in culture for this time contain totipotent cells, at least a proportion of which retain their original haploid chromosome complement. In comparison, only a small percentage of the plants regenerated by Nitsch, Nitsch and Hamon (1969) from N. sylvestris cell-lines after only eight weeks in culture were haploid. In addition, Facciotti and Pilet (1979) regenerated only diploid plants from protoplast cultures of haploid N. sylvestris plants.

The ability to regenerate haploid plants from cell suspensions is potentially a very useful one as inheritance of a variant phenotype in a cell culture can thus be studied. However, the inability of the N. sylvestris cell cultures described in this work to grow on a nitrogen source other than nitrate effectively precluded their use in the isolation of nitrate reductase-minus cell-lines, as such cell-lines could not be maintained in the absence of any utilisable nitrogen. No further work was therefore carried out with this species.

N. tabacum cell culture.

In contrast to N. sylvestris, dispersed cell suspensions

could be quickly raised from pith of dihaploid N. tabacum plants on nitrate, amino-acid or glutamine media, thus fulfilling the requirement for a culture system consisting of single cells or small aggregates growing on a nitrogen source other than nitrate (p. 59). The growth of these cell suspensions on these three different nitrogen sources was studied (Figures 19 - 22) and nitrate reductase activity was measured in cell-free extracts from the cells (see below). Before using these cell suspensions for selection of chlorate-resistant and nitrate reductase-minus cell-lines, it was firstly necessary to check whether the cells retained their dihaploid chromosome complement in culture (p. 67).

Extraction of nitrate reductase activity from N. tabacum cell cultures.

Nitrate reductase activity was originally extracted from N. tabacum cultured cells in a buffer containing 0.05 M-KH_2PO_4, pH 7.5 with 0.1 mM-EDTA, 1 mM-cysteine and 10 µM-FAD, which was found suitable for extraction of nitrate reductase activity from Hordeum vulgare leaf tissue (Small and Wray, 1980). However, N. tabacum nitrate reductase extracted in this buffer was found to be unstable in vitro at 0°C (p. 60) so the choice of buffer required investigation, with a view towards increasing the stability of the extracted enzyme.

Nitrate reductase has a pH optimum of 7.5 in most higher plant systems, including N. tabacum cell cultures (Sanderson and Cocking, 1964; Beevers, Flesher and Hageman, 1964; Filner, 1966) and may require flavin (Spencer, 1959; Wray and Filner, 1970; Rosa, Castillo, Mendez and Palacian, 1976) and EDTA (Notton and Hewitt, 1979) in the buffer for maximal extraction. In some systems, again including N. tabacum cell cultures, there is an absolute requirement for a

sulphydryl-group protecting reagent, e.g. cysteine, in the buffer for extraction of nitrate reductase activity (Sanderson and Cocking, 1964; Filner, 1966). It would appear then that the buffer used above was well suited to the extraction and assay of nitrate reductase activity from N. tabacum cell cultures, but its instability in this buffer suggests that some other stabilising agent was required.

Filner (1966) found that nitrate reductase activity in extracts from N. tabacum cells was greater in Tris than in phosphate buffer. However, when compared in this laboratory, there was little difference between the two (Table 13), but the addition of 3% BSA to either buffer caused a marked increase in the total activity extracted and its subsequent in vitro stability (Table 13), Schrader, Cataldo and Peterson (1974) showed that the addition of BSA to extraction buffers greatly increased the efficiency of extraction and stability of nitrate reductase activity in cell-free extracts from several species. BSA probably acts by protecting the enzyme from specific or non-specific proteases released during preparation, whether by providing an excess of alternative substrate or by some more specific mechanism, for example binding to the enzyme and protecting labile bonds from protease attack. Furthermore, Brown, Small and Wray (1981) have shown that the increased lability of nitrate reductase in extracts from more mature Hordeum leaves could be overcome by adding BSA to the extraction buffer, again presumably by protecting the enzyme from protease attack.

If the mechanism is not yet fully understood, then at least the effect is clear. With the addition of 3% BSA to the buffer of Small and Wray (1980), extracted nitrate reductase activity

was stable enough to allow reliable and reproduceable assay.

Ploidy of N. tabacum cell cultures.

The ploidy of cell suspension cultures derived from dihaploid N. tabacum plants was found to be unstable but, although amphidiploid and polyploid cells outgrew dihaploid cells, a variable percentage of dihaploid cells could be maintained, even after up to six months in culture (Tables 14 and 15).

Plant cells are known to be genetically unstable in culture. Due to endomitosis, a failure of telophase and cytokinesis following metaphase and anaphase, leading to a restitution nucleus, polyploidy often develops (D'amato, 1977). Aneuploidy may also be common due to chromosome loss, lagging chromosomes or multipolar mitoses (Bayliss, 1973) and cell suspensions can soon develop a wide range of ploidy. It has been noted that polyploidisation happens more rapidly in initially haploid cell cultures than in diploid cultures (Sacristán, 1971), thus causing the percentage of haploid cells in an initially haploid cell suspension to decline rapidly. Such polyploidisation appeared to be occurring in the N. tabacum cultures studied in this work (Tables 14 and 15). If this polyploidisation could be prevented, or at least limited, then suspension cultures containing a greater percentage of dihaploid cells would result. Rates of polyploidisation can, however, be very unpredictable.

Dix (1975) found the rate of polyploidisation amongst N. sylvestris cell-lines to vary greatly and in Oryza some predominantly haploid cell cultures have been maintained for three years whilst others have become rapidly polyploid (Niizeki and Oono, 1971). Variations in the apparent rates of polyploidisation may be due to variable levels of somatic polyploidy in normal plant tissues (D'amato, 1965) which is

species and tissue dependent, (Partanen, 1965) and it has been shown that in N. tabacum the type of explant chosen influences the ploidy levels of the callus developed from it (Novak and Vyskot, 1975). All the cell cultures in this work were initiated from pith explants taken from within 10 cm of the apex of dihaploid N. tabacum plants where the incidence of mixed ploidy is least (Murashige and Nakano, 1967). However, attempts to achieve stable single cell clones from haploid cells have not been successful in the past, such cultures also becoming polyploid and aneuploid.

Control of polyploidisation.

A number of factors have been implicated as affecting the rate of polyploidisation in cell cultures, particularly phytohormone levels in culture media. Evidence for this, however, is conflicting. 2,4-D at high levels was reported to disturb mitotic events in root-tips (Bayliss, 1973), and in cell suspensions (Bayliss, 1977) of Daucus carota. Conversely, Singh and Harvey (1975) reported that the higher the level of 2,4-D the lower was the frequency of mitotic irregularities in Haplopappus gracilis suspensions. It seems probable though, that the effect of normally used concentrations of 2,4-D is too slight to be of import.

Rashid and Street (1974) found that high levels of kinetin caused rapid polyploidisation in Atropa belladonna cultures and in a wider study Nitsch, Nitsch and Hamon (1969) investigated the effect of various culture medium cytokinin levels on the ploidy of plants regenerated from cultures of N. tabacum. Nitsch et al (1969) found that the greatest proportion of dihaploid plants was obtained with cytokinin-free medium and, as time was not available to experimentally determine the most

suitable hormone levels for minimising polyploidisation in N. tabacum cell cultures in this laboratory, the results of Nitsch et al, (1969) were accepted as the best guide available and kinetin was subsequently omitted from B_5 media. Omission of kinetin did not affect culture growth.

Another factor implicated as influencing the rate of polyploidisation is the frequency of subculture (Bayliss, 1975). Avoiding the incidence of nutrient limitation seemed to minimise the rate, possibly because polyploid cells are at a disadvantage during logarithmic phase or because nutrient limitation allows polyploid cells a selective advantage or allows DNA replication but not mitosis. In the absence of sufficient time to determine the effect of subculture frequency on polyploidisation of N. tabacum cell cultures in this laboratory, the times between subculture were kept to a minimum as a precaution.

Having made these efforts to limit polyploidisation and somatic polyploidy in the dihaploid N. tabacum cell-lines, the percentage of dihaploid cells remaining after a given time in culture ought to be higher than those reported above (Tables 14,15). It is reasonable to expect that cultures two months after initiation should contain around 50% dihaploid cells and should thus be suitable for selection of variant cell-lines.

N. tabacum as an amphidiploid species.

As indicated previously (p. 10), the isolation of non-leaky auxotrophic cell-lines probably requires a population of wild-type cells carrying single copies of the relevant genes, i.e. haploid cell-lines. Having established dispersed cell suspensions containing around 50% "haploid" cells on a nitrogen source other than nitrate, it should be possible to use these cell-lines for selection of chlorate-resistant and nitrate

reductase-minus cell-lines. One additional factor, however, which requires consideration, is whether dihaploid N. tabacum cells do in fact carry only single genomes.

N. tabacum is thought to be a hybrid of N. sylvestris and N. tomentosiformis and cells of N. tabacum are amphidiploid (2n=48) (Gray, Kung, Wildman and Sheen, 1974). Having such a large number of small chromosomes makes genetic analysis difficult but, more importantly, N. tabacum cells theoretically carry duplicate copies of two genomes rather than one. N. tabacum cells derived from anther culture and carrying half the normal number of chromosomes are thus more accurately described as dihaploid, rather than simply haploid. Carrying two, possibly very similar genome copies, such cells are not truly haploid and might not be suitable for attempts to isolate auxotropic cell-lines.

Auxotropic cell-lines from N. tabacum.

Carlson (1970) was the first to isolate auxotropic cell-lines from dihaploid N. tabacum cell suspensions but unfortunately all these were "leaky". This suggested either that the selection procedure used did not allow isolation of non-leaky auxotropic cell-lines, or that such cell-lines could not be obtained by a single mutational event because of a lack of functional haploidy.

Assuming that N. tabacum cells are amphidiploid, it is still possible that since the initial hybridisation event which gave rise to the species, much of the genome has become functionally diploid due to chromosome loss or modification. Smith (1968) noticed that most morphological mutants in N. tabacum act as if the species were functionally diploid, and Stines and Mann (1960) found N. tabacum to be functionally diploid for a number of characteristics. Conversely, however, Clausen and Cameron (1944)

showed that certain morphological characteristics were each determined by duplicate factors belonging to different parental genomes, and more recently, Legg, Chaplin and Collins (1969) and Legg and Collins (1971) have obtained conclusive evidence of duplicated genetic material. It would appear then that N. tabacum is functionally diploid in some but not all of its genome.

In support of this, Müller and Grafe (1978) have isolated non-leaky nitrate reductase-minus mutant cell-lines and Malmberg (1979) has obtained non-leaky temperature-sensitive mutant cell-lines, both from N. tabacum dihaploid cell suspensions. This suggests that the affected genes are present as single copies in dihaploid cells. Alternative explanations, based on the presence of duplicate genes, require that duplicate genes mutate simultaneously, or that one of the duplicate genes is inactive in a least a fraction of the cells due to prior mutation or chromosome loss. Both are possible, but seem less likely.

In conclusion then, it seems likely that the dihaploid N. tabacum cell-lines, raised as on p. 58, are functionally haploid in parts of their genome, and it should be possible to isolate auxotrophic cell-lines from them, provided the genes involved are amongst those which are functionally haploid.

Chapter 4. The isolation and partial characterisation of chlorate-resistant and nitrate reductase-minus cell-lines from dihaploid *N. tabacum* cell cultures.

INTRODUCTION

Having established cell suspension cultures of dihaploid N. tabacum, from which it should theoretically be possible to isolate nitrate reductase-minus cell-lines, it is then necessary to work out a suitable procedure for the successful isolation of such cell-lines.

It was anticipated that a mutagenic agent would be required in order to increase the frequency of variant cell-line occurrence. A safe and effective treatment protocol for its use must therefore be developed.

In the first instance, selection will be for chlorate-resistance in mutagenised cells plated in petri-dishes containing agar medium and the selective agent, chlorate. The selective medium must be formulated to totally inhibit wild-type cell growth whilst allowing chlorate-resistant cells to form calli. Chlorate must firstly be shown to inhibit wild-type cell growth and then the most suitable chlorate concentration determined.

It will be necessary to isolate chlorate-resistant cell-lines on a medium containing an N source other than nitrate. Assuming for the moment that chlorate toxicity is dependent on nitrate reductase activity (p. 15), wild-type cells growing on amino-acid medium might be less sensitive to chlorate than on nitrate medium, as their nitrate reductase levels will be lower (Figures 19,21). The selective medium should therefore be chosen to maximise cellular nitrate reductase under the culture conditions for chlorate-resistant cell-line isolation. Efficient selection may require the addition of low concentrations of nitrate to the amino-acid/chlorate medium.

After these details of the selection procedure have been settled, it should then be possible to proceed with efforts to isolate chlorate-resistant cell-lines. Chlorate-resistant cell-lines will then be screened for growth on nitrate medium and for nitrate reductase activity.

RESULTS

Inhibition of growth of N. tabacum cell cultures by chlorate.

Before attempting to select chlorate-resistant cell-lines from the dihaploid N. tabacum cell cultures described in Chapter three, it was first necessary to determine the extent to which wild-type cell growth is inhibited by chlorate.

Stationary phase suspension cultures grown in amino-acid medium were therefore subcultured into fresh amino-acid medium with or without 20 mM-chlorate. Similar cultures grown in nitrate medium were subcultured into nitrate medium with and without chlorate.

Chlorate was shown to inhibit culture growth whether in nitrate or amino-acid medium (Figures 23,24) but in amino-acid medium some growth was noted in the presence of chlorate after an eight to ten day lag period. Cells were brown, however, and the stationary phase fresh weight was only 37% of that in the absence of chlorate. When, after twenty days in amino-acid medium with chlorate, these cells were subcultured into amino-acid medium without chlorate, no growth ensued, suggesting that the cells were dead by this time.

Wild-type dihaploid N. tabacum cell suspensions (logarithmic phase in amino-acid medium) were also plated in petri-dishes (p. 27) containing amino-acid medium with the following additions,

a, no additions.

b, 20 mM-chlorate.

c, 40 mM-chlorate.

d, 80 mM-chlorate.

e, 40 mM-chlorate, 5 mM-nitrate.

f, 40 mM-chlorate, 10 mM-nitrate.

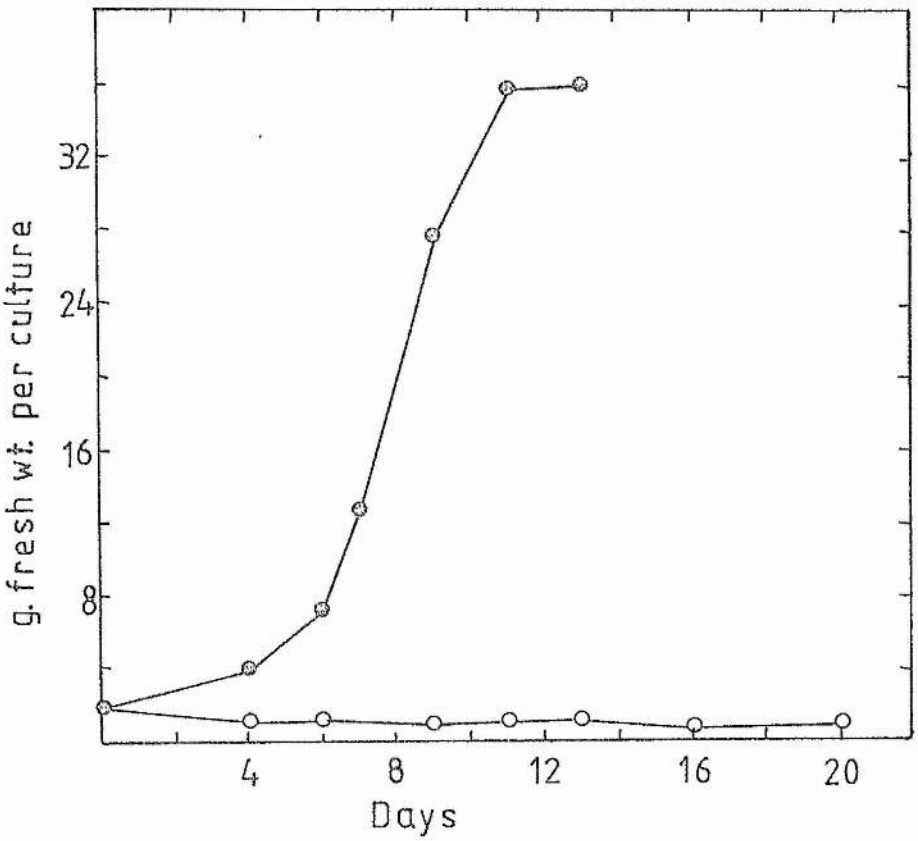


Figure 23 Growth of wild-type cells subcultured into nitrate medium containing (O) or not containing (◎) 20mM-chlorate.

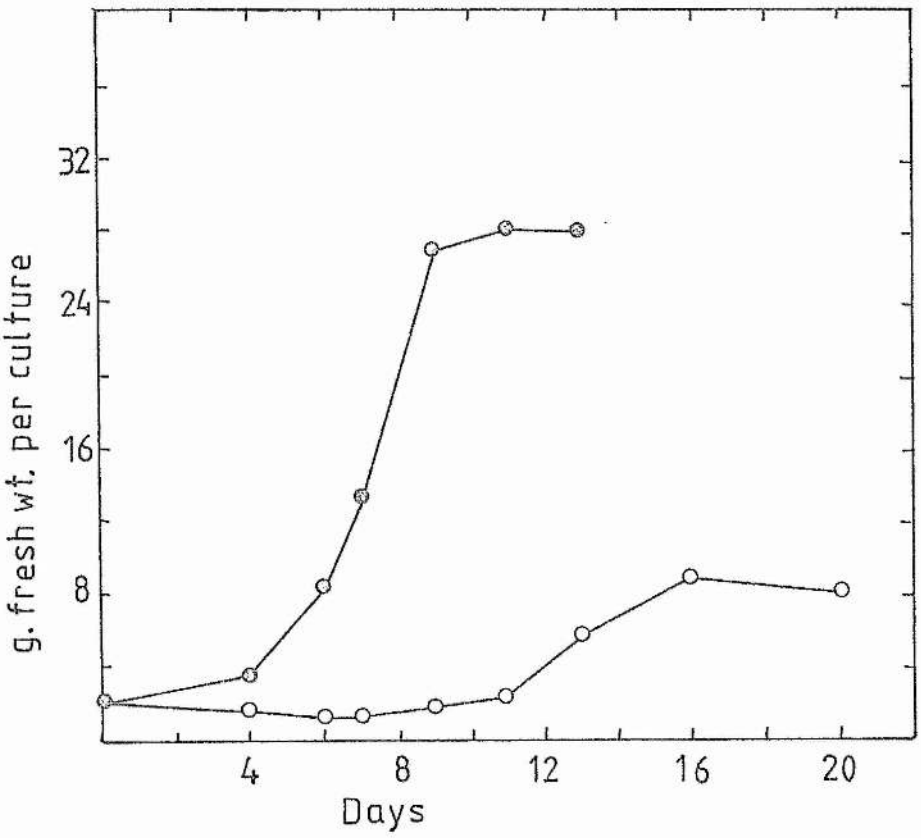


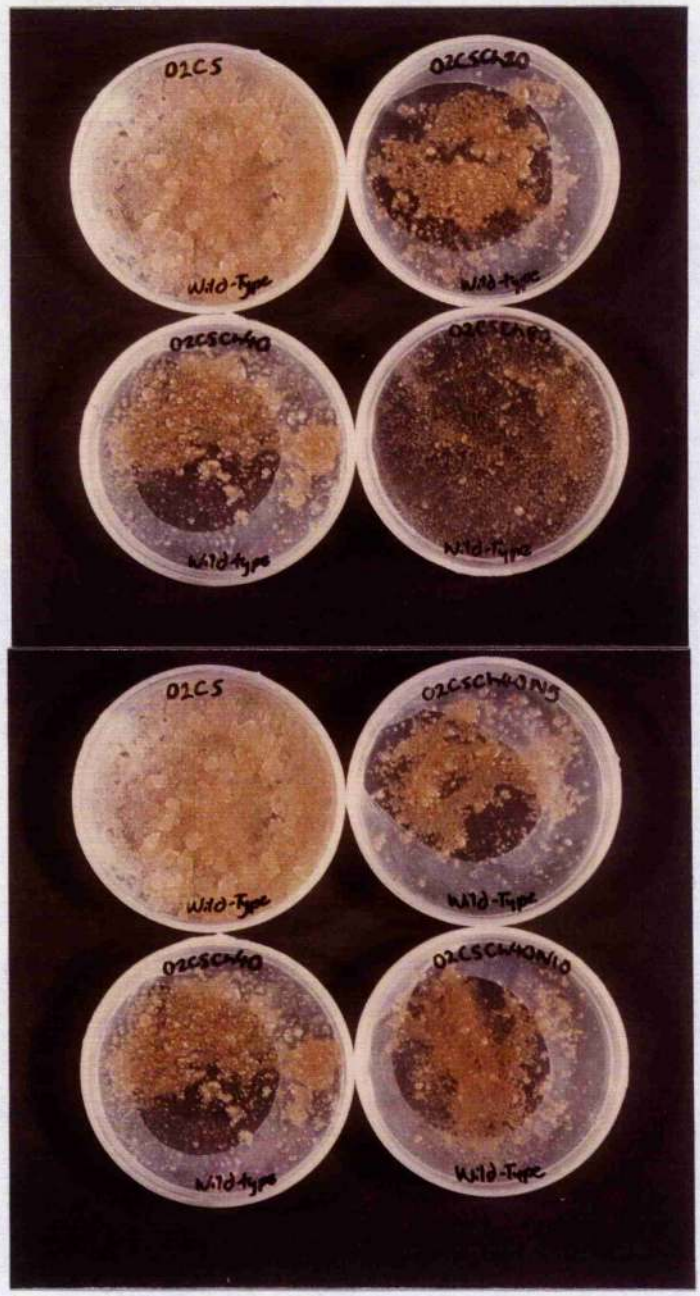
Figure 24 Growth of wild-type cells subcultured into amino-acid medium containing (O) or not containing (⊙) 20mM-chlorate.

no chlorate
no nitrate

20mM-chlorate
no nitrate

40mM-chlorate
no nitrate

80mM-chlorate
no nitrate



no chlorate
no nitrate

40mM-chlorate
5mM-nitrate

40mM-chlorate
no nitrate

40mM-chlorate
10mM-nitrate

Figure 25 Growth of wild-type callus on amino-acid medium with differing levels of chlorate and nitrate.

Nitrate was included in media e, and f, in order to raise cell nitrate reductase levels. It was thought that this might increase their susceptibility to chlorate (p. 72).

There was no observable growth in any of the dishes containing chlorate when examined after twenty days, showing that 20 mM-chlorate is sufficient to inhibit cell growth under these conditions also (Figure 25). After four or five weeks, however, some growth was noted but callus was brown and growth, like that seen in suspension culture (Figure 24), was less extensive than in dishes containing amino-acid medium without chlorate.

Nitrate reductase in N. tabacum cells grown in amino-acid medium with or without added nitrate.

There is much evidence to support a link between nitrate reductase and chlorate toxicity in higher plants (p. 15) It was therefore desirable to determine whether wild-type dihaploid N. tabacum cell cultures possessed nitrate reductase activity under the conditions to be used for isolation of chlorate-resistant cell-lines; i.e. plated in petri-dishes containing amino-acid medium with chlorate and with or without added nitrate.

This, however, was impractical as chlorate has been shown to be toxic to these cells (Figures 23-25) and assaying the enzyme in dying cells could give misleading results.

An experiment was therefore designed to determine the nitrate reductase levels in cells plated in amino-acid medium with or without added nitrate, but without chlorate. This would give some indication of the likely nitrate reductase levels in cells plated on amino-acid medium with or without

added nitrate and also including chlorate.

A homogeneous suspension of wild-type dihaploid N. tabacum cells was subcultured into flasks containing amino-acid medium. After five days, cells were harvested from every second flask and resuspended in flasks containing nitrate medium. After a further 24h cells were harvested from both these and the remainder of the flasks and plated on amino-acid medium with 0, 2.5, 5 or 10 mM-nitrate.

Nitrate reductase activity was measured in cell-free extracts, prepared from cells harvested at the time of plating and three and ten days after plating. All these extracts possessed nitrate reductase activity (Table 16), even those from cells grown in the absence of nitrate. However, as little as 2.5 mM-nitrate produced a greatly elevated nitrate reductase level, especially after ten days. Resuspension in nitrate medium for twenty-four hours had caused a twenty-fold increase in the nitrate reductase levels at the time of plating, but after plating the difference was much less.

In all cases, nitrate reductase levels fell between three and ten days after plating, and the highest level after ten days was found in those cells not previously exposed to nitrate and plated in amino-acid medium with 10 mM-nitrate.

As cells would be plated for longer than ten days during selection for chlorate-resistant cell-lines, a further experiment was carried out. Nitrate reductase activity was measured in cell-free extracts prepared from N. tabacum cells at the time of plating onto amino-acid medium with 10 mM-nitrate and three, ten, seventeen and twenty-four days after plating. Nitrate reductase activity steadily declined with time but was still present even after 24 days (Table 17).

Table 16 Nitrate reductase activity in dihaploid *N. tabacum* cells plated in amino-acid medium with or without added nitrate.

a, Cells plated directly from amino-acid medium.

Nitrate added to amino-acid medium (mM)	Nitrate reductase activity Days after plating 0	3	10
0	0.033	0.193	0.052
2.5	"	0.239	0.154
5	"	0.301	0.310
10	"	0.421	0.327

b, Cells plated after 24h in nitrate medium.

Nitrate added to amino-acid medium (mM)	Nitrate reductase activity Days after plating 0	3	10
0	0.640	0.272	0.080
2.5	"	0.354	0.174
5	"	0.416	0.214
10	"	0.480	0.234

Nitrate reductase activity is given in µmol nitrite produced/h per g. fresh wt. and is the mean from duplicate petri-dishes.

Table 17 Nitrate reductase activity in N. tabacum cells plated in amino-acid medium with 10 mM-nitrate.

Days after plating	Nitrate reductase activity (μmol nitrite produced/h per g. fresh wt)
0	0.041
3	0.371
10	0.216
17	0.163
24	0.099

Values given are the mean from duplicate petri-dishes.

Viability of N. tabacum cells after treatment with ethyl methane sulphonate.

Ethyl methane sulphonate (EMS) was chosen as a suitable mutagenic agent for increasing the frequency of variant cell-line occurence in dihaploid N tabacum cell cultures (p. 87) and Turtoczky and Ehrenberg (1969) showed that in E. coli, the degree of exposure to EMS which caused death of 50% of the cells in a treated population, gave the highest mutagenic efficiency. Efforts were thus made to determine what this degree of exposure would be for N. tabacum cell cultures.

EMS was added to logarithmic phase suspension cultures of wild-type dihaploid N. tabacum cells up to final concentrations of 0, 0.4, 0.8 and 1.2%. After two hours, cells were harvested, washed and resuspended in amino-acid medium without EMS. After a further two days the viability of the cultured cells was assessed (p. 29).

Although EMS was shown to decrease the number of viable cells under these conditions, a linear relationship was not obtained with respect to EMS concentration and cell viability (Table 18). A treatment with 0.8% EMS for two hours was necessary to kill approximately 50% of the cells.

Cultures were similarly treated with 0.4% EMS for varying times. Again no linear relationship was achieved with respect to treatment time and cell viability (Table 19), indeed a two hour treatment had almost as much effect on cell viability as a four hour one.

Table 18 Viability of dihaploid *N. tabacum* cell cultures after treatment with differing concentrations of EMS for two hours.

EMS (%)	Live cells (% of all cells in cultures.)	Live cells (% of live cells in control culture.)	Cells killed by EMS (%)
0 (control)	78	-	-
0.2	49	63	37
0.4	45	58	42
0.8	40	51	49
1.2	40	51	49

Approximately 10^3 cells were screened for viability in each flask. Figures given are the mean of duplicate flasks.

Table 19 Viability of dihaploid N. tabacum cell cultures after treatment with 0.4% EMS for varying times.

Duration of EMS treatment (h)	Live cells (% of all cells in cultures.)	Live cells (% of live cells in control culture)	Cells killed by EMS (%)
0 (control)	82	-	-
1	66	80	20
2	51	62	38
3	52	63	37
4	48	59	41

Approximately 10^3 cells were screened for viability in each flask. Figures given are the mean of duplicate flasks.

Isolation of Chlorate-resistant cell-lines.

Having carried out these preliminary experiments, a series of ten attempts was then made to isolate chlorate-resistant cell-lines from the dihaploid N. tabacum cell-lines described in Chapter three. As these cell-lines contained a percentage of dihaploid cells which was likely to decrease with increasing time in culture (p. 63), only recently established cell-lines were used.

Cell cultures were treated with EMS within about eight weeks of initiation from pith explants (p. 25). After a further week in culture in the absence of EMS to allow treated cells to express themselves phenotypically, they were plated in petri-dishes containing amino-acid medium with differing levels of chlorate and nitrate (Table 20). After another three weeks, the 126 discrete light-coloured calli which had developed on the selective plates (Figure 26) were removed and transferred to agar slopes in universal bottles. Many of the calli were too small to survive the transfer and cell-lines were established from only 39 of the 126 calli removed from the selective plates.

In many of the selective plates, but especially those in Experiments 4 and 5, a generalised callus growth appeared four-five weeks after plating. Like that described on p. 74 callus was brown rather than pale yellow in colour, and its growth was limited, but it did indicate that not all wild-type cells had been killed by this time. Although discrete chlorate-resistant calli had been removed before this background growth appeared, it was possible that cells responsible for this growth were removed with them.

Cell-lines were routinely maintained in amino-acid medium

both with and without 40 mM-chlorate. All the cell-lines retained their ability to grow in the presence of chlorate after having been grown in its absence for up to one year. This suggests that the cell-lines were not contaminated with wild-type cells, and shows that they are stable chlorate-resistant cell-lines.

Figure 26 Development of chlorate-resistant calli in a petri-dish containing amino-acid medium with 20mM-chlorate and 5mM-nitrate.

Table 20 Treatment of dihaploid N. tabacum cell-lines with EMS and plating on selective media.

a) Cells in Experiment 1 were resuspended in nitrate medium for one day prior to plating on selective media so as to maximise their nitrate reductase activity.

b) To determine whether EMS was having any effect on the number of chlorate-resistant and nitrate reductase-minus cell-lines obtained, EMS was omitted in Experiment 2.

c) In order to reduce the possibility of metabolic co-operation between cells in aggregates, cell suspensions in some experiments were filtered through nylon gauze prior to mutagenic treatment. As shown by microscopic examination, the filtrate contained only single cells and aggregates of two-three cells. Cell aggregates remaining on the filter were resuspended and also treated with EMS.

d) Several formulations of selective media were used since it was not known which would be most suitable for isolation of variant cell-lines. The media were amino-acid medium (Tables 4,5) with the following additions: A - 20 mM-chlorate; B - 20 mM-chlorate, 5 mM-nitrate; C - 20 mM-chlorate, 10 mM-nitrate; D - 40 mM-chlorate; E - 40 mM-chlorate, 2.5 mM-nitrate.

Experiment	Filtration of cells(c)	Days since pith excision	EMS treatment		Plates of selective media(d)				
			%	hr	A	B	C	D	E
1(a)	unfiltered	57	0.4	1	3	12	-	-	-
2(b)	unfiltered	57	-	-	2	2	-	-	-
3	unfiltered	66	0.4	1	-	1	-	-	-
4	filter residue	32	0.4	1	1	1	9	-	-
5	filter residue	32	0.25	1	-	6	-	-	-
6	filtrate	40	0.25	1	-	4	4	-	-
7	filtrate	40	0.25	1	-	-	-	2	2
8	filtrate	40	0.25	1	2	2	-	4	6
9	filtrate	32	0.25	1	1	-	-	3	3
10	filtrate	32	0.25	2	1	1	-	3	3

Isolation of nitrate reductase-minus cell-lines.

Several times during the six months after their isolation from selective plates, chlorate-resistant cell-lines were subcultured into medium containing nitrate as the sole nitrogen source (nitrate medium). 32 of these cell-lines grew as well on nitrate medium as they had done on amino-acid medium with or without chlorate, producing pale yellow friable callus. These cell-lines were clearly not lacking in nitrate reductase.

After a lag period of several weeks, three of the other seven cell-lines also began to grow on nitrate medium, and when subcultured again, grew as well on nitrate medium as on amino-acid medium. These cell-lines too, were not lacking nitrate reductase.

Only four cell-lines, all produced in Experiment 4, consistently failed to grow on nitrate medium (Tables 4,5). None of these were of the "wet" phenotype. When these four cell-lines (042, P12, P31 and P47) were subcultured from amino-acid medium into nitrate medium, no nitrate reductase activity could be detected in cell-free extracts prepared from callus 24h afterwards. This was carried out several times over the course of a year and each time the result was the same: subcultured calli showed no growth and gradually turned brown. Under these conditions wild-type callus was pale yellow and friable and possessed nitrate reductase activity of up to 1 µmol nitrite produced/h per gram fresh wt. No nitrate reductase activity could be detected in cell-lines 042, P12, P31 and P47 when grown on amino-acid or glutamine media either, but all of them possessed nitrite reductase activity, even in the absence of nitrate. (see Addendum, p. 92).

Table 21 Isolation of chlorate-resistant and nitrate reductase-minus cell-lines from EMS-treated wild-type dihaploid N. tabacum cell-lines on various selective media (see also Table 20).

a) A total of 126 calli were removed from petri-dishes containing selective media A to E (see footnote d. to Table 20). 39 chlorate-resistant cell-lines were established from these calli.

b) 15 of the chlorate-resistant cell-lines exhibited an unusual phenotype. Callus was "wet" in appearance and bright yellow in colour. As these characteristics were never found in wild-type callus it is possible that they are connected with a mechanism for chlorate-resistance.

c) No nitrate reductase-minus cell-lines were established from plates containing selective media A, B, D or E.

Experiment	Colonies removed from plates (a)					Chlorate-resistant cell-lines established (a)					Nitrate reductase-minus cell-lines established (c)
	Selective media					Selective media					Selective medium
	A	B	C	D	E	A	B	C	D	E	C
1	5	23	-	-	-	3	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-
3	-	1	-	-	-	-	1	-	-	-	-
4	-	-	30	-	-	-	-	16	-	-	4
5	-	8	-	-	-	-	2	-	-	-	-
6	-	-	9	-	-	-	-	2	-	-	-
7	-	-	-	5	11	-	-	-	1	6	-
8	5	6	-	6	17	-	-	-	5	3	-
9	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-

Cell-lines 042, P12, P31 and P47 would thus appear to be stable variant cell-lines lacking nitrate reductase.

Callus culture of chlorate-resistant cell-lines.

Chlorate-resistant cell-lines were routinely maintained as callus cultures in universal bottles, firstly on the same selective medium as the plates from which they were isolated (Table 20), then on both amino-acid medium and amino-acid medium containing 40 mM-chlorate (hereafter referred to as amino-acid/chlorate medium). Callus was also tested regularly for growth on nitrate medium.

Although callus growth was too irregular to quantitatively compare the growth of calli subcultured onto these three media, the overall growth pattern was clear (Figure 27 a - f); all cell-lines grew as well on amino-acid/chlorate medium as on amino-acid medium, but only those chlorate-resistant cell-lines which were not also nitrate reductase-minus grew on nitrate medium. This confirms that there are two quite distinct types of chlorate-resistant cell-line: those which are also nitrate reductase-minus and those which are not.

Suspension culture of nitrate reductase-minus cell-lines.

Nitrate reductase-minus cell-lines 042, P12, P31 and P47 were also maintained in suspension culture in amino-acid and amino-acid /chlorate media. As later experiments in this chapter involved measurement of nitrate reductase levels in these and wild-type cell-lines, a less complex and more fully-defined nitrogen supplement was desirable. These cell-lines were, therefore, subsequently also maintained on glutamine medium and glutamine medium containing 40 mM-chlorate (glutamine/chlorate medium.)

Figure 27 a-g Growth of chlorate-resistant cell-lines on amino-acid, amino-acid/chlorate and nitrate media.

a,042

amino-acid/ chlorate | amino-acid | nitrate

b,P12

amino-acid/ chlorate | amino-acid | nitrate

c, P31

amino-acid/ chlorate | amino-acid | nitrate

d, P47

amino-acid/ chlorate | amino-acid | nitrate

e, 041

amino-acid/ chlorate | amino-acid | nitrate

f, P21

amino-acid/ chlorate | amino-acid | nitrate

Effect of chlorate on nitrate reductase-minus and wild-type cell-lines.

The effect of chlorate on the growth of the four nitrate reductase-minus cell-lines was then compared with that on the wild-type cell-line from which they were derived. Stationary phase cell suspensions grown in amino-acid medium were subcultured into fresh amino-acid medium with or without 20 mM-chlorate and, whilst wild-type cell growth was greatly inhibited by chlorate (Figure 28a), the nitrate reductase-minus cell-lines were almost unaffected by it (Figure 28 b-e). On harvesting, wild-type cells were brown in appearance, whereas cells of the nitrate reductase-minus cell-lines were a pale yellow colour.

Logarithmic phase suspension cultures of nitrate reductase-minus cell-line 042 were also plated in petri-dishes (p. 27) containing amino-acid medium with the following additions.

a) No additions.

b) 20 mM-chlorate.

c) 40 mM-chlorate.

d) 80 mM-chlorate.

e) 40 mM-chlorate, 5 mM-nitrate.

f) 40 mM-chlorate, 10 mM-nitrate.

There was substantial growth in all of the petri-dishes when examined twenty days later (Figure 29). 042 cells were thus shown to tolerate up to 80 mM-chlorate in the culture medium without inhibition of growth. Growth of wild-type cells was shown previously to be inhibited by only 20 mM-chlorate (p. 74).

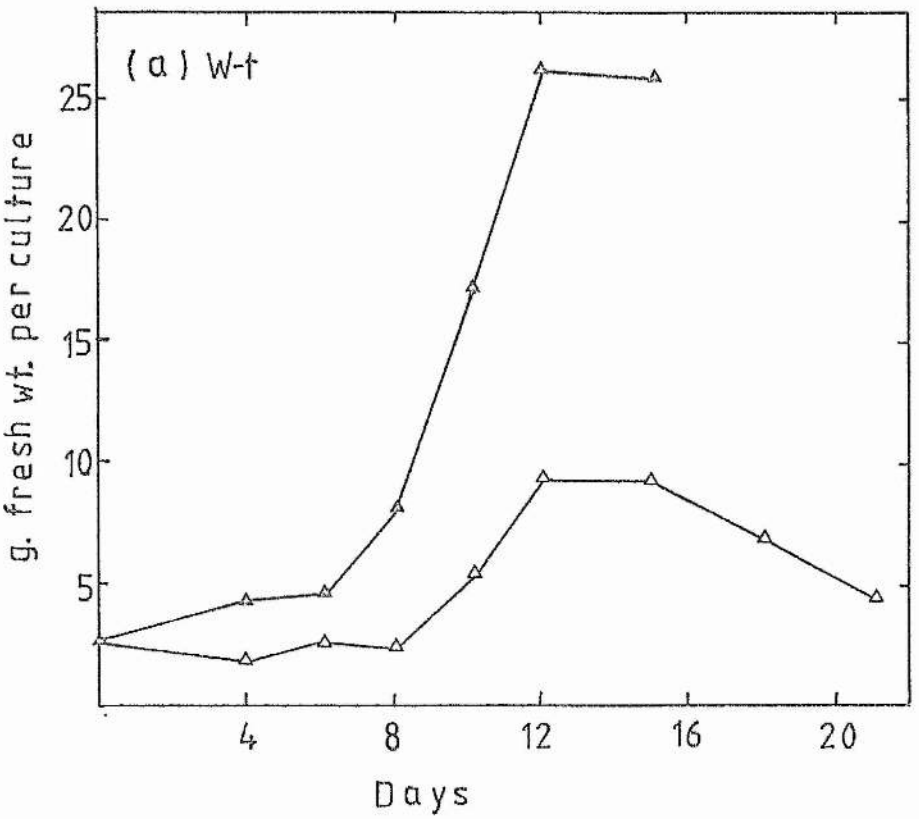
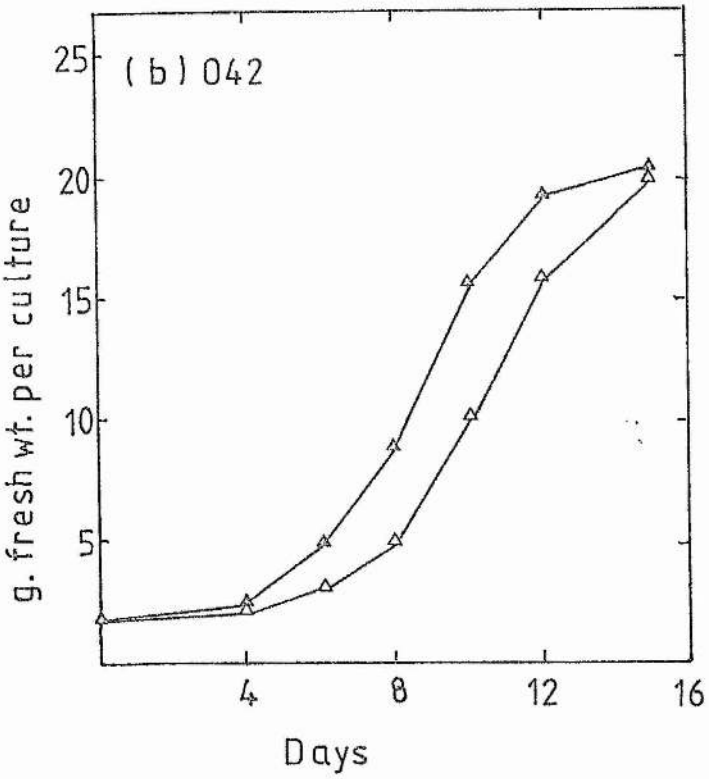
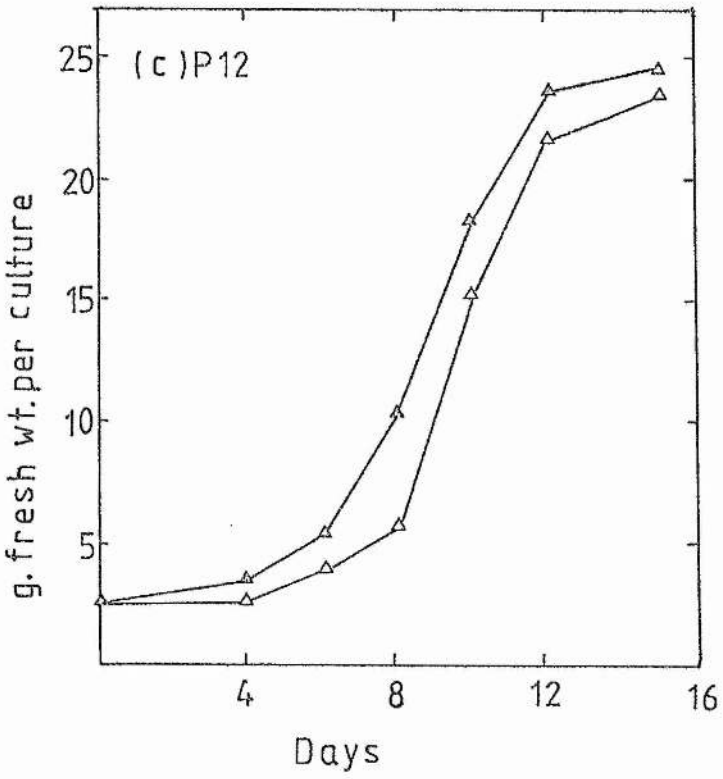
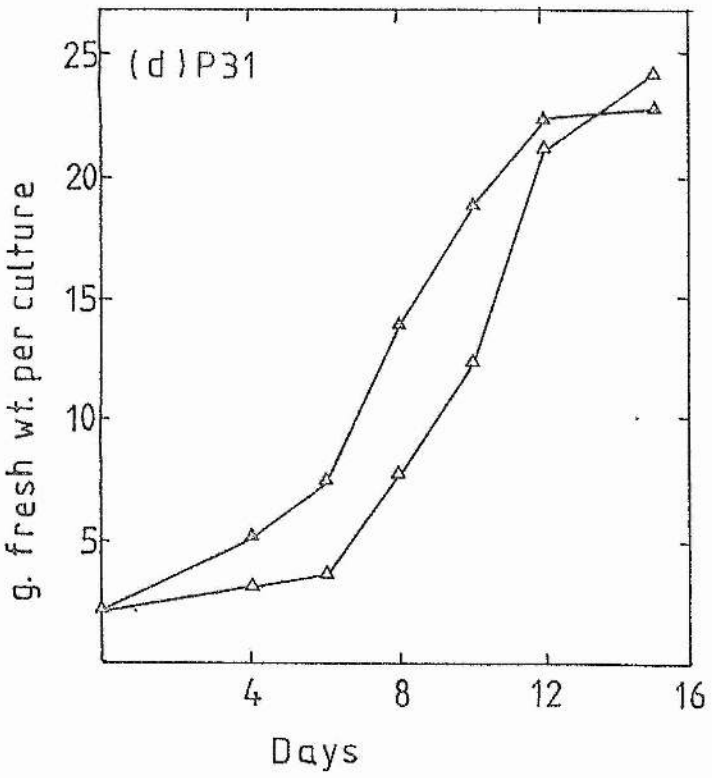
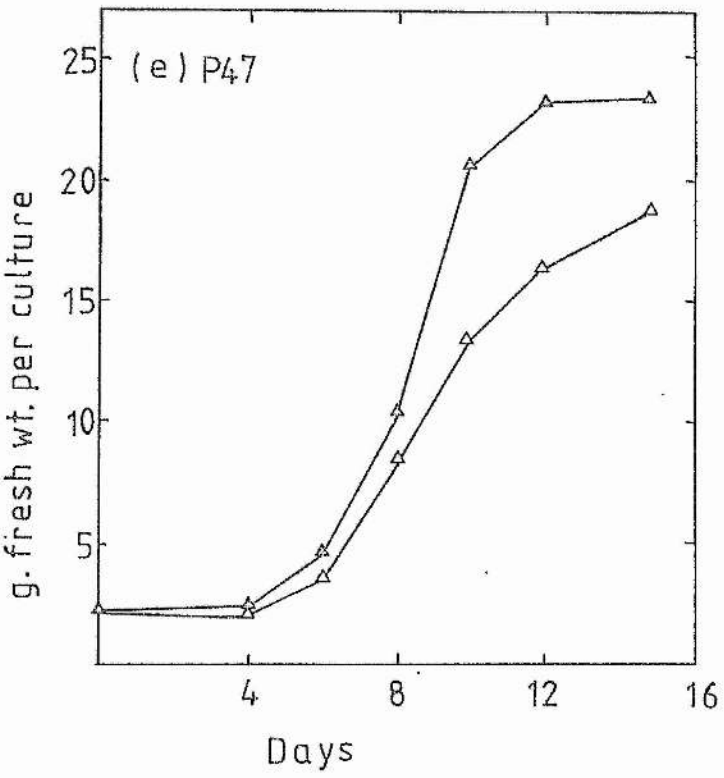


Figure 28 a-e Growth of a wild-type cell-line and nitrate reductase-minus cell-lines 042, P12, P31 and P47 on amino-acid medium in the absence (▲) or presence (△) of 40mM-chlorate.


(b) 042
25
20
15
10
5
g. fresh wt. per culture
4
8
12
16
Days

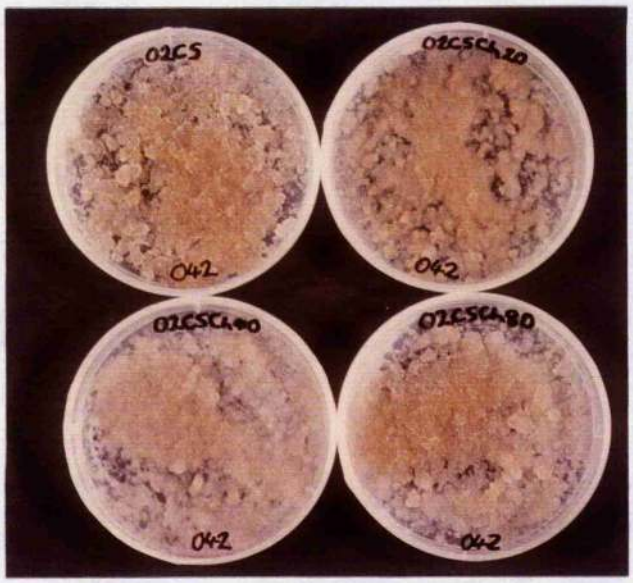

(c) P12
25
20
15
10
5
g. fresh wt. per culture
4
8
12
16
Days


(d) P31
g. fresh wt. per culture
25
20
15
10
5
4
8
12
16
Days


(e) P47
g. fresh wt. per culture
25
20
15
10
5
4
8
12
16
Days

no chlorate
no nitrate

20mM-chlorate
no nitrate

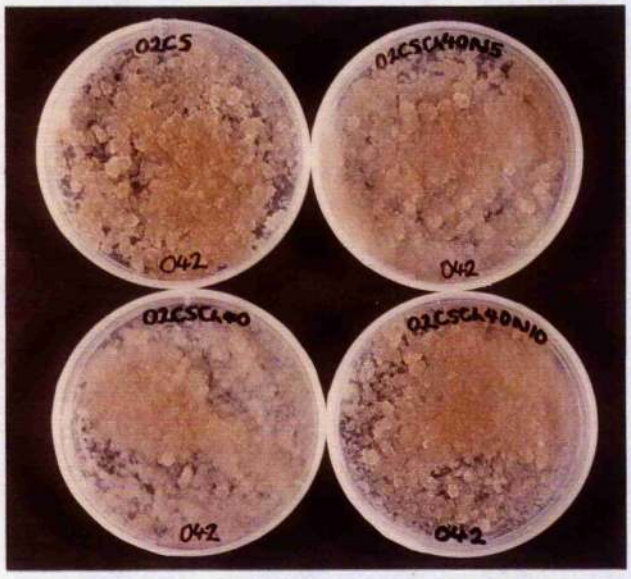


40mM-chlorate
no nitrate

80mM-chlorate
no nitrate

no chlorate
no nitrate

40mM-chlorate
5mM-nitrate



40mM-chlorate
no nitrate

40mM-chlorate
10mM-nitrate

Figure 29 Growth of nitrate reductase-minus cell-line 042 on amino-acid medium with differing levels of chlorate and nitrate.

Nitrate in nitrate reductase-minus and wild-type cell-lines after subculture into nitrate medium.

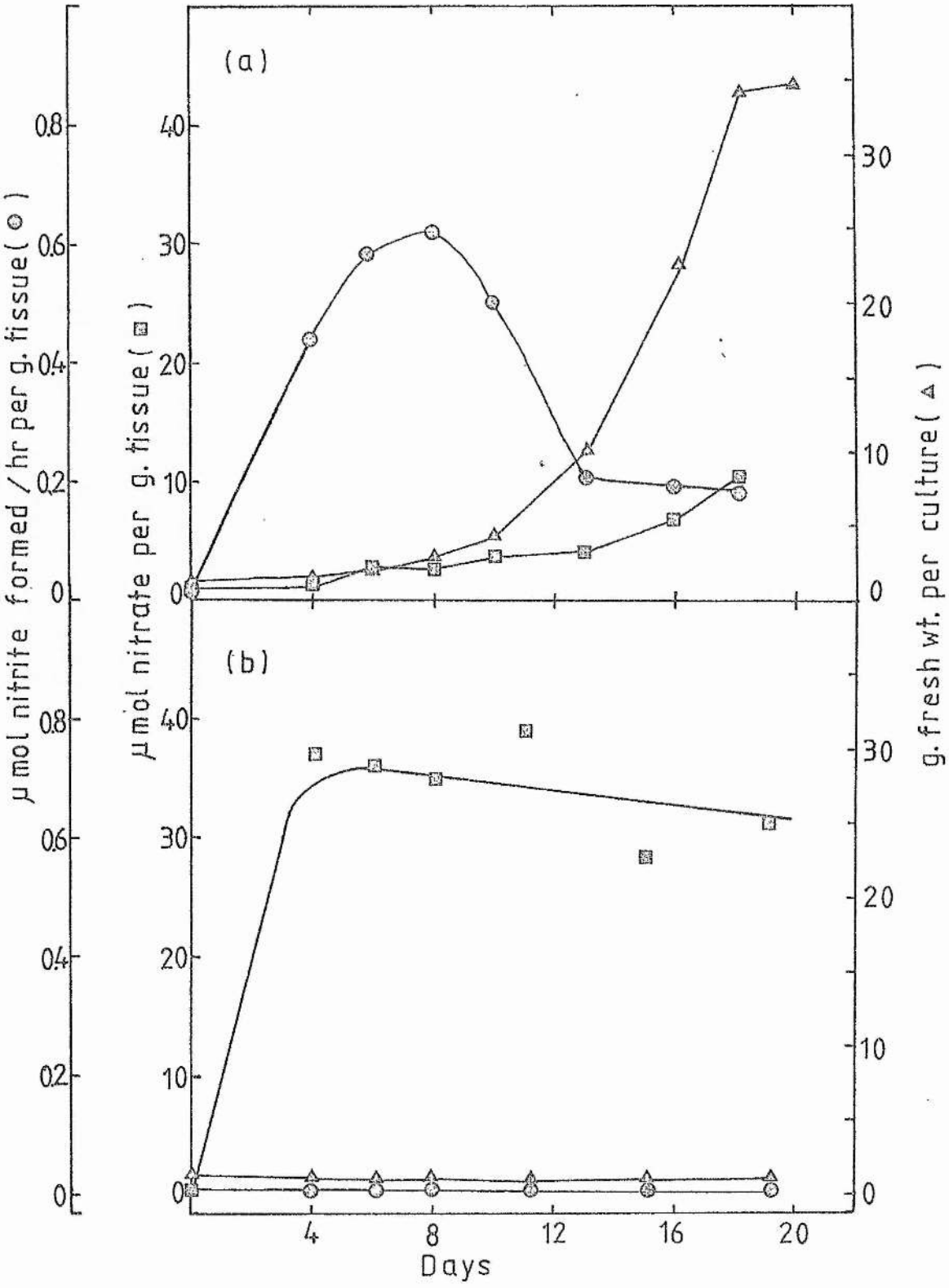
Nitrate reductase could not be detected in cell-lines 042, P12, P31 and P47 when subcultured into nitrate medium (p. 80). It was important to show whether or not this, and their inability to grow on nitrate medium, was due simply to a defect in nitrate uptake.

Suspension cultures of cell-line 042 and the wild-type cell-line from which it was derived (both stationary phase in glutamine medium) were subcultured into nitrate medium. At intervals thereafter, cell nitrate and nitrate reductase levels were measured.

After a short lag period, wild-type cultures rapidly increased in total fresh weight, reaching a maximum of 35 g in stationary phase (Figure 30a). Nitrate reductase activity rose sharply at first then declined, forming a recognisable peak at the early exponential phase of culture growth. Internal nitrate levels were low, rising more rapidly as cultures approached stationary phase, coinciding with a drop in nitrate reductase activity.

042 cultures showed a slow decrease in total fresh weight (Figure 30b), accompanied by browning of the cells. Nitrate reductase could not be detected but cells quickly built up much higher internal nitrate levels than wild-type cells, showing that the inability of 042 cells to grow on nitrate medium and their lack of nitrate reductase activity is not due to a defect in nitrate uptake.

Figure 30 Growth (▲), nitrate content (■) and nitrate reductase activity (●) of a wild-type cell-line (a) and nitrate reductase-minus cell-line 042 (b) after subculture into nitrate medium.


(a)
(b)
µmol nitrite formed / hr per g. tissue (○)
µmol nitrate per g. tissue (□)
g. fresh wt. per culture (△)
Days
0
0.2
0.4
0.6
0.8
10
20
30
40
4
8
12
16
20

Nitrate toxicity in nitrate reductase-minus cell-lines.

Nitrate reductase-minus cell-lines should not be able to grow on nitrate as a sole nitrogen source and cells would be expected to die slowly through nitrogen starvation. However, cells of the nitrate reductase-minus cell-line 042 turned brown within a week of subculture into nitrate medium, whilst at the same time building up high internal nitrate levels (Figure 30b). Perhaps these cells were dying not from nitrogen starvation but from a toxic effect exerted by these high levels of nitrate. To show whether this was the case, suspension cultures of nitrate reductase-minus cell-lines 042, P12, P31 and P47, and a wild-type cell-line (all stationary phase in glutamine medium) were subcultured into nitrate medium and a similar medium where the nitrate had been replaced by an equimolar concentration of KCl (nitrogen-free medium).

When the four nitrate reductase-minus cell-lines were subcultured into nitrate medium, nitrate built up inside the cells (Figure 31 b - e). This confirmed that in none of these cell-lines was the inability to grow on this medium due to a defect in nitrate uptake. It would appear then that nitrate was building up in these cells because they lacked nitrate reductase. Nitrate build-up was accompanied by cell browning and a decrease in total fresh weight of the cultures. However, when subcultured into nitrogen-free medium, cells remained light in colour and the total fresh weight of the cultures remained unchanged. This suggested that rapid cell death in nitrate medium was indeed due to some toxic effect of nitrate and not simply to nitrogen starvation.

The wild-type cell-line behaved similarly to the nitrate reductase-minus cell-lines when subcultured into nitrogen-free

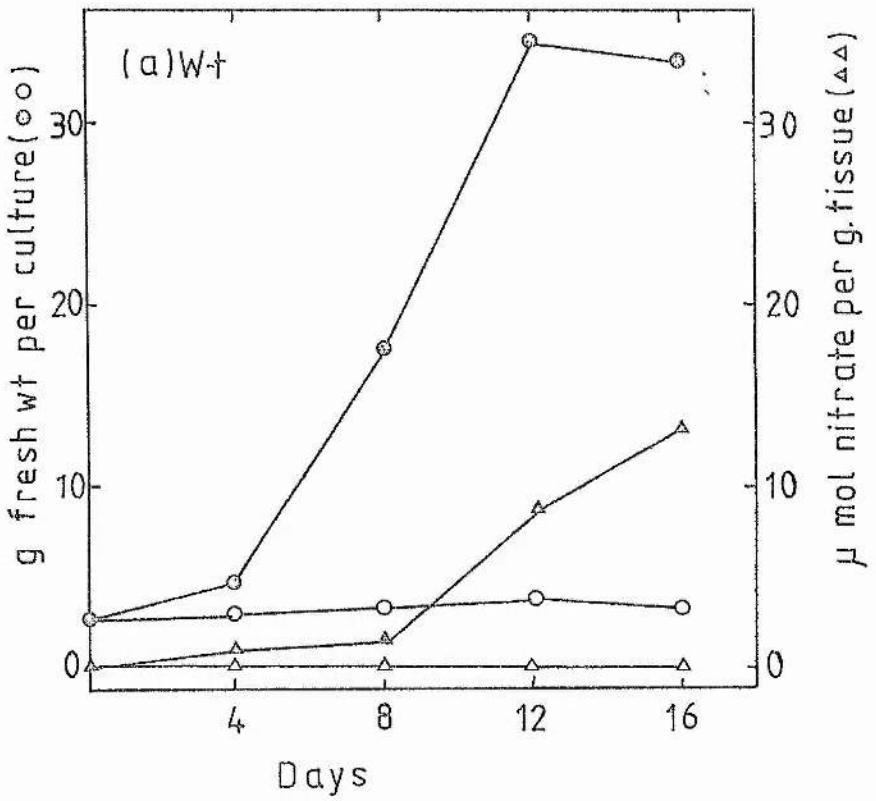
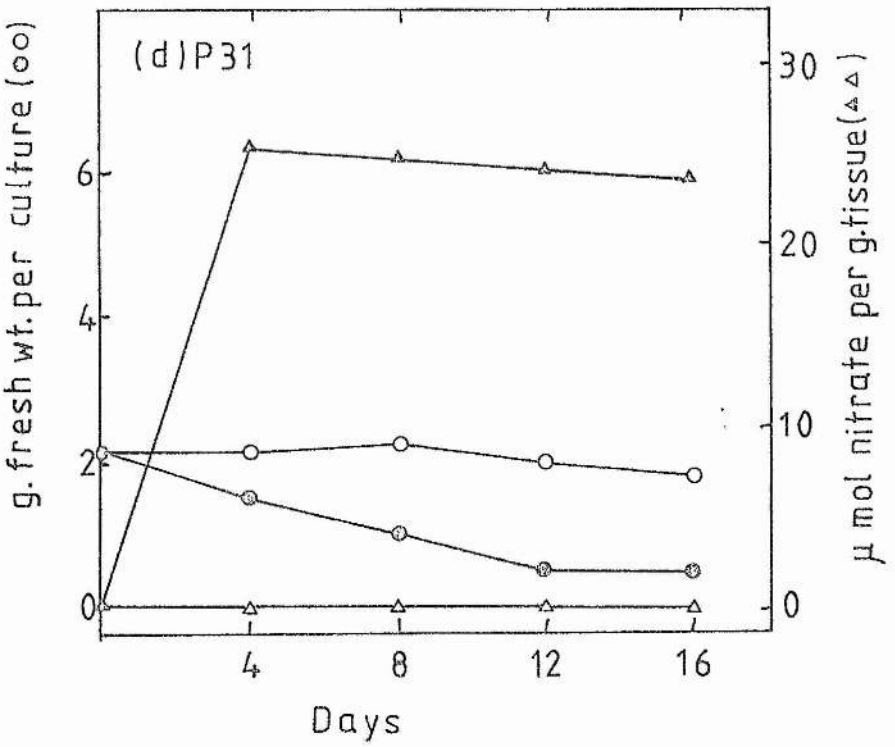
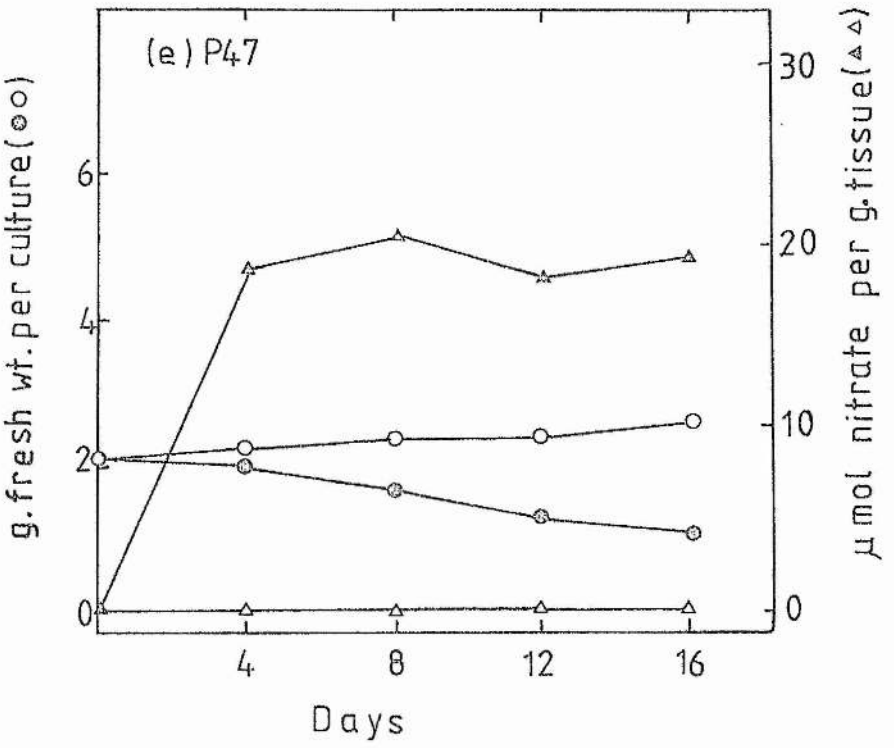


Figure 31 a-e Growth (◎ ○) and nitrate content (△ △) of a wild-type cell-line (a) and nitrate reductase-minus cell-lines 042, P12, P31 and P47 (b-e) after subculture into nitrate (closed symbols) and nitrogen-free (open symbols) media.

(b) 042
g. fresh wt. per culture (o o)
μ mol nitrate per g. tissue (Δ Δ)
Days
0
2
4
6
10
20
30
4
8
12
16

(c) P12
g. fresh wt. per culture (o o)
μ mol nitrate per g. tissue (Δ Δ)
Days
0
2
4
6
10
20
30
4
8
12
16


(d) P31
g. fresh wt. per culture (oo)
μ mol nitrate per g.tissue (△△)
0
2
4
6
10
20
30
4
8
12
16
Days


(e) P47
g. fresh wt. per culture (oo)
μ mol nitrate per g.tissue (△△)
0
2
4
6
10
20
30
4
8
12
16
Days

medium (Figure 31a) but grew well on nitrate medium and did not build up high levels of nitrate.

Cell viability in a nitrate reductase-minus cell-line after subculture into nitrate and nitrogen-free media.

In order to show conclusively whether nitrate was exerting a toxic effect on the nitrate reductase-minus cell-lines, suspension cultures of 042 cell-line (stationary phase in glutamine medium) were subcultured into nitrate and nitrogen-free media. The percentage of viable cells in each culture was determined by the phenosafranine dye exclusion method (p. 29) at three and six days after subculture.

In nitrate medium the percentage of viable cells dropped by 44% within six days but in nitrogen-free medium the corresponding drop was only 8% (Table 22).

When, after six days, glutamine was added to the remaining cultures to a final concentration of 8 mM, cell growth in the nitrogen-free flasks was rapid, but in the nitrate flasks there was no growth.

It would appear then that nitrate, as it builds up in the nitrate reductase-minus cell-lines subcultured into nitrate medium, is toxic to the cells.

Table 22 Viability of 042 nitrate reductase-minus cells after subculture into nitrate and nitrogen-free media, as determined by phenosafranine dye exclusion.

Medium	% viable cells		
	0 days	3 days	6 days
Nitrate	71 (100)	55 (77.4)	40 (56.3)
Nitrogen-free	71 (100)	65 (91.5)	66 (92)

Figures in parentheses show the percentage of viable cells as a percentage of that at the time of subculture (0 days.)

DISCUSSION

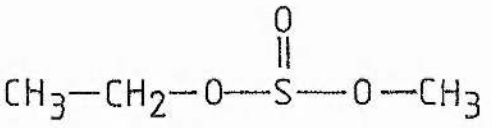
As a first step towards isolating chlorate-resistant cell-lines, the response of wild-type dihaploid N. tabacum cell cultures to chlorate was determined. 20 mM-chlorate was found to inhibit culture growth whether in amino-acid (Figure 24) or nitrate (Figure 23) medium, but in the case of amino-acid medium there was some growth after a lag of eight to ten days. Similar growth was not found in nitrate medium, suggesting that cells were more susceptible to chlorate toxicity in nitrate than in amino-acid medium. In view of the connection (p. 15) between nitrate reductase activity and chlorate toxicity, it was thought that this greater susceptibility might be due to the higher levels of nitrate reductase activity found in cells growing in nitrate as opposed to amino-acid medium (Figures 19 and 21) However, it was necessary to raise chlorate-resistant cell-lines on an N source other than nitrate (p. 55), so by way of a compromise low levels of nitrate were added to amino-acid/chlorate medium. Wild-type cells plated on this medium did indeed possess higher levels of nitrate reductase than those plated on the same medium without nitrate (Table 16) and their growth should therefore be more fully inhibited by chlorate.

Since the optimum levels of chlorate and nitrate for allowing growth of chlorate-resistant calli whilst suppressing growth of wild-type cells could not be predicted in advance, a number of different formulations of selective media were used in the chlorate-resistant cell-line isolation experiments that followed (p. 78).

The use of EMS to raise the mutation rate in N. tabacum cell cultures.

Mutation rates in plant cell cultures are in the order of 10^{-6} (Widholm, 1974; Maliga, 1976) and most workers who have attempted to isolate auxotrophic or recessive mutants from plant cell-lines have found it necessary to use mutagenic agents to increase the incidence of variation in the wild-type cell population prior to application of selection pressure. It was therefore reasonable to assume that a mutagenic agent would be required in this work also.

A commonly used mutagenic agent in plant systems is ethyl methane sulphonate (EMS), an alkanesulphonic ester of structure



The mutagenic action of EMS has been thoroughly investigated by Ehrenberg, Lundquist, Osterman and Sparrman (1966) and Osterman - Golkar, Ehrenberg and Wachtmeister (1970). It is thought to act by alkylation of the unesterified phosphate oxygens of DNA (Ross, 1962) and it will also alkylate proteins due to their high nucleophilicity. EMS exerts its mutagenic effect whilst producing only a small amount of chromosomal aberration (Moutschen, 1965) and has been used successfully in the production of variant cell-lines by Carlson (1970) and Nabors, Daniels, Nadolny and Brown (1975) in N. tabacum, by Dix and Street (1976) in N. sylvestris, and by Savage, King and

Gamborg (1979) in Datura innoxia. Although certain other chemical mutagens are reported to have a higher mutagenic efficiency than EMS (Ehrenberg, 1970), they are for the most part more hazardous to handle. Given the laboratory conditions available, it was decided on balance to use EMS in this work.

Working on barley seeds, Ehrenberg et al (1966) found that the mutagenic efficiency of EMS was greatest at a degree of exposure which inhibited 50% of the treated cells from germinating. This was supported by work on E. coli (Turtoczky and Ehrenberg, 1969), where the degree of exposure which caused the death of 50% of the cells gave the highest mutagenic efficiency. Efforts were thus made to determine what this degree of exposure would be for wild-type dihaploid N. tabacum cell cultures (p. 77).

Although a linear relationship was not obtained with regard to either EMS concentration or duration of treatment against cell killing (Tables 18, 19), a two hour treatment with 0.8% EMS was required to kill 50% of the cells in the cultures. Parallels in literature are scarce but Dix (1975) found that a one hour incubation with 0.75% EMS killed 70-80% of the cells in a largely diploid population of N. sylvestris cells. Species or varietal differences might explain the discrepancy but it is also possible that the medium in which the cells were treated is involved.

Osterman -Golkar et al (1970) showed that EMS will react with amino-groups in solution ($t^{\frac{1}{2}} = 1.5h.mol^{-1}$), effectively reducing the EMS concentration. The half-life of EMS in distilled water at 25°C is 48h (Ehrenberg, 1970), but the half-life in the amino-acid medium in which the dihaploid

N. tabacum cells were cultured might be substantially less than this because of reaction with amino-groups.

Effect of EMS on cultures of mixed ploidy.

One other very important factor which must be taken into account is the ploidy of the treated cells. As explained in Chapter three, the dihaploid N. tabacum cell suspensions used in this work are in fact mixoploid, containing a variable percentage of dihaploid cells but also amphidiploid, polyploid and aneuploid cells. Cells of differing ploides might be affected by mutagenic agents to differing extents. Single mutations which would be masked by duplicated genetic material in a polyploid cell, for example, might be lethal in a haploid cell. A level of EMS which killed 50% of the cells in a mixoploid culture might therefore represent selective killing of cells of lower ploidy.

Indeed, the "tailing-off" of the effect of increasing exposure to EMS (Tables 18, 19) might indicate that a sub-population of cells was being affected disproportionately. This sub-population might represent the dihaploid cells in the cultures. As mutations leading to a lack of nitrate reductase are unlikely to be expressed in diploid or polyploid cells (p. 10), such a selective killing would reduce the chances of isolating nitrate reductase-minus cell-lines. A lower degree of exposure to EMS than the 2h treatment with 0.8% EMS which killed 50% of the wild-type N. tabacum cells (Table 18) was therefore indicated for isolation of variant cell-lines.

Chlorate-resistant and nitrate reductase-minus cell-lines.

Aberg (1947) postulated that, in wheat, chlorate is made toxic on reduction to chlorite by nitrate reductase. Applying his theory to tobacco, we would therefore expect to find nitrate reductase-minus cell-lines amongst the chlorate-resistant N. tabacum cell-lines isolated, and indeed this proved to be the case, four out of 39 chlorate-resistant cell-lines being nitrate reductase-minus. The chances of this happening by coincidence are very small.

The other 35 chlorate-resistant cell-lines grew on medium containing nitrate as a sole N source, and, by inference, possessed nitrate reductase activity. Their resistance to chlorate requires, therefore, some other explanation. The most obvious one is that in these cell-lines there is some restriction on chlorate uptake, but there are other possible explanations. Cove (1976a) suggested that, in Aspergillus, chlorate mimics nitrate, via nitrate reductase and a regulatory gene, in producing a shut-down of nitrogen catabolism, causing death by nitrogen starvation. Applying this theory to the N. tabacum cell-lines, chlorate resistance in the presence of nitrate reductase could be explained by deletion or modification of this regulatory gene.

Whatever the explanation it is clear that we have at least two classes of chlorate-resistant cell-line, only one of which can be due to a lack of nitrate reductase activity.

Nitrate reductase-minus cell-line isolation in different experiments.

Although ten isolation experiments were carried out, all four nitrate reductase-minus cell-lines were produced in the same experiment, Experiment 4. It is pertinent, then, to ask why

this experiment was successful whilst the others were not.

There were many minor procedural differences between experiments and many variables (Table 20) so to attribute their success or failure to any one aspect is very difficult. Several formulations of selective media were used. The nitrate reductase-minus cell-lines all developed on that containing 20 mM-chlorate and 10 mM-nitrate. This, however may have been only coincidence as most of the selective plates in Experiment 4 contained this medium and in other experiments where this medium was used no nitrate reductase-minus cell-lines were isolated. It seems unlikely to me that the small variations in the selective media could be responsible for the success or failure of the experiments anyhow. Some other explanation is needed.

N. tabacum cells grow in suspension as isolated cells and small aggregates (p. 25). As cell aggregation might result in metabolic co-operation, making isolation of variant cell-lines difficult (Zryd, 1978), attempts were made to separate off the larger of these aggregates. Nylon gauze filters were used to strain the cell suspensions and produce suspensions containing only single cells and very small aggregates. Aggregates which were held back by the filter were resuspended in fresh medium, producing suspensions containing mostly aggregates. These suspensions were also treated with EMS, and it was from these, used in Experiment 4, that all four nitrate reductase-minus cell-lines were isolated. It might appear then, that these larger aggregates were more, rather than less, suitable than free cells for the isolation of variant cell-lines.

One possible explanation is that the cell aggregates

contained a greater proportion of dihaploid cells, more suitable than higher ploidy cells for the isolation of variant cell-lines (p. 10), than the filtered cell suspensions. Haploid cells are, on average, smaller than diploid and polyploid cells (Kimber and Riley, 1963), and for kinetic reasons small cells are more likely to be in aggregates than larger ones, so in a suspension containing both haploid and diploid cells, cell aggregates are likely to contain a greater percentage of haploid cells than a population of free cells in suspension. Filtering off the aggregates from the "dihaploid" cell suspensions (Table 20). might then have the effect of making them dihaploid-depleted, leaving the aggregates dihaploid-enriched.

Addendum (see foot of p. 80).

Cell-lines 042, P12, P31 and P47 possessed similar nitrite reductase levels to wild-type cells; i.e., about 1 µmol nitrite reduced/h per gram fresh wt. when grown on glutamine medium.

Chapter 5. Further characterisation of nitrate reductase-minus cell-lines.

INTRODUCTION

Having succeeded in raising four, apparently stable, nitrate reductase-minus cell-lines, the next step was to study them in some detail in order to determine what genetic defects were responsible for their lack of this enzyme activity.

Partial activities of the nitrate reductase molecule.

At present, the best model of the genetic control of nitrate reductase production is in the fungus Aspergillus nidulans (Cove, 1979). There nitrate reductase consists of a number of similar apoprotein sub-units which possess NADPH-cytochrome c reductase activity. There, sub-units associate with a molybdenum-containing co-factor (Mo-Co) to form a nitrate reductase holoprotein which also possesses NADPH-cytochrome c reductase activity. This Mo-Co is shared with a number of molybdoenzymes including xanthine dehydrogenase (Pateman, Cove, Rever and Roberts, 1964). Mutations at the gene loci coding for the nitrate reductase apoprotein, for Mo-Co synthesis and processing, and for regulatory enzymes can all cause loss of nitrate reductase activity. By assaying for the activities associated with the nitrate reductase molecule, Pateman, Rever and Cove (1967) have established the genetic basis of the defects in numerous nitrate reductase-minus Aspergillus cell-lines.

Higher plant nitrate reductases are similar in many respects to that in Aspergillus. As in Aspergillus, spinach nitrate reductase has been shown to contain FAD, a b-type cytochrome and molybdenum (Hewitt, 1975; Notton, Fido and Hewitt, 1977; Notton and Hewitt, 1971) and barley nitrate reductase has been shown to possess cytochrome c reductase activity (Wray and Filner, 1970; Small and Wray, 1980).

The plan was, therefore, to determine the genetic basis of the defects in the four nitrate reductase-minus N. tabacum cell-lines (042, P12, P31 and P47) using a similar approach to that used successfully by Cove and co-workers with Aspergillus.

If, as in Aspergillus, N. tabacum nitrate reductase consists of apoprotein sub-units with NAD(P)H-cytochrome c reductase activity and a molybdenum-containing co-factor (Mo-Co) shared with xanthine dehydrogenase, then, by assaying nitrate reductase-associated NADH cytochrome c reductase activity in cell-free extracts from the nitrate reductase-minus cell-lines, we could determine whether these cell-lines produced a nitrate reductase apoprotein. Similarly, if these extracts lacked xanthine dehydrogenase activity and extracts from wild-type cells did not, then it would suggest that the nitrate reductase-minus cell-lines possessed a defective Mo-Co.

RESULTS

Xanthine dehydrogenase activity in nitrate reductase-minus and wild-type cell-lines.

If, as discussed above, N. tabacum nitrate reductase shares a common component with the enzyme xanthine dehydrogenase, then it is possible that some of the nitrate reductase-minus cell-lines also lack xanthine dehydrogenase. Attempts were therefore made to measure xanthine dehydrogenase activity in cell-free extracts from nitrate reductase-minus and wild-type cell-lines.

The only quantitative assay for xanthine dehydrogenase which has been published is that of Scazzochio, Holl and Foguelman (1973). This involves the assay of xanthine dehydrogenase-dependent reduction of cytochrome c by NADH. However, when attempted in this laboratory, other cytochrome c reductase activities in the extracts made it difficult to measure any xanthine dehydrogenase-dependent activity.

As an alternative, a modification of the qualitative-only assay of Mendel and Müller (1976) was used (p. 33). Using this procedure, xanthine dehydrogenase was detected in wild-type cells whether grown on glutamine or nitrate medium (Table 23) but no xanthine dehydrogenase could be detected in any of the four nitrate reductase-minus cell-lines.

Table 23 Xanthine dehydrogenase activity in wild-type and nitrate reductase-minus cell lines.

Cell-line	glutamine medium	nitrate medium *
Wild-type	+	+
O42	-	
P12	-	
P31	-	
P47	-	

* Cell-lines O42, P12, P31 and P47 were incapable of growth in nitrate medium.

Nitrate reductase-associated NADH-cytochrome c reductase activity in N. tabacum cell-lines.

a) NADH-cytochrome c reductase in a nitrate reductase-minus and a wild-type cell-line.

Higher plant nitrate reductases have been shown, in some species at least, to possess NADH-cytochrome c reductase activity (p. 8). It would be useful, therefore, to compare the NADH-cytochrome c reductase activity of a nitrate reductase-minus cell-line with that of a wild-type cell-line.

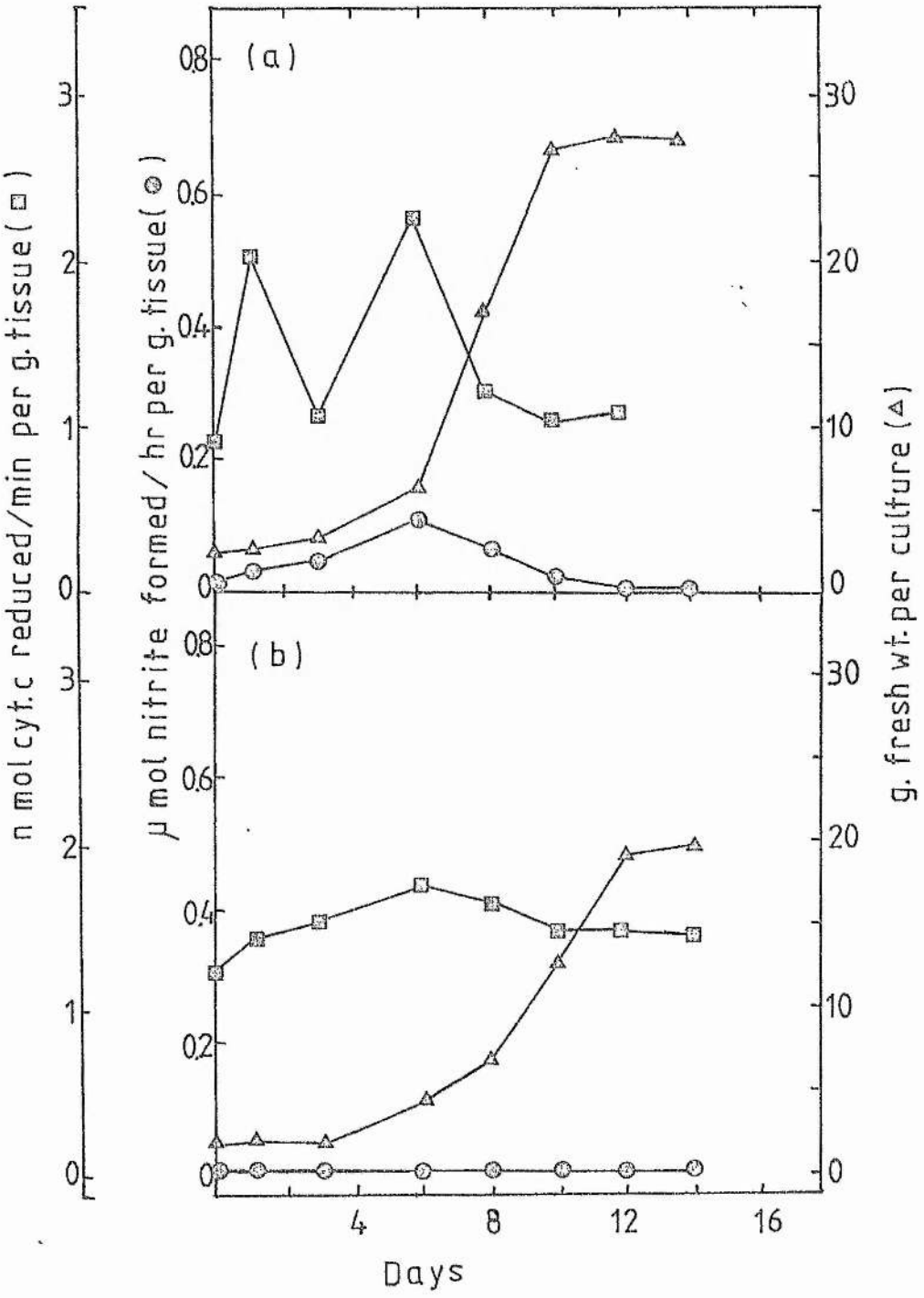
Suspension cultures of nitrate reductase-minus cell-line 042 and a wild-type cell-line (both stationary phase in glutamine medium) were subcultured into fresh glutamine medium. The two cell-lines proved to have similar levels of NADH-cytochrome c reductase activity even although only the wild-type cell-line possessed nitrate reductase activity. (Figure 32).

b) Stimulation of NADH-cytochrome c reductase activity by nitrate.

If nitrate causes an increase in both nitrate reductase and NADH cytochrome c reductase activities in extracts from wild-type cells, then this would support the idea that both activities are carried on the same protein. Furthermore if nitrate increases NADH-cytochrome c reductase activity in the nitrate reductase-minus cell-lines, then this might indicate that these cell-lines possess an incomplete or inactive form of nitrate reductase.

Suspension cultures of nitrate reductase-minus cell-lines 042, P12 and P31 and a wild-type cell-line (all stationary phase in glutamine medium) were therefore subcultured into nitrate medium and a nitrogen-free medium.

Figure 32 Growth (▲), nitrate reductase (●) and NADH-cytochrome c reductase (■) activities of a wild-type cell-line (a) and nitrate reductase-minus cell-line 042 (b) after subculture into glutamine medium.


(a)
(b)
n mol cyt.c reduced/min per g.tissue (□)
μ mol nitrite formed/hr per g.tissue (○)
g. fresh wt. per culture (△)
Days
0
0.2
0.4
0.6
0.8
1
2
3
4
8
10
12
16
20
30

In the wild-type cell-line, nitrate caused a marked stimulation of NADH-cytochrome c reductase activity. No comparable increase occurred in nitrogen-free medium (Figure 33a). There was a close correlation between NADH-cytochrome c reductase and nitrate reductase activities in this cell-line.

In the nitrate reductase-minus cell-lines, nitrate produced an increase in NADH-cytochrome c reductase activity which was greatest after two days (Figure 33 b,c,d). A smaller increase occurred in nitrogen-free medium. Although the net increase caused by nitrate was short-lived, this can be explained by the toxic effect of the high levels of nitrate which built up in the cells within two-three days.

c) Fractionation of protein in cell-free extracts.

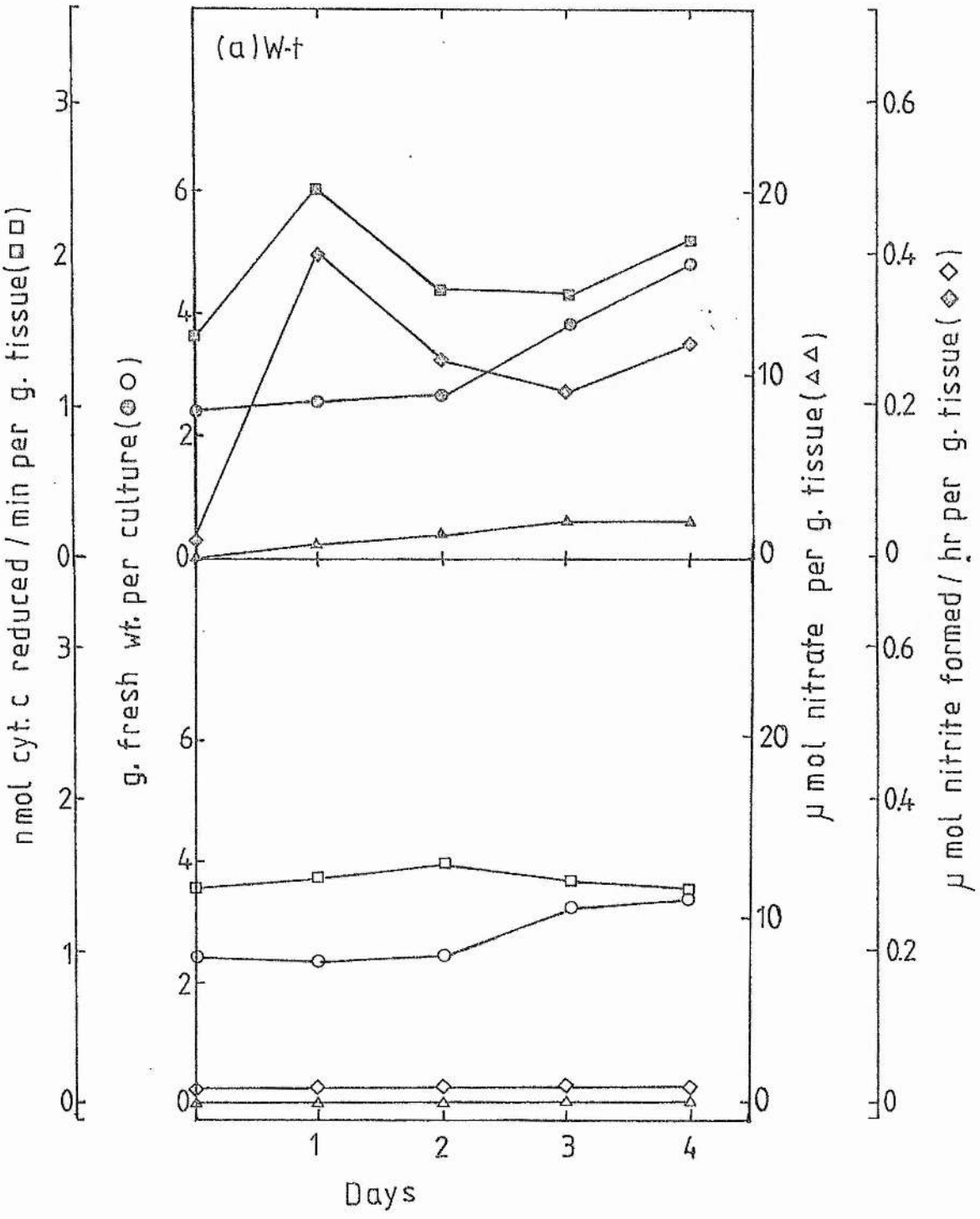
In order to find out what proportion of the total NADH-cytochrome c reductase activity in N. tabacum cells is associated with nitrate reductase, protein in cell-free extracts was fractionated and the two activities were measured in the fractions.

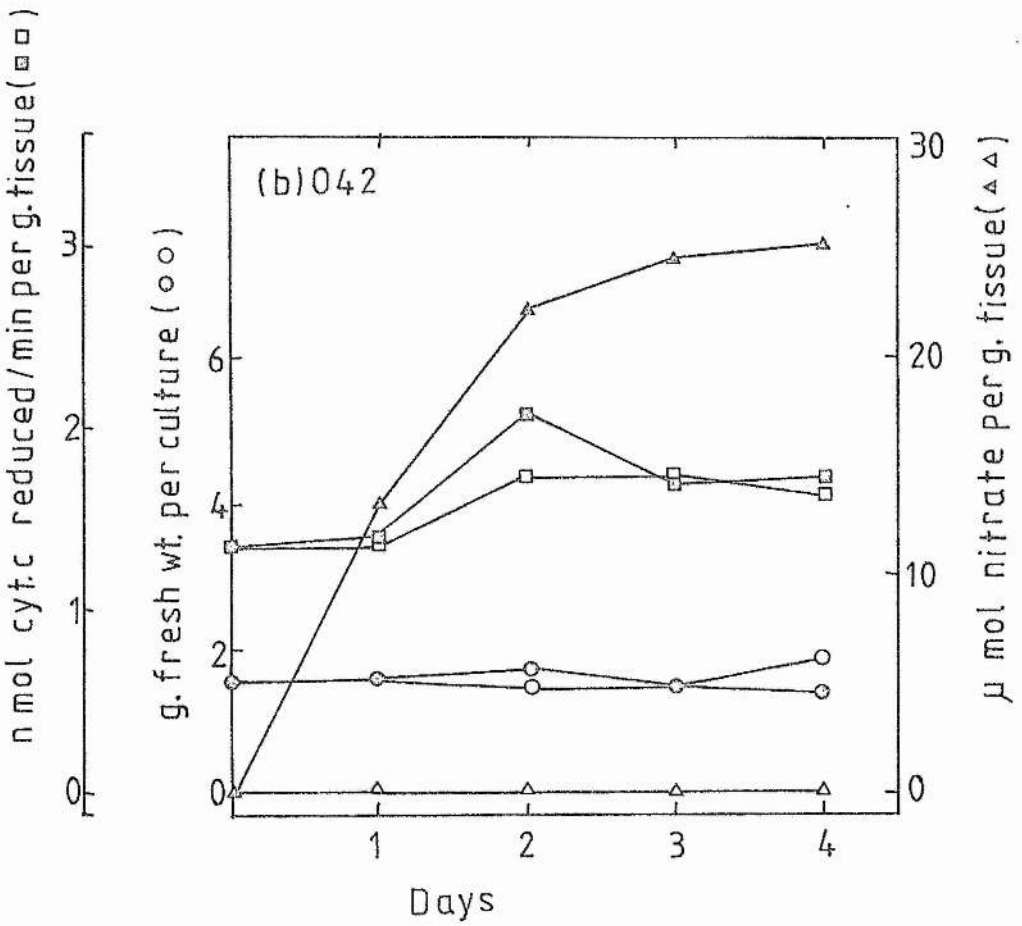
Protein in cell-free extracts from a wild-type cell-line (logarithmic phase in nitrate medium) was sequentially precipitated by adding ammonium sulphate to increasing percentage saturation levels. Four fractions were produced containing the protein which was precipitated by increasing the saturation level of ammonium sulphate from 0 to 30, 30 to 45, 45 to 60 and from 60 to 100 percent, respectively. The average molecular weight of the proteins in the fractions should be inversely proportional to the percentage saturation.

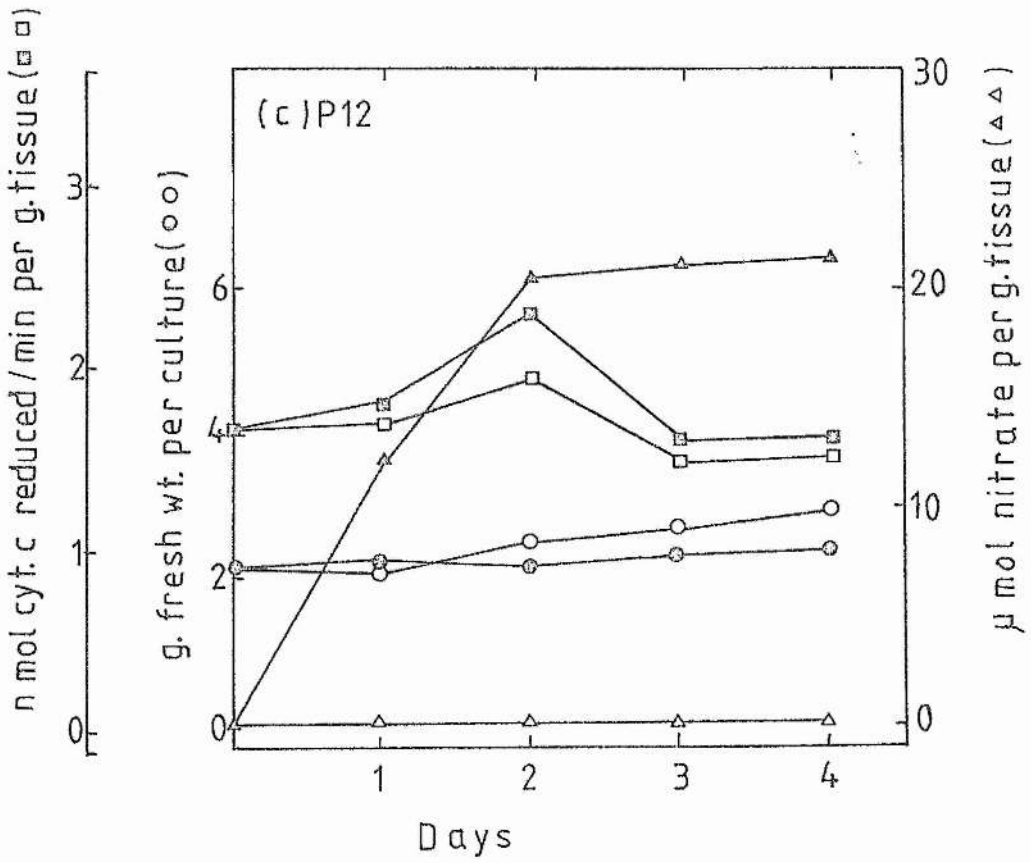
The 30-45% saturation fraction proved to possess most (60%) of the total nitrate reductase activity in the original extract but a much smaller proportion (22%) of the NADH-cytochrome c

reductase activity, most of which (53%) was precipitated at between 45 and 60% saturation (Table 24) and thus of lower molecular weight. Only a very small proportion (3.5%) of the nitrate reductase activity was found in this fraction. This showed that perhaps only a quarter of the NADH-cytochrome c reductase activity in the original cell-free extract can possibly be associated with active nitrate reductase.

Figure 33 a-d Growth (● ○), nitrate content (▲ △) and nitrate reductase (◆ ◇) and cytochrome c reductase (■ □) activities of a wild-type cell-line (a) and nitrate reductase-minus cell-lines 042, P12 and P31 (b-d) after subculture into nitrate (closed symbols) and nitrogen-free (open symbols) media.


(a) W-t
nmol cyt. c reduced / min per g. tissue(□□)
g. fresh wt. per culture(○○)
μ mol nitrate per g. tissue(△△)
μ mol nitrite formed / hr per g. tissue(◇◇)
Days
0
1
2
3
4
6
20
0.2
0.4
0.6


(b)042
n mol cyt.c reduced/min per g.tissue(□□)
g. fresh wt. per culture (○○)
μ mol nitrate per g. tissue(△△)
Days
0
1
2
3
4
6
10
20
30


(c) P12
n mol cyt. c reduced / min per g. tissue (□ □)
g. fresh wt. per culture (○ ○)
μ mol nitrate per g. tissue (△ △)
Days
0
1
2
3
4
0
2
4
6
0
10
20
30

(d)P31
n mol cyt.c reduced/min per g. tissue(□□)
g. fresh wt. per culture (●○)
μ mol nitrate per g. tissue (△△)
Days
0
1
2
3
4
6
10
20
30

Table 24 Nitrate reductase and NADH-cytochrome c reductase activities in ammonium sulphate fractions of extracts from wild-type cells.

Fractions	% of total nitrate reductase activity	% of total NADH-cytochrome c reductase activity.
0 - 30%	36	19
30 - 45%	60	22
45 - 60%	3.5	53
60 - 100%	1.5	6

Fractions contained the protein precipitated between the two percentage saturation levels of ammonium sulphate indicated above. Protein in each fraction was suspended in 1 ml extraction buffer.

d) Sucrose density gradient centrifugation of extracts from N. tabacum cell-lines.

If N. tabacum nitrate reductase also possesses NADH-cytochrome c reductase activity (p. 8), then the two activities should co-sediment in sucrose density gradients. Cell-free extracts were therefore prepared from wild-type cell suspensions (logarithmic phase) and subjected to centrifugation on sucrose density gradients, essentially as described by Small and Wray (1980).

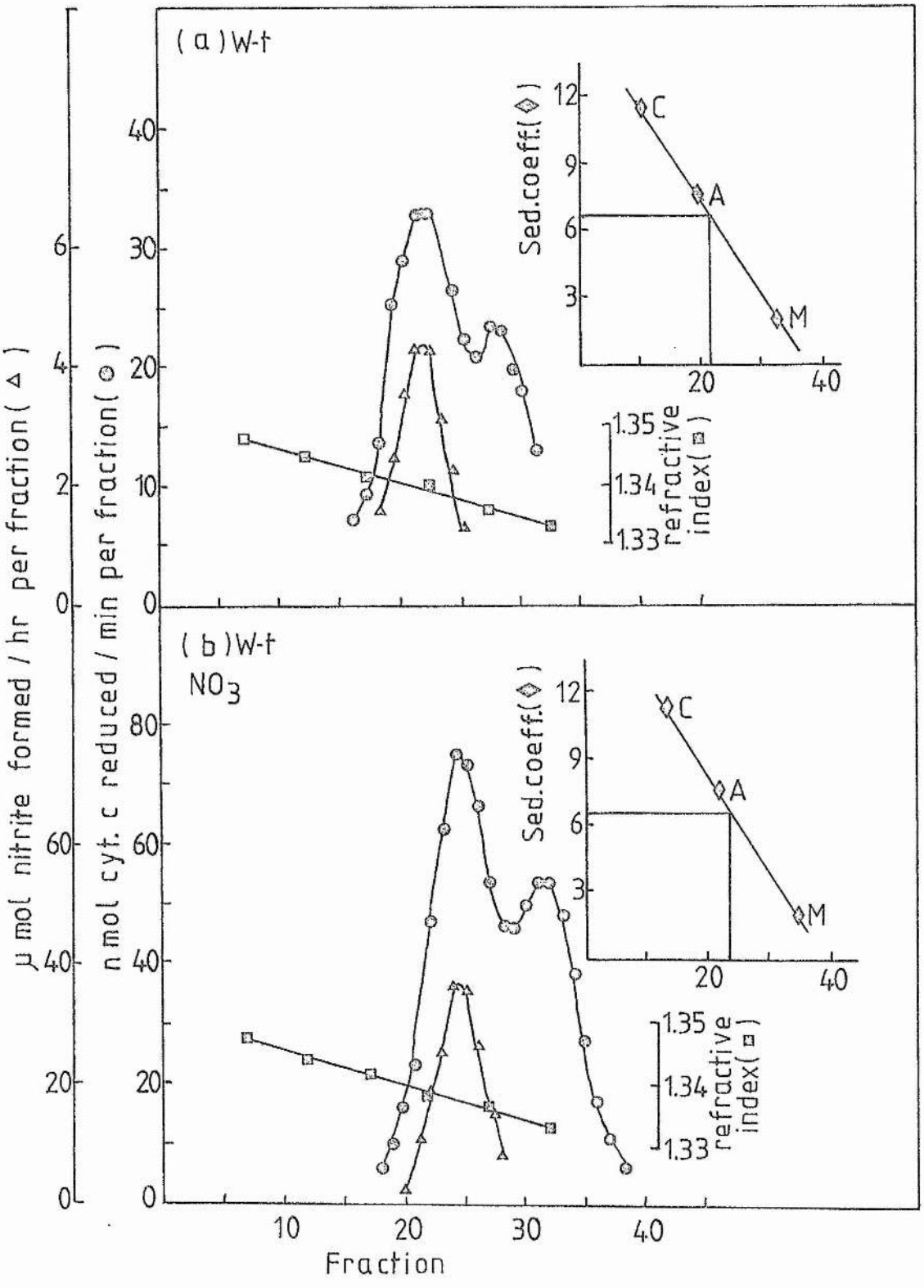
Initial attempts to measure nitrate reductase and NADH-cytochrome c reductase activities in the fractionated gradients were only partly successful. Enzyme breakdown appeared to be occurring during centrifugation and it was also apparent that some prior purification of the cell-free extracts was required.

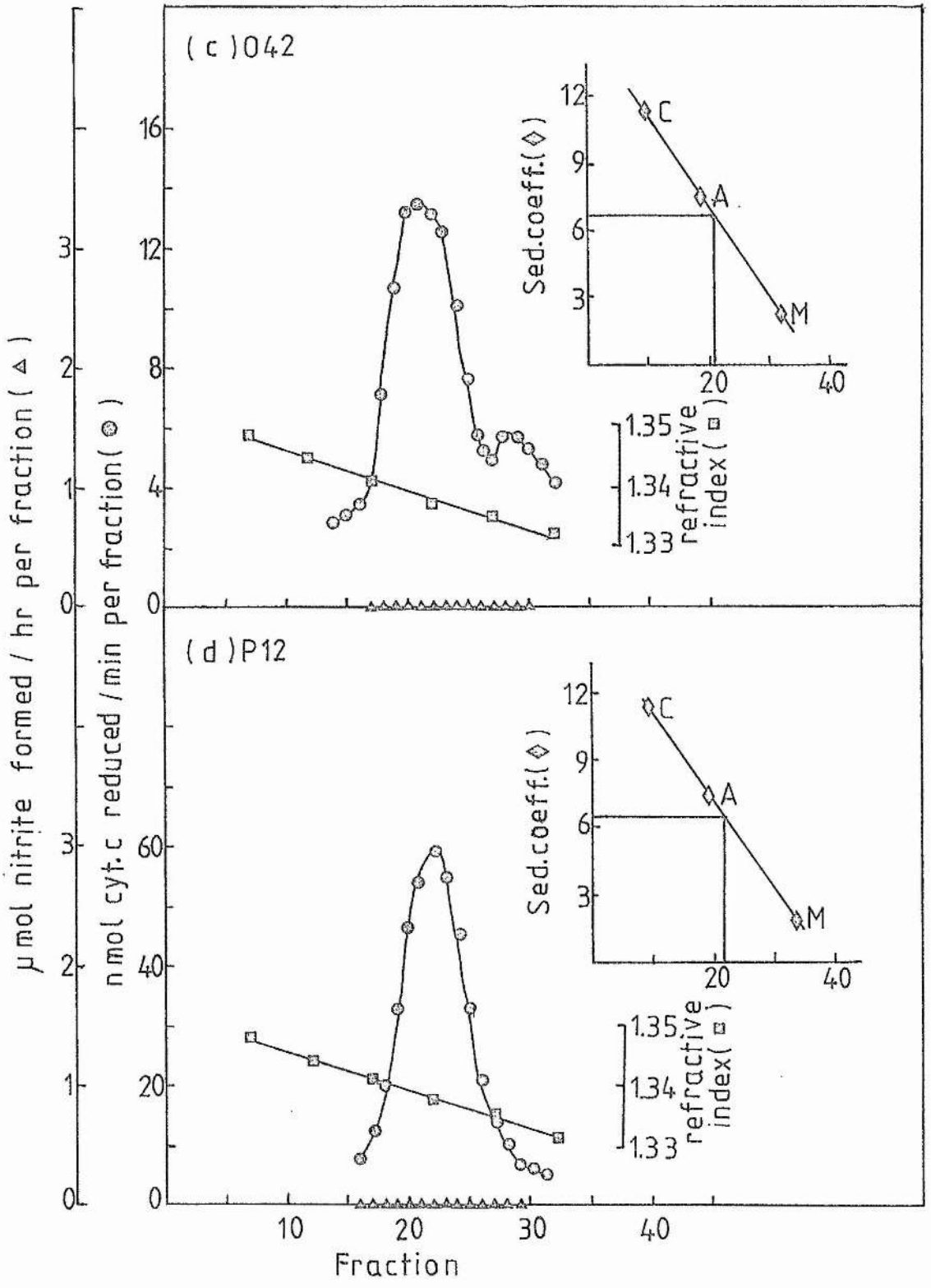
Cell-free extracts were therefore subsequently prepared from 10g fresh weight of cells in a buffer containing 12 mM β-mercaptoethanol (to inhibit enzyme breakdown). Protein in the extracts was precipitated by adding ammonium sulphate and that protein precipitated between 30 and 45% saturation with ammonium sulphate was resuspended in 1ml of buffer and applied to the gradients, which also contained β-mercaptoethanol.

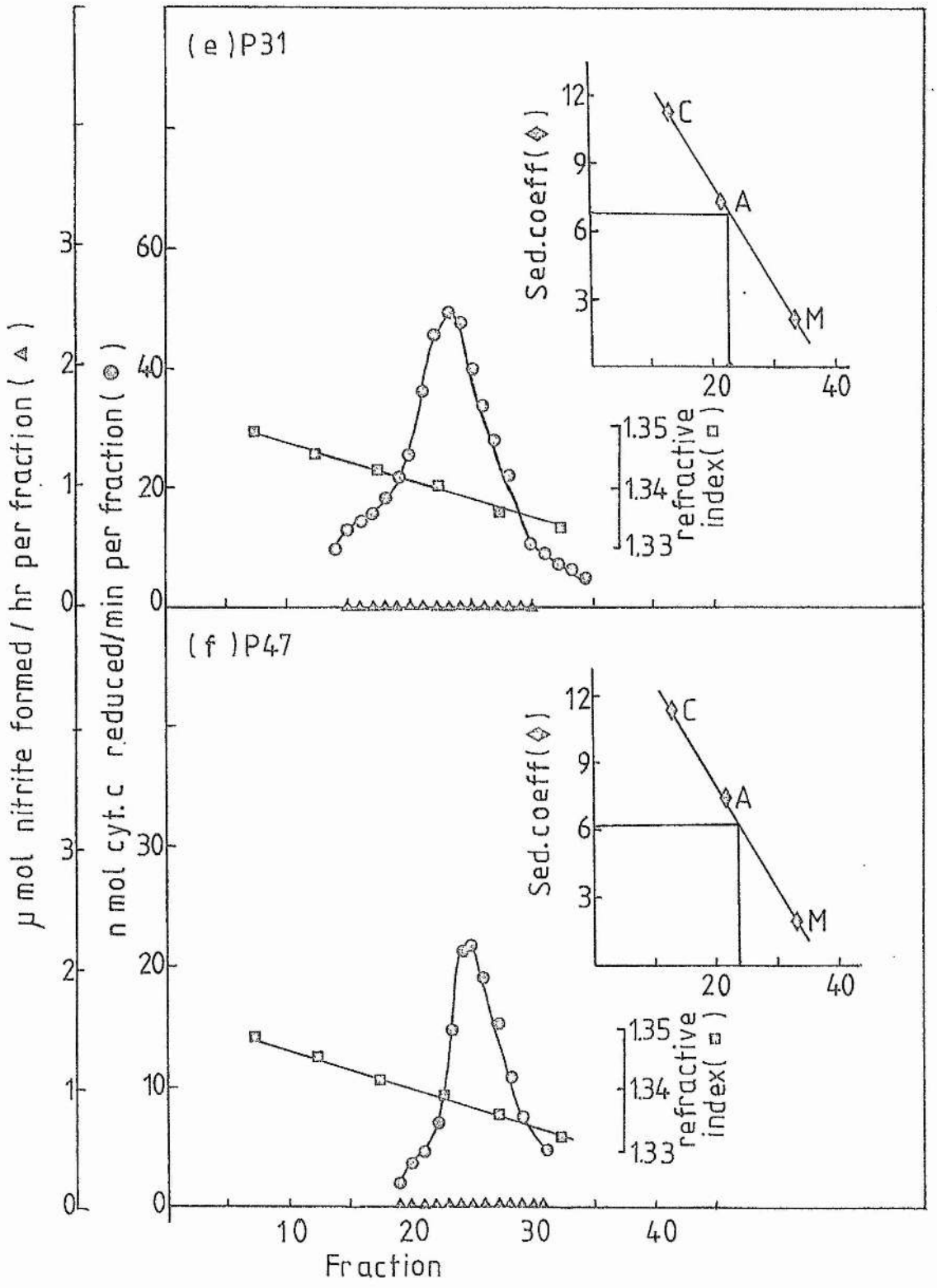
Extracts from wild-type cells growing on glutamine and nitrate media and nitrate reductase-minus cell-lines O42, P12, P31 and P47 growing on glutamine medium only, were prepared in this way and centrifuged on the sucrose gradients as before. Nitrate reductase and NADH-cytochrome c reductase were then measured in the fractionated gradients.

In the wild-type cell-line, a peak of nitrate reductase activity co-sedimented with a peak of NADH-cytochrome c reductase activity, having a sedimentation coefficient of 6.6S.

Figure 34 a-f Distribution of nitrate reductase (▲) and cytochrome c reductase (●) activities after sucrose density gradient centrifugation of the 30 - 45% ammonium sulphate fraction from cell-free extracts of a, wild-type cells; c, line 042; d, line P12; e, line P31 and f, line P47, all grown on glutamine medium, and b, wild-type cells grown on nitrate medium. (■) indicates refractive indices of fractions. Insets - Relationship between sedimentation coefficient of reference proteins, C - catalase (11.3S), A - alcohol dehydrogenase (7.4S) and M - myoglobin (2.04S), and their positions after centrifugation. Intersecting axes indicate the major cytochrome c reductase species present and also nitrate reductase where present.


(a) W-t
(b) W-t
NO3
μ mol nitrite formed / hr per fraction (△)
n mol cyt. c reduced / min per fraction (○)
Sed.coeff.(◇)
refractive index(□)
C
A
M
1.35
1.34
1.33
Fraction
10
20
30
40


(c) O42
(d) P12
Sed.coeff.(◇)
C
A
M
refractive index(□)
1.35
1.34
1.33
μmol nitrite formed / hr per fraction (△)
nmol cyt.c reduced / min per fraction (○)
Fraction


(e)P31
(f)P47
μ mol nitrite formed / hr per fraction (▲)
n mol cyt.c reduced/min per fraction (●)
Sed.coeff(◇)
refractive index(□)
C
A
M
1.35
1.34
1.33
Fraction

Both activities were greater in nitrate than in glutamine medium (Figure 34 a, b).

No nitrate reductase activity could be detected in any of the fractions from the nitrate reductase-minus cell-lines (Figure 34 c -f), but all possessed an NADH-cytochrome c reductase species of approximately the same sedimentation coefficient as nitrate reductase from wild-type cells.

Attempts to repair nitrate reductase-minus cell-lines with sodium molybdate.

Nitrate reductase-minus cell-lines 042, P12, P31 and P47 are similar to *cnx* mutants in *Aspergillus* in that they lack xanthine dehydrogenase activity and possess nitrate reductase-associated NAD(P)H-cytochrome c reductase activity. A feature of some *cnx* mutants is that by growing cells in a medium containing a high concentration of molybdenum, nitrate reductase activity can be restored. Attempts were thus made to see whether nitrate reductase could be similarly restored in cell-lines 042, P12, P31 and P47.

Cell-line 042 was therefore subcultured into nitrate medium containing 0, 1, 5, 10 and 30 mM-sodium molybdate. Within two days, cells in 10 and 30 mM-molybdate had turned brown and those in 1 and 5 mM-molybdate had turned yellow. Those not exposed to molybdate remained pale yellow. As there was also a rapid decline in fresh weight of those cultures with 10 and 30 mM-molybdate, these levels would appear to be toxic, but although those cultures with 1 and 5 mM-molybdate remained stable with respect to fresh weight, nitrate reductase could not be detected in any of the cultures.

All four nitrate reductase-minus cell-lines were then

subcultured into medium containing only 5 mM-nitrate (as nitrate build-up in these cell-lines had been shown to be detrimental to them) with 1 mM-molybdate. None of the four cell-lines regained their ability to grow and *in vitro* nitrate reductase activity could not be detected after 3 and 7 days. However, cells possessed higher levels of NADH-cytochrome c reductase in the presence of molybdate (Figure 35 b - e), and the decline of fresh weight experienced after subculture into nitrate medium was reduced. Under these conditions, molybdate inhibited the growth of wild-type cells (Figure 35a).

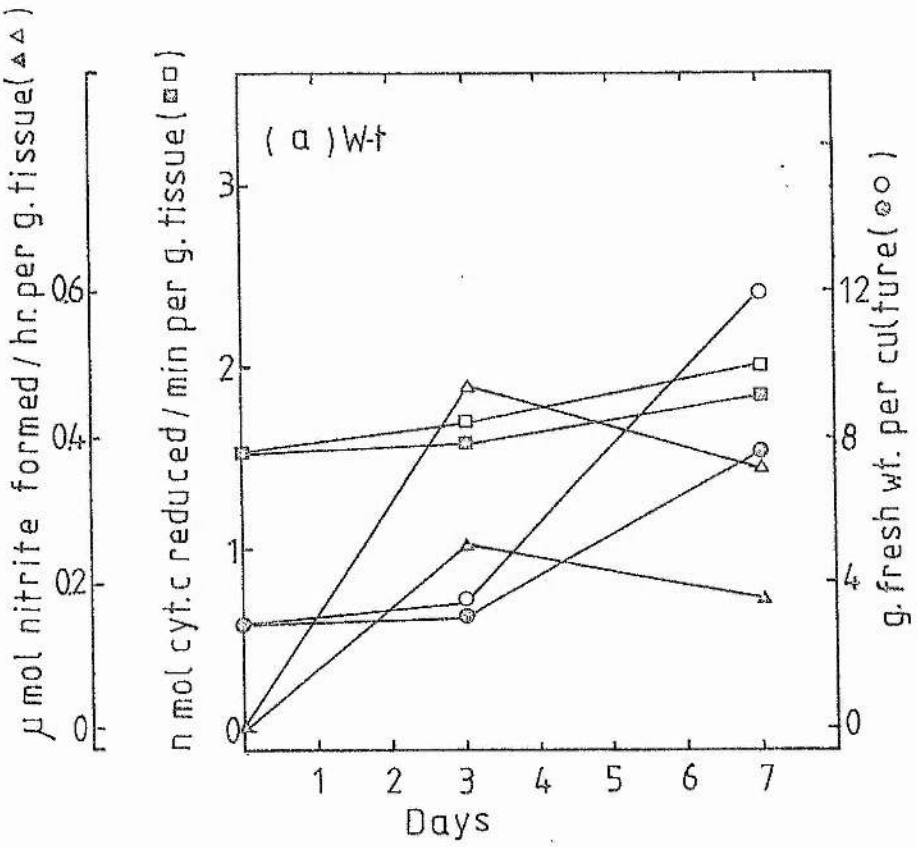


Figure 35 a-e Growth (● ○) and nitrate reductase (▲ △) and cytochrome c reductase (■ □) activities of a wild-type cell-line (a) and nitrate reductase-minus cell-lines 042, P12, P31 and P47 (b-e) after subculture into nitrate medium with (closed symbols) or without (open symbols) 1mM-sodium molybdate.

(b) 042
μ mol nitrite formed/hr per g.tissue(△△)
n mol cyt.c reduced/min per g.tissue(□□)
g. fresh wt. per culture(○○)
0
0.2
0.4
0.6
1
2
3
4
5
6
7
Days

(c) P12
μ mol nitrite formed/hr per g.tissue(△△)
n mol cyt.c reduced/min per g.tissue(□□)
g. fresh wt. per culture(○○)
0
0.2
0.4
0.6
1
2
3
4
5
6
7
Days

(d) P31
μ mol nitrite formed/hr per g. tissue (▲▲)
n mol cyt. c reduced/min per g. tissue (■□)
g. fresh wt. per culture (○○)
Days

(e) P47
μ mol nitrite formed/hr per g. tissue (▲▲)
n mol cyt. c reduced/min per g. tissue (■□)
g. fresh wt. per culture (○○)
Days

DISCUSSION

Xanthine dehydrogenase in nitrate reductase-minus and wild-type cell-lines.

Whilst xanthine dehydrogenase activity was found in wild-type N. tabacum cells whether grown in glutamine or nitrate medium, no activity was detected in any of the four nitrate reductase-minus cell-lines (Table 24). If the situation in N. tabacum is analogous to that in Aspergillus where xanthine dehydrogenase and nitrate reductase share a common molybdenum-containing co-factor (Mo-Co), then the simultaneous lack of both enzymes in an otherwise normal cell-line is likely to be due to a defect in that common component. It seems probable then, that all four nitrate reductase-minus cell-lines are defective in this molybdenum-containing co-factor.

NADH-cytochrome c reductase activity in nitrate reductase-minus and wild-type cell-lines.

There is now much evidence to support the observation of Wray and Filner (1970) that higher plant nitrate reductase possesses NADH-cytochrome c reductase activity and may do so even when inactive and/or incomplete, (Wallace and Johnson, 1978 ; Small and Wray,1980). When N. tabacum cell-lines were assayed for NADH-cytochrome c reductase activity, nitrate reductase-minus cell-line 042 was found to possess roughly similar levels to the wild-type cell-line from which it was derived (Figure 32 a, b). Although this then might suggest that 042 cell-line possessed a defective nitrate reductase which still possessed NADH-cytochrome c reductase activity, it must first be established whether the NADH-cytochrome c reductase measured is associated with the nitrate reductase molecule .

In an attempt to show whether or not this was the case an experiment was carried out to find out if the NADH-cytochrome c reductase activity in a wild-type cell-line, like that of nitrate reductase, increases in the presence of nitrate. Nitrate did, in fact, cause an increase in both activities (Figure 33a) and there was a close correlation between them as they fluctuated over the period of the experiment. However the level of NADH-cytochrome c reductase activity was relatively much higher than the level of nitrate reductase activity in the absence of nitrate and whilst nitrate produced a twenty-fold increase in nitrate reductase activity after one day, NADH-cytochrome c reductase activity barely doubled. This showed either that a large proportion of the total NADH-cytochrome c reductase activity is not associated with nitrate reductase or is associated with an inactive form. More importantly, however, it does suggest that a proportion of the total NADH-cytochrome c reductase activity is associated with nitrate reductase.

Nitrate also produced an increase in the NADH-cytochrome c reductase activity of nitrate reductase-minus cell-lines O42, P12 and P31. In all cases the increase was small but significant, and suggested that these cell-lines do possess nitrate reductase-associated NADH-cytochrome c reductase activity. It is possible then, that in these cell-lines, this activity is associated with an inactive nitrate reductase.

NADH-cytochrome c reductase and nitrate reductase activities in cell-free extracts after sucrose density gradient centrifugation.

When protein in cell-free extracts from wild-type cells was fractionated by sequential precipitation with ammonium sulphate (p. 30) most of the NADH-cytochrome c reductase activity was found in a fraction which contained very little of the

nitrate reductase activity, confirming that most of the NADH-cytochrome c reductase activity in the extracts is not associated with active nitrate reductase. The fraction which contained most of the nitrate reductase activity should also possess most of the NADH-cytochrome c reductase activity which is postulated to be carried on the same protein. This fraction, containing the protein precipitated between 30 and 45% saturation with ammonium sulphate, was therefore further fractionated by centrifugation on sucrose density gradients (p. 36).

In the wild-type cell-line, two NADH-cytochrome c reductase species were identified. The larger of these co-sedimented with the single nitrate reductase species, having a sedimentation coefficient of 6.6S. It is very likely therefore that the same protein possesses both activities. The fact that both activities were greater in nitrate than in glutamine medium supports this conclusion. The smaller NADH-cytochrome c reductase species had a sedimentation coefficient of 3.6S and it too showed more activity in nitrate medium. This suggests that this species is also associated in some way with nitrate reductase, possibly representing inactive fragments of the molecule. Similar small NADH-cytochrome c reductase species in barley have been shown to be associated with nitrate reductase, probably representing subunits or domains cleaved off the enzyme by endogenous proteinases (Wray and Filner, 1980; Small and Wray, 1980; Wray and Kirk, 1981).

Nitrate reductase-minus cell-lines 042, P12 and P31 also possessed an NADH-cytochrome c reductase species of the same sedimentation coefficient (6.6S) as nitrate reductase in wild-type cells, whilst that in cell-line P47 was only slightly

smaller (6.2S). This would suggest that these cell-lines still possess nitrate reductase but in an inactive form.

Partial activities of nitrate reductase in nitrate reductase cell-lines.

Nitrate reductase-minus cell-lines 042, P12, P31 and P47 have now been shown to simultaneously lack nitrate reductase and xanthine dehydrogenase.

Following the nomenclature adopted by Pateman et al (1964) for Aspergillus mutants, these cell-lines are therefore cnx types, being defective at a cnx locus, responsible for the production of molybdenum co-factor (Mo-co).

In Neurospora (Ketchum et al, 1970) and in Aspergillus (Cove, 1979), molybdenum co-factor is thought to play an essential role in the aggregation of the haemoflavoprotein sub-units of nitrate reductase. If a similar situation occurs in plants then the demonstration in the cnx tobacco cell-lines of an NADH-cytochrome c reductase species of approximately the same sedimentation coefficient as wild-type nitrate reductase (Figure 34) indicates that the Mo-co in these cell-lines, though defective, is still able to effect this dimerisation.

In Aspergillus, cnx E mutants are of this type (McDonald et al, 1974). The cnx gene is thought to code for a protein which allows insertion of molybdenum into its co-factor (Arst et al, 1970). A feature of cnx E mutants is the partial restoration of their nitrate reductase (Pateman et al, 1964) and xanthine dehydrogenase (Arst et al, 1970) activities when grown in the presence of a high concentration of molybdate. However, such an effect could not be produced in the cnx tobacco cell-lines (Figure 35) suggesting that they are not of the cnx E type.

Table 25 Properties of N.tabacum variant cell-lines 042, P12, P31 and P47 and the corresponding wild-type cell-line .

	Wild type	042	P12	P31	P47
1) Chlorate resistance	-	+	+	+	+
2) Growth on nitrate as sole N. source.	+	-	-	-	-
3) Nitrate reductase activity.	+	-	-	-	-
4) Nitrate accumulated when on nitrate medium.	-	+	+	+	+
5) Nitrite reductase activity.	+	+	+	+	+
6) Xanthine dehydrogenase activity	+	-	-	-	-
7) NADH-cytochrome c reductase induced by nitrate and of same sed. coeff. as wild-type nitrate reductase.	+	+	+	+	+
8) Nitrate reductase repaired with high molybdenum.	Not applicable	-	-	-	-

Summary.

The properties of N. tabacum variant cell-lines 042, P12, P31 and P47 and the corresponding wild-type cell-line, as determined in the preceding work, are summarized in Table 25. From these results we can conclude that cell-lines 042, P12, P31 and P47 are cnx - type variants (Pateman et al, 1964), lacking nitrate reductase due to the synthesis of a defective molybdenum co-factor (Mo-co) which allows dimerisation of nitrate reductase sub-units but cannot be repaired in vivo by growth in a high concentration of molybdate. These cell-lines are therefore not of the cnx E type and are thus different from other molybdenum co-factor-defective cell-lines so far described in N. tabacum.

General Discussion.

Experience with N. sylvestris.

Although N. sylvestris appeared to be a very promising species from which to try to isolate nitrate reductase-minus cell-lines (p. 12), the obstacles to its use for this purpose were not overcome (p. 64). Attempts to culture N. sylvestris protoplasts were unsuccessful and friable cell suspensions could not be obtained on a nitrogen source other than nitrate. It is likely that continued attempts to obtain friable suspensions would have led to an accelerated loss of haploidy as it has been reported that increased friability of N. sylvestris cell cultures is linked to a rise in polyploidy and aneuploidy (Dix, 1975). Such attempts might therefore be unproductive as the object was to obtain friable suspension cultures of mostly haploid cells (p. 12).

The protocol published by Nagy and Maliga (1976) for the protoplast culture and whole plant regeneration of N. sylvestris was thought by the authors to provide a suitable culture system from which to isolate auxotrophic cell-lines. However, no such cell-lines have since been produced from this species, and, despite their laboratories broad experience with N. sylvestris, Marton et al (1982a) chose another Nicotiana species, N. plumbaginifolia for their work on the isolation of nitrate reductase-minus cell-lines. Furthermore, other workers have reported considerable difficulties in obtaining reproducibly high yields of N. sylvestris protoplasts (Durand, 1979; Van Slogteren et al, 1980).

This suggests to me that other workers, as well as myself, have experienced practical problems in the culture of N. sylvestris, whether for the purpose of auxotrophic cell-line

isolation or otherwise.

Attempts to regenerate plants from nitrate reductase-minus cell-lines.

In a study of this kind, regeneration of sporophyte plants from variant cell-lines is desirable for two reasons. Firstly, callus and cell suspension cultures are cytologically unstable and inevitably undergo genetic change, tending to become progressively polyploid and aneuploid (D'amato, 1977). Secondly, conventional crossing studies with fertile regenerated variant plants enable determination of whether the variations are due to mutations, as well as allowing classification of mutants into different complementation groups.

Cell cultures of many species, including N. tabacum, have been shown to lose their morphogenic potential with prolonged culture (Murashige and Nakano, 1965; Reinart et al, 1977). It has been generally assumed that the accumulation of genetic changes due to the cytological instability of cell cultures is connected with the decrease in morphogenic potential. This is supported by the findings that, in several species, only diploid plants can be regenerated from suspensions of mixed ploidy (Reinart et al, 1977), although aneuploid N. tabacum plants have been regenerated by Sacristan and Lutz (1971). Whatever the reason, it is important to regenerate plants from variant cell-lines after as short a time in culture as possible.

Many workers have successfully regenerated plants from N. tabacum cell cultures (Skoog and Miller, 1957; Maliga et al, 1977; Müller and Grafe, 1978; Malmberg, 1979). In each case, shoot formation was firstly achieved by increasing the cytokinin / auxin ratio in the culture medium, followed by

root formation in a hormone-free medium.

However, despite repeated attempts with hormone combinations used successfully by the above groups of workers, shoot formation could not be achieved with any of the four nitrate reductase-minus cell-lines described in this work. The reason for this is not clear although there is evidence from other species that continuous growth in the presence of 2,4-D decreases morphogenic potential (Kamada and Harada, 1979). Cytological investigation of the N. tabacum nitrate reductase-minus cell-lines after six months in culture showed that they were composed mostly of polyploid and aneuploid cells. This also might explain their lack of morphogenic potential.

Perhaps the most likely explanation, however, is that their lack of morphogenic potential is associated in some way with the fact that they are cnx - type variants (p. 106). Márton et al (1982a) were able to regenerate plants only from "leaky" cnx variants and could not regenerate plants from "tight" cnx variants. Furthermore, plants could be regenerated from only one of Müller's cnx N. tabacum variants and these were grossly abnormal, non-fertile and thus quite unsuitable for conventional crossing studies (A Müller, personal communication to J. L. Wray).

Are the nitrate reductase-minus cell-lines of mutational origin ?

The most satisfactory evidence for a mutational event in a cell-line relies on the regeneration of fertile plants from the variant cell-line and transmission of the variant trait in sexual crosses (Maliga, 1976). Since regeneration of plants from the nitrate reductase-minus cell-lines could not be achieved (above), it was unfortunately not possible to determine whether these variations were of mutational origin.

There are, however, several other criteria which, if fulfilled, indicate a mutational origin, though they are not unequivocal evidence for it (Maliga, 1976). The first is the stability of the variant phenotype in the absence of selective pressure. In this respect the four nitrate reductase-minus cell-lines described in this thesis would qualify as their variant phenotypes have been stable in the absence of chlorate for almost three years. The second is concerned with the frequency of variant cell-line occurrence. If the frequency

is low and increased by mutagenic treatment, then this is a strong indicator of a mutagenic origin.

Based on an approximate cell number of 10^6 per petri-dish (1g. fresh wt.), 39 chlorate-resistant cell-lines (4 of which were also nitrate reductase-minus) were established from 7.4×10^7 EMS-treated cells and no variant cell-lines were established from 4×10^6 cells not treated with EMS. In the single experiment where nitrate reductase-minus cell-lines were produced (Table 21) the mutation frequency was about 10^{-6}, a figure given by Widholm (1974) and Maliga (1976) as the expected mutation frequency in higher plant cells. This does not, of course, firmly indicate that the four nitrate reductase-minus variants described here have a mutagenic origin but shows at least that there is a good possibility of this being the case.

Are the four nitrate reductase-minus cell-lines derived from a common variant clone.

The four nitrate reductase-minus cell-lines obtained have many similarities: they have similar cultural characteristics and their simultaneous lack of nitrate reductase and xanthine dehydrogenase is not repaired by high molybdenum. They may, then, fall into the same complementation group of *cnx* variants.

Since all four variant cell-lines were derived in the same experiment from the same EMS-treated cell suspension, it is therefore possible that the cell-lines have their origin in the same single mutagenic event. However, the cell suspension was divided into two flasks immediately after EMS-treatment; cell-line O42 was derived from one of these flasks and cell-lines P12, P31 and P47 were derived from the other. If all

four variant cell-lines were derived from the same mutagenic event then this would have to have occurred prior to EMS treatment. Given that no variant cell-lines were produced without EMS treatment, this seems unlikely.

Further thoughts on the mechanism of chlorate toxicity.

The view that chlorate is rendered toxic upon nitrate reductase-mediated reduction to chlorite, is widely held (p.15). However, in this work only 10% of chlorate-resistant cell-lines isolated were nitrate reductase-minus (Table 21). Furthermore, Murphy and Imbrie (1981) have isolated numerous chlorate-resistant cell-lines from Rosa damascena suspensions, only 15% of which were nitrate reductase-minus and only 18% of the chlorate-resistant N. plumbaginifolia cell-lines of Màrton et al (1982a) were fully deficient in nitrate reductase. These results might therefore appear on first inspection to support the observation of Cove (1976a), that chlorate toxicity in Aspergillus is not primarily dependent on its nitrate reductase-catalysed conversion to chlorite. Amongst the evidence for this observation was the discovery of nitrate reductase-minus mutants of Aspergillus which were not chlorate-resistant. In Aspergillus, chlorate has been postulated to exert its toxic effect primarily by preventing the catabolism of nitrogenous compounds (Cove 1976a).

However, amongst higher plants, there have been no reported cases of nitrate reductase-minus plants or cell-lines which are not also chlorate-resistant. Since most of these were initially selected for resistance to chlorate, this is perhaps not surprising, but nitrate reductase-minus cell-lines of Hyoscyamus muticus, which were isolated according to their nutritional requirements, have also proved to be chlorate-

resistant (Fankhauser and King,1982). The numerous chlorate-resistant, nitrate-utilising cell-lines described in this thesis may simply have a defect in chlorate uptake. Similar cell-lines of Rosa damascena may have a mechanism for tolerating chlorate and its reduction products (Murphy and Imbrie, 1981).

In short, there is no evidence from higher plant systems that chlorate toxicity is not wholly dependent on nitrate reductase-mediated reduction to chlorite and there is no reason why the theory of Aberg (p. 90) should not apply in all the higher plant systems thus far studied.

Chlorate-resistant cell-line 041.

Since this work was completed, one of the chlorate-resistant cell-lines isolated here which was capable of growth on nitrate (p. 80) has been studied further. 041 cell-line was shown to possess nitrate reductase, nitrite reductase and xanthine dehydrogenase activities (Qureshi et al, 1982). When cells which had been continuously maintained on glutamine medium were subcultured into nitrate medium, 041 cells grew only after a lag phase of 15 days, unlike wild-type cells where the lag phase was only 4 days. However, upon second subculture into nitrate, after having been grown on nitrate for 8 weeks, the lag phase of 041 cells was reduced to that of wild-type cells. The reason why line 041 is chlorate-resistant and is unable to grow normally when first presented with nitrate is as yet unknown, but the cell-line appears superficially similar to the CRUN mutants described by Cove 1976b). Mutation at the crnA locus probably leads to the production of an altered nitrate permease (Tomsett and Cove, 1979).

Size of the nitrate reductase molecule in N. tabacum cell cultures.

Wild-type nitrate reductase from higher plants, including tobacco, has a sedimentation coefficient of around 7.7S (Notton et al, 1977; Small and Wray, 1980; Mendel and Müller, 1979). However, in this work, the nitrate reductase species (and its associated cytochrome c reductase activity) present in the wild-type N. tabacum cells, grown on either glutamine (Figure 34a) or nitrate medium (Figure 34b), was repeatedly found to have a sedimentation coefficient of about 6.6S. The most likely explanation for this is that these 6.6S species are derived from a 7.7S nitrate reductase by proteolytic cleavage (Wray and Kirk, 1981), even though BSA was included in the extraction buffer (Brown et al, 1981).

Recent reports of nitrate reductase-minus cell-lines isolated from higher plant systems.

In recent years several groups of workers have succeeded in raising nitrate reductase-minus cell-lines of higher plants. The first and most complete study of such cell-lines has been carried out by Müller and co-workers at Gatersleben.

Müller and Grafe (1978) described the isolation of seven conditionally-lethal nitrate reductase-minus N. tabacum cell-lines by selection for chlorate-resistance. Numerous other cell-lines exhibited varying degrees of chlorate-resistance, often associated with lower than normal nitrate reductase levels. The seven nitrate reductase cell-lines were classified according to whether they possessed xanthine dehydrogenase activity into 6 nia and 1 cnx cell-lines, following the nomenclature adopted by Pateman et al (1964). nia and cnx mutants were shown to

complement each other *in vitro* to produce nitrate reductase activity (Mendel and Müller, 1978) and *in vivo* to restore the ability to grow on nitrate as a sole nitrogen source.

A total of 36 *nia* mutants have now been isolated. Plants have been regenerated from many of these and all 16 analysed by sexual hybridisation have been shown to be homozygous double mutants. The two mutations are unlinked and define duplicate loci (*nia* A1, *nia* A2) which are probably on homologous chromosomes. These loci appear to be the structural genes for nitrate reductase apoprotein (Müller, 1982).

Four *cnx* mutants are now known (Mendel *et al*, 1981). These mutants have been shown to exhibit nitrate reductase-associated cytochrome c reductase activity (Mendel and Müller, 1979) and to possess an inactive Mo-co which can be repaired *in vivo* by growth in a high (1 - 10mM) molybdenum concentration, partially restoring nitrate reductase and xanthine dehydrogenase activity (Mendel *et al*, 1981). *In vitro* repair was also demonstrated by regained ability to complement *Neurospora nit* - 1 mycelium and restore the fungal nitrate reductase activity (Nason *et al*, 1971) in the presence of 10 - 30mM molybdate. These mutants therefore lack only catalytically-active molybdenum, the structural moiety of the co-factor being apparently intact (Mendel *et al*, 1981). Assembly of the nitrate reductase sub-units is also unaffected by the *cnx* mutation (Mendel and Müller, 1980; Mendel, 1980).

Of the *Aspergillus cnx* mutants, only in *cnx* E mutants is a nitrate reductase-associated cytochrome c reductase species present and is nitrate reductase activity partially restored *in vivo* by growth in a high concentration of molybdate (Cove, 1979). The Gatersleben tobacco mutants would thus

appear to be analogous to Aspergillus cnx E mutants. Grafe and Müller (1982) have recently shown that their four cnx tobacco mutants are in the same complementation group (cnx A) and are hence allelic to each other.

Nicotiana plumbaginifolia.

Several nitrate reductase-minus cell-lines have now been isolated from protoplast cultures of diploid N. plumbaginifolia, using selection for chlorate resistance (Màrton et al, 1982a). All five nia-type (XDH^+) lines were shown to be allelic to each other but the four cnx-types (XDH^-) fell into three different complementation groups (Màrton et al, 1982b). Nitrate reductase activity could be restored in one of these cnx-type lines with a high molybdate concentration in the culture medium. This cnx-type line is therefore similar to the tobacco cnx A mutants of Mendel et al (1981) and to Aspergillus cnx E mutants (Cove, 1979). The other two cnx-type lines, at least one of which is unable to complement Neurospora nit - 1 mycelia in vitro (Mendel, Màrton and Wray, 1982), must represent new types (Table 26).

Datura innoxia.

Using selection for chlorate-resistance, King and Khanna (1980) isolated a nitrate reductase-minus cell-line from Datura innoxia suspension cultures. This cell-line possessed nitrate reductase-associated cytochrome c reductase activity but xanthine dehydrogenase activity was not measured. Though this nitrate reductase-minus cell-line was unable to reduce nitrate to nitrite, nitrite reductase remained inducible by nitrate, reinforcing the widely-held view (Hewitt, 1975) that nitrate is the main inducer of the nitrate assimilation

pathway, not nitrite as some have suggested (Kaplan et al, 1974). Conversely, in this thesis, N. tabacum wild-type cells were shown to possess nitrate reductase activity (Figure 22) when grown on glutamine medium. This lends further support to the idea that nitrate is not an obligatory inducer of nitrate reductase.

Rosa damascena.

Murphy and Imbrie (1981) describe the isolation of nitrate reductase-minus cell-lines from UV-irradiated Rosa damascena cultures, using selection for chlorate-resistance. Only a small minority of the chlorate-resistant cell-lines isolated were nitrate reductase-minus and these were not characterised further with regard to partial activities of nitrate reductase.

Whole plant nitrate reductase-deficient mutants.

The first nitrate reductase-deficient whole plants reported were the Arabidopsis thaliana B 2-1 mutants (Oostindier - Braaksma and Feenstra, 1973; Braaksma and Feenstra, 1975). One of these mutants (B25) has almost no nitrate reductase activity and may be a cnx-type mutant (Braaksma and Feenstra, 1982). These Arabidopsis mutants were selected initially for chlorate-resistance but Warner et al (1976) subsequently succeeded in isolating nitrate reductase-deficient Hordeum plants by nutritional means. However, these and subsequent Hordeum mutants (Kleinhofs et al, 1980), are "leaky", possessing low levels of nitrate reductase and able to grow on nitrate as a sole nitrogen source. Other Hordeum mutants, selected for partial resistance to chlorate (Tokarev and Shummy, 1977) are also "leaky" and it is only recently that conditionally-lethal nitrate reductase-deficient whole plant

mutants, as opposed to those derived from tissue cultures, have been isolated (Bright *et al*, 1983)

The defect in these *Hordeum* plants has been shown to be due to a single recessive mutation and, as they possess nitrate-inducible cytochrome c reductase activity and lack xanthine dehydrogenase activity, this is probably a *cnx*-type mutation (Bright *et al*, 1983).

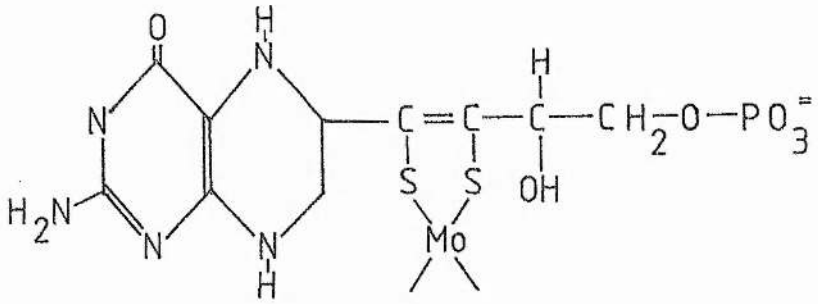
The nature of the molybdenum co-factor (Mo-co) in nitrate reductase and other molybdoenzymes.

Our knowledge of the nature of the molybdenum co-factor (Mo-co) found in higher plant, fungal and algal nitrate reductase has been extended considerably over the past few years. In fungal nitrate reductase at least, Mo-co is a small (mol. wt. 1000), dialysable molecule (Ketchum and Swarin, 1973; Lee *et al*, 1974), which plays a role in the dimerisation of the haemoflavoprotein sub-units to which it is non-covealently bound (Nason et al, 1971), as well as being involved in electron transport (Nason *et al*, 1970).

Since Pateman *et al* (1964) showed that *cnx Aspergillus* mutants lacked both nitrate reductase and xanthine dehydrogenase activity and Arst *et al* (1970) produced convincing evidence that Mo-co was common to both enzymes, the fungal co-factor has been shown to possess similar properties to molybdenum co-factors in bovine milk xanthine oxidase, rat and chicken liver sulphite oxidase, rabbit liver aldehyde oxidase and *E.coli* nitrate reductase and formate dehydrogenase (Johnson *et al*, 1977; Amy and Rajagopalan, 1979; Johnson 1980; Amy, 1982), in fact all molybdoenzymes except nitrogenase (Pienkos *et al*, 1977). Combined sulphite oxidase and xanthine dehydrogenase deficiency has even been demonstrated in a human patient

(Johnson et al, 1980a). Data is presented which indicates that the patient lacks an active Mo-co.

Mo-co from several sources has been shown to contain a novel pterin, reduced in the active state, and an unidentified 6-alkyl side chain (Johnson et al, 1980b). The co-factor is extremely labile in the presence of oxygen (Pienkos et al, 1977). Based on current knowledge, Rajagopalan et al (1982) have put forward a tentative model for the structure of active Mo-co



Pterins are known to take part in oxidation/reduction reactions (Kaufman, 1963) so the pterin may be involved in electron transport (Johnson et al, 1980b).

Higher plant Mo-co has not yet been shown to possess the above structure but considering its ability to complement Neurospora nit - 1 mycelia in vitro, it seems very likely that higher plant Mo-co has a very similar structure to that in other organisms.

Molybdenum co-factor in nitrate reductase-minus cell-lines 042, P12, P31 and P47. Evidence for new cnx variants.

Subculture of Müller's cnx A tobacco mutants onto medium supplemented with 1mM-sodium molybdate partially restored both nitrate reductase and xanthine dehydrogenase activities (Mendel et al, 1981). They are therefore similar to Aspergillus cnx E mutants (Arst et al, 1970). It has been shown that nitrate reductase is not restored by such treatment in the four

cnx cell-lines isolated in this work (Figure 35b-e). Further attempts have now been made to show whether nitrate reductase and also xanthine dehydrogenase could be restored by an identical procedure to that used by Mendel et al (1981). Results showed that neither nitrate reductase nor xanthine dehydrogenase (Figure 36) activities could be restored (Buchanan-and Wray, 1982) under conditions in which those activities were restored in Müller's cnx A mutants.

Further to this, it has now also been shown that heat-treated (Mendel, Alikulov and Müller, 1982) extracts from cnx cell-lines 042, P12, P31 and P47 are capable of complementing Neurospora nit - 1 mycelia in vitro in a high molybdenum concentration and that synthesis of the inactive Mo-co in these cell-lines is inducible by nitrate (Mendel, Màrton and Wray, 1982). Müller's cnx A mutants can also complement nit - 1 mycelium but in their case synthesis of the inactive Mo-co has been shown to be derepressed (Mendel et al, 1981).

This suggests that the cnx cell-lines described in this thesis are different from those previously described in N. tabacum (Table 26). Recent somatic hybridisation experiments at Gatersleben have confirmed that the cnx cell-lines isolated in this work do indeed belong to a different complementation group to those of Müller and are hence not allelic to them (R-R. Mendel, personal communication to J.L.Wray). Work is in progress to show whether cnx cell-lines 042, P12, P31 and P47 are allelic to each other or whether two or more genes are involved. We can conclude however that, as in lower organisms, more than one gene locus is involved in the formation of a functional Mo-co in N. tabacum nitrate reductase.

Figure 36 Presence of xanthine dehydrogenase activity after acrylamide gel electrophoresis of cell-free extracts and activity staining. Wild-type and nitrate reductase-minus cell-lines were subcultured into glutamine medium with (F-J) or without (A-E) 1mM-sodium molybdate and cell-free extracts were prepared 24h later. (A,F)-wild-type; (B,G)-line 042; (C,H)-line P12; (D,I)-line P31; (E,J)-line P47 (+) represents the presence of hypoxanthine, (-) is a control where hypoxanthine is omitted (Buchanan and Wray, 1982).

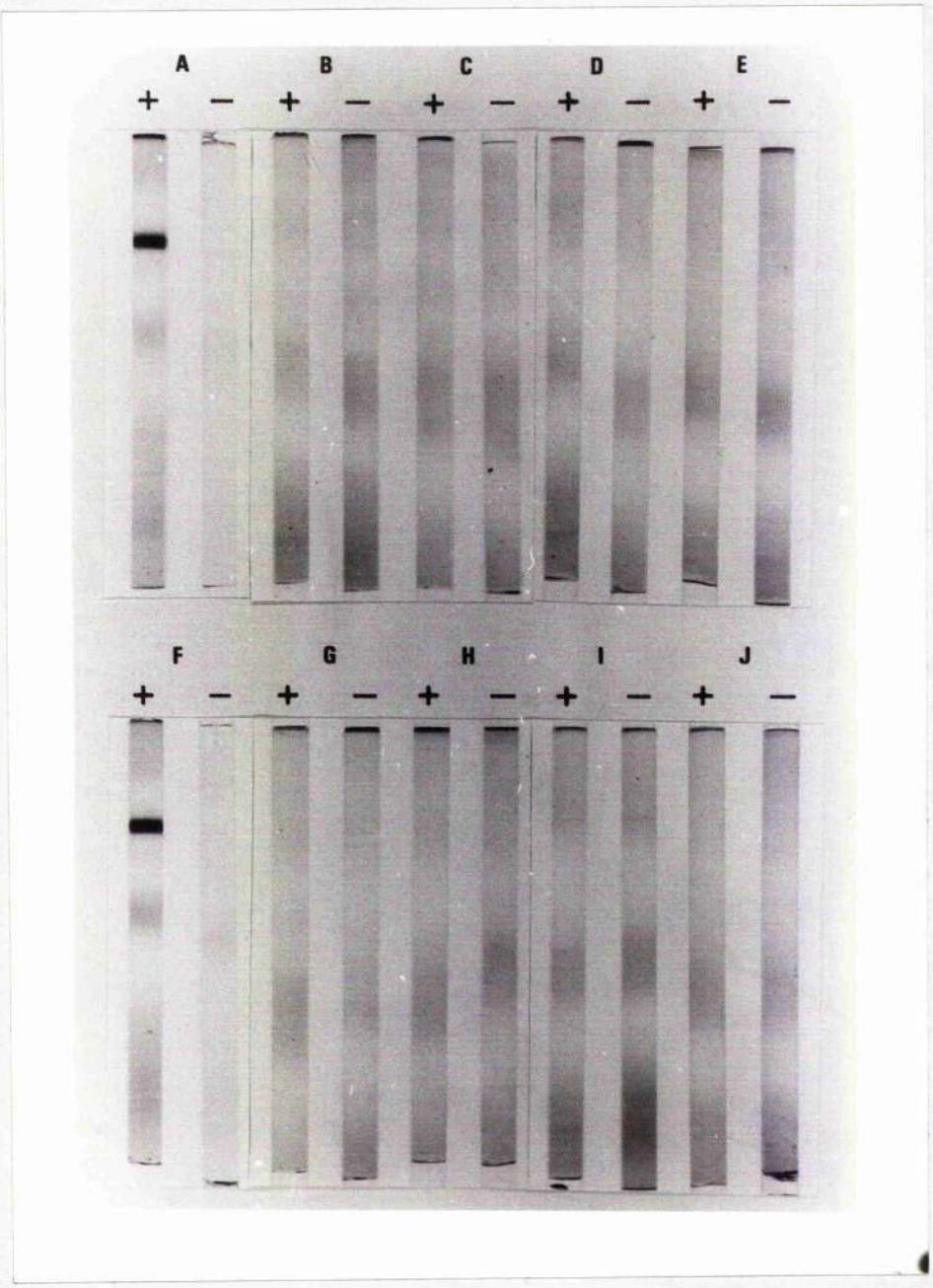

A
B
C
D
E
+ − + − + − + − + −
F
G
H
I
J
+ − + − + − + − + −

Table 26 Comparison of cell-lines O42, P12, P31 and P47 with other *Nicotiana* *cnx*-type (NR^-, XDH^-) lines and with *Aspergillus* *cnx* mutants.

Species	cnx-type line	NR and XDH restored in vivo with high [Mo]	Neurospora nit-1 complementation in vitro	Nitrate reductase-associated cytochrome c reductase activity	References
N.tabacum	042	-	+	+	This thesis
"	P12	-	+	+	
"	P31	-	+	+	
"	P47	-	+	+	
N.tabacum	cnx A	+	+	+	Mendel et al, 1981 Mendel, Mârton and Wray, 1982
N.plumbaginifolia	NX 1, NX 9	+	+	+	Marton et al, 1982b Mendel, Mârton and Wray, 1982
"	NX 21	-	-	+	
"	NX 24	-	?	?	
Aspergillus nidulans	cnx A-5	-	-	-	MacDonald et al, 1974 Mendel, 1982
"	cnx B-11	-	-	-	
"	cnx C-3	-	-	-	
"	cnx E-14	+	+	+	
"	cnx F-8	-	-	-	
"	cnx F-9	-	+	+	
"	cnx G-2	-	+	+	
"	cnx G-4	-	-	-	
"	cnx H-4	-	-	-	

Of the Aspergillus cnx mutants in which nitrate reductase cannot be partially restored in vivo by growth in a high concentration of molybdenum (Table 26), only cnx F-9 and cnx G-2 have been shown to be capable of restoring nitrate reductase activity to Neurospora nit - 1 mycelia (Mendel, 1982). These two cnx mutants also possess nitrate reductase-associated cytochrome c reductase activity, though only in the presence of nitrate, and are thus capable of allowing formation of an inactive nitrate reductase from its apoprotein sub-units (MacDonald et al, 1974). In these respects Aspergillus cnx F-9 and cnx G-2 mutants resemble the tobacco cnx cell-lines isolated in this work.

For the future it would be interesting to show whether all or any of cnx cell-lines 042, P12, P31 and P47 belong to the same complementation group as either of the two non-repairable N. plumbaginifolia cnx cell-lines (Màrton et al, 1982b).

References

ABERG, B. (1947) Kungl. Lantbrukshogkolans Ann. 15 : 37 - 107.

ALBERSHEIM, P. (1974) in "Tissue Culture and Plant Science" (Street, H. E. ed.) pp. 379 - 404 Academic Press, London.

ALLARD, R. W. (1960) Principles of Plant Breeding, Wiley, New York.

AMY, N. K. and RAJAGOPALAN, K. V. (1979) J. Bacteriol. 140 : 114 - 124.

AMY, N. K. (1982) Communicated at the Seventh Internat. Symp. on Pteridines and Folic Acid Derivatives - Chemical, Biological and Clinical Aspects, 21 - 24 Sep., 1982, St. Andrews.

ARST, H. N., MacDONALD, D. W. and COVE, D. J. (1970) Molec. Gen. Genet. 108 : 129 - 145.

AVIRAM, E. (1975) Isr. Chem. Soc. 43rd. Ann. Meeting Proc: 100.

AVIV, D. and GALUN, E. (1977) Z. Pflanzenphysiol. 83 : 267 - 273.

BANKS, M. S. and EVANS, P. K. (1976) Plant Sci. Lett. 7 : 409 - 416.

BARZ, R. and UMIEL, N. (1977) Z. Pflanzenphysiol. 83: 437 - 447.

BAYLISS, M. W. (1973) Nature 246 : 529 - 530.

BAYLISS, M. W. (1975) Chromosoma 51 : 401 - 411.

BAYLISS, M. W. (1977) Plant Sci. Lett. 8 : 99 - 103.

BEERS, R. F. and SIZER, I. W. (1952) J. Biol. Chem. 195 : 133 - 140.

BEEVERS, L., FLESHER, D. and HAGEMAN, R. H. (1964) Biochim. Biophys. Acta. 89 : 453 - 464.

BEEVERS, L. and HAGEMAN, R.H. (1969) Ann. Rev. Plant. Physiol. 20 : 495 - 522.

BEHREND, J. and MATELES, R.I. (1976) Plant Physiol. 58 : 510 - 512.

BOURGIN, J-P. and NITSCH, J. P. (1967) Ann. Physiol. Vég. 9 : 377 - 382.

BOURGIN J-P., MISSONIER, C. and CHUPEAU, Y. (1976) C. R. Acad. Sci. Paris 282 : 1853 - 1856.

BOURGIN, J-P. and MISSONIER, C. (1978) Z. Pflanzenphysiol. 87 S : 55 - 64.

BRAAKSMA, F. J. and FEENSTRA, W. J. (1975) Arabidopsis Inf. Serv. 12 : 16 - 17.

BRAAKSMA, F. J. and FEENSTRA, W. J. (1982) Theor. Appl. Genet. 61 : 263 - 271.

BRIGHT, S. W. J., NORBURY, P. B., FRANKLIN, J., KIRK, D. W. and WRAY , J. L. (1983) Molec. Gen. Genet. (in press).

BROWN, J., SMALL, I. S. and WRAY, J. L. (1981) Phytochemistry 20 : 389 - 398.

BUCHANAN, R. J. and WRAY, J. L. (1982) Molec. Gen. Genet. 188 : 228 - 234.

CAMPBELL, J. McA. and WRAY, J. L. (1982) Proc. Int. Symp. on Nitrate Assimilation - molecular and Genetic Aspects, Gatersleben, DDR, P25.

CARLSON, P.S. (1970) Science 168 : 487 - 489.

CARLSON, P.S. (1973) Science 180 : 1366 - 1368.

CHALEFF, R. S. and CARLSON, P. S. (1975) in "Genetic Manipulation with Plant Material" (Ledoux, L. ed.) pp.351 - 363, Plenum, New York.

CLAPHAM, D. (1973) Z. Pflanzenzüchtg. 69 : 142 - 155.

CLAUSEN, R. and CAMERON, D. (1944) Genetics 29 : 447.

CODDINGTON, A. (1976) Molec. Gen. Genet. 145 : 195 - 206.

COLLINS, G. B., LEGG, P. D. and KASPERBAUER, M. J. (1972) J. Hered. 63 : 113 - 118.

COLLINS, G. B., DUNWELL, J. M. and SUNDERLAND, N. (1974) Protoplasma 82 : 365 - 378.

COLLINS, G. B. and SUNDERLAND, N. (1974) J. Exp. Bot. 25 : 1030 - 1039.

COLLINS, G. B. and LEGG, P.D. (1980) in "The Plant Genome" (Davies, D. R. and Hopwood, D. A. eds.) pp. 197 - 213. John Innes Charity, Norwich.

COVE, D. J. and CODDINGTON, A. (1965) Biochim. Biophys. Acta 110 : 312 - 318.

COVE D.J. and PATEMAN, J. A. (1969) J. Bacteriol. 97 : 1374 - 1378.

COVE, D. J. (1976a) Molec. Gen. Genet. 146 : 147 - 159.

COVE, D. J. (1976b) Heredity 36 : 191 - 203.

COVE, D. J. (1979) Biol. Rev. 54 : 291 - 327.

D'AMATO, F. (1965) Proc. Internat. Conf. Plant Tissue Culture, Penn. State Univ. (White, P. R. ed.) pp. 449 - 462, McCutchan, Berkeley, U.S.A.

D'AMATO, F. (1977) in "Applied and Fundamental Aspects of Plant Cell Tissue and Organ Culture" (Reinart, J. and Bajaj, Y. P. S. eds.) pp. 343 - 357, Springer, Berlin.

DAWSON, R. M. C., ELLIOT, D. C. and JONES, K. M. (1969) Data for Biochemical Research. Ox. Univ. Press.

DIX, P. J. (1975) PhD thesis, Univ. Leicester.

DIX, P. J. and STREET, H. E. (1976) Ann. Bot. 40 : 903 - 910.

DOWNEY, R. J. (1971) J. Bacteriol. 105 : 759 - 768.

DOWNEY, R. J. (1973) Biochem. Biophys. Res. Commun. 50 : 920 - 925.

DOWNEY, R. J. and FOCHT, W. J. (1974) Microbios. 11A : 61 - 70.

DOWNEY, R.J. and STEINER, F. X. (1979) J. Bacteriol. 137 : 105 - 114.

DUNN-COLEMAN, N.S., TOMSETT, A. B. and GARRETT, R. H. (1979) J. Bacteriol. 139 : 697 - 700.

DUNN-COLEMAN, N. S. and GARRETT, R. H. (1980) Molec. Gen. Genet. 179 : 25 - 32.

DURAND, J. (1979) Z. Pflanzenphysiol. 93 : 283 - 288.

EHRENBERG, L., LUNDQUIST, V., OSTERMAN, S. and SPARRMAN, B. (1966) Hereditas 56 : 277 - 305.

EHRENBERG, L. (1970) in "Chemical Mutagens" (Hollander, E. ed.) pp. 365 - 386.

EVANS, P. K. and COCKING, E. C. (1977) in "Plant Tissue and Cell Culture, " 2nd. edition (Street, H. E. ed.) pp. 103 - 136, Blackwell, Oxford.

FACCIOTI, D and PILET, P-E. (1979) Plant Sci. Lett. 15 : 1 - 6.

FACKLAM, T. J. and MARZLUF, G. A. (1978) Biochem. Genet. 16 : 343 - 355.

FANKHAUSER, H. and KING, P.J. (1982) Proc. Int. Symp. on Nitrate Assimilation - Molecular and Genetic Aspects, Gatersleben, DDR, P2.

FILNER, P. (1966) Biochim. Biophys. Acta. 118 : 299 - 310.

de FOSSARD, R. A. (1974) in "Haploids in Higher Plants : Advances and Potential" (Kasha, K. V. ed.) pp. 145 - 150, Univ. Guelph Press, Canada.

FREARSON, E. M., POWER, J. B. and COCKING, E. C. (1973) Developmental Biol. 33 : 130 - 137.

FUKUNAGA, Y., KING, J. and CHILD, J. J. (1978) Planta 139 : 199 - 202.

GAMBORG, O. L., MILLER, R. A. and OJIMA, K. (1968) Exp. Cell Res. 50 : 151 - 158.

GARRETT, R. H. and NASON, A. (1967) Proc. Nat. Acad. Sci. USA. 58 : 1603 - 1610.

GARRETT, R. H. and NASON, A. (1969) J. Biol. Chem. 244 : 2870 - 2882.

GARRETT, R. H. and COVE, D. J. (1976) Molec. Gen. Genet. 149 : 179 - 186.

GARRETT, R. H. and AMY, N. K. (1978) Adv. Microb. Physiol. 18 : 1 - 65.

GIRI, L. and RAMADOSS, C. S. (1979) J. Biol. Chem. 254 : 11703 - 11712.

GLIMELIUS, K., ERIKSSON, T., GRAFE, R. and MULLER, A. J. (1978) Physiol. Plant. 44 : 273 - 277.

GRAFE, R. and MULLER, A. J. (1982) Proc. Int. Symp. on Nitrate Assimilation - Molecular and Genetic Aspects, Gatersleben, DDR, P14.

GRAY, J. C., KUNG, S. D., WILDMAN, S. G. and SHEEN, S. J. (1974) Nature 252 : 266 - 267.

GROVE, G, and MARZLUF, G. A. (1981) J. Biol. Chem. 256 : 463 - 470.

GUERRERO, M. and GUTIERREZ, M. (1977) Biochim. Biophys. Acta 482 : 272 - 285.

GUHA, S. and MAHESHWARI, S. C. (1964) Nature 204 : 497.

HEWITT, E. J. (1975) Ann. Rev. Plant Physiol. 26 : 73 - 100.

HOFSTRA, J. J. (1977) Physiol. Plant, 41 : 65 - 69.

JOHNSON, J. L., JONES, H. P. and RAJAGOPALAN, K. V. (1977) J. Biol. Chem. 252 : 4994 - 5003.

JOHNSON, J. L. (1980) in "Molybdenum and Molybdenum- containing Enzymes (Coughlan, M. P. ed.) pp. 345 - 383, Pergamon Press, Oxford.

JOHNSON, J. L., WAUD, W. R., RAJAGOPALAN, K. V., DURAN, M., BEEMER, F. A., and WADMAN, S. K. (1980a) Proc. Natl. Acad. Sci. USA. 77 : 3715 - 3719.

JOHNSON, J. L., HAINLINE, B. E. and RAJAGOPALAN, K. V. (1980b) J. Biol. Chem. 255 : 1783 - 1786.

KAMADA, H. and HARADA, H. (1979) Z. Pflanzenphysiol. Bd. 91 S : 255 - 266.

KAO, K. N., CONSTABEL, F., MICHALYUK, M. R. and GAMBORG, O. L. (1974) Planta 120 : 215 - 227.

KAPLAN, D., ROTH-BEJERANO, N. and LIPS, H. (1974) Eur. J. Biochem. 49 : 393 - 398.

KASPERBAUER, M. J. and COLLINS, G. B. (1972) Crop Sci. 12 : 98 - 101.

KAUFMAN, S. (1963) Proc. Natl. Acad. Sci. USA. 50 : 1085 - 1093.

KETCHUM, P. A., CAMBIER, H. Y., FRAZIER, W. A., MADANSKY, C. H. and NASON, A. (1970) Proc. Natl. Acad. Sci. USA. 66 : 1016 - 1023.

KETCHUM, P.A. and SWARIN, R. S. (1973) Biochem. Biophys. Res. Commun. 52 : 1450 - 1456.

KIMBER, G. and RILEY, R. (1963) Bot. Rev. 29 : 490 - 531.

KING, J. and KHANNA, V. (1980) Plant Physiol. 66 : 632 - 636.

KLEINHOFS, A., KUO, T. and WARNER, R. L. (1980) Molec. Gen. Genet. 177 : 421 - 425.

KUO, T., KLEINHOFS, A. and WARNER, R. L. (1980) Plant Sci. Lett. 17 : 371 - 381.

LEE, K-Y., ERICKSON, R., PAN, S-S., JONES, G., MAY, F. and NASON, A. (1974) J. Biol. Chem. 249 : 3953 - 3959.

LEGG, P.D., CHAPLIN, J. F. and COLLINS, G. B. (1969) J. Hered. 60 : 213 - 217.

LEGG, P. D. and COLLINS, G. B. (1971) Can. J. Genet. Cytol. 13 : 287 -291.

LESCURE, A. M. (1973) Plant Sci. Lett. 1 : 375 - 383.

LEWIS, G. M. and FINCHAM, J. R. S. (1970) Genet. Res. Camb. 16 : 151 - 163.

MacDONALD, D. W. and CODDINGTON, A. (1974) Eur. J. Biochem. 46 : 169 - 178.

MacDONALD, D. W., COVE, D. J. and CODDINGTON, A. (1974) Molec. Gen. Genet. 128 : 187 - 199.

MALIGA, P., SZ-BREZNOVITS, A. and MARTON, L. (1973) Nature New Biol. 244 : 29 - 30.

MALIGA, P. (1976) in "Cell Genetics in Higher Plants" (Dudits, D., Farkas, G. L., Maliga, P. eds.) pp. 59 - 76. Akademiai Kiado, Budapest.

MALIGA, P., LAZAR, G., JOO, F., NAGY, A. H. and MENCZEL, L. (1977) Molec. Gen. Genet. 157 : 291 - 296.

MALMBERG, R. L. (1979) Genetics 92 : 215 - 221.

MARTON, L., DUNG, T. M., MENDEL, R-R. and MALIGA, P. (1982a) Molec. Gen. Genet. 182 : 301 - 304.

MARTON, L., SIDOROV, V., BIASINI, G. and MALIGA, P. (1982b) Mol. Gen. Genet. 187 : 1 - 3.

MENDEL, R-R. and MULLER, A. J. (1976) Biochem. Physiol. Pflanzen 170 : 538 - 541.

MENDEL, R-R. and MULLER, A. J. (1978) Molec. Gen. Genet. 161 : 77 - 80.

MENDEL, R-R. and MULLER, A. J. (1979) Molec. Gen. Genet. 177 : 145 - 153.

MENDEL, R-R. (1980) Biochem. Physiol. Pflanzen. 175 : 216 - 227.

MENDEL, R-R. and MULLER, A. J. (1980) Plant Sci. Lett. 18 : 277 - 288.

MENDEL, R-R., ALIKULOV, Z. A., LVOV, N. P. and MULLER, A. J. (1981) Molec. Gen. Genet. 181 : 395 - 399.

MENDEL, R-R. (1982) Proc. Int. Symp. on Nitrate Assimilation - Molecular and Genetic Aspects, Gatersleben, DDR, P12.

MENDEL, R-R., ALIKULOV, Z. A. and MULLER, A. J. (1982) Plant Sci. Lett. 25 : 67 - 72.

MENDEL, R-R., MARTON, L. and WRAY, J. L. (1982) Proc. Int. Symp. on Nitrate Assimilation - Molecular and Genetic Aspects, Gatersleben, DDR, P16.

MICHALYUK, M. R. and KAO, K. N. (1975) Z. Pflanzenphysiol. 75 : 181 - 185.

MOUTSCHEN, J. (1965) Mem. Soc. Roy. Sci. Liege. Sev. 6 11 Fasc. 2 : 1 - 296.

MULLER, A. J. and GRAFE, R. (1978) Mole. Gen. Genet. 161 : 67 - 76.

MULLER, A. J. (1982) Proc. Int. Symp. on Nitrate Assimilation - Molecular and Genetic Aspects, Gatersleben, DDR, P9.

MURASHIGE, T. and SKOOG, F. (1962) Physiologia Pl. 15 : 473 - 497.

MURASHIGE, T. and NAKANO, R. (1965) Amer. J. Bot. 52 : 819 - 827.

MURASHIGE, T. and NAKANO, R. (1967) Amer. J. Bot. 54 : 963 - 970.

MURPHY, J. M. and IMBRIE, C. W. (1981) Plant Physiol. 67 : 910 - 916.

NABORS, M. W., DANIELS, A., NADOLNY, L. and BROWN, C. (1975) Plant Sci. Lett. 4 : 155 - 159.

NAGATA, T. and TAKEBE, I. (1970) Planta 92 : 301 - 308.

NAGATA, T. and TAKEBE, I. (1971) Planta 99 : 12 - 20.

NAGY, J. I. and MALIGA, P. (1976) Z. Pflanzenphysiol. 78 : 453 - 455.

NASON, A., ANTOINE, D. D., KETCHUM, P. A., FRAZIER, W. A., and LEE, D. K. (1970) Proc. Nat. Acad. Sci. USA. 65 : 133 - 144.

NASON, A., KEE, K-Y., PAN, S-S., KETCHUM, P. A., LAMBERTI, A. and DeVries, J. (1971) Proc. Nat. Acad. Sci. USA. 68 : 3242 - 3246.

NICHOLS, G. L. and SYRETT, P. J. (1978) J. Gen. Micro. 108 : 71 - 77.

NIIZEKI, H. and OONO, H. (1971) Colloques Internat. du CNRS Paris 193 : 251 - 257.

NITSCH, J. P. (1969) Phytomorphology 19 : 389 - 404.

NITSCH, J. P. and NITSCH, C. (1969) Science 163 : 85 - 87.

NITSCH, J. P., NITSCH, C. and HAMON, S. (1969) C. R. Acad. Sci. Paris. 269 : 1275 - 1278.

NITSCH, J. P. (1972) Z. Pflanzenzüchtg. 67 : 3 - 18.

NITSCH, J. P. (1974) in "Haploids and Higher Plants: Advances and Potential" (Kasha, K. J. ed.) pp. 123 - 135, Univ. Guelph Press, Canada.

NOTTON, B. A. and HEWITT, E. J. (1971) Plant and Cell Physiol. 12 : 465 - 477.

NOTTON, B.A., FIDO, R. J. and HEWITT, E. J. (1977) Plant Sci. Lett. 8 : 165 - 170.

NOTTON, B. A. and HEWITT, E. J. (1979) in "Nitrogen assimilation of plants" (Cutting, C. V. and Hewitt, E. J. eds.) pp. 227 - 244, Academic Press, London.

NOVAK, F. J. and VYSKOT, B. (1975) Z. Pflanzenzüchtg. 75 : 62 - 70.

OOSTINDIER- BRAAKSMA, F. J. and FEENSTRA, W. J. (1973) Mutation Res. 19 : 175 - 185.

OSTERMAN - GOLKAR, S., EHRENBERG, L. and WACHTMEISTER, C. A. (1970) Radiation Bot. 10 : 303 - 327.

PAN, S-S. and NASON, A. (1978) Biochem. Biophys. Acta. 128 : 202 - 204.

PARTENEN, C. R. (1965) Am. J. Bot. 52 : 204 - 209.

PATEMAN, J. A., COVE, D. J., REVER, B. M. and ROBERTS, D. B. (1964) Nature 201 : 58 - 60.

PATEMAN, J. A., REVER, B. M. and COVE, D. J. (1967) Biochem. J. 104 : 103 - 111.

PICARD, E. and de BUYSER, J. (1973) C. R. Acad. Sci. Paris 277D : 1463 - 1466.

PICHINOTY, F. (1966) Physiol. Vég. 12 : 97 - 104.

PIECHAUD, M., PUIG, J., PICHINOTY, F., AZOULAY, E. and LeMINOR, L. (1967) Ann. Inst. Pasteur 112 : 24 - 57.

PIENKOS, P. T., SHAH, V. K. and BRILL, W. J. (1977) Proc. Natl. Acad. Sci. USA. 74 : 5468 - 5471.

POWER, J. B. and COCKING, E.C. (1969) Biochem. J. 111 Proc. Biochem. Soc: 33P.

POWER, J. B., FREARSON, E. M. and HAYWARD, C. (1974) in "The use of protoplasts from fungi and higher plants as genetic systems" (Cocking, E.C. and Peberdy, J. F. eds.) pp. 18 - 25. Univ. Nottingham Botany Dept.

PREMAKUMAR, R., SORGER, G. J. and GOODEN, D. (1979) J. Bacteriol. 137 : 1119 - 1126.

PURVIS, M. J., COLLIER, D. C. and WALLS, D. (1966) Laboratory Techniques in Botany 2nd edition. p163, Butterworths, London.

QURESHI, J. A., BUCHANAN, R. J. and WRAY, J. L. (1982) Proc. Int. Symp. on Nitrate Assimilation - Molecular and Genetic Aspects, Gatersleben, DDR, P22.

RAJAGOPALAN, K. V., JOHNSON, J. L. and HAINLINE, B. E. (1982) Fed. Proc. 41 : 2608 - 2612.

RASHID, A. and STREET, H. E. (1974) Plant Sci. Lett. 2 : 89 - 94

REDINBAUGH, M. G. and CAMPBELL, W. J. (1981) Plant. Physiol. 68 : 115 - 120.

REINERT, J., BAJAJ, Y. P. S. and ZBELL, B. (1977) in "Plant Tissue and Cell Culture," 2nd edition (Street, H. E. ed.) pp. 389 - 428, Blackwell, Oxford.

RENOSTO, F., ORNITZ, D. M., PETERSON, D. and SEGEL, I. H. (1981) J. Biol. Chem. 256 : 8616 - 8625.

ROSA, F. F. de la, CASTILLO, F., MENDEZ. J. M. and PALACIAN, E. (1976) FEBS. Lett. 64 : 293 - 295.

ROSS, W. C. J. (1962) Biological Alkylating agents. Butterworths, London.

SACRISTAN, M. D. (1971) Chromosoma 33 : 273 - 283.

SACRISTAN, M. D. and LUTZ, A. (1971) C. R. Acad. Sci. Paris, 270 : 1334 - 1336.

SADASIVAIAH, R. S. (1974) in "Haploids in Higher Plants : Advances and Potential" (Kasha, K. J. ed.) pp. 355 - 386. Univ. Guelph Press, Canada.

SANDERSON, G. W. and COCKING, E. C. (1964) Plant Physiol. 39 : 423 - 431.

SAVAGE, A. D., KING, J. and GAMBORG, O. L. (1979) Plant Sci. Lett. 16 : 367 - 376.

SCAZZOCHIO, C., HOLL, F. B. and FOGUELMAN, A. I. (1973) Eur. J. Biochem. 36 : 428 - 445.

SCHRADER, L. E., CATALDO, D. A. and PETERSON, D. M. (1974) Plant Physiol. 53 : 688 - 690.

SHARP. W. R., DOUGALL, D. K. and PADDOCK, E. F. (1971) Bull. Torrey. Bot. Club 98 : 219 - 222.

SIEGEL, L. M. and MONTY, K. J. (1966) Biochim. Biophys. Acta 112 : 346.

SINGH, B. D. and HARVEY, B. L. (1975) Experientia 31 : 785 - 787.

SKOOG, F. and MILLER, C. O. (1957) Symp. Soc. Exp. Biol. 11 : 118 - 131.

SMALL, I. S. and WRAY, J. L. (1980) Phytochemistry 19 : 387 - 394.

SMITH, H. (1968) Adv. Genet. 14 : 1.

SNELL, F. D. and SNELL, C. T. (1949) in "Colorimetric Methods of Analysis" p. 804, Van Nostrand, New York.

SOLOMONSON, L..P. and VENNESLAND, B. (1972) Biochim. Biophys. Acta 267 : 544 - 551.

SOLOMONSON, L. P., LORIMER, G. H., HALL, T. L., BORCHERS, R. and BAILEY, J. L. (1975) J. Biol. Chem. 250 : 4120 - 4127.

SOLOMONSON, L. P. (1982) Proc. Int. Symp. on Nitrate Assimilation - Molecular and Genetic Aspects, Gatersleben, DDR, L12.

SORGER, G. J. (1965) Biochim. Biophys. Acta. 99 : 234 - 245.

SORGER, G. J. (1966) Biochim. Biophys. Acta. 118 : 484 - 492.

SOSA, F. H., ORTEGA, T. and BAREA, J. L. (1978) Plant Sci. Lett. 11 : 51 - 58.

SPENCER, D. (1959) Aust. J. Biol. Sci. 12 : 181 - 191.

STEINBERG, R. A. (1939) J. Agr. Res. 57 : 569 - 574.

STINES, B. J. and MANN, T. J. (1960) Hereditas 51 : 222 - 227.

STREET, H. E., HENSHAW, G. G. and BUIATTI, M. C. (1965) Chem. Ind. 9 : 27 - 31.

STREET, H. E. (1977) in "Plant Tissue and Cell Culture" 2nd edition (Street, H, E. ed.) pp. 207 - 222, Blackwell, Oxford.

SUNDERLAND, N. (1970) New Scient. 47 : 142 - 144.

SUNDERLAND, N. (1971) Sci. Prog. Oxf. 59 : 527 - 549.

SUNDERLAND, N. and DUNWELL, J. M. (1977) in "Plant Tissue and Cell Culture," 2nd edition (Street, H. E. ed.) pp. 223 - 265, Blackwell, Oxford.

SUNDERLAND, N. (1980) in "The Plant Genome" (Davies, D. R. and Hopwood, D. A. eds.) pp. 171 - 184, John Innes Charity, Norwich.

TAKEBE, I., OTSUKI, Y. and AOKI, S. (1968) Plant and Cell Physiol. 9 : 115 - 124.

TOKAREV, B. I. and SHUMMY, V. K. (1977) Genetika (Moskva) 13 : 2097 - 2103.

TOMSETT, A. B. and COVE, D. J. (1979) Genet. Res. 34 : 19 - 32.

TOMSETT, A. B. and GARRETT, R. H. (1980) Genetics 95 : 649 - 660.

TOMSETT, A. B. and GARRETT, R. H. (1981) Mol. Gen. Genet. 184 : 183 - 190.

TRIBE, H. T. (1955) Ann. Bot. 19 : 351 - 371.

TULECKE, W. R. (1953) Science 117 : 599 - 600.

TULECKE, W. R. (1957) Am. J. Bot. 44 : 602 - 608.

TURTOCZKY, I. and EHRENBERG, L. (1969) Mutation Res. 8 : 229 - 238.

UCHIMIYA, H. and MURASHIGE, T. (1974) Plant Physiol. 54 : 936 - 944.

VALLEE, B. L. and HOCH, F. L. (1955) Proc. Natl. Acad. Sci. U.S. 41 : 327 - 338.

VAN SLOGTEREN, G. M. S., PLANQUE, K. and LEKKERK, J. (1980) Plant Sci. Lett. 20 : 35 - 45.

VASIL, I. K. and VASIL, V. (1972) In Vitro. 8 : 117 - 125.

WAGNER, R. P. and MITCHELL, H. K. (1964) Genetics and Metabolism, Wiley, New York.

WALLACE, W. and JOHNSON, C. B. (1978) Plant Physiol. 61 : 748 - 752.

WANG, Y. Y., SUN, C. S., WANG, C. C. and CHIEN, N. F. (1973) Scientia Sinica 16 : 147 - 151.

WARNER, R. L., LIN, C. J. and KLEINHOFS, A. (1977) Nature 269 : 406 - 407.

WHITE, P. K. (1943) Growth 7 : 53 - 65.

WIDHOLM, J. M. (1972) Stain Technology 47 : 189 - 194.

WIDHOLM, J. M. (1974) in "Tissue Culture and Plant Science" (Street, H. E. ed.) pp. 288 - 299. Academic Press, London.

WRAY, J. L. and FILNER, P. (1970) Biochem. J. 119 : 715 - 725.

WRAY, J. L. and KIRK, D. W. (1981) Plant Sci. Lett. 23 : 207 - 213.

WRAY, J. L. (1982) Proc. Int. Symp. on Nitrate Assimilation - Molecular and Genetic Aspects, Gatersleben, DDR, L6.

YEOMAN, M. M. and MacLEOD, A. J. (1977) in "Plant Tissue and Cell Culture" 2nd edition, (Street, H. E. ed.) pp. 31 - 60. Blackwell, Oxford.

ZENK, M. H. (1974) in "Haploids in Higher Plants : Advances and Potential" (Kasha, K. J. ed.) pp. 339 - 354, Univ. Guelph Press, Canada.

ZRYD, J-P. (1978) Physiol. Vég. 16(3) : 353 - 363.