

THE USE OF HAPLOID SYSTEMS
IN PLANT GENETIC MANIPULATION

by

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ABSTRACT

In the present study the use of haploid plants and tissues was considered in relation to plant genetic manipulation. Haploid plants can be exploited directly, in the synthesis of true breeding lines. Alternatively, haploid plants and tissues may provide material for further experimentation involving protoplast fusion. Both approaches were investigated.

Cyclamen persicum, an attractive flowering plant is grown commercially from seed produced following open cross-pollination. As a result, Cyclamen is highly heterozygous, but the resulting variation is commercially undesirable. Inbreeding depression prevents the recovery of commercial inbred lines. Anther culture as an alternative approach for the recovery of true breeding lines was attempted. In order to test the efficiency of the culture procedure and conditions, anther culture of N. tabacum was also attempted, since this species is known to be highly responsive to anther culture. Despite the recovery of very many allodihaploid N. tabacum plants from anther culture, no success was achieved with Cyclamen, and the possible reasons for this are discussed.

It has recently been proposed that limited gene transfer might be achieved by somatic hybridisation if diploid protoplasts of a crop species were fused with haploid protoplasts of a wild type species, and novel allotriploid somatic hybrid plants recovered. Haploid protoplasts can be isolated from anther culture derived plants, however the range of species responsive to anther culture is limited. Tetrads, formed as a result of meiosis in the pollen mother cells, were investigated as an alternative source of haploid protoplasts for fusion studies.

Somatic hybrids were recovered following fusion between N. tabacum leaf mesophyll (2n) and N. glutinosa tetrad (n) protoplasts. The somatic hybrids were fertile, and the progeny of the first backcross to N. tabacum were obtained. These results, and potential limitations to somatic hybridisation are considered in the context of plant breeding.



Cyclamen persicum

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NR ⁻	nitrate reductase deficient
oz	ounce
P.E.G.	polyethylene glycol
pers. comm.	personal communication
pH	$-\log (H^+)$
p.I.	isoelectric focusing point
P.M.C.	pollen mother cell
p.s.i.g.	pounds per square inch
r DNA	ribosomal DNA
r.p.m.	revolutions per minute
R.I.T.C.	rhodamine isothiocyanate
RNA	ribonucleic acid
r RNA	ribosomal RNA
TEMED	NNN'N' tetramethylethylene diamine
Tris	tris (hydroxymethyl) amino methane
U.V.	ultra violet
% (v/v)	% ratio of volume of solute to solvent
% (w/v)	% ratio of weight of solute to solvent
μ	micro

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

The discovery that within cultured anthers of Datura innoxia, pollen grains can undergo sustained division giving rise to haploid embryos, and plants (Guha and Maheshwari, 1964 and 1966) led to speculation that anther culture might become a simple and widely applicable procedure for obtaining large numbers of haploid plants. Haploid plants and cell cultures can be used in a variety of ways. Haploids are invaluable in mutant selection, since single recessive mutations will be immediately expressed. An excellent example of the use of anther culture derived haploid cell cultures for mutant selection, is the production of nitrate reductase deficient (NR^-) cell lines, and in some cases plants which have been obtained in Nicotiana tabacum (Muller and Grafe, 1978), Datura innoxia (King and Khanna, 1980), Hyocymus muticus (Strauss et al., 1981), N. plumbaginifolia (Marton et al., 1982) and Oryza sativa (Wakasa et al., 1984). Such mutants are of great interest in studies on nitrate metabolism, DNA transformation and somatic hybridisation (Maliga, 1984).

The ability to obtain diploid plants following the application of chromosome doubling techniques to haploids offers an alternative method for the development of true breeding lines. Usually extensive inbreeding is necessary to obtain complete homozygosity, whereas the haploid approach achieves this directly. This procedure can be applied to hybrids between varieties, allowing new lines to be rapidly developed. In this way improved varieties of N. tabacum (Nakamura et

al., 1974) and O. sativa (Hu et al., 1978) have been obtained.

In spite of these examples, and the optimism and interest expressed for the use of haploids at the first international symposium on haploids in higher plants (Kasha, 1974) they have so far failed to significantly contribute to the genetic manipulation and improvement of crop plants. The reasons for this are complex, but can largely be attributed to our inability to obtain an anther culture response in most crop plants. Even when haploid plants can be obtained, the response is often dependent on the genotype of the donor plant, restricting their use still further. In cereals even greater problems must be overcome, since albino plants of little use in breeding programmes, are frequently produced by anther culture.

The haploid approach to the production of uniform true breeding lines would be advantageous in the breeding of Cyclamen persicum, a horticulturally important flowering plant. Inbreeding over several generations would be time consuming, and inbreeding depression has been reported (Wellensiek, 1959), suggesting that commercial inbred lines may be difficult to obtain. Previous unsuccessful attempts at Cyclamen anther culture have concentrated on manipulating the culture media and environment (Geier, 1978). An alternative approach, based on the following survey of the literature, was developed. Emphasis was placed on stress treatments performed on excised buds prior to the dissection and culture of the anthers. To evaluate the efficiency of this approach, parallel experiments were performed using anthers from a Nicotiana tabacum variety, White Burley, from which haploid embryos can readily be obtained by anther culture.

A second application for haploid cell systems to be evaluated, was the use of haploid protoplasts in fusion studies. Plant protoplasts can be released by degrading the cell wall with enzymes,

usually of fungal origin (Cocking, 1960). Isolated protoplasts can undergo sustained division giving a small cell colony capable of plant regeneration (Takebe et al., 1971). Protoplasts isolated from different species can be induced to fuse, and the resulting heterokaryon may also be capable of sustained division and plant regeneration. Novel somatic hybrid plants can be created in this way, which differ from sexual hybrids in a number of ways. Somatic hybrids originate from a heterokaryon which possesses the mitochondria and chloroplasts of both fusion partners. The retention, independent segregation and/or genome recombinations which may result can give rise to a wide range of different organelle combinations (Davey and Kumar, 1983). In contrast sexual hybridisation usually results in uniparental (usually maternal) inheritance of organelles. Nuclear fusion in the heterokaryon may result in somatic hybrids which possess the expected amphiploid chromosome number consisting of a summation of the chromosome complements of the two fusion partners. Chromosome elimination may occur following nuclear fusion, giving rise to a partial hybrid. In some cases chromosome elimination may be extreme, in which case only a limited gene transfer may occur. Failure of nuclear fusion may give rise to cybrids which contain a mixed cytoplasm, but the nucleus of only one fusion partner.

Somatic hybridisation has been demonstrated between sexually compatible species (Carlson et al., 1972), but successful somatic hybridisation between sexually incompatible species (Melchers et al., 1978) has aroused more interest. It has been suggested that the ability to overcome sexual incompatibility by somatic hybridisation will augment conventional breeding programmes, permitting a free exchange of genetic material. Few somatic hybrids have been produced between sexually incompatible species, and those which have been

obtained display a range of morphological abnormalities, and are sterile. Protoplast systems essential for somatic hybridisation are not yet available in many crop species, and it remains to be seen to what extent somatic hybridisation can contribute to crop improvement.

It has recently been proposed that the synthesis of triploid somatic hybrid plants by fusion of diploid protoplasts of a crop plant with haploid protoplasts of a wild type species could facilitate a limited genetic exchange from the wild type species into the crop species (Pental and Cocking, 1985). In triploid cells introgression could occur either by somatic crossing over, by transposition, or more probably by trivalent formation at meiosis. Unpaired wild type chromosomes might be lost at meiosis or randomly segregate to give gametes and ultimately offspring with a reduced wild type chromosome complement. By repeated backcrossing to the diploid parent, chromosomes of the wild type would be eliminated. Resulting plants and their progeny would need to be assessed for the introgression of novel genes of wild type origin.

Protoplasts can be isolated from anther culture derived haploid plants, but as has already been noted, the range of species in which haploids can be obtained in this way is limited. Tetrads formed as a result of meiosis in the pollen mother cells consist of four haploid spores bound within a thick callose wall, and may be an alternative source of haploid protoplasts for use in fusion studies.

In the genus Nicotiana somatic hybrid plants have been recovered between nitrate reductase deficient (NR^-) Nicotiana tabacum leaf mesophyll protoplasts ($2n = 4x = 48$) and N. glutinosa cell suspension protoplasts ($2n = 2x = 24$). The selection of hybrids was based on the restoration of nitrate reductase activity, green colour and regeneration capacity (Cooper-Bland et al., 1985a). This species

combination was chosen to investigate if haploid N. glutinosa protoplasts could be used in fusions with NR⁻ N. tabacum leaf mesophyll protoplasts and allopolyploid (functionally triploid) somatic hybrid plants recovered. The use of anther culture to generate haploid plants of N. glutinosa suitable for protoplast isolation was attempted. Tetrads as an alternative source of haploid protoplasts were also investigated.

1.2 ANTHOR AND POLLEN CULTURE

Following the discovery that haploid embryos can be obtained directly from pollen grains in cultured anthers of Datura innoxia (Guha and Maheshwari, 1964 and 1966), similar studies have been undertaken for many other species. An androgenetic response ranging from the development of pollen derived calli to the production of haploid plants has been recorded in at least 171 species, including some sexual hybrids, belonging to 60 genera and 26 families of Angiosperms (Maheshwari et al., 1982). The majority of these examples however, fall into just two families the Solanaceae and Gramineae.

The mechanism by which the young microspore is switched from its normal gametophytic pathway to a sporophytic one is poorly understood. Comparisons have been made between pollen development in vitro and in vivo. It is becoming increasingly apparent that conditions preceding anther culture may have more significance in the induction of pollen embryogenesis than the culture conditions, which are seen to be important only in maintaining further embryogenic development (Sunderland, 1981). There are few examples of successful pollen culture, in isolation from the anther. It has been proposed that the somatic anther tissues may play a role in inducing or maintaining pollen embryogenesis, possibly contributing substances to the culture medium (Sunderland and Roberts, 1977). In anther culture, conditions must be such that the development of the pollen within the anther is stimulated without proliferation of the somatic anther tissues. Growth and regeneration from the somatic anther tissue will result in the recovery of diploid heterozygous plants. Such plants would need to be separated from haploid and homozygous diploid plants of pollen origin.

1.2.1 Pollen Development in vivo

The developmental sequence by which mature pollen forms from pollen mother cells is summarised in Figure 1.1. Premiotic development of the male meiocytes, or pollen mother cells (P.M.C.'s) occurs within the anther locus. Mitotic division of the P.M.C.'s gives rise to a large number of P.M.C.'s which may be arranged within the anther locus such that they all have direct contact with the tapetal wall (e.g. Hordeum vulgare). Alternatively, the P.M.C.'s may be arranged in rows several cells thick such that some P.M.C.'s have no surface contact with the tapetal cells (e.g. Nicotiana tabacum). The association between the P.M.C.'s and tapetal cells may be significant in the control of pollen development (Sunderland et al., 1984).

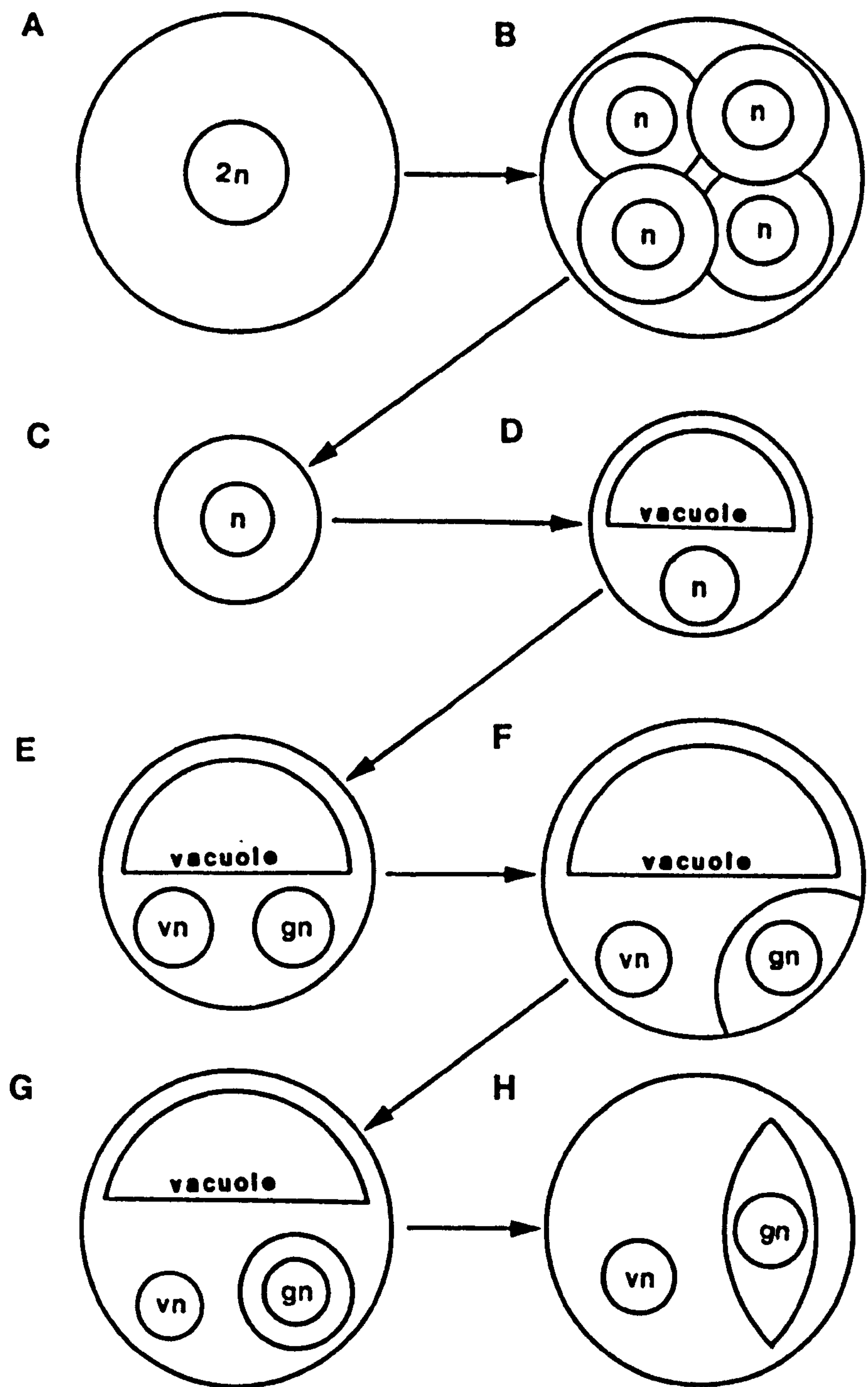
Sporogenesis begins at the onset of meiosis (Bennet, 1976). Meiosis is a highly co-ordinated event, all P.M.C.'s enter meiosis synchronously, possibly due to the high degree of cytoplasmic continuity between individual P.M.C.'s. Cytoplasmic continuity in the P.M.C.'s is established by the presence of cytomictic channels (Heslop-Harrison, 1966). The meiotic division results in the formation of a tetrad consisting of four microspores bound within a thick callose wall (Figure 1.1-b). Subsequent deposition of sporopollenin occurs, initiating the development of an individual wall surrounding each microspore. Release of the microspores from the tetrad occurs as a result of the enzymatic degradation of the callose wall (Figure 1.1-c). Following release the microspores enlarge considerably and further wall development occurs. A vacuole begins to form, which pushes the haploid nucleus, and most of the cytoplasm towards one pole of the developing microspore (Figure 1.1-d). First pollen mitosis occurs giving rise to the formation of the haploid vegetative and generative nuclei (Figure 1.1-e). The first pollen

FIGURE 1.1

Schematic representation of pollen development in vivo.

- 1.1 a Diploid pollen mother cell.
- 1.1 b Tetrad consisting of four haploid spores within a thick callose wall.
- 1.1 c Uninucleate microspore resulting from the release of the spores from the tetrad.
- 1.1 d Uninucleate microspore in which vacuole formation has occurred.
- 1.1 e Mitosis - resulting in the formation of the vegetative and generative nuclei.
- 1.1 f Early binucleate pollen grain, in which wall formation has occurred around the generative nucleus.
- 1.1 g The generative cell is completely free within the pollen grain, and the vacuole degenerates.
- 1.1 h A mature pollen grain.

The formation of a binucleate structure as a result of the first pollen mitosis (1.1 d - 1.1 e) marks the transition from a microspore, to a pollen grain. However, in this study, consistent with all previous reports on anther culture, the term pollen will be used to describe any of the stages of spore development from uninucleate microspore to mature pollen grains (1.1 c - 1.1 h).



Key

$2n$ - diploid nucleus

n - haploid nucleus

v - Vegetative

g - generative

mitosis is an asymmetric division. One daughter nucleus, the vegetative nucleus, remains near the centre of the microspore. The generative nucleus remains close to the microspore wall, and a hemispherical cell wall develops surrounding the generative nucleus and attaching it to the microspore wall (Figure 1.1-f). Eventually the generative cell wall is constricted, pinching the generative cell away from the microspore wall (Figure 1.1-g). Free within the cytoplasm of the microspore, the generative cell enlarges, becoming elongated. The generative nucleus enters prophase. Little if any cytoplasmic synthesis occurs within the generative cell (Mascarenhas, 1975).

During the first pollen mitosis there is an unequal division of the cytoplasm, with most of the plastids, mitochondria, and lipid bodies being incorporated into the vegetative cell. The vegetative cell becomes active with much cytoplasmic synthesis occurring. The vacuole declines, and starch deposition occurs within the plastids. The changes occurring in the vegetative cell are in preparation for the rapid synthetic capability required during pollen tube formation (Mascarenhas, 1975).

A successful anther culture response is usually only achieved when anthers selected for culture contain pollen at stages of development between release as the uninucleate microspores from the tetrad (Figure 1.1-c) and just after the first pollen mitosis (Figure 1.1-e). A standard convention has been proposed, numbering the stages of pollen development (Sunderland, 1974), and this was adopted in this study. The stages of pollen development are as follows :-

Stage 1 : Tetrads or young microspores just released from the callose wall. G1 of the cell cycle.

Stage 2 : Midphase microspores. Exine well developed.

Vacuole present but nucleus still in G1.

Stage 3 : Late phase microspore. Vacuole present.

Nucleus in S phase or G2.

Stage 4 : Pollen mitosis.

Stage 5 : Generative and vegetative nuclei present.

Generative nucleus cut off by a wall.

Microspore vacuole still present but often obscured by the synthesis of gametophytic cytoplasm.

Stage 6 : Starch deposition commencing. No vacuole.

The pollen stains densely with acetocarmine.

1.2.2 Pollen Development in vitro

The switch from a gametophytic to a sporophytic mode of development may involve the sustained division of either the vegetative cell (Rashid and Street, 1974) or generative cell (Raghavan, 1975). Alternatively, the microspore may undergo an equal first pollen division, giving rise to two cells which continue to divide (Reinert and Bajaj, 1977). In cultured anthers of Nicotiana tabacum two populations of pollen grains occur. In addition to large pollen grains which stain densely with acetocarmine, smaller less densely stained grains occur. It is from this latter type of pollen that embryoids originate (Sunderland and Wicks, 1971, Wernicke et al., 1978). Such embryogenic like pollen grains have also been observed in vivo, and have been reported to give rise to multinuclear structures within the anther (Sunderland, 1974; Dale, 1975; Horner and Street, 1978). The correlation between the occurrence of pollen

dimorphism in vivo and an anther culture response in vitro may indicate that pollen grains capable of undergoing pollen embryogenesis pre-exist within the anther prior to culture (Sunderland, 1980). The subsequent yield of pollen derived plants would therefore be a function of the size of the existing pool of embryogenic pollen grains ('E' grains) and the limitations imposed on the subsequent development of these 'E' grains by the culture conditions. Several factors influence the size of the 'E' grain pool in vivo, including the genotype (Sunderland, 1980) and growth conditions of the donor plant (Heberle-Bors and Reinert, 1980; Heberle-Bors, 1982a). The size of the 'E' grain pool may well be determined very early in pollen development. Pre-meiotic treatments have been shown to influence the size of the 'E' grain pool in N. tabacum (Heberle-Bors, 1982b). Despite the evidence that pollen grains capable of embryogenic development are formed in the anther, and that culture permits the expression of this capacity, it is not necessarily true that those pollen grains visually identified to be embryogenic are the only ones capable of further development in this way. Apparently normal pollen grains have been observed to undergo additional divisions (Sunderland and Evans, 1980). The 'E' grain pool is not always visually identifiable at the optimum time for anther culture (Sunderland, 1981).

1.2.3 Factors Influencing the Response of Pollen within Cultured Anthers

a) Bud Stress Treatments Prior to Anther Culture

The emphasis on inducing an embryogenically competent fraction of pollen grains prior to culture has led to the development of bud pre-treatments designed to increase the fraction of induced

pollen. In spite of evidence that the size of the 'E' grain pool is determined very early in pollen development (Heberle-Bors, 1982b), most pre-treatments are performed immediately prior to anther culture. The observation that incubation of buds at 4°C prior to culture increases the embryogenic response of the pollen in culture (Nitsch and Noreel, 1973), has led to the development of a variety of temperature stress pre-treatments. It has been demonstrated in N. tabacum that an interaction exists between the pre-treatment temperature, and its duration (Sunderland and Roberts, 1979). Lower temperatures give an optimum response when used for short periods, whereas longer pre-treatment times are necessary at higher temperatures. The optimum pre-treatment conditions may also vary according to the stage of pollen development within the buds at the beginning of pre-treatment (Sunderland and Wildon, 1979). The genotype of the donor plant may also influence the optimum pre-treatment conditions (Dunwell et al., 1985). The stress pre-treatments are usually carried out on excised buds maintained in such a way as to minimize water loss. In Brassica anther cultures the incubation of the anthers at an unusually high temperature for the first few days of culture stimulates pollen embryogenesis (Keller and Stringham, 1978). Such a treatment can also be considered as a stress pre-treatment.

The mechanism by which temperature stress pre-treatments of excised buds bring about an increase in pollen embryogenesis is not clear. An increase in the frequency of pollen grains undergoing an equal first pollen mitosis has been reported following low temperature treatments (Nitsch, 1974). Cold temperature pre-treatments may prevent the formation of polarity within the microspore by disturbing microtubule assembly (Maheshwari et al., 1980). Low temperature treatments are known to cause dissolution of microtubules (Hepler

and Palevitz, 1974). Centrifugation may also cause the dissolution of microtubules, and has also been reported to increase the frequency of pollen embryogenesis (Sangwan-Norreel, 1977). Whatever the mechanism by which stress pre-treatments influence the yield of pollen derived embryos, it is likely that a balance exists between the rate at which embryogenic pollen grains are induced, and the rate at which normal and induced pollen grains die. This balance will determine the optimum duration and conditions necessary for the pre-treatment of anthers from a given species or cultivar (Sunderland, 1980).

Although it is assumed that stress pre-treatments induce otherwise normal pollen grains to become embryogenic, it is not proven that this is the case. Alternatively, the stress pre-treatments may act by permitting a larger fraction of a pre-existing 'E' grain pool to express their embryogenic potential. Further experimentation will be required to distinguish between these two alternatives.

b) The Stage of Pollen Development within the Anther Prior to Culture

The stage of pollen development within the anther prior to culture is extremely important (Sunderland, 1974). Successful anther culture may only be achieved when the pollen is at the correct developmental stage. Three classes of plants can be determined according to the developmental stage of the pollen within anthers which gives an optimum culture response.

Class I plants include Nyctagynus niger, Hordeum vulgare and Lolium multiflorum which respond best when cultured anthers contain pollen at stage 2.

Class II plants include N. tabacum and Datura innoxia, which respond best when cultured anthers contain pollen at stages 3 and 4.

Class III plants respond best when cultured anthers contain pollen at stage 5, and include N. sylvestris, N. paniculata and Atropa belladonna (Sunderland, 1980).

The optimum developmental stage for anther culture may depend on the genotype of the donor plant. Different varieties of N. tabacum have been classified as class II plants (Sunderland and Roberts, 1979) and class III plants (Heberle-Bors and Reinert, 1979). Class III plants are more likely to produce embryos or callus having a higher than haploid chromosome number (Engvild, 1974).

The culture of anthers which contain pollen outside of the optimum developmental stage, at best, may result in a much reduced yield of pollen embryos or calli, or at worst may not elicit a response.

c) The Influence of the Genotype of the Donor Plant

The fact that the genotype of the donor plant can influence the size of the 'E' grain pool has already been noted. In addition there are several examples of genotypic variation in anther culture where no data is given on the frequency of pollen dimorphism, or potential 'E' grains. These include Hordeum vulgare (Feroughi-Weir et al., 1982) and Triticum aestivum (Bullock et al., 1982; Lazar et al., 1984). In Arabidopsis thaliana only 3 out of 18 genotypes tested responded in anther culture (Gresshof and Doy, 1972a) and similarly low success rates were achieved in a range of Lycopersicon esculentum and Vitis vinifera genotypes (Gresshof and Doy, 1972b and 1974). Attempts have been made to recombine genotypes exhibiting a high degree of responsiveness in anther culture, to further increase the yield of haploid plants both in Solanum tuberosum (Jacobson and Sopory, 1978) and Oryza sativa (Miah et al., 1985). The product-

ivity of tobacco anthers cultured under standard conditions has also been found to be under genotypic control. Genotype also influences the effect of environmental treatments designed to increase anther productivity (Heberle-Bors, 1984). Standard conditions are often used to evaluate the responsiveness of anthers from a range of genotypes. The apparent lack of response recorded for many genotypes may be due in part to their different culture requirements. Probably because of this, conditions have been determined by which Arabidopsis thaliana cultivars previously found unresponsive to culture (Gresshof and Doy, 1972a) will give rise to pollen derived calli (Ames and Scholl, 1978).

d) The Growth Conditions of the Donor Plant

The growth of the donor plant also effects the subsequent anther response in culture, and again it is not always clear if this is due to an increase in pollen dimorphism and therefore the size of the potential 'E' grain pool, or whether some other mechanism is operating. Both the light intensity and the photoperiod under which the donor plants are grown have been shown to influence the anther culture response in N. tabacum (Dunwell, 1976). The photoperiod has also been shown to influence the frequency of pollen dimorphism in vivo (Heberle-Bors and Reinert, 1979). The temperature at which the donor plant is grown can also influence the anther culture response, with a higher than normal growth temperature (20-30°C) favouring an anther culture response in N. knightiana whilst the opposite is true for Brassica napus (Keller and Stringham, 1978).

Contradictory reports about the effect of the nutritional status of the donor plant on anther culture response have been published. In different varieties of N. tabacum, nitrogen starvation, and nitrogen supplements have been shown to stimulate

the response of anthers (Sunderland, 1978; Heberle-Bors and Reinert, 1979). Differences in the nutritional status between buds from the same plant may be responsible for the observation that the first flower buds harvested from Brassica napus (Thurling and Chay, 1984), and N. tabacum respond best in culture (Dunwell, 1976).

e) Anther Culture Conditions

A variety of culture media have been successful in supporting embryogenic development of pollen in cultured anthers. The nutritional requirements of developing tobacco pollen grains are relatively simple. Pollen derived embryos are capable of development on a simple M.S. based medium (Murashige and Skoog, 1962 - see Appendix 1) solidified with 0.8%(w/v) agar and containing 3%(w/v) sucrose as a carbon source. Many other members of the Solanaceae also respond well to anther culture under similar conditions. An enhanced response may be obtained when agar-solidified media are replaced with liquid media (Wernicke and Kohlenbach, 1976). An improved method has been reported by which free embryos can be obtained from anthers which are floated on the surface of a liquid medium. As culture proceeds, the anthers dehisce and induced pollen grains begin embryogenic development. The anthers can be serially transferred to several petri dishes, such that the embryos develop in isolation from the anthers (Sunderland and Roberts, 1977). The beneficial effect of liquid as opposed to agar solidified media is not fully understood. Better diffusion of inhibitory substances away from the anther wall, or of nutrients into the anther may be facilitated (Wernicke and Kohlenbach, 1976). The observation that activated charcoal added to agar solidified media, but removed by filtration prior to anther culture, stimulates the culture response may indicate

that substances inhibitory to an anther culture response are present in the media, possibly in the agar (Johansson, 1983). This is further supported by the fact that agarose, a more refined form of agar, also stimulates the yield of pollen derived embryos, when compared to the use of agar (Wernick and Kohlenbach, 1976). Activated charcoal also stimulated the culture response when liquid media are employed (Tyagi et al., 1980). For this reason overcoming any inhibitory effects of agar cannot be the only beneficial role of activated charcoal in anther culture. An alternative role may be the absorption of phenolic compounds originating from senescing somatic anther tissues (Johansson, 1983).

The addition of plant growth hormones to the culture media has induced or enhanced the anther culture response for many species. Auxins may stimulate pollen embryogenesis, either in addition with a cytokinin or alone. Many members of the Solanaceae respond in anther culture without the addition of plant growth hormones. In some cases pollen embryogenesis has been disrupted by the addition of hormones to the culture media. Organic extracts are often used in place of plant growth hormones. Coconut milk and vegetable or fruit extracts have been particularly useful in cereal anther culture (Chih Ching Chu, 1982). Organic extracts may be subject to seasonal fluctuation in their content, which may result in unpredictable and even unreproducible results being obtained. The use of a defined culture medium is always preferable, if the same results can be achieved.

Sucrose is usually used as a carbon source, at a concentration of 2-3% (w/v). Higher levels have been used, with 6-12% (w/v) being most effective in Hordeum vulgare (Clapham, 1973), Brassica campestris (Keller et al., 1975), and Solanum tuberosum (Sopory, et al., 1978). High sucrose levels act not only as a carbon source, but also in

favouring callus formation from the pollen grains, while inhibiting callus formation from the somatic anther tissue (Ouyang et al., 1973). Further development of pollen derived calli may require their transfer to media containing a lower sucrose concentration (Keller et al., 1975).

The influence of the physical culture environment has not been critically examined. The light intensity has been shown to influence the culture response for several species. Continuous light proved beneficial for Datura metel anther cultures (Narayanaswamy and Chandy, 1971) but inhibited the response of Anemone virginiana anthers (Johansson and Erikson, 1977). Conditions necessary for the initiation of pollen embryogenesis may differ from those required for further embryo or callus development. Initial culture of Vitis vinifera anthers in the light, followed by their transfer to the dark gave an optimal response (Gresshof and Doy, 1974), whereas the reverse was found to be true for N. tabacum (Sunderland and Roberts, 1977).

The effect of the incubation temperature has not been examined critically, and usually lies between 20 and 30°C. Higher initial culture temperatures may be required for successful anther culture in Brassica species (Keller and Armstrong, 1979).

The volume of the culture vessel atmosphere has been shown to influence the response of N. tabacum anthers in culture (Dunwell, 1979). The possible mechanisms by which the gaseous environment may influence the anther culture response have not been studied in detail. Ethylene has been proposed as the most likely active component (Dunwell, 1979) but previous studies have failed to correlate the anther culture response to ethylene concentration (Horner et al., 1977).

1.2.4 Cytological Analysis of Anther Culture Derived Plants

The majority of plants derived from anther cultures of Nicotiana species have been shown to be haploid (Sunderland, 1971; Sharma et al., 1983). Usually the ploidy is determined by cytological examination of mitosis in actively dividing cells. Alternatively, morphological characteristics are used which distinguish between haploid and diploid plants, such as the number of chloroplasts per guard cell in the stomata (Nitzsche and Wenzel, 1977). More recently, the use of a fluorescence activated cell sorter has enabled the DNA content of small samples as anther derived embryos to be analysed. Using this method, 75.8% of N. sylvestris, and 94.4% of N. paniculata embryos were found to be haploid (Sharma et al., 1983). In spite of the high recovery of haploid plants in Nicotiana species, non-haploid plants, including diploids, polyploids and aneuploids have been found for nearly all species which have been tested (McComb, 1978). Diploid plants may be the result of nuclear fusion or endoreduplication early in pollen embryogenesis. Diploid plants resulting in this way would be homozygous. Heterozygous diploid plants may also be obtained following the development of unreduced microspores (Wenzel et al., 1976). For breeding purposes haploid plants must be diploidised and this can be readily achieved using colchicine treatments of the developing inflorescence (Nakamura et al., 1974), or young anther derived plants (Chowdhury, 1984).

1.2.5 Variation Arising in Anther Culture Derived Plants

Dihaploid plants developed from anther culture of homozygous lines should be identical to the donor plants. However, dihaploid plants derived from inbred flue cured cultivars of N. tabacum show a reduction in vigour when compared to their donor plants (Burk and

Matzinger, 1976; Arcia et al., 1978). In a similar study using Burley tobacco cultivars no significant reduction in plant vigour was reported (Deaton et al., 1982). These conflicting reports may be explained in terms of genetic differences between the flue cured, and Burley tobacco cultivars. Dihaploid plants of the flue cured tobacco cultivars were generated using colchicine treatments. In the study using Burley tobacco cultivars, an alternative chromosome doubling technique exploiting natural chromosome doubling in the leaf veins of haploid plants was used. Colchicine is known to be mutagenic (De Paepe et al., 1977) and may be responsible for the reduction in vigour of the dihaploid flue cured tobacco cultivars. The possibility that anther culture is intrinsically mutagenic appears unlikely. Mutations are mostly deleterious, yet dihaploid plants of the Burley tobacco cultivars showed no reduction in vigour, compared to the donor inbred lines (Deaton et al., 1982).

One example of variation in anther culture derived plants, which is influenced by the culture conditions, is the regeneration of albino plants. Albino's are frequently formed in anther cultures of members of the Gramineae, with 5-100% of pollen derived plants being affected. (Chi-Ching Chu, 1982). The proportion of albino plants obtained varies according to the genotype and physiological state of the donor plant, as well as the anther culture conditions used (Bullock et al., 1982; Ouyang et al., 1983). Albino plants have been shown to contain proplastids, thought to be precursors of chloroplasts (Clapham, 1977). Albino's do not therefore appear to be derived from the generative cell in which the plastids may be entirely absent (Schroder, 1985). The absence of 23 S and 16 S r RNA as well as Fraction 1 protein which are entirely or partly coded for by the chloroplast genome suggests an alteration in the expression or

structure of the chloroplast encoded genes (Sun et al., 1979). Chloroplast DNA has been extracted from albino wheat plants and found to have extensive deletions when compared to chloroplast DNA extracted from green plants also obtained from anther culture (Day and Ellis, 1984). Chloroplast DNA deletions may be a normal part of pollen development, since organelle alterations have been proposed as a possible mechanism for the maternal inheritance of chloroplasts (Vaughn et al., 1980). Alternative possible mechanisms for maternal inheritance have also been proposed, including the physical exclusion of plastids from the generative cell (Schroder, 1985). It remains to be demonstrated if chloroplast DNA deletions are a normal part of pollen development in vivo or merely an artifact induced in vitro.

Modification of another cytoplasmically encoded characteristic, a maternally inherited temperature sensitive mutant of N. tabacum has also been reported. This indicates that changes in organelle encoded information may occur following anther culture even when apparently normal green plants are generated (Matzinger and Burk, 1984).

1.2.6 Applications of Anther Derived Haploid Plants and Tissues

a) Mutant Selection

The potential advantages of using haploid cell cultures for mutant selection are based on the fact that recessive mutations will be expressed immediately. In addition, very large numbers of cells can be screened enabling infrequent events to be detected. The selection of nitrate reductase deficient mutants in several species has already been discussed in the general introduction. Not only are such mutants useful in studies on nitrate assimilation, but are also extremely valuable in the development of selection schemes for the recovery of somatic hybrids. This latter point will be discussed in

some detail in the next section. Auxotrophic mutants of Hyocymus muticus requiring amino acids, and vitamins have been obtained using anther culture derived haploid cells (Gebhardt et al., 1981). Similarly amino acid auxotrophs of N. plumbaginifolia have been obtained (Negrutiu et al., 1985).

There remains one problem in the use of haploid cell cultures for mutant selection. Once mutant cell lines have been established, their plant regeneration capacity has often been lost. Because of this extensive genetic analysis by studying the progeny of mutant plants cannot be performed. To a limited extent, somatic cell fusion can be used to detect genetic complementation. A rapid assay has been developed to detect complementation between nitrate reductase deficient cell lines (Biasini and Marton, 1985).

b) Plant Breeding

Although some culture-induced variation may occur in dihaploid plants, their potential use in plant breeding has already been demonstrated. Improved cultivars of tobacco (Nakamura et al., 1974), rice and wheat (Hu et al., 1978) have been obtained. The recovery of anther culture derived plants from interspecific diploid hybrids between the potato and its wild relatives may also contribute to the improvement of this crop species (Cappadocia et al., 1984). Several Brassica species respond well to anther culture, but it remains to be seen if this will lead to the development of improved Brassica cultivars (Keller and Stringham, 1978; Keller and De la Roche, 1983).

However, anther culture has not been successfully achieved for the majority of crop species. In those species for which an anther culture response has been reported, this is often limited to the production of calli which fail to regenerate. In many species of the

Gramineae regeneration gives rise to albino plants of little use in plant breeding. Successful anther culture is frequently limited to a few highly responsive genotypes. Until a better understanding of the mechanism underlying pollen embryogenesis is achieved, progress in the application of the anther culture technique to plant breeding will continue to be slow.

1.2.7 Anther Culture in *Cyclamen persicum*

From the preceding discussion, the following general conclusions can be drawn. Anther culture is widely applicable within the Solanaceae, but attempts to utilise this technique in other families has led to limited success. Pollen embryogenesis is poorly understood. While the influence of externally applied treatments has been widely reported, few attempts have been made to critically examine their effect. Contradictory reports about the effect of different treatments designed to increase the response of anthers in culture have been reported. There would appear to be great differences in the response of different species, and even between cultivars of the same species.

In general, it would appear that there are two stages in anther culture. The first stage involves the induction of pollen into an embryogenic state. The second stage involves the maintenance of embryo development. Induction can be brought about prior to anther culture. Physical stress treatments are extensively used to induce embryogenic pollen in anthers prior to culture. Such treatments can greatly increase the yield of pollen derived embryos, or result in their recovery when previously untreated anthers had failed to respond in culture. The induction phase may, or may not involve the culture medium. However, it is clear that the culture medium,

and physical culture conditions strongly influence the second stage, that of maintaining the development of pollen derived embryos.

The advantages of anther culture for the development of true breeding lines in Cyclamen persicum have been discussed in the general introduction. Previous unsuccessful attempts at anther culture in Cyclamen have concentrated on the influence of the culture medium. An alternative approach would be to concentrate on physical treatments prior to anther culture, in order to induce embryogenic pollen. The results of such pre-treatment may be detected by microscopic observation of the pollen following pre-treatments, or after a few days of anther culture. Alternatively, the development of macroscopic structures arising from the cultured anthers following pre-treatments may permit large numbers of anthers to be screened for a culture response.

At the same time studies on the development of somatic tissue in vitro may give valuable information about the culture requirements of Cyclamen tissues. This information may be applied to stage two of anther culture - the maintenance of embryogenic development.

To assess the efficiency of such an approach, parallel experiments were performed to see if an improvement in the culture response of N. tabacum anthers can be achieved.

A more systematic approach to Cyclamen anther culture may result in a successful anther culture response.

1.3 HAPLOID PROTOPLASTS IN SOMATIC HYBRIDISATION

Haploid protoplasts have only very rarely been used in studies on somatic hybridisation. However, the recent suggestion that the production of novel triploid somatic hybrid plants might facilitate a limited gene transfer from a wild type species to a crop plant (Pental and Cocking, 1985) (See 1.1, General Introduction) encourages the view that haploid protoplasts can contribute significantly to somatic hybridisation.

The advantages of somatic hybridisation over sexual hybridisation have been discussed in the general introduction. For somatic hybridisation to contribute to breeding programmes many problems must be overcome. The range of crop species in which protoplast systems are available is still limited. Selection schemes for the recovery of somatic hybrid plants and tissues must be developed. For seed plants fertility is essential, if the products of somatic hybridisation are to be integrated into plant breeding programmes. Somatic hybrids between sexually incompatible species have so far proved sterile.

Somatic hybridisation can be divided into distinct stages. Suitable methods by which large numbers of viable protoplasts can be isolated must be developed. Conditions under which these protoplasts will divide and ultimately give rise to complete plants must be ascertained. For somatic hybrids to be produced protoplast fusion must be brought about. Following fusion, a method by which the relatively small number of surviving hybrid colonies can be recovered from the majority of colonies of parental or homokaryon origin must be applied. Hybrid colonies must be regenerated, and the somatic hybrid plant characterised. Each of these stages is essential to any somatic hybridisation programme, and will be considered in some detail.

1.3.1 Protoplast Isolation

The isolation of large numbers of plant protoplasts is brought about by the enzymatic degradation of the plant cell wall (Cocking, 1960). The cell wall has three major components: cellulose - a linear polymer of D-glucose with β -1, 4 linkages, hemicellulose - a mixed polymer of glucose, galactose and xylose with β -1, 3 and β -1, 4 linkages, and pectic substances - pectin being a methyl -D-galacturonate with α -1, 4 linkages.

Successful protoplast release is usually achieved using a combination of commercially available enzyme preparations, usually of fungal origin, which have a range of cellulytic, hemicellulytic and pectolytic activities (Power and Cocking, 1968). The combination and concentration of enzymes used must usually be determined empirically (Patnaik and Cocking, 1982). Partial purification of the enzyme preparation by desalting (Slobas et al., 1980) or elution through biogel (Patnaik et al., 1981) may be necessary. Purification removes substances harmful to protoplast viability including compound of fungal origin, and other enzyme activities (e.g. proteases, ribonucleases). Highly purified enzyme solutions may however, be less effective at protoplast release, indicating that a range of enzyme activities may be required for effective cell wall degradation (Davey and Kumar, 1983).

Isolated protoplasts must be separated from the enzyme solution and any remaining cellular debris. Highly vacuolated protoplasts may be separated by flotation on sucrose solutions (Gregory and Cocking, 1965). Density gradients of 'percol' (Scowcroft and Larkin, 1980) and two phase systems employing sodium metrazoate and 'ficol' (Larkin 1976), have also been used.

Solutions used in the isolation and purification of protoplasts,

must be osmotically stabilised to prevent excess plasmolysis, or bursting of the protoplasts. Mannitol, a sugar alcohol cannot be metabolised by the protoplast and is frequently used in this respect.

Protoplasts can be isolated from a wide range of somatic plant tissues, including roots (Xu et al., 1982), cotyledons (Lu et al., 1982), coleoptiles (Hall and Cocking, 1971), stem pith (Harms et al., 1979), and petals (Flick and Evans, 1983). Protoplasts of gametic origin have also been isolated from tetrads (Bohjwani and Cocking, 1972), and germinating pollen tubes (Condeelis, 1974). For consistently high yields of uniform protoplasts, leaf mesophyll tissue (Takebe et al., 1968), and cell suspension cultures have been used most extensively. One disadvantage of using cell cultures as a source of protoplasts is the observed chromosomal instability arising in such cultures. Most long term cultures contain a mixture of diploid, polyploid, and aneuploid cells (Bayliss, 1980). Conditions have, however, been described which enable the long term maintenance of chromosomally stable suspension cultures of N. tabacum suitable for protoplast isolation (Evans and Gamborg, 1982).

Methods by which large numbers of viable protoplasts can be routinely isolated must be established empirically for each system. Generally, young actively growing cells or tissues are selected. Plants grown in the greenhouse are subject to environmental fluctuations which can influence the yield and viability of isolated protoplasts (Shepard and Totten, 1977; Tal and Watts, 1979). The growth of plants under controlled conditions can be manipulated to give more reproducible results (Santos et al., 1980). Similarly, the growth conditions of cultured cells must be manipulated and standardised to give reproducibly high yields of viable protoplasts.

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1.3.2 Protoplast Culture

Isolated protoplasts are usually cultured in media modified from those used in cell and tissue culture by the addition of a suitable osmoticum. The regeneration of a cell wall (Nagata and Takebe, 1970), and sustained division of protoplast derived cells (Kao et al., 1970) is followed by the regeneration of whole plants from the protoplast derived calli (Takebe et al., 1971).

Cell wall synthesis may be initiated after a very short delay, as was observed in Vicia hajastana (Williamson et al., 1977) or after a lag phase which may last 8 hours in N. tabacum (Burgess et al., 1978). Although nuclear division has been observed in the absence of cell wall synthesis (Reinert and Hellman, 1971; Galbraith and Shields, 1982), the formation of a cell wall is essential for cell division (Meyer and Abel, 1975; Schilde-Renshcher, 1977).

Division of protoplast derived cells usually occurs after 2-7 days of culture. Sustained cell division leads to the formation of colonies as undifferentiated cells. Suitable manipulation of the hormone regime may induce differentiation and ultimately plant regeneration. Protoplast culture at high density is usually required to initiate division in protoplast derived cells. Once initiated, cell division can proceed at a much reduced density (Caboche, 1980). This can be exploited in selection schemes designed to recover mutant or somatic hybrid colonies. Conditions which only permit the survival of mutant or hybrid colonies can be imposed once cell division has been initiated. The few surviving mutant or hybrid colonies would be capable of sustained division even at the much reduced density.

The growth conditions of the donor plant (Cassels and Cocker, 1982) as well as the culture media, hormone combination and physical culture conditions (photoperiod and light intensity, temperature) can

influence the response of cultured protoplasts (Davey, 1983). However, genetic differences between species and cultivars usually determine the protoplast response in culture. Only 61 species have been reported to regenerate plants from cultured protoplasts, many of these species are in the Solanaceae, and there are few examples of economically important crop plants (Binding et al., 1981; Davey and Kumar, 1983).

1.3.3 Protoplast Fusion

A variety of chemical treatments, as well as electrical stimuli have been reported to induce protoplast fusion. The first report of chemically induced protoplast fusion used sodium nitrate (Power et al., 1970). This method was subsequently used in the production of the first interspecific somatic hybrid plant, between Nicotiana glauca and N. langsdorffii (Carlson et al., 1972). Deterioration of protoplasts exposed to sodium nitrate has also been reported (Power and Frearson, 1973) and this led to the development of less damaging chemical fusogens.

The use of a high concentration of calcium ions (50 mM CaCl_2), buffered at high pH (pH 10.5) causes protoplast fusion without excess damage (Keller and Melchers, 1973). Similarly polyethylene glycol (P.E.G.) has also been reported to promote protoplast fusion (Kao and Michayluk, 1974; Wallin, et al., 1974). Subsequently enhanced fusion frequencies have been obtained using a combination of high pH/ Ca^{2+} and P.E.G. (Kao et al., 1974). The majority of somatic hybrids reported to date result from the use of high pH/ Ca^{2+} or P.E.G. as fusogen, either alone, or in combination.

A recent advance is the development of electrically induced fusion, as an alternative to chemical treatments (Zimmerman and Scheurich, 1981; Vienken et al., 1981). Protoplasts are aligned in

an A.C. field, and induced to fuse by a short D.C. pulse. Using this technique interspecific somatic hybrids between N. tabacum and N. plumbaginifolia have recently been recovered (Bates and Hasenkampf, 1985).

Chemical and physical treatments designed to induce protoplast fusion also cause considerable damage. High fusion frequencies are often correlated with a reduction in the viability of the protoplasts (Ward et al., 1979). It has recently been proposed that high fusion frequencies might be achieved without the subsequent loss of viability, if protoplast systems which readily undergo fusion are exploited (Boss et al., 1984). Tetrad protoplasts readily undergo fusion without the application of a specific fusogen (Bhojwani and Cocking, 1972). This may be of considerable advantage if tetrad protoplasts are to be used in attempts at somatic hybridisation.

The mechanism of protoplast fusion is not fully understood. The plasma membrane of plant protoplasts usually carries a net negative electropotential charge, causing protoplasts to repel one another under normal conditions (Grout et al., 1972). The electropotential charge of -25 to -35 mV for normal tobacco mesophyll protoplasts is neutralised when the protoplasts are suspended in a solution containing a high concentration of calcium ions. This neutralisation of the surface potential is associated with protoplast aggregation and fusion (Nagata and Melchers, 1978), and may explain the action of high pH/Ca²⁺ fusogens. It is not known if the electropotential charge carried by the plasma membrane of tetrad protoplasts is different from that of mesophyll protoplasts. If it is significantly lower, this might explain the ease with which tetrad protoplasts undergo fusion.

It is not clear how P.E.G. brings about fusion. It has been

suggested that P.E.G. might link protoplasts via bonds between positively charged cations and the negatively charged plasma membrane (Kao and Michayluk, 1974). In addition the strong affinity of P.E.G. for water may cause local dehydration of the plasma membrane, increasing fluidity and encouraging fusion (Borochoy and Borochoy, 1979). Commercial P.E.G. preparations contain traces of α -tocopherol to reduce oxidation. It has been observed that α -tocopherol also stimulates fusion synergistically with P.E.G. Highly purified P.E.G. causes agglutination but not fusion of human erythrocytes. This has led to speculation that P.E.G. alone only causes agglutination, and that fusion is stimulated by the presence of α -tocopherol (Honda et al., 1980; Honda et al., 1981).

Fusion between plant protoplasts isolated from very different sources, such as Daucus carota and Hordeum vulgare has been reported (Dudits et al., 1976). Following fusion between protoplasts with visually distinct cytoplasm, cytoplasmic mixing can be observed. Nuclear fusion has been observed in heterokaryons formed between Pisum sativum and Glycine max within the first day of culture, and prior to mitosis (Constabel et al., 1975). Similarly nuclear fusion was observed in heterokaryons formed between D. carota and H. vulgare (Dudits et al., 1976), suggesting that the formation of a heterokaryon in which a hybrid nucleus exists does not limit our ability to create somatic hybrids. Limitations must be due to factors operating subsequently to fusion.

1.3.4 Selection Schemes for the Recovery of Somatic Hybrid Cell Lines and Plants

Following fusion, the populations of protoplasts usually includes between 0.1 and 5.0% heterokaryons. In the unlikely event

that heterokaryons divide with the same frequency as protoplasts of parental origin, the cell colonies recovered will consist of a similarly low percentage of hybrid material. As will be discussed later, in reality the recovery of hybrid colonies may be much lower than predicted by this assumption. The ability to recognise hybrid material amongst material of parental origin is therefore a crucial step in any somatic hybridisation programme.

a) Mass Selection

It has been suggested that somatic hybrid plants might be identified among a large population of plants regenerated from colonies recovered following a fusion experiment. Using this approach somatic hybrids have been recovered between N. tabacum and N. glutinosa. Somatic hybrids were identified following biochemical analysis performed on a large number of individual plants (Uchimaya, 1982). Using a similar approach putative somatic hybrids between Lycopersicon esculentum and L. peruvianum have been identified based on the morphology of the regenerated plants. Hybridity in this case must be established biochemically (Kinsara, pers. comm.). Mass selection in this way will probably be of limited use, since somatic hybrids may be so few in number that very large numbers of plants would need to be regenerated to recover them.

b) Single Heterokaryon Isolation

Visual identification and mechanical isolation of single heterokaryons has been proposed as a simple and universally applicable selection method. Fusion between protoplasts from different sources often results in heterokaryons which are visually distinct from either protoplast type. The presence of chloroplasts (from mesophyll

protoplasts) and cytoplasmic strands (from cultured cell protoplasts) has enabled the identification of heterokaryons between Glycine max and N. glauca. Following dilution and inoculation of 25 µl aliquots of post fusion protoplast suspension into individual wells, those wells identified to contain single heterokaryons were registered and subsequently somatic hybrid colonies recovered (Kao, 1977). Using similar methods somatic hybrid tissues have been recovered between Arabidopsis thaliana and Brassica campestris (Gleba and Hoffman, 1978) and also Atropa belladonna and N. chinensis (Gleba et al., 1982).

Frequently heterokaryons have been physically isolated and placed in a suitable nurse culture to permit their growth. Auxotrophic mutants or albinos against which selection can be applied are cultured at the normal density. The small number of heterokaryons manually isolated are added to this culture. Thus hybrids between nitrate reductase deficient (NR^-) N. tabacum and a wild type N. paniculata and wild type N. sylvestris have been recovered following the use of the same NR^- N. tabacum line as a nurse culture. Hybrids were subsequently recovered from the nurse culture on the basis of their ability to utilise nitrate as sole nitrogen source (Hein et al., 1983). Hybrids have also been recovered between N. knightiana and N. sylvestris following heterokaryon isolation and nurse culture in albino N. sylvestris protoplasts (Menczel et al., 1978). Similarly somatic hybrids between N. tabacum and N. rustica have been recovered, in this case the nurse culture consisted of albino Petunia hybrida protoplasts (Hamill et al., 1984). In both cases green somatic hybrid colonies were selected from a mass of colourless albino colonies.

The visual identification of heterokaryons between protoplasts of different cell types cannot always be unequivocally ascertained,

and labelling protoplasts with fluorescent dyes may aid manual isolation (Patnaik et al., 1982; Kanchanapoom et al., 1985).

The use of fluorescent dyes has been extensively used in animal systems where the mechanical isolation of heterokaryons is automated using a fluorescence activated cell sorter (Herzenberg et al., 1976). This technique may be applied to plant protoplast fusion studies (Redenbaugh et al., 1982), and although somatic hybrids have yet to be recovered in this way, protoplasts can survive sorting in such an instrument (Galbraith et al., 1984) and future success seems likely.

Automation would be a significant advance for mechanical isolation. The success of single heterokaryon isolation largely depends on the number of heterokaryons selected, and the frequency with which they subsequently form calli and ultimately plants. Although the throughput of heterokaryons to hybrid plants may be high in interspecific hybrids within the Nicotiana (Hamill et al., 1984), a much lower success rate has been achieved in other systems (Power et al., 1977). This may severely limit the applicability of manual heterokaryon isolation.

c) Differential Growth Characteristics

The use of media on which a preferential growth of hybrid cells occurs was used in the production of the first interspecific somatic hybrid, between N. glauca and N. langsdorffii (Carlson et al., 1972). Selection was based on the observation that the sexual hybrid between these two species shows genetic tumour formation. Colonies obtained following fusion were selected on a medium deficient in auxin. Colonies capable of sustained growth were regenerated, and the somatic hybrid nature of the plants verified cytologically and biochemically. The ability of protoplasts isolated from the sexual hybrid between

Petunia parodii and P. hybrida to grow on a defined medium which would not support the growth of either parent was also used as the basis for selection of somatic hybrids between these two species (Power et al., 1977). Similarly the knowledge that only the sexual hybrid between Solanum tuberosum and S. chacoense regenerated on a given medium was used to select somatic hybrids in this species combination (Butenko and Kuchko, 1980). Selection schemes which require a knowledge of the culture requirements of the sexual hybrid between a given species combination are obviously of limited use. Somatic hybridisation may well be used only in cases where the sexual hybrid cannot be produced, and so a knowledge of the culture requirements of the somatic hybrid cannot be obtained.

d) Metabolic Inactivation

The metabolic inactivation of protoplasts prior to fusion has been successfully used in the recovery of somatic hybrids. Iodoacetate inactivated Petunia hybrida protoplasts have been fused with diethylpyrocarbonate inactivated Solanum nigrum protoplasts, and hybrid colonies recovered (Nehls, 1978). Similarly x-irradiated N. tabacum protoplasts have been fused with iodoacetate inactivated N. plumbaginifolia protoplasts, and somatic hybrids recovered (Siderov et al., 1981). The use of irradiation to inactivate one partner in protoplast fusion has been extensively used in studies designed to recover cybrids-hybrids having the nucleus of one species in a foreign or mixed cytoplasm (Fluhr et al., 1984).

e) Gene-Based Complementation Selection Schemes

The majority of somatic hybrid plants and tissues produced so far have involved the use of gene-based complementation schemes

(Pelletier and Chupaeu, 1984). Non allelic albinos, auxotrophic mutants and resistance to metabolic inhibitors have been used frequently in combination with a knowledge of the growth characteristics of the species to be fused. A lack of growth or regeneration in one of the fusion partners is particularly useful and can be used in combination with mutant systems.

e)

(I) The Use of Albinos

The observation that wild type Petunia parodii protoplasts were incapable of sustained growth on a medium which supported sustained growth of albino P. hybrida and albino P. parviflora protoplasts has been exploited in the hybridisation of P. parodii with each of these species. Green colonies were selected on the medium preventing P. parodii alone from undergoing sustained division. These were later confirmed to be somatic hybrids (Cocking et al., 1977; Power et al., 1979 and 1980). Similarly the limited development of wild type Datura discolor and D. stramonium protoplasts on a medium supporting sustained development of albino D. innoxia protoplasts has also facilitated somatic hybrid plant production between these two species (Schieder, 1978).

The use of an albino as half selection, and the morphology of regenerated green shoots to identify somatic hybrids has been used in the combination of albino D. innoxia with wild type Atropa belladonna (Krumbiegel and Schieder, 1979), albino N. tabacum with wild type N. nesophylla and wild type N. stocktonii (Evans et al., 1981), and also between albino Solanum nigrum and wild type S. tuberosum (Binding et al., 1982).

Non allelic haploid light sensitive (chlorophyll deficient)

mutants of N. tabacum have been fused, and green intraspecific somatic hybrid plants recovered as a result of complementation (Melchers and Labib, 1974). Green plants have also been recovered as a result of complementation between non-allelic albinos following fusion between albino N. tabacum and albino N. sylvestris (Melchers 1977) and also between N. tabacum and N. rustica (Douglas et al., 1981a).

e)

(II) The Use of Stable Auxotrophic Mutants

Few stable auxotrophic mutants have been isolated and characterised in higher plants (Maliga 1980, 1984). Nitrate reductase deficient (NR^-) mutants are, however, a notable exception (Muller and Grafe, 1978), and have been used extensively in somatic hybridisation. Non-allelic NR^- N. tabacum cell lines have been fused and intraspecific hybrids produced based on the ability of hybrid colonies to utilise nitrate as sole source of nitrogen (Glimelius et al., 1978). Interspecific somatic hybrids have also been recovered by complementation of non-allelic NR^- mutants, including hybrids between N. tabacum and Hyocymus muticus (Potrykus et al., 1983).

Somatic hybrids have frequently been obtained using NR^- lines as half selection. Fusion of NR^- N. tabacum with wild type N. paniculata and also wild type N. sylvestris followed by single heterokaryon isolation and nurse culture in the same NR^- N. tabacum line has enabled the recovery of somatic hybrids between these species (Hein et al., 1983). Somatic hybrids have also been recovered following fusion between NR^- N. tabacum and wild type N. glutinosa. Selection was based on the restoration of nitrate reductase activity coupled with the regeneration ability of N. tabacum. N. glutinosa

colonies were pale in colour and failed to regenerate on the medium used (Cooper-Bland et al., 1985a). The use of irradiation to prevent division of the wild type fusion partner has also been used and somatic hybrids obtained between NR^- N. tabacum and irradiated wild type Datura innoxia as well as with irradiated wild type Physalis minima (Gupta et al., 1983). Similarly hybrids between NR^- N. tabacum and irradiated N. glutinosa have been recovered (Cooper-Bland et al., 1985b).

Recently nitrate reductase deficiency has been combined with streptomycin resistance by sexual crosses (Hamill et al., 1983) and also by somatic hybridisation (Hamill et al., 1984). Such an NR^- , streptomycin resistant N. tabacum mutant has proved useful in the recovery of somatic hybrid plants with wild type N. rustica (Hamill, 1983; Pental et al., 1984) and also somatic hybrid cell lines with wild type Petunia hybrida, one of which regenerated after extensive loss of N. tabacum chromosomes (Pental et al., 1985).

e)

(III) The Use of Resistance to Metabolic Inhibitors

Resistance to normally toxic amino-acid analogues can also be used in somatic hybridisation as a selective marker. Intraspecific hybrids between non-allelic amino acid analogue resistant N. sylvestris lines has been reported (White and Vasil, 1979). Interspecific somatic hybrids have also been recovered between an amino acid analogue resistant Daucus carota cell line unable to regenerate, and a D. capillofolium line capable of regeneration (Kameya et al., 1981).

Naturally occurring differential drug sensitivities between species have also been described (Cocking et al., 1974), and incorp-

orated into selection schemes. Hybrids have been recovered following protoplast fusion between Petunia parodii resistant to Actinomycin-D but unable to undergo sustained division on a medium which supports the growth of Actinomycin-D sensitive P. hybrida protoplasts. Selection was based on sustained growth in the presence of Actinomycin-D (Power et al., 1976). Drug resistance can be obtained by selection in vitro. Resistance to streptomycin (Maliga et al., 1973) and lincomycin (Cseplo and Maliga, 1984) has been obtained in this way. Both streptomycin and lincomycin resistance has been exploited in somatic hybridisation (Menczel et al., 1981; Cseplo et al., 1984).

Advances in the manipulation of genetically engineered Agrobacterium tumefaciens Ti plasmid have resulted in the regeneration of plants expressing foreign genes (Herrera-Estrella et al., 1983; Barton et al., 1983). The ability to transform plant cells using vectors conferring resistance factors has also been demonstrated (Bevan, Flavel and Chilton, 1983). It is likely that this will be used as a tool for manipulating selective markers in protoplast systems prior to somatic hybridisation attempts (Negrutiu et al., 1984).

1.3.5 The Efficiency of Selection Schemes

The frequency with which somatic hybrid plants are recovered following protoplast fusion depends mostly on the species combination chosen, but also on the efficiency of the selection scheme employed.

Somatic hybrids between N. tabacum and N. rustica have been recovered by single heterokaryon isolation, as well as by complementation selection using an NR^- , streptomycin resistant double mutant of N. tabacum. 20% of heterokaryons selected manually gave rise to somatic hybrid plants, compared to a 7% recovery using complementation

selection (Hamill et al., 1984; Pental et al., 1984). Using the same double mutant of N. tabacum somatic hybrid colonies were also recovered following fusion experiments with Petunia hybrida. Approximately 0.1 - 1.2% of heterokaryons gave rise to hybrid colonies. Biochemical analysis suggested a gradual loss of N. tabacum chromosomes from cell lines established from these colonies. Of six somatic hybrid cell lines studied in detail, one eventually regenerated giving a frequency of hybrid plant recovery from heterokaryons of only 0.02 - 0.2% (Pental et al., 1985).

Somatic hybrids between P. hybrida and P. parodii have also been reported. Using different selection schemes the frequency of hybrid plant recovery from heterokaryons formed varied between 0.001 and 0.09% (Power et al., 1977).

Clearly the use of manual isolation would be inappropriate if, for biological reasons, the throughput of heterokaryons to somatic hybrid plants is very low. Only a limited number of heterokaryons can be isolated manually, although automation by the use of a fluorescence activated cell sorter would overcome this problem. Gene based complementation selection schemes enable very large numbers of heterokaryons to be screened. This may overcome the problem of low recovery rates of hybrid plants from heterokaryons.

1.3.6 Analysis of the Products of Somatic Hybridisation

Protoplast fusion may allow the combination of genomes from widely divergent species and genera with the potential of overcoming sexual incompatibility barriers. Sexual reproduction usually results in uniparental (maternal) segregation of cytoplasmically inherited characteristics. Cytoplasmic mixing following protoplast fusion may permit novel combination of nuclear and cytoplasmic encoded information

to be recovered. The nuclear and cytoplasmic combinations which may arise as a result of fusion are extensive (Davey and Kumar, 1983). The retention of a mixed organelle population, independent organelle segregation, and mitochondrial and chloroplast genome recombinations have been reported.

Heterokaryons initially possess the nuclear genomes of both fusion partners. These may be retained giving rise to balanced somatic hybrid plants with a stable amphiploid chromosome complement, as was found in somatic hybrids between N. tabacum and N. glauca (Evans et al., 1980). Chromosome elimination may result in a partial or asymmetric hybrid with all or most of one of the contributing genomes, but a much reduced contribution from the other fusion partner. Such a situation was observed in hybrid cell lines between Glycine max and N. glauca, which gradually lost N. glauca chromosomes over a 6 month period in culture (Kao, 1977). Only very few N. glauca chromosomes were retained after 36 months in culture (Wetter and Kao, 1980). The complete elimination of one set of chromosomes has also been reported following fusion between Petunia hybrida and Parthenocissus tricuspidata. In spite of the loss of all P. hybrida chromosomes, a P. hybrida specific isoperoxidase was still present in this cell line (Power et al., 1975). In this way protoplast fusion can bring about a limited gene transfer.

Protoplast fusion does not always result in nuclear fusion within the heterokaryon. This may result in the development of somatic hybrids possessing the nucleus of only one fusion partner, but having a mixed cytoplasm. Such hybrids, termed cybrids may be particularly useful in the manipulation of mitochondrial and chloroplast encoded characteristics (Fluhr, 1983).

Analysis of putative somatic hybrids, both to confirm hybridity,

and to determine their genetic make-up has included studies on the morphology of regenerated plants, especially where identifiable genetic markers have been used. Somatic hybrid cell lines and plants can also be analysed cytologically and biochemically. Recently the analysis of organelle D.N.A. restriction patterns, and the use of nuclear D.N.A. probes has added to our understanding of the consequences of somatic hybridisation (Harms, 1983a).

a) Morphological Studies

Examination of the morphology of regenerated plants, following the fusion of Petunia parodii protoplasts with P. hybrida, P. inflata and P. parviflora has permitted the identification of hybrids on the basis of intermediate leaf size and shape, flower morphology and pigmentation, petiole and pedicel length, and trichome morphology in comparison with the parental types (Power et al., 1976, 1979, 1980). However, morphological studies may be of limited use in the identification of somatic hybrids. Partial hybrids, or cases of limited gene transfer may well mostly resemble one parent. Intermediate morphology is not always observed in balanced somatic hybrids. Somatic hybrid plants between N. tabacum and N. rustica exhibit increased vigour when compared to both parents (Douglas et al., 1981a; Pental et al., 1984). Somatic hybrid plants formed between Lycopersicon esculentum and Solanum tuberosum (Melchers et al., 1978) and also between Arabidopsis thaliana and Brassica campestris (Gleba and Hoffman, 1980) showed grossly abnormal morphologies.

b) The Cytology of Somatic Hybrid Plants and Tissues

The cytological examination of somatic hybrid cell lines and plants has also assisted in their identification, and has contributed to our understanding of processes occurring following fusion. The

chromosome number of somatic hybrid plants may correspond to the summation of the parental chromosome numbers, giving the expected amphiploid condition. Such a situation was found in somatic hybrids between N. glauca and N. langsdorffii (Evans et al., 1982), and in several other combinations.

However, variation from the expected amphiploid chromosome number is frequently observed in somatic hybrids. For example, following fusion between two non-allelic haploid light sensitive mutants of N. tabacum intraspecific somatic hybrids have been produced of which, 54% possessed the expected amphiploid number ($2n = 48$), with a further 12% having numbers close to this value. In addition 23% were triploid ($3n = 72$) and 12% tetraploid ($4n = 96$). The remainder were aneuploids close to the triploid or tetraploid number (Melchers and Labib, 1974). More recently somatic hybrids between N. tabacum and N. rustica have been reported. Only 1 plant out of 15 tested was found to have the expected amphiploid chromosome number of 96. The rest had lower chromosome numbers ranging from 68 to 95. A variation in the chromosome number was reported between plants arising from the same hybrid colony (Douglas et al., 1981b). Variations from the expected amphiploid chromosome number may arise due to variation pre-existing at the time of fusion. Multiple fusions may also occur, and the changes associated with long term culture may also arise during the tissue culture phase between protoplast fusion and hybrid plant regeneration. Variation in the chromosome number between different cells of the same root tip has been demonstrated in somatic hybrids between N. tabacum and N. knightiana indicating that plant regeneration does not necessarily fix a stable chromosome number (Maliga et al., 1978).

Chromosome eliminations are not necessarily random. Somatic

hybrid cell lines between Arabidopsis thaliana and Brassica campestris have been reported to show extreme variations in chromosome number and ploidy levels (Gleba and Hoffman, 1978). Although there was no evidence of specific chromosome loss in the cell lines, or one of the regenerated plants obtained, a second regenerated plant showed the complete loss of identifiable Brassica chromosomes, although evidence of Brassica specific gene expression was still present (Gleba and Hoffman, 1979 and 1980). Unidirectional chromosome loss has been reported for somatic hybrid plants between Datura innoxia and Atropa belladonna, which possess only a few chromosomes from Atropa but a full complement of Datura chromosomes (Krumbiegel and Schieder, 1981). Similarly the gradual loss of N. glauca chromosomes from somatic hybrid cell lines between Glycine max and N. glauca (Kao, 1977; Wetter and Kao, 1980), and also N. tabacum chromosomes from somatic hybrid cell lines between G. max and N. tabacum (Chien et al., 1982) has been reported.

Chromosome loss may be extreme, as was reported for somatic hybrid cell lines between Petunia hybrida and Parthenocissus tricuspidata (Power et al., 1975). In this case complete elimination of Petunia chromosomes was observed in spite of the presence of Petunia specific iso-peroxidases found in extracts of this tissue.

Although chromosome loss is frequently observed in somatic hybrids between widely divergent species, it is not necessarily inevitable. Hybrid callus tissue between Atropa belladonna and N. chinensis has been shown to retain both chromosome sets (Gleba et al., 1982). Although chromosome elimination frequently precedes plant regeneration, it is not yet clear if it facilitates plant regeneration.

Chromosomal rearrangements are known to occur in cultured cells

of celery (Murata and Orton, 1983), and restructuring of the chromosomes has been observed in somatic hybrids between Glycine max and N. glauca (Kao, 1977). Such rearrangements or somatic recombinations, if they occur between chromosomes of the different fusion partners, may explain the persistence of gene activity specific to a fusion partner for which complete chromosome elimination has occurred. For example, the persistence of an iso-peroxidase specific to Petunia hybrida in hybrid cell lines between Petunia hybrida and Parthenocissus tricuspidata which have lost all Petunia chromosomes may be explained in this way. Similarly the correction of nuclear albinism in Daucus carota by fusion with Aegipodium podagraria protoplasts persisted despite the elimination of all identifiable Aegipodium chromosomes (Dudits et al., 1979). The use of irradiation to direct unidirectional chromosome elimination (and simplify selection) has been proposed (Harms, 1983a). Chromosome fragmentation as a result of irradiation may facilitate elimination. Fusion between nuclear albino Daucus carota and irradiated Petroselinum hortense protoplasts resulted in the incomplete loss of Petroselinum chromosomes in the regenerated plants (Dudits et al., 1980). No non-irradiated fusions were performed, and the possibility remains that irradiation alone did not bring about the elimination of Petroselinum chromosomes. Chromosome fragments have been observed in somatic hybrids where one fusion partner has been irradiated (Itoh and Futsuhara, 1983; Cooper-Bland et al., 1985b), suggesting that irradiation does not necessarily bring about complete chromosome elimination. This complicates the interpretation of experiments in which the correction of nitrate reductase deficiency in N. tabacum has been achieved by fusion with irradiated Physalis maxima and irradiated Datura innoxia protoplasts, but cytological analysis has not been performed (Gupta et al., 1983).

c) Biochemical Characterisation of Somatic Hybrid
Cell Lines and Plants

Neither morphological or cytological methods are sufficient to confidently determine the hybridity of putative somatic hybrid tissues or plants. For this reason, more definitive methods have been sought, and biochemical methods are usually used to confirm hybridity. Isoenzymes, multiple molecular forms of an enzyme which exhibit similar substrate specificity (Scandalios, 1969) have frequently been used for this purpose. Following electrophoresis, species specific banding patterns are frequently obtained with gels stained for a particular family of isoenzymes. Somatic hybrids may be represented by the summation of the parental banding patterns (Carlson et al., 1972; Power et al., 1976), or as a summation of the parental banding patterns, with the addition of novel bands not present in either parent (Maliga et al., 1978; Gleba and Hoffman, 1978, 1979). Isoenzyme patterns may change due to the gradual loss of chromosomes in hybrid tissues (Power et al., 1975; Wetter, 1977; Pental et al., 1985). Care must be taken in the interpretation of isoenzyme data since the expression of isoenzymes may be influenced differently in somatic hybrid tissues under the same conditions as the parental tissues used for comparison.

Fraction 1 protein (ribulose biphosphate carboxylase/oxygenase) is the most abundant soluble protein in plant leaves, and comprises of nuclear encoded small subunits and chloroplast encoded large subunits (Kung, 1976). Analysis of the subunit polypeptide composition of Fraction 1 protein therefore gives information on the nuclear and chloroplast composition of somatic hybrids between species which have characteristically different large and small subunit polypeptide compositions. Fraction 1 protein is not subject to the

same environmental variations in gene expression which may confound isoenzyme studies, as it is produced in only one form. Analysis of the Fraction 1 protein polypeptides from somatic hybrid plants between N. glauca and N. langsdorffii (Carlson et al., 1972) indicated the presence of the small subunit polypeptides (nuclear encoded) from both parental species, but only the large subunit polypeptides (chloroplast encoded) of N. langsdorffii (Kung et al., 1975). More detailed analysis of a population of somatic hybrid plants between N. glauca and N. langsdorffii has confirmed the presence of the nuclear encoded subunits from both species, but only the chloroplast encoded subunits of one of the parental species. An almost equal number of plants possessed the chloroplast encoded subunits of either N. glauca or N. langsdorffii (Smith et al., 1976; Chen et al., 1977).

The segregation of chloroplast types, indicated by Fraction 1 protein analysis has been reported in several other somatic hybrid combinations, including Petunia parodii and P. parviflora (Power et al., 1980), N. tabacum and N. rustica (Douglas et al., 1981c; Hamill et al., 1984), and N. tabacum and N. glutinosa (Uchimaya, 1982; Cooper-Bland et al., 1985a).

Chloroplast segregation does not always occur at an early stage of development of somatic hybrid plants. This can result in the regeneration or subsequent development of chimearas with different cell lineages possessing different chloroplast types. A mixed population of chloroplasts may be retained. In somatic hybrids between N. tabacum and N. sylvestris chloroplast segregation was still found to occur in the second generation progeny of this fertile somatic hybrid (Fluhr et al., 1983). Similarly "plastome heterozygotes" have been detected in somatic hybrids between N. tabacum and

N. glauca as well as between N. tabacum and N. tabacum lines bearing a foreign cytoplasm (that of N. suaveoleus, N. undulata, N. repanda or N. plumbaginifolia) (Gleba et al., 1985).

Where selective markers associated with the chloroplast are used in the isolation of somatic hybrids, the chloroplast type is inevitably that of the type favoured by selection. Thus in a comparison between single heterokaryon isolation and the use of mutant complementation incorporating a chloroplast encoded streptomycin resistance, in producing somatic hybrids between N. tabacum and N. rustica, random chloroplast segregation occurred in the absence of selection pressure. In contrast hybrids obtained on the basis of streptomycin resistance were found to possess only the N. tabacum streptomycin resistant chloroplast type (Hamill, 1983).

Although random chloroplast segregation or a mixed chloroplast population may result following the selection of hybrids in the absence of selection pressure in favour of either chloroplast type, one chloroplast type may still be preferentially incorporated into somatic hybrid plants (Kumar et al., 1982; Bonnett and Glimelius, 1983). This suggests that some chloroplasts naturally possess a selective advantage in spite of the absence of applied selection pressure.

d) Molecular Approaches to the Analysis of Somatic Hybrids

A more accurate method by which the chloroplast or mitochondrial genome can be identified is the analysis of restriction endonuclease cleavage patterns for isolated chloroplast or mitochondrial D.N.A. Using this method chloroplast segregation was found to have occurred in intraspecific somatic hybrids of N. tabacum (Belliard et al., 1978). Similarly segregation of

chloroplast types occurred in somatic hybrids between Lycopersicon esculentum and Solanum tuberosum and was identified in this way (Schiller et al., 1982). Recently recombination has been reported between the chloroplasts of a lincomycin resistant N. plumbaginifolia, and streptomycin resistant, chlorophyll deficient mutant of N. tabacum. Green lincomycin and streptomycin resistant somatic hybrids were found to have novel chloroplast types in which restriction endonuclease restriction sites specific to both parents were present (Medgyesy et al., 1985). Failure to detect chloroplast recombination in other studies (Fluhr et al., 1984) and in all somatic hybrids examined previously suggests chloroplast recombination is an infrequent event. Success in this recent investigation was probably due to the strong selective pressure in favour of the recombinant chloroplast type.

In contrast to the infrequent occurrence of chloroplast genome recombination, extensive rearrangements of the mitochondrial genome have been found in many somatic hybrids examined (Belliard et al., 1979; Nagy et al., 1981; Boeshore et al., 1983; Chetrit et al., 1985). The association of cytoplasmic male sterility (C.M.S.) with the mitochondrial genome (Galun et al., 1982) has been used as a marker for the mitochondrial genome in Nicotiana cybrids (Menczel et al., 1983).

The use of labelled D.N.A. probes has also enabled the identification and characterisation of somatic hybrids. Species specific differences in the nuclear D.N.A. restriction pattern probed with labelled r D.N.A. can be used to demonstrate nuclear hybridity (Uchimaya, 1982), and similar methods can be used with chloroplast and mitochondrial gene probes. Using these methods a regenerated plant obtained following fusion between N. tabacum and Petunia hybrida was

found to possess the chloroplast type of N. tabacum but only the nuclear r D.N.A. hybridisation pattern specific to P. hybrida. Isoenzyme analysis performed at various stages after fusion already indicated the gradual loss of N. tabacum chromosomes (Pental et al., 1985).

Species specific D.N.A. probes have also been used in the identification of a somatic hybrid cell line between Lycopersicon esculentum and L. penellii (O'Connell and Hanson, 1985).

1.3.7 Commercial Applications for Somatic Hybrid Plants

Polyploidy has played a major role in plant evolution, and at least one-third of flowering plants are thought to be of polyploid origin. The evolutionary success of polyploids is considered to be due, in part, to genetic heterozygosity incorporated into the hybrid genome (Stebbins, 1979). Allopolyploid plants are hybrids resulting from a cross between two species whose chromosomes do not pair at meiosis. After chromosome doubling an allopolyploid nucleus contains at least two distinct diploid genomes which distribute themselves independently at meiosis. Thus allopolyploid plants acquire the genetic information and diversity represented in each complete progenitor genome.

Allopolyploids are analagous with balanced somatic hybrid plants. Somatic hybridisation may contribute to plant breeding by permitting the generation of a wider range of allopolyploids since fertility between species is not required.

Although sexually fertile somatic hybrids have been generated between sexually compatible species by somatic hybridisation, somatic hybrids between sexually incompatible species have so far proved sterile (Melchers et al., 1978; Poulsen et al., 1980; Power et al.,

1980). Such plants also frequently display gross morphological abnormalities, which may be linked to chromosomal instability (Harms, 1983).

For somatic hybrids to contribute to plant breeding, fertility is an essential requirement. The observation that chromosomal instability, and unidirectional chromosome loss may lead to plant regeneration has led to speculation that limited gene transfer might be a more realistic approach to the use of somatic hybridisation. If, following fusion gene transfer can occur between the two genomes, prior to the complete elimination of one of the genomes, valuable genetic heterogeneity may be retained, and the regenerated plants may well be fertile (Negrutui et al., 1984). For this reason, asymmetric hybrids, and hybrids in which complete unidirectional chromosome elimination has occurred may be of considerable interest.

Somatic hybridisation does not always result in the formation of a hybrid nucleus. Recently the herbicide (atrazine) resistant chloroplasts from Brassica campestris, and cytoplasmic male sterility (C.M.S.) from Raphanus sativus have been combined with the nucleus of Brassica napus by protoplast fusion. This was achieved in two steps. Firstly B. napus protoplasts were fused with R. sativus protoplasts, and plants resembling B. napus but possessing male sterility were selected. C.M.S. is thought to be encoded by the mitochondrial genome (Galun et al., 1982). Protoplasts of the male sterile hybrid B. napus plants were then fused with protoplasts from B. campestris and ultimately B. napus cybrid plants possessing male sterility and atrazine resistance selected (Pelletier et al., 1983). Herbicide resistance and C.M.S. in these novel cybrids is of commercial interest in Brassica breeding programmes.

Protoplast systems are so far only available in a few crop

species. For these to be used in attempts at somatic hybridisation, suitable selection schemes must be developed for the recovery of somatic hybrid colonies or plants. Although a commercial application for cybrids has been noted, somatic hybrids have yet to contribute to the improvement of crop plants.

1.3.8 Somatic Hybridisation between N. tabacum (2n) and N. glutinosa (n)

That the synthesis of novel triploid somatic hybrids may facilitate a limited gene transfer has recently been proposed and the combination of N. tabacum (2n) with N. glutinosa (n) was chosen to test this hypothesis (See the General Introduction - 1.1). Haploid protoplasts may be obtained from anther culture derived plants, or from tetrads, formed as a result of meiosis in pollen mother cells. Both sources were investigated. However, tetrad protoplasts would appear to offer certain advantages over haploid protoplasts isolated from anther culture derived haploid plants.

Tetrad protoplasts are known to undergo fusion without the addition of specific fusogens (Bhojwani and Cocking, 1972). It has been proposed that the use of highly fusogenic protoplasts might permit high fusion frequencies to be obtained, without causing excess damage or loss of viability (Boss et al., 1984).

Haploid protoplasts isolated from anther culture derived N. tabacum plants are capable of sustained division (Caboche, 1980). Although the culture response of N. glutinosa haploid protoplasts isolated in this way has not been reported, it is likely that these too will be capable of sustained division. If this is the case, selection against the haploid protoplasts must be developed. In contrast, tetrad protoplasts are not capable of sustained division

(Bhojwani and Cocking, 1972; Bajaj, 1974), and no selection is therefore required to eliminate them.

Haploid plants from anther culture are only available for a few responsive species. In contrast, tetrads form in all fertile dicotyledonous species. It is possible therefore, that tetrads will be a widely available source of haploid protoplasts for fusion studies. Somatic hybrids resulting from protoplast fusion experiments involving tetrad protoplasts have not been reported previously. Somatic hybridisation between tetrad protoplasts and leaf mesophyll protoplasts will be attempted in this study.

CHAPTER 2

TISSUE CULTURE OF CYCLAMEN PERSICUM

2.1 INTRODUCTION

Cyclamen persicum is an attractive flowering plant grown horticulturally throughout Europe, and to a limited extent in North America. Originating from Palestine and the islands around the Aegean and eastern Mediterranean seas, Cyclamen was introduced into Europe in the 17th Century.

Cyclamen species derived their name from the Greek Kyklos - or circle, apparently referring to the round shape of the corm. The taxonomy of the genus Cyclamen remains largely unresolved due to extensive heterogeneity, however, at least 15 species have been recognised (De Haan and Doorenbos, 1951). Of these, Cyclamen persicum is the only widely grown commercial variety.

Cyclamen persicum is a herbaceous plant, with a cluster of blueish/green heart shaped ovate leaves, with silvery markings and dentate margins, on long petioles arising from flattened tubers or corms. Attractive sympetalous, five parted, strongly reflexed flowers are born above the leaf canopy on peduncles arising from the corm. Cyclamen is a pseudomonocot, in that only one cotyledon is found in the embryo. The cotyledon strongly resembles the true leaves (Widmer, 1980). In their native habitat, Cyclamen are dormant during the hot, dry summers, and new foliage develops in response to autumn rain and lower temperatures. Flowering follows, and continues until terminated by dry summer heat.

Cyclamen persicum has been the subject of several tissue

culture studies. Early reports suggested that cultured explants, originating from the corm, were capable of giving rise to shoots and roots as well as sporadic plant regeneration (Mayer, 1956; Stichel, 1959). Internal microbial contamination of the corm tissue was also noted, and antibiotics used to combat this problem (Stichel, 1959). Contamination has not always been successfully overcome with antibiotics, and alternative treatments have been developed which include curing explants prior to culture with a minimal surface area in contact with the nutrient medium (Okumoto and Takabayashi, 1969). This approach also met with limited success. In spite of the problems associated with initiating contamination free cultures of corm tissue, the long term growth of callus derived from corm explants has been reported (Leowenberg, 1969). Even after six years, the callus retained its morphogenic capability, with sporadic organ formation occurring 2-4 months after subculture.

Microbial contamination can largely be avoided using explants from leaves and petioles. Plant regeneration from petiole explants has been reported, although incomplete details were given about the procedure, and the frequency with which this response was achieved (Morel, 1975). In a comparative study between the response of corm explants, and leaf and somatic anther tissues, corm explants were found to give a superior morphogenic response. Although shoot and root formation occurred on callus derived from all three sources, only the shoots formed on corm callus were directly associated with a root system. The frequency with which regenerated structures were observed was also higher with callus derived from corm explants (Geier, 1977). Somatic anther tissues were cultured, only after the failure to stimulate pollen embryogenesis. With the addition of hormones to the culture medium, only the somatic anther tissues were

found to proliferate (Geier, 1978).

The regeneration of whole plants from Cyclamen tissue cultures led to speculation that Cyclamen might be multiplied by mass clonal propagation (Okumoto and Takabayashi, 1969; Morel, 1975; Fersing et al., 1982). The small number of plants obtained, and the overall lack of control over morphogenesis has led to the conclusion that "mass clonal propagation by tissue culture such as is possible in other plants is however, not likely to be achieved in Cyclamen, at least in the near future" (Geier et al., 1979).

Recently the regenerated structures obtained in Cyclamen tissue cultures have been examined histologically. It has been proposed that they all arise from a single embryogenic pathway. Embryogenesis may result in a normally formed bipolar corm. Alternatively, callusing may occur, resulting in a unipolar corm, which may have vascular connections with the surrounding callus tissue. If the developing embryo begins to callus before the development of a corm, shoots or roots may arise directly from the surrounding callus (Wicart et al., 1984). The regenerated structures studied were found to occur throughout Cyclamen tissue cultures in an apparently random fusion, not determined by the hormone regime under which the explants and callus were grown.

There would appear to be considerable variation in the reported response of Cyclamen tissues cultured in vitro. Plant regeneration has been achieved (Morel, 1975; Geier, 1977; Fersing et al., 1982), but Cyclamen has also been described as an "object recalcitrant in morphogenic respects" (Kohlenbach, 1976). Seasonal variations in the response of cultured Cyclamen explants have been reported (Geier, 1979). Different commercial varieties of Cyclamen have also been used for tissue culture studies, and it is likely that the genotype

of the experimental material also influences the reported response of explants in vitro.

The purpose of this study was to gain a better understanding of the culture response of explants from Cyclamen persicum cv. T.R. "mini". A knowledge of the culture requirements of somatic Cyclamen tissues may be useful in developing suitable media which will support the growth of pollen derived embryos or calli, obtained from anther culture. The response of Nicotiana tabacum and N. glutinosa tissues was also briefly assessed, since these species were also to be the subject of anther culture studies.

The isolation and culture of protoplasts from Cyclamen leaf mesophyll and cell suspension tissues was also assessed.

2.2 MATERIALS AND METHODS

2.2.1 Source of Plant Material

Cyclamen persicum cv. T.R. "mini" seed and plants were originally obtained from Thomas Rochford and Sons Ltd., Broxbourne, Herts. Seed was subsequently collected following open cross pollination in the greenhouse.

Nicotiana tabacum cv. White Burley seed was originally obtained from Rothamsted Experimental Station, and N. glutinosa L. seed from the Botanical Gardens, University of Birmingham. Seed was subsequently collected following self-pollination of bagged flower heads.

2.2.2 Growth of Plant Material

Originally batches of 50-150 mature Cyclamen persicum cv. T.R. "mini" plants, having 2-3 open flowers, were provided by Thomas Rochfords and Sons Ltd. The plants had been cultivated following standard commercial procedures, and were maintained on arrival in the controlled environment of a growth room (S.B. Refrigeration, U.K.) at $15 \pm 2^{\circ}\text{C}$ with an 8 hour photoperiod. Light was provided by daylight fluorescent tubes (Thorne, U.K.) at an intensity of 5,000-6,000 lux, measured at shelf level. Young plants, 4-6 months old, with a corm 1-2 cm in diameter and having 6-8 leaves were also provided by Thomas Rochford and Sons Ltd. This supply was augmented with plants grown from seed in Leavingtons soil-less compost (Fisons, U.K.), and maintained in the greenhouse in $3\frac{1}{2}$ inch pots. Young plants were also transferred into the growth room after bud initiation.

N. tabacum cv. White Burley, and N. glutinosa L. plants were also grown from seed in Leavingtons soil-less compost. Plants were grown in the greenhouse under a 16 hour photoperiod of approximately

10,000 lux (minimum), supplemented by daylight fluorescent tubes (Thorne, U.K.) at 23-30°C.

2.2.3 Contamination Control

Infestation of Cyclamen plants with red spider mites was prevented using 'Torque' a fenbutin oxide treatment (Albright and Wilson, U.K.). Mushroom fly larvae were efficiently controlled using 'Basudin' granules containing diazinon (Ciba-Geigy, U.K.).

Periodic infestation of N. tabacum and N. glutinosa plants with whitefly and aphids was controlled using a permethrin (Mitchel and Cotts, U.K.) and nicotine (Bentleys, U.K.) spray. Fumigation of the greenhouses with 'Fumite' permethrin (Octavius Hunt, U.K.) or nicotine (Bentleys, U.K.) was also performed as required.

All pesticides were applied following the manufacturers recommended dosages, and procedures.

2.2.4 Media Preparation and Sterilisation

Full details of media constituents are given in Appendix 1. Media based on M.S. (Murashige and Skoog, 1962) or B5 (Gamborg et al., 1968) salts were made using commercially available preparations (Flow Labs., U.K.). Other media were prepared from refrigerated stock solutions. Vitamins, hormones, a carbon source, and any organic supplements were added as appropriate. The pH was adjusted to 5.8 using 1 M KOH or 1 M HCl. For solid media 0.8% (w/v) agar (type IV, Sigma, U.K.) was dissolved in the medium.

Media were dispensed into appropriate containers and sterilised by autoclaving at 121°C with 15 psig nominal steam pressure for 15 minutes, or 115°C with 10 psig nominal steam pressure for 10 minutes.

Alternatively, media containing heat labile components were prepared at double strength and filter-sterilised using a Millipore or Sartorius 'sterilitest' filtration system with a pore size of 0.2 or 0.45 μm . In this case agar solidified media were prepared by mixing equal proportions of 1.6% (w/v) sterile agar solidified water with double strength filter-sterilised media.

2.2.5 Enzyme Preparation and Storage

Enzyme solutions were prepared by mixing weighed quantities of the relevant enzymes with CPW salts (Frearson et al., 1973; See Appendix 1), with the addition of 9 or 13% (w/v) mannitol. For protoplast isolation from greenhouse material antibiotics (400 mg/L ampicillin, Boots, U.K.; 10 mg/L gentamycin, Sigma, U.K. and 10 mg/L tetracycline, Sigma, U.K.) were also added to the enzyme solution. The pH was adjusted to 5.8, and the enzyme solution filter-sterilised, as described previously (2.2.4). Sterile enzyme solutions were dispensed into appropriate sterile containers, and stored at -20°C until use.

2.2.6 Surface Sterilisation of Experimental Material

a) Seeds

Seeds were surface sterilised by immersion in 20% (v/v) 'Domestos' (Lever Bros., U.K.) for 20-30 minutes. Surface sterilisation was followed by four washes in sterile tap water.

b) Leaves and Petioles

Leaves and petioles from greenhouse grown material were surface sterilised in 8% (v/v) 'Domestos' for 20-25 minutes. Surface sterilisation was followed by four washes in sterile tap water.

'Domestos' consists of 10.5% (v/v) commercial sodium hypochlorite, 10% (w/v) sodium chloride, 0.5% (w/v) sodium hydroxide, 0.3% (w/v) sodium carbonate, and a patented thickener, made up to volume with softened water.

2.2.7 Preparation of Explants

a) Greenhouse Grown Material

Leaves no longer than 50 mm, and their attached petioles were removed from flowering Cyclamen plants, maintained in the growth room as described previously (2.2.2). After surface sterilisation, petiole explants 10 mm long were cut, and cultured horizontally on the surface of the culture medium. Leaf explants, approximately 5 mm by 15 mm were cut, traversing the mid-vein of the leaf, following the methods of Geier, (1977).

Young fully expanded leaves of N. tabacum and N. glutinosa L. were surface sterilised and explants 10 mm by 20 mm cut and placed on the surface of the culture medium.

b) In vitro Germinated Seedlings

Surface sterilised seeds of Cyclamen persicum were placed on M.S-0 medium (See Appendix 1) and germinated in the dark at $20 \pm 1^\circ\text{C}$. Following germination, the seedlings were grown in the light (200 - 1,050 lux, $22 \pm 2^\circ\text{C}$). Young seedlings were used as a source of explants after a further 6-8 weeks of growth. Fragments of the root system 10-15 mm in length, pieces of corm approximately 3 x 3 x 5 mm, sections of petiole 10 mm in length, and leaf fragments approximately 5 x 10 mm were used as explants. No attempt was made to distinguish between the cotyledon, and the true leaves.

Germination of Cyclamen seeds was slow, taking 4-8 weeks. The effect of gibberellic acid on germination was assessed by imbibing

surface sterilised Cyclamen seed for 1 or 2 hours in a solution containing 200 mg/L gibberellic acid.

2.2.8 Culture Preparation and Conditions

A variety of different culture vessels were used, including 30 ml universal bottles, and 2 oz or 6 oz powder jars (Beatson-Clark, U.K.), which were filled with 10, 25 or 50 ml of agar solidified media respectively. Single explants were cultured in universal bottles, whereas 3 or 4 explants were cultured in 2 oz or 6 oz powder jars.

Cultures were generally maintained at $22 \pm 2^{\circ}\text{C}$ with constant illumination (200 - 1,050 lux), $25 \pm 2^{\circ}\text{C}$ with constant illumination (2,300 - 2,400 lux) or at $25 \pm 2^{\circ}\text{C}$ in the dark. A range of other culture conditions were also tested (See Results). Illumination was provided by cool white fluorescent tubes (Thorne, U.K.).

The culture media were based on those previously used for tissue culture of Cyclamen persicum, or were based on media used for callus initiation and morphogenesis of a wide range of other species.

2.2.9 Culture Response and Subculture

Cultured explants either died, or gave rise to a proliferation of callus tissue. Callus was classed as being either friable (f), in which case it was easily broken up with a spatula. Alternatively, dense callus (d) was obtained which had to be cut with a scalpel when subcultured. The callusing response of leaf and petiole explants from mature Cyclamen plants was assessed, on a variety of media, and under different culture conditions. Similarly the callusing response of root, corm, petiole and leaf explants of aseptically germinated Cyclamen seedlings was also assessed.

Callus derived from leaf and petiole explants of mature Cyclamen plants was subcultured by transferring 1-2 g fresh weight of callus to fresh medium, in 2 oz or 6 oz powder jars. The occurrence of dense nodular organised tissue (n) and the regeneration of shoots, roots or isolated leafy structures was noted. The response of friable callus derived from leaf explants to a variety of different media and hormone regimes was examined. Similarly the growth of callus derived from explants from aseptically germinated Cyclamen seedlings was also recorded.

Care was taken when subculturing callus to avoid transferring any of the original explant material.

Friable leaf callus was also used to initiate a cell suspension culture. Friable callus (5 g fresh weight) was broken up and transferred to 80 ml of liquid medium in a 250 ml erlenmeyer flask. Suspension cultures were maintained at $22 \pm 2^{\circ}\text{C}$ in the light (1,300 - 2,300 lux) on a rotary shaker (80 cycles/minutes). Suspensions, when established, were subcultured by transferring approximately 8-12 g fresh weight of cells to 70-80 ml of fresh liquid medium. This was performed every 7-10 days.

2.2.10 Protoplast Isolation and Culture

Fully expanded leaves from greenhouse grown young Cyclamen plants, were surface sterilised, and the lower epidermis removed by peeling. Peeled leaf pieces were plasmolysed for at least 90 minutes in CPW salt solution containing 13% (w/v) mannitol (CPW 13M - See Appendix 1). The CPW 13M was replaced with 10 ml of enzyme solution/ 9 cm petri dish (Sterilin Ltd., U.K.) (4 ml/5 cm petri dish [A/S Nunc, Kamstrup, Denmark]). Petri dishes were sealed with nescofilm (Nippon Shoji Kaisha Ltd., Japan) and incubated overnight (14-16 hours)

at $22 \pm 2^{\circ}\text{C}$ on a rotary shaker (30 cycles/minute) in the dark.

Following incubation, leaf pieces were teased to release the protoplasts into the enzyme solution, which was then transferred to a screw top centrifuge tube, and centrifuged at $80 \times g$ for 5 minutes. The supernatant was removed and replaced with CPW salt solution containing 21% (w/v) sucrose (CPW 21S). Following resuspension of the pellet, and centrifugation at $100 \times g$ for 8 minutes, protoplasts were collected from the surface of the CPW 21S solution, and resuspended in a known volume of CPW 13M. The protoplast density was calculated using a haemocytometer (Hawksley U.K., Model number BS 748).

Cell suspensions were also used for protoplast isolation. Cultures 3-4 days after subculture were left stationary to allow the cells to settle, and the medium removed using a pipette. 50 ml of CPW 13M was added to plasmolyse the cells, and was removed after 30-60 minutes. 20 ml of enzyme solution was added and incubation carried out overnight (14-16 hours) at $22 \pm 2^{\circ}\text{C}$ on a rotary shaker (30 cycles/minute) in the dark.

Following incubation the enzyme was passed through a $250 \mu\text{m}$ sieve, followed by a $100 \mu\text{m}$ sieve, before being transferred to a centrifuge tube. Suspension cell protoplasts were purified by flotation on CPW 21S solution as described for leaf mesophyll protoplasts.

Protoplasts isolated from leaf mesophyll tissue, and cell suspensions were cultured in liquid media, agar solidified media and also in agarose solidified media at a range of densities between 1×10^4 and 1×10^5 protoplasts/ml medium. Protoplasts were cultured in 5 cm plastic petri dishes (A/S Nunc, Kamstrup, Denmark), containing 4 ml of medium. Cultures were maintained at $25 \pm 2^{\circ}\text{C}$ in the light (2,100 - 2,300 lux) or in the dark.

2.2.11 Fluorescein Diacetate (FDA) Staining of Isolated Protoplasts

A stock solution of fluorescein diacetate (FDA, Sigma) was prepared by dissolving 5 mg in 1 ml of acetone. This solution was stored at -20°C in the dark, until used. Approximately 50 µl of this stock solution was added to 5 ml of CPW 13M and 1 drop of this solution was added to 1 drop of CPW 13M containing freshly isolated protoplasts. After five minutes at room temperature the protoplasts were observed using a Vickers M41 photoplan microscope (mercury vapour lamp HBO 200) fitted with suppressor filter GG 475 and a Balzer FITC 5 exciter filter. FDA which enters the protoplasts is broken down to fluorescein by the action of esterases. In protoplasts with an intact plasma membrane, fluorescein accumulates, giving rise to a yellow green fluorescence when excited by U.V. light (Larkin, 1976). An intact plasma membrane is an indication of viability.

2.3 RESULTS

2.3.1 Callus Initiation from Leaf and Petiole Explants of Flowering Cyclamen Plants

Leaf and petiole explants were cultured in 2 oz or 6 oz powder jars as described, and the presence or absence of callus growth recorded after 28 days. The number of explants responding, and type of callus growth produced was also noted. The results are present in Table 2.3.1 and 2.3.2.

Culture media based on full strength M.S. salts mostly failed to permit callus growth, with the exception of limited friable callus growth obtained on UM medium. The use of culture media based on M.S. salts at reduced strength was most successful for callus initiation. Failed explants were invariably blackened, and some degree of browning was also observed in most of the responding cultures.

The frequency of explants giving rise to callus was similar in the light (2,300 - 2,400 lux) and the dark. However, cultured explants incubated at 15 ± 2 or $30 \pm 2^\circ\text{C}$ gave rise to callus less frequently than explants cultured at 22 ± 2 or $25 \pm 2^\circ\text{C}$. The degree of callus growth was similar under all conditions tested, and in all cases was very limited, however, the type of callus obtained varied. Friable callus, easily broken up with a spatula, proliferated from the cut edges of the explants. Friable callus was obtained more frequently when explants were cultured in the dark, and was always obtained on UM medium. Dense callus, also originating from the cut edges of the explants was also obtained. When subcultured, this type of callus could only be broken up by cutting with a scalpel. Dense callus developed more frequently when explants were cultured in the light, and was always obtained on MS-D and N69-D3 media.

Leaf and petiole explants showed a similar response in culture, although petiole explants gave rise to callus proliferation slightly less frequently than leaf explants. Dense and friable callus was obtained from both leaf and petiole explants. Leaf and petiole explants were taken from ten Cyclamen plants selected on the basis of their widely different flower colour and leaf shape. When cultured on MS-A1, MS-A2, MS-B, MS-C and MS-D media explants taken from the ten plants were found to respond quite differently. Explants from five of the ten plants tested failed to give rise to callus growth, and the frequency with which explants responded and the degree of callus growth obtained varied considerably for cultured explants taken from the other five plants. However, when repeated using the same ten plants, a very low frequency of callus initiation was obtained. Explants from only two plants gave rise to limited callus growth. Previously explants from these two plants had given a similar response, but at a higher frequency (8 and 6/16 as opposed to 3 and 4/16).

2.3.2 Subculture and Maintenance of Callus Cultures Initiated from Leaf Explants of Flowering Cyclamen Plants

Callus initiated from leaf explants after 28 days of culture was either transferred to fresh medium in 2 oz or 6 oz powder jars, or was left for a further 14-28 days under the same conditions. Cultures left in this way continued to grow, although severe browning of the callus and discolouration of the medium occurred. Occasional roots, shoots and isolated leafy structures arose from these cultures (see Fig. 2.3.1). The frequency with which regenerated structures were obtained was low. Shoots or isolated leafy structures were obtained in 3/25 cultures, roots in 5/25 cultures. Roots and shoots were occasionally found in the same culture, but were not usually connected.

Both roots and shoots occurred on MS-A1 and MS-D media. Regenerated structures originated from areas of dense callus. No regenerated structures were observed to arise from areas of friable callus.

Friable callus when subcultured onto MS-A1, MS-A2 and MS-D media continued to proliferate, but no regenerated structures were observed. Dense callus when subcultured continued to proliferate on MS-A1, MS-A2, MS-B and MS-D media. Both friable callus and dense callus was obtained. Occasional shoots and roots regenerated from areas of dense callus. Cultures were either subcultured regularly every 28 days, or were left to grow for 35-49 days before subculture. When frequently subcultured, the dense callus became friable and no regeneration was obtained. When cultures were left to grow for upto 49 days, isolated roots, shoots and leafy structures were observed. When subcultured friable and dense sectors of callus proliferated. Further root and shoot regeneration was observed from dense callus after 28-49 days growth. Regenerated structures were obtained on all the media tested which supported the proliferation of dense callus (MS-A1, MS-A2 and MS-D).

In all cases regenerated shoots were not directly connected with a root system. Shoots were transferred to MS-0, MS-R1 and MS-R2 media in attempts to induce rooting. Although rooting occurred on MS-R1 all the shoots died.

Friable callus derived from leaf explants was used to test the response of cultured Cyclamen tissues to a variety of culture media, different carbon sources and hormone regimes. Four replicate 2 oz powder jars each with one initial callus explant of approximately 0.25g fresh weight were used. Cultures were maintained at $22 \pm 2^{\circ}\text{C}$ in the light (200 - 1,050 lux). Callus growth was visually assessed after 28 days, and the arbitrary scale of callus growth subsequently

quantified by weighing a minimum of 8 callus pieces for each size class. The results are presented in Fig. 2.3.3.

Modified M.S. based media at reduced strength, and N69 salt based media proved most effective for Cyclamen callus growth. Callus growth was promoted more effectively by combinations of 1-naphthyl acetic acid (N.A.A.) and 6-benzyl amino purine (B.A.P.) than by 3-indole acetic acid (I.A.A.) and 6-furfuryl-amino purine (kinetin). A combination of 1.0 mg/L N.A.A. and 1.0 mg/L B.A.P. was found to be most effective at stimulating Cyclamen callus growth. The addition of adenine sulphate (75 mg/L) slightly enhanced Cyclamen callus growth. Sucrose at 3% (w.v) was found to be more effective than glucose at 1,3 and 5% (w/v) and sucrose at 1 and 5% (w/v), at promoting Cyclamen callus growth.

2.3.3 Subculture and Maintenance of Callus Cultures Initiated from Petiole Explants of Flowering Cyclamen Plants

When callus initiated from leaf explants after 28 days was either transferred to fresh medium, or was left for a further 14-28 days under the same conditions, the response was much the same as that noted for leaf derived callus. When subcultured, friable callus proliferated as friable callus and no regeneration was observed. Dense callus gave rise to some dense, some friable sectors of callus. When preferentially subcultured the dense callus occasionally gave rise to regenerated shoots, roots and isolated leafy organs, after 28-49 days of culture. Shoots were not connected with a root system, and failed to root on MS-0, MS-R1 and MS-R2.

The growth of dense callus was slow, when compared to friable callus, and frequently browning of the callus and discolouration of the medium occurred, although this did not affect the regeneration

capability of the callus.

2.3.4 Establishing Cyclamen Cell Suspensions

Friable leaf callus (10g fresh weight) was transferred from MS-A1 and MS-A2 to 80 ml UM, MS-A1 or MS-A2 liquid media, and actively growing cells (approximately 10g fresh weight) transferred to fresh medium every 7-10 days. In MS-A1 and MS-A2 significant discolouration of the cell clumps occurred, and the cell cultures lost. In UM medium, similar discolouration occurred but pale actively growing clumps of cells were also observed, and preferentially transferred when sub-cultured. After 6 subcultures an actively growing pale yellow/brown cell suspension was achieved. The cells were in small clumps 2-6 mm in diameter. When transferred to solid UM media the cell clumps proliferated as friable callus. When transferred to solid MS-A1, MS-A2, MS-B, MS-C and MS-D, the clumps of cells proliferated only on MS-A1, MS-A2 and MS-D media. The callus growth was friable and no regenerated structures were observed.

2.3.5 Callus Initiation from Root, Corm, Petiole and Leaf Explants of Axenically Grown Cyclamen Seedlings

Root, corm, petiole and leaf explants were prepared and cultured as described in the Materials and Methods. Callus initiation was assessed after 28 days as for leaf and petiole explants from flowering Cyclamen plants, described previously. 10-12 explants in four replicate 2 oz jars were examined. The results are presented in Table 2.3.4.

Root explants gave rise to friable callus which proliferated on MS-A2 and MS-D media. On subsequent transfers to these, and other media, no organised structures were observed, and the callus remained

friable, (see Table 2.3.5). Explants of corm tissue responded poorly in culture, with very little callus growth, and considerable discolouration of both explant and culture medium. When subcultured the callus failed to proliferate on MS-A2 medium.

Explants of petiole and leaf tissue responded well in culture giving rise to prolific callus growth on MS-A2 medium. The callus consisted of friable and dense sectors and was pale red brown in colour, with occasional white or green sectors. The callus was transferred to MS-A1, MS-A2, MS-B, MS-C, MS-D media but prolific callus growth only occurred on MS-A1, MS-A2 and MS-D media. Subsequently, regeneration of shoots and roots occurred on a variety of media tested including MS-A1, MS-A2, MS-D, MS-E and MS-F. Although roots and shoots were occasionally found on the same callus, the shoots were not directly associated with a root system. Shoots failed to root when transferred to MS-0, MS-R1 and MS-R2 media. Regenerated structures were observed 28-42 days after subculture.

As was found previously, the regenerated structures only arose from areas of dense callus. In some cases the dense callus was more organised, consisting of nodular tissue. No regeneration was observed from friable callus.

The results of subculturing root, corm, petiole and leaf explants are presented in Table 2.3.5.

2.3.6 Callus Initiation and Morphogenesis in *Nicotiana tabacum* and *N. glutinosa*

Leaf explants taken from young greenhouse grown plants of *N. tabacum* and *N. glutinosa* were cultured as described in the Materials and Methods, and the response noted after 28 days growth. The results are presented in Table 2.3.6.

Prolific callus growth occurred from leaf explants of N. tabacum on MS-P1, MS-D3, MS-D4, MS-A1, MS-A2, MS-B and MS-D media. Shoot regeneration also occurred on MS-D3, MS-D4, MS-Z, MS-A1, MS-B and MS-D media. Slight callusing and rooting occurred on MS-P2 and MS-C media. In contrast to the results obtained with cultured Cyclamen leaf explants, all the cultured N. tabacum explants responded in culture, and the degree of callus growth obtained was very much greater. No discolouration of the explants or media was observed.

Similarly, all the cultured N. glutinosa explants responded giving rise to varying degrees of callus growth. Prolific callusing occurred on MS-P1, whereas slight callusing occurred on MS-Z, MS-D3, MS-D4, MS-A1, MS-A2, MS-B and MS-D. Slight callusing and root regeneration occurred on MS-P2 and MS-C media, and slight callusing with some shoot regeneration occurred on UM medium.

2.3.7 Cyclamen Protoplast Isolation and Culture

A range of concentrations of meicelase P (Meiji Seika Kaisha Ltd., Japan) or Cellulase R10 (Yakult Honsha Ltd., Japan) were tested in combination with macerozyme R10 (Yakult Seika Kaisha Ltd., Japan) for their ability to release leaf mesophyll protoplasts from leaves taken from young Cyclamen plants. Peeled and plasmolysed leaf pieces were floated on 4 ml of enzyme solution in a 5 cm petri dish, and incubated as described in the Materials and Methods section. To assess the efficiency with which the enzyme combinations were capable of releasing mesophyll protoplasts, the leaf tissue was gently teased to release the protoplasts into the enzyme solution. Protoplast release was assessed visually and ranked according to an arbitrary scale. The results of this assessment are presented in Table 2.3.7.

An enzyme mixture consisting of 0.5% (w/v) Cellulase R10 and

0.05% (w/v) macerozyme R10 was chosen on the basis of the preliminary assessment for use in large scale protoplast isolation experiments. Using this enzyme combination the yield ranged between 4.4×10^4 and 1.5×10^5 protoplasts/g leaf material. Protoplast viability varied independantly of yield, and was usually between 28-52% as determined by fluorescence in the presence of F.D.A. Leaf mesophyll protoplasts were cultured in MS-P19M, MS-A19M, KM-8P and KP-8 media as described in the Materials and Methods. No protoplast division was observed. When cultured in the light (2,100 - 2,300 lux) the protoplasts became bleached within two days of culture. After six days protoplasts had collapsed, and when treated with F.D.A. no longer accumulated fluorescein, indicating that a loss of membrane integrity and thus viability had occurred.

Protoplast isolation from Cyclamen cell suspensions was also investigated. A range of concentrations of pectolyase Y23 (Seishin Pharmaceutical Co. Ltd., Japan) in combination with Cellulase RS (Yakult Honsha Co. Ltd., Japan) or Cellulase R10 with the addition of 2% (w/v) driselase (Kyowa-Hakko Kogyo Co. Ltd., Japan) or Cellulase R10 with the addition of 2% (w/v) rhozyme HP150 (Rohm and Haas, U.S.A.) were tested. 2-3g wet weight of plasmolysed cell suspension cells were incubated in 4 ml enzyme/5 cm petri dish, as described in the Materials and Methods section. Following incubation, the cells were gently teased to release the protoplasts into the enzyme solution, and protoplast release visually assessed according to an arbitrary scale. The results are presented in Table 2.3.8.

A combination of 0.5% (w/v) pectolyase Y23 and 0.1% (w/v) Cellulase RS was chosen for larger scale protoplast isolations, as described in the Materials and Methods section. The protoplast yield was between 0.8 and 6.0×10^6 protoplasts/flask (approximately

5.7×10^4 - 4.3×10^5 protoplasts/g wet weight of cells). The protoplast viability was higher than found with Cyclamen mesophyll protoplasts, ranging between 35 and 65% as determined by F.D.A. When cultured in MS-P19M, MS-A19M, KM-8P and KP-8 as described in the Materials and Methods Section, no division was observed. Protoplasts remained spherical for up to 4 days, indicating no cell wall synthesis had occurred, but by 6 days of culture had collapsed, and were no longer viable as determined by F.D.A.

FIGURE 2.3.1

Shoots and isolated leaf-like structures regenerating from leaf explants of mature Cyclamen plants.

- 2.3.1 - A : Isolated leaf-like structures arising from a leaf explant after 7 weeks culture on MS-A2 medium. Note the limited callus proliferation from the cut edge of the explant, which is still visible, and green.
(X 1.4)
- 2.3.1 - B : Shoot regeneration from leaf callus subcultured on MS-D medium and grown for a further 6 weeks. Note the browning of the callus.
(X 1.5)
- 2.3.1 - C : Shoot and root regeneration from leaf callus subcultured on MS-A1 medium and grown for a further 7 weeks.
(X 1.5)
- 2.3.1 - D : Shoot regeneration from a leaf explant after 7 weeks on MS-A1 medium.
(X 2.0)

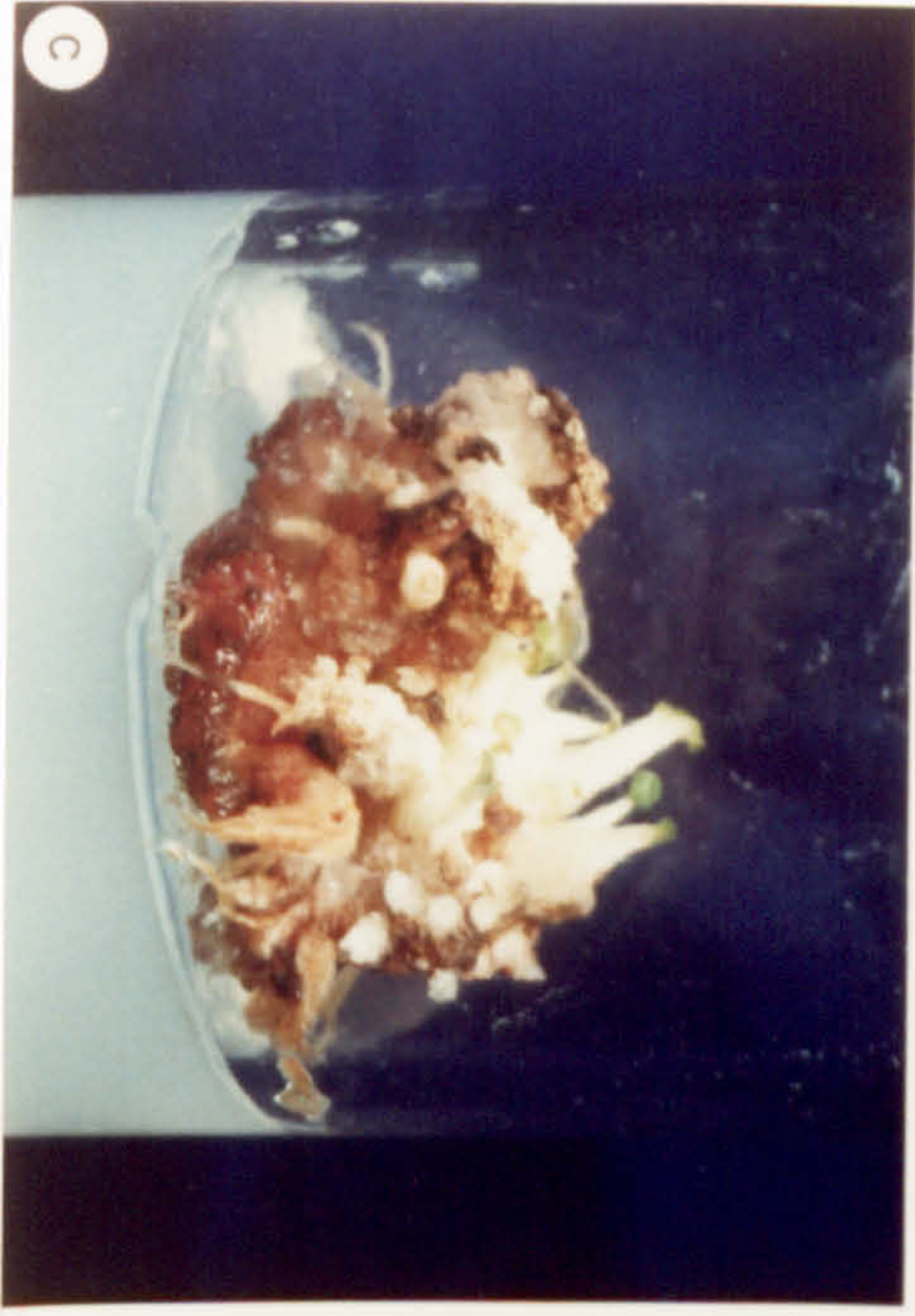


FIGURE 2.3.2

Callus growth and shoot regeneration from cultured leaf and petiole explants of aseptically germinated Cyclamen seedlings.

2.3.2 - A : Friable callus from a leaf explant after two passages on MS-A1 medium.

(X 1.5)

2.3.2 - B : Nodular callus from a leaf explant after two passages on MS-E medium. Note the apparent shoot formation on some of the nodular structures.

(X 1.5)

2.3.2 - C : Nodular callus from a petiole explant after three passages on MS-D medium. Again, note the apparent shoot or leaf regeneration from some of the nodular structures.

(X 1.5)

2.3.2 - D : Shoot regeneration from nodular leaf callus after three passages on MS-E medium.

(X 1.5)

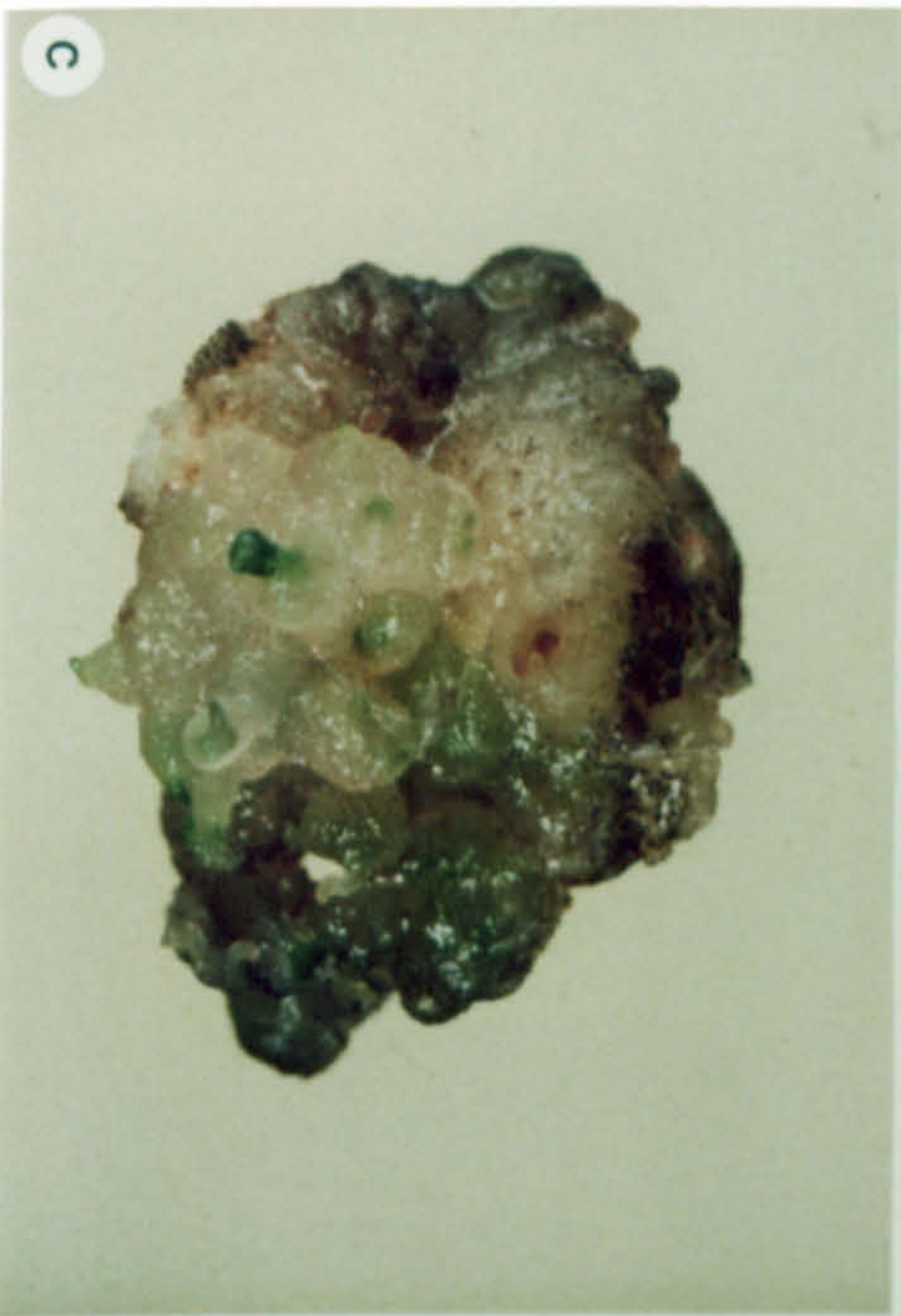
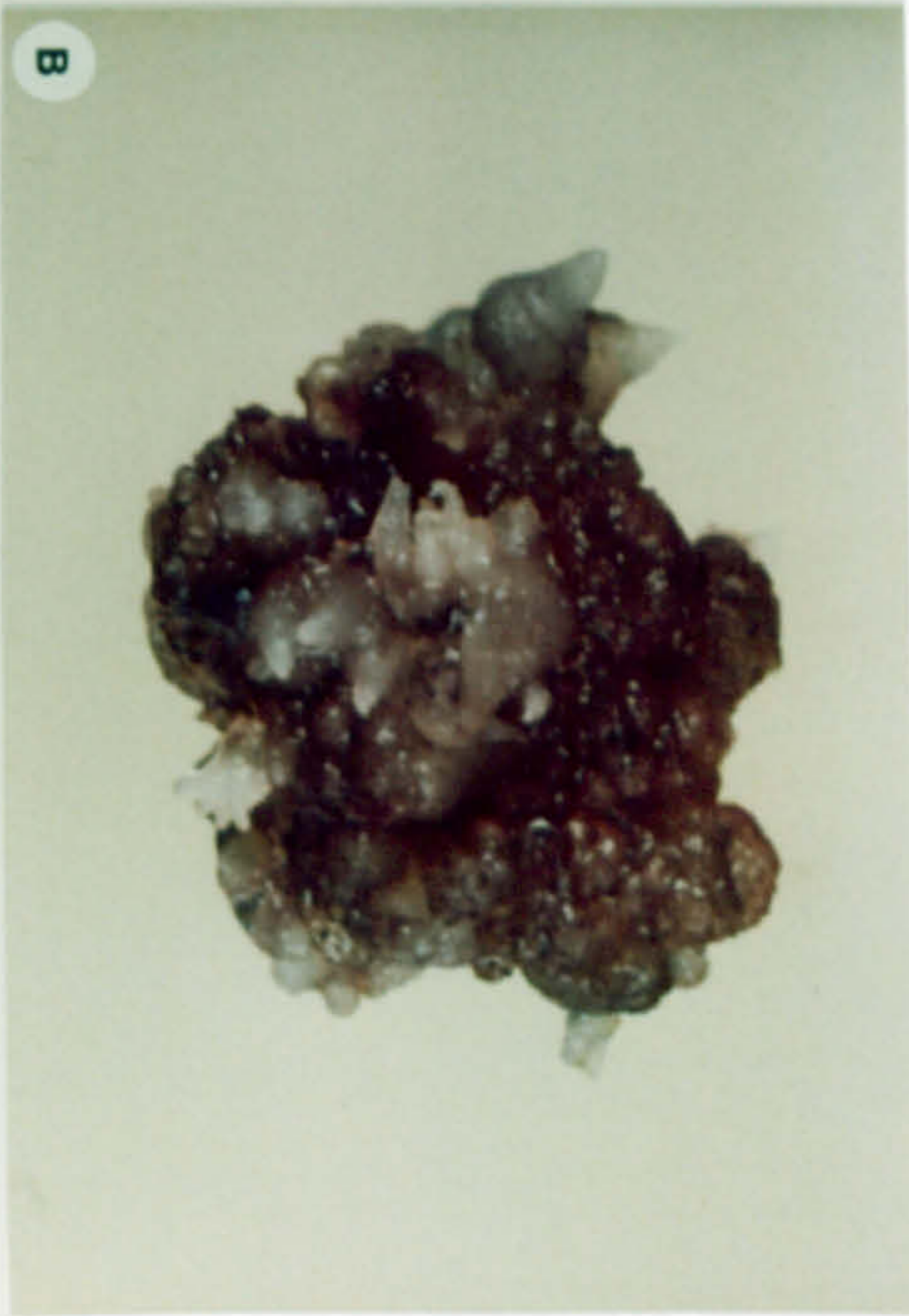
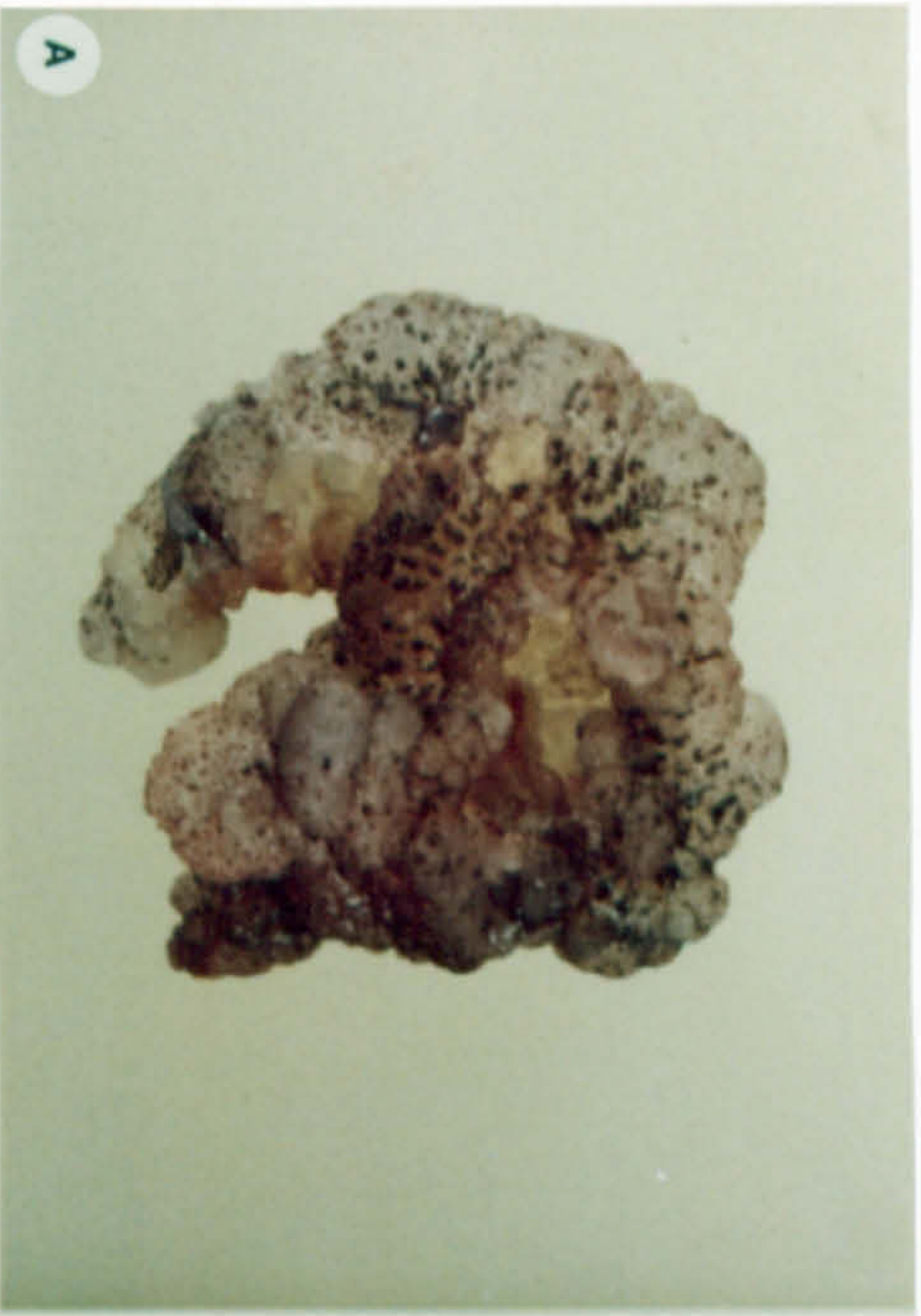


Table 2.3.1 : Callus initiation from leaf and petiole explants from flowering Cyclamen plants after 28 days of culture.

a) 25 \pm 2°C in the light (2,300 - 2,400 lux).

Medium	Leaf			Petiole		
	Response	Frequency	Type	Response	Frequency	Type
MS-0	-			-		
MS-P1	-			-		
MS-D3	-			-		
MS-D4	-			-		
UM	+	2/18	f	+	3/18	f
N69-P1	+	2/12	d	+	2/12	f
N69-D3	+	1/12	d	-		
MS-A1	+	4/12	d	+	3/11	f
MS-A2	+	5/12	d	+	4/12	d
MS-B	-			-		
MS-C	+	2/12	f	-		
MS-D	+	4/12	d	+	2/12	d

b) 25 \pm 2°C in the dark

Medium	Leaf			Petiole		
	Response	Frequency	Type	Response	Frequency	Type
MS-0	-					
MS-P1	-			-		
MS-D3	-			-		
MS-D4	-			-		
UM	+	3/17	f	+	1/18	f
N69-P1	+	3/12	f	+	2/11	f
N69-D3	+	1/12	d	-		
MS-A1	+	4/12	f	+	3/12	f
MS-A2	+	4/12	f	+	2/12	f
MS-B	-			-		
MS-C	-			-		
MS-D	+	3/12	d	+	4/12	d

Table 2.3.2 : Callus initiation from leaf explants from flowering
Cyclamen plants after 28 days of culture.

Temperature(°C) Light intensity(lux)	Leaf - MS-A2			Leaf - MS-D		
	Response	Frequency	Type	Response	Frequency	Type
15 ± 2 0 }	+	1/12	f	+	2/12	f
22 ± 2 0 }	+	5/12	f/d	+	3/12	d
25 ± 2 0 }	+	5/12	d	+	2/12	d
30 ± 2 0 }	-			-		
22 ± 2 200 - 1,050 }	+	4/12	d	+	3/12	d
25 ± 2 2,300 - 2,400 }	+	3/12	d	+	2/12	d
30 ± 3 3,200 - 3,400 }	+	1/12	d	-		

Table 2.3.3 : The growth response of Cyclamen callus derived from cultured leaf explants from flowering Cyclamen plants.

A) The effect of the media composition.

Medium	Hormone Regime	
	A2	D
MS (modified)	5,5,5,4	4,4,3,4
MW	1 2 2 1	3 1 3 3
B5	0 1 1 1	1 2 0 1
N69	3 4 4 5	3 3 4 4
L	1 2 0 2	3 0 1 2

B) The effect of the hormone regime and combination, with and without the addition of adenine sulphate (75 mg/L).

+ Adenine sulphate		NAA (mg/L)			
		0.01	0.1	1.0	10.0
BAP (mg/L)	0.01	1	1	1	1
	0.1	2	4	3	1
	1.0	2	4	5	1
	10.0	1	1	1	1

- Adenine sulphate		NAA (mg/L)			
		0.01	0.1	1.0	10.0
BAP (mg/L)	0.01	0	1	1	1
	0.1	1	2	3	1
	1.0	1	3	5	1
	10.0	1	0	1	1

(The result indicates the overall response when all four replicates were considered)

Table 2.3.3 : continued ...

+ Adenine sulphate		IAA (mg/L)			
		0.01	0.1	1.0	10.0
Kinetin (mg/L)	0.01	0	1	0	0
	0.1	1	1	2	1
	1.0	1	2	2	1
	10.0	1	2	1	1

- Adenine sulphate		IAA (mg/L)			
		0.01	0.1	1.0	10.0
Kinetin (mg/L)	0.01	0	0	0	0
	0.1	1	1	1	1
	1.0	1	1	2	1
	10.0	1	1	1	0

C) The effect of the carbon source (callus growth on MS-A2).

Carbon Source	Concentration % w/v	Growth Response
Sucrose	1	1,3,2,1
Sucrose	3	4,5,4,5
Sucrose	5	4,3,3,4
Glucose	1	2,1,2,2
Glucose	3	3,3,4,4
Glucose	5	3,2,3,2

D) Key to the growth response.

Code	Mean Fresh Weight (g)	S.D.
1	0.3	0.1
2	0.8	0.2
3	1.3	0.3
4	2.2	0.1
5	4.0	0.2

Table 2.3.5 : The response of root, corm, petiole and leaf callus initiated on MS-A1 media to subsequent subculture.

a) Root Callus

Passage number	Medium					
	MS-A1	MS-B	MS-C	MS-D	MS-E	MS-F
1	f	-	-	-	-	-
2	f	-	-	-	-	-
3	f	f	x	f	-	-
4	f	-	-	f	f	x
5	f	-	-	f	f	-

b) Corm

Passage number	Medium					
	MS-A1	MS-B	MS-C	MS-D	MS-E	MS-F
1	F	-	-	X	-	-
2	X	-	-	-	-	-

c) Petiole

Passage number	Medium					
	MS-A1	MS-B	MS-C	MS-D	MS-E	MS-F
1	d					
2	d/n					
3	n/l	n/l	n/r	n	-	-
4	n/l	n	d	n/l	n/l	-
5	n/r/l	-	-	n/s	n/s/r	n/s/r
6	f/n/s	-	-	n/s/r	n/s/r	d/n/s/r

Table 2.3.5 continued ...

d) Leaf

Passage number	Medium					
	MS-A1	MS-B	MS-C	MS-D	MS-E	MS-F
1	d	-	-	-	-	-
2	d	f	f	d	-	-
3	d	f	f	d	-	-
4	d	-	-	d	n/r	n/r
5	d	-	-	d/s/r	n/s/r	d
6	d/r	-	-	d/s/r	n/s/l	n/s/l

KEY

Callus response	f	-	friable	
	d	-	dense	s - shoots
	n	-	nodular	r - roots
				l - isolated leafy structures
	x	-	no growth	
	-	-	not tested	

Callus initiated on MS-A1 medium was transferred to MS-B, MS-C and MS-D. Callus proliferating on MS-D was subsequently transferred to MS-E and MS-F.

Table 2.3.7 : The effect of different enzyme combinations and concentrations on the release of Cyclamen leaf mesophyll protoplasts.

		Meicelase P % (w/v)			
		0.5	1.0	2.0	3.0
macerozyme R10 % (w/v)	0.01	-	+	++	+
	0.05	++	++	+	-
	0.10	+	++	++	+++
	0.15	+++	+++	++	++

		Cellulase R10 % (w/v)			
		0.5	1.0	2.0	3.0
macerozyme R10 % (w/v)	0.01	+	++	+++	+++
	0.05	++	+++	+++	+++
	0.10	++	+++	+++	+++
	0.15	++	+++	+++	+++

KEY

-	no protoplast release	
+	minimum	} protoplast release
++		
+++	maximum	

Table 2.3.8 : The effect of different enzyme combinations and concentrations on the release of Cyclamen cell suspension protoplasts.

		Pectolyase Y23 % (w/v)				
		0.01	0.1	0.5		
Cellulase RS	0.1	+	++	+++		
% (w/v)	0.5	+	++	+++		
	1.0	+	++	+++		
Cellulase R10	0.1	++	++	+++	}	+ 2 % (w/v) driselase
% (w/v)	0.5	+	++	++		
	1.0	++	++	+++		
Cellulase R10	0.1	+	+	+	}	+ 2 % (w/v) rhozyme HP 150
% (w/v)	0.5	+	+	+		
	1.0	+	+	++		

KEY

- no protoplast release
- +

minimum
- ++
- +++

maximum
- } protoplast release

2.4 CONCLUSION

Leaf and petiole explants taken from flowering Cyclamen plants responded poorly to tissue culture. The frequency with which callus initiation was obtained was low, and the callus grew very slowly. Significant browning of the callus and discolouration of the medium was observed. Callus obtained from leaf and petiole explants was either friable, and was not observed to undergo regeneration, or was dense, and regenerated shoots, roots and isolated leaf-like organs at low frequency. There did not appear to be a close correlation between root or shoot regeneration and the hormone regime under which the callus was grown. Although roots and shoots were obtained from the same culture, the shoots were not directly associated with a root system, and attempts to root the shoots failed.

Explants taken from axenically grown Cyclamen seedlings were more responsive in culture, giving rise to callus more frequently. The callus growth was faster, and the tendency to undergo browning, and for media discolouration to occur was much reduced. Occasional roots and shoots were also regenerated, however, as was the case with explants from flowering Cyclamen plants. Shoots were not directly associated with a root system, and rooting was not successfully achieved. The callus growth was either friable, or dense, and the dense callus often gave rise to nodular organised tissue.

All Cyclamen tissues were found to grow best in culture on media based on modified M.S. salts at reduced strength, or N69 medium. A cell suspension was successfully established using liquid UM medium based on full strength M.S. salts.

Cyclamen leaf mesophyll and cell suspension protoplasts were successfully isolated, but did not undergo division under the media and culture conditions tested.

CHAPTER 3

ANTHER CULTURE OF CYCLAMEN PERSICUM, NICOTIANA TABACUM AND N. GLUTINOSA

3.1 INTRODUCTION

Anther culture is the most effective method by which normal pollen development can be disrupted, and pollen derived haploid plants recovered (Chih-Ching Chu, 1982). The advantage of using anther culture derived haploid and dihaploid plants in the production of a more uniform Cyclamen crop has been discussed in the General Introduction. In previous attempts at recovering haploid Cyclamen plants by anther culture, particular attention has been given to the composition of the anther culture medium. However, no androgenetic response was observed (Geier, 1978). As an alternative approach in this study, emphasis was placed on stress treatments performed on excised buds prior to anther culture. Such stress pre-treatments are frequently reported in successful accounts of anther culture in other species, and usually take the form of low or high temperature incubation periods (see Chapter 1.2.3a). Many factors are thought to influence the response of anthers in culture. To assess the efficiency of the basic approach to Cyclamen anther culture, and the culture procedures and conditions employed, parallel experiments were performed using anthers from Nicotiana tabacum cv. White Burley. This variety was chosen since it is known to be highly responsive to anther culture, and the influence of bud pre-treatment and culture conditions on the recovery of pollen derived plants has been reported (Dunwell, 1979; Sunderland and Roberts, 1979). In N. tabacum, the stage of pollen development within the anthers prior to culture, or bud stress pre-treatments, is known to

influence the subsequent anther culture response (Dunwell, 1976; Sunderland and Roberts, 1979; see also Section 1.2.3b). Pollen development was therefore studied in Cyclamen and N. tabacum, in order that anthers containing pollen at a known developmental stage were cultured, or subjected to pre-treatments within the bud.

The potential use of haploid protoplasts in somatic hybridisation was also discussed in the General Introduction. Although tetrad protoplasts would appear to be ideal for protoplast fusion studies, they have not been shown to undergo sustained division, and no somatic hybrid plants have been reported using tetrad protoplasts as one fusion partner. Attempts at recovering somatic hybrids between N. tabacum leaf mesophyll protoplasts (2n) and N. glutinosa tetrad protoplasts (n) will be discussed in Chapter 5. Haploid N. glutinosa plants have been recovered by anther culture (Nitsch, 1972; Nakamura and Itagaki, 1973; Nakamura et al., 1974; Tomes and Collins, 1976). For this reason anther culture was also attempted with N. glutinosa in order to recover haploid plants which may be suitable for protoplast isolation.

3.2 MATERIALS AND METHODS

3.2.1 Source and Growth of Plant Material

Flowering Cyclamen persicum cv. T.R. "mini" plants were grown from seed, or provided by Thomas Rockford and Sons Limited. The plants were grown and subsequently maintained in the controlled environment of a growth room as described in Chapter 2.2.2.

3.2.2 Analysis of Pollen Development

Pollen development was examined in Cyclamen, N. tabacum and N. glutinosa. Buds were removed and measured before the anthers were individually removed and gently squashed and stained in acetocarmine. The development stage of the pollen was assessed under bright field illumination of a Vickers M41 fotoplan microscope. The correlation between bud length and pollen developmental stage was assessed, and the synchrony of pollen development between the anthers from same bud noted. The time course of pollen development was estimated by measuring a minimum of eight buds over a number of days. The buds were initially chosen at the pollen mother cell or tetrad stage of development, as judged by the bud length.

3.2.3 Preparation of Acetocarmine Stain

A 2.5% solution of acetocarmine stain was prepared by the addition of 2.5g carmine (B.D.H.) to 100 ml of 45% (v/v) glacial acetic acid. This solution was thoroughly shaken and boiled for 90 minutes in a conical flask fitted with a reflux condensor. The stain solution was filtered through Whatman number 1 filter paper and stored at room temperature until use. Developing pollen grains were stained by gently squashing the anther contents into 2 drops of acetocarmine solution on a microscope slide. The pollen was observed after five minutes.

3.2.4 Media Preparation and Sterilisation

Full details of media constituents are given in Appendix 1. Media based on M.S. salts (Murashige and Skoog, 1962) and Nitsch's medium 'H' (Nitsch and Nitsch, 1969) were prepared using commercially available preparations (Flow Labs., U.K.). Additional vitamins, hormones, a carbon source and activated charcoal (Sigma, neutralised) were added as appropriate. The pH was adjusted, and media dispensed into 100 ml medical flats, and sterilised as described in Chapter 2.2.4.

3.2.5 Bud Selection, Bud Pretreatment and Anther Culture Conditions

Buds containing pollen at the appropriate stages of development for anther culture or bud pre-treatment were selected on the basis of bud length. The buds were harvested, measured, and grouped according to size prior to surface sterilisation in 10% domestos (Lever Bros., U.K.) for 20 minutes, followed by four washes in sterile tap water. The surface sterilised buds were either dissected, and the anthers cultured, or were placed intact in 9 cm petri dishes (Sterilin Ltd., U.K.) 5-10 buds per dish. The dishes were carefully sealed with nesco-film (Nippon Shoji Kaisha Ltd., Japan) and incubated in the dark at the appropriate pre-treatment temperature for 4, 8 and 12 days. For each experiment four replicates were usually initiated. Bud harvests were performed sequentially over a 16 day period such that a typical experiment would start with the selection of buds for 12 day pre-treatments on days 1-4. Buds for 8 day pre-treatments were selected on days 5-8 and for 4 day pre-treatments on days 9-12. On days 13-16 buds were selected for direct anther culture, and these in addition to buds pre-treated for 4, 8 and 12 days were dissected and the anthers cultured. In this way, over the 16 day period four replicate experiments were initiated such that all the anthers within each replicate were cultured

on the same day permitting direct comparison between the affect of different bud pre-treatment times.

Anthers were cultured individually in the wells of plastic multi-well grids (5 x 5 format 100 mm square) (Sterilin Ltd., U.K.) each well containing 2.5 ml of agar solidified medium. Initially anthers were cultured in the dark at $25 \pm 2^{\circ}\text{C}$ for 28 days, followed by a further 28 days at $25 \pm 2^{\circ}\text{C}$ in the light (2,100 - 2,300 lux).

3.2.6 Data Collection and Recovery of Anther Culture Derived Plants

After 28 days in the light, the cultured anthers were examined under a dissecting microscope for the presence of macroscopic embryos or plantlets. The number of anthers giving rise to embryos or plantlets was recorded, as was the actual number of embryos or plantlets emerging from each responding anther. Anthers which failed to respond in culture were sampled, and the anther contents stained in acetocarmine and examined microscopically.

In the case of N. tabacum, a random sample of 100 emerging plantlets were individually transferred to solid MS-0 medium in 6 oz powder jars. These plantlets grew rapidly, and a number were examined cytologically to determine their somatic chromosome complement.

3.2.7 Cytological Observations

Young plants derived from anther culture and maintained on MS-0 medium under a constant illumination of 2,300 lux at $25 \pm 2^{\circ}\text{C}$ were examined. Under these conditions the plants developed a fine but rapidly growing root system. The uppermost 3-4 cm of these plants was aseptically removed, and transferred to fresh MS-0 medium, in which the cut stem rapidly grew a new root system. The remaining roots in the previous jar were carefully extracted from the agar and placed in 3 ml

of a solution consisting of 0.03% (w/v) 8-hydroxyquinoline (Sigma) and 0.05% colchicine (Sigma). The roots were incubated in this solution at 15°C for 5-6 hours, and then at 4°C for a further 2 hours. The roots were subsequently fixed overnight in freshly prepared acetic ethanol (1:3). Fixed roots were hydrolysed for 6-8 minutes in 5 M HCl at room temperature, and were transferred to a small volume of feulgen solution. After 30-40 minutes the extreme 2 mm of the root tips, stained purple, were removed and placed on a microscope slide in two drops of acetocarmine stain. A cover slip was placed on top of the root tips, and gentle pressure applied to spread the cells. The acetocarmine helped in the location of the root tip squashes when examined microscopically under bright field illumination.

Chromosomes were stained purple as a result of the complexing of aldehyde groups (from hydrolysed DNA) with the feulgen reagent. The somatic chromosome number was determined by counting the number of chromosomes contained within the area of the cell. Several chromosome counts were made for each plant assessed, and where possible chromosome squashes photographed.

3.2.8 Preparation of Feulgen Stain

0.5 g of basic fuchsin (B.D.H.) was added to 100 ml of boiling water, and stirred until dissolved. This solution was cooled to 50°C and 1.0 g of sodium metabisulphite, and 10 ml of 1M HCl were added. This solution was shaken and left to stand overnight in a loosely stoppered bottle. The following morning 1.0 g of activated charcoal was added, the solution shaken, and then filtered through Whatman number 1 filter paper, giving a colourless liquid. The feulgen stain thus prepared was stored at 4°C in the dark for up to 6 weeks.

3.3 RESULTS

3.3.1 Pollen Development in Cyclamen persicum, Nicotiana tabacum and N. glutinosa

Pollen development in Cyclamen, N. tabacum and N. glutinosa followed the typical pattern as described in the introduction (Chapter 1.2.1). Meiosis occurred within the pollen mother cells with a very high degree of synchrony, and resulted in the formation of tetrads consisting of four haploid spores bound within a thick wall presumably composed of callose. In N. tabacum and N. glutinosa tetrad formation was highly uniform, and no aberrant structures were observed. In most of the Cyclamen buds examined at the tetrad stage, tetrad formation was also very uniform. However, aberrant structures were occasionally observed at high frequency within particular buds. Dyads, consisting of apparently undreduced spores, and 'tetrads' containing additional microspores occurred (see Figure 3.3.1). All three species possess five anthers in each bud. At meiosis all five anthers were found to contain pollen mother cells at a similar stage of development. Where tetrads were observed, all five anthers were usually found to contain tetrads. N. tabacum and N. glutinosa tetrads were similar in size, between 30-40 μm in diameter. Cyclamen tetrads were somewhat smaller, at 16-18 μm in diameter.

The dissolution of the presumably callose wall of the tetrad resulted in the release of uninucleate microspores which initially lacked a vacuole. At this stage, Cyclamen microspores possessed a prominent centrally located nucleus. Vacuole formation causes the nucleus and most of the cytoplasm to be displaced to one pole of the developing microspore. In N. tabacum and N. glutinosa the microspore became oval in shape, and prominent sculpturing of the wall was observed. The uninucleate (stage 2-3) microspores stained poorly with

acetocarmine.

First pollen mitosis resulted in the formation of the generative and vegetative nuclei. This division was much less synchronous than meiosis, with uninucleate, early binucleate and mitotic microspores present within the same anther of Cyclamen and N. tabacum. Mitosis was not observed in N. glutinosa. The synchrony between the anthers within a Cyclamen bud was poor at this stage, whereas various stages of mitosis were usually observed in all five anthers within an N. tabacum bud.

In N. tabacum and N. glutinosa the early binucleate pollen grains (stage 5) were much more heavily stained with acetocarmine, and the presence of a degenerating vacuole, and two distinct nuclei could not always be observed. Similarly in Cyclamen, the increased staining and small size of the pollen made observation difficult. More mature binucleate pollen grains (stage 6) were however, characterised by an apparently uniform densely stained cytoplasm. At this stage N. tabacum and N. glutinosa pollen measured 32-36 μm in diameter, and Cyclamen pollen 15-16 μm in diameter. Cyclamen pollen was found to be much more numerous, with each anther containing $1.0 - 1.4 \times 10^6$ pollen grains. N. tabacum and N. glutinosa anthers contained $3.8 - 4.2 \times 10^4$ pollen grains.

The development of Cyclamen, N. tabacum and N. glutinosa pollen is illustrated in Figures 3.3.1, 3.3.2, 3.3.3 and 3.3.4.

The correlation between bud length, and stage of pollen development was poor with Cyclamen. Buds measuring 12 mm in length contained pollen ranging from tetrads (stage 1) to early binucleate pollen (stage 5). In general, however, buds measuring 6-12 mm were most likely to contain tetrads or early uninucleate microspores (stage 1), buds 12-14 mm uninucleate pollen (stages 2-3) and buds 14-22 mm mitotic microspores, and binucleate pollen (stages 4-6). Based on these

observations, and the growth of Cyclamen buds against time (see Figure 3.3.5) the sequence of development from tetrads to binucleate pollen grains occurred in approximately 12 days.

The correlation between bud length and stage of pollen development, was much more accurate in N. tabacum and N. glutinosa. In N. tabacum, tetrads were found in buds 11-13 mm long, uninucleate microspores in buds 14-17 mm long, and pollen at mitosis in buds 18-19 mm long. Early binucleate pollen was observed in buds 18-24 mm in length, and mature binucleate pollen in buds 23 mm and larger. The development from tetrads (stage 1) to binucleate pollen (stage 6) took approximately 4-5 days based on the growth of N. tabacum buds (see Figure 3.3.5). Similarly, N. glutinosa buds 13-15 mm in length contained tetrads, 15-17 mm in length uninucleate microspores, 17-20 mm in length early binucleate microspores, and 19 mm and greater mature binucleate pollen. The sequence of development from tetrads (stage 1) to binucleate pollen (stage 6) took approximately 5 days, again based on bud growth (Figure 3.3.5).

3.3.2 Anther Culture of *Nicotiana tabacum*

Four replicate experiments were performed to assess the culture response of N. tabacum anthers following 0,4,8 and 12 day pre-treatments at 4 - 6.5°C. Buds were selected on the basis of length, and two groups identified. The first, measuring 17.9 ± 1.0 mm contained pollen at the uninucleate stage, or mitosis (stages 2-4). The second group, measuring 20.3 ± 1.4 mm contained early binucleate pollen grains (stage 5). Anthers were cultured on solid NH medium as described in the Materials and Methods.

After culture for 28 days in the dark, followed by 28 days in the light, the anthers were examined. The total number of plated anthers

(T.A.), number of anthers responding in culture(T.R.),and number of macroscopic embryos/plantlets emerging from each responding anther (T.E.)was recorded. From this information three values were calculated which describe the anther culture response :

- 1) Induction Frequency (I.F.) - The frequency with which anthers gave rise to a culture response, expressed as a percentage of the total number of plated anthers.
- 2) Anther Productivity (A.P.) - The mean number of embryos/plantlets emerging from each responding anther.
- 3) Efficiency (E) - The product of the induction frequency and anther productivity, gives an indication of the expected recovery of anther culture derived embryos/plantlets for 100 cultured anthers.

The results of the four replicate experiments are presented in Table 3.3.1. Wide variation was observed between the four replicates. The values of the induction frequency, anther productivity and efficiency have also been calculated, based on the cumulative data of total anthers, total responding anthers and total number of embryos/plantlets. This information is also presented in Table 3.3.1, and graphically in Figure 3.3.6.

From the results, it is clear that an anther culture response was achieved for both of the bud size classes chosen, with and without bud pre-treatment at 4 - 6.5°C for up to 12 days. The data obtained for each of the four replicates of an experimental treatment shows considerable variation, especially when the efficiency is considered.

From the cumulative results given in Figure 3.3.6 it would appear that anthers containing uninucleate or mitotic microspores gave a culture response superior to that obtained with anthers containing

early binucleate pollen, when the anthers were directly cultured, or pre-treated for 12 days. However, the reverse was true when the anthers were cultured after 4 or 8 days pre-treatment. Anthers containing binucleate pollen when pre-treated for 8 days at 4 - 6.5°C gave an efficiency which was almost twice the value obtained for anthers containing uninucleate or mitotic microspores under the same conditions. The majority of responding anthers gave rise to 1-10 embryos, as can be seen from the frequency histogram (Figure 3.3.7).

To clarify the effect of the stage of pollen development on the induction frequency, anther productivity and efficiency after 8 days pre-treatment at 4 - 6.5°C a wider range of bud sizes were selected for culture. A minimum of ten anthers for each size class were floated on the surface of 2.5 ml of liquid NH medium individually in the wells of plastic multiwell grids, and were cultured as described in the Materials and Methods. Liquid culture medium was chosen, since it enabled the number of embryos/plantlets to be counted more easily. Using this method embryos of various stages of development could be identified after 28 days of culture in the dark (see Figure 3.3.9 A, B and C). After a further 28 days in the light, the cultures were examined, and the number of anthers responding and embryos/plantlets per responding anther noted. The results are presented in Table 3.3.2, and Figure 3.3.8.

The greatest induction frequency was achieved for buds 25.0 ± 1.0 mm in length, and highest yield for buds 20.8 ± 1.1 mm in length. When the efficiency is considered, a very sharp peak in the response was observed for buds 20.8 ± 1.1 mm in length, corresponding to early binucleate pollen.

3.3.3 Characterisation of the Plantlets Derived from N. tabacum

Anther Culture

100 small plantlets were randomly selected from the N. tabacum anther cultures, and individually grown on solid MS-0 medium in 6 oz powder jars. Of these, 44 were examined cytologically. 43 were found to possess the allodihaploid somatic chromosome number of $n = 2x = 24$. One plant was found to possess the normal allotetraploid somatic chromosome number of $2n = 4x = 48$ (See Figure 3.3.10).

10 of the allodihaploid plants were selected at random and transferred to soil-less compost in 3½ inch pots, and were maintained in a mist propagator for 7 days prior to being grown to maturity in the open greenhouse. The 10 plants flowered, but no seed set was observed, and no functional pollen was produced. When compared to normal allotetraploid N. tabacum cv. White Burley plants, the allodihaploids were found to vary in a number of ways. When flowering plants were compared the allodihaploids were considerably shorter, measuring 84-95 cm, as compared to 108-116 cm for the normal allotetraploids (see Figure 3.3.9 E). The flowers were also smaller, measuring 4.9 ± 0.2 cm and 6.1 ± 0.1 cm respectively. However, the flower colour was the same (Royal Horticultural Society colour chart number 62 A), and the flower corolla width/flower length ratio was very similar (0.16 ± 0.02 and 0.15 ± 0.01 for the allodihaploid and allotetraploid plants respectively). The leaf shape of the allodihaploids was quite different to the allotetraploids, being much more narrow (width/length ratio of 0.35 ± 0.02 as compared to 0.55 ± 0.06 for the allotetraploid).

After flowering, the plants were cut back and repotted to encourage side shoot development. When the plants again came to flowering, one of the flowering shoots was found to be fertile, and set seed.

3.3.4 Anther Culture in *Cyclamen persicum*

Four replicate experiments were performed to test the culture response of Cyclamen anthers following 0, 4, 8 and 12 days pre-treatment at a temperature of 4 - 6.5°C. Due to the poor correlation between bud size and the stage of pollen development within the bud, one size group was chosen. Buds measuring between 12 and 16 mm were selected since buds of this size group were most likely to contain pollen between the uninucleate and early binucleate stages of development (2-5). Anthers were cultured on solid NH medium, or solid NH medium with the addition of 1.0% (w/v) activated charcoal (Sigma, neutralised), or solid MS-R1 medium, as described in the Materials and Methods.

Four replicate experiments were also performed to test the culture response of Cyclamen anthers following 0, 4, 8 and 12 days of pre-treatment at a temperature of $15 \pm 2^\circ \text{C}$ and also $25 \pm 2^\circ \text{C}$. Buds selected for culture also measured between 12 and 16 mm, and the anthers were cultured on solid MS-R1 medium.

After 28 days of culture in the dark, followed by 28 days culture in the light, the anthers were examined for evidence of macroscopic embryos/plantlets. A random selection of five anthers which showed no culture response was made, and the anther contents gently squashed into acetocarmine, and examined microscopically.

The results of this investigation are presented in Table 3.3.3. Data for all four replicates has been combined.

No macroscopic embryos or plantlets were observed emerging from the cultured Cyclamen anthers. Many of the cultured anthers were brown, and discolouration of the medium frequently occurred. The addition of activated charcoal (1.0% (w/v)) to the medium reduced the browning, but did not permit any anther culture response. When the

anther contents of five randomly selected anthers was examined, no microscopic embryo-like structures were observed. Only dead pollen, devoid of internal structure was observed. This was true for all of the experimental treatments applied.

3.3.5 Anther Culture of *Nicotiana glutinosa*

Two replicate experiments were performed to test the culture response of *N. glutinosa* anthers following 0, 4 and 8 days of pre-treatment at 4 - 6.5°C. Buds were selected on the basis of bud length, and were grouped as follows :

- 1) Bud length 14.4 ± 1.9 mm; Stage 1
- 2) Bud length 15.8 ± 1.4 mm; Stage 2-3
- 3) Bud length 17.3 ± 1.2 mm; Stage 5
- 4) Bud length 21.2 ± 2.1 mm; Stage 5-6

Anthers were cultured on solid NH medium, solid NH medium with the addition of 1.0% (w/v) activated charcoal, or solid NH medium with the addition of 0.1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-d). Anthers were cultured as described in the Materials and Methods. After four weeks culture in the light followed by four weeks culture in the dark, the anthers were examined for evidence of macroscopic embryos/plantlets.

The results are presented in Table 3.3.4. The data for the two replicate experiments has been combined.

Out of a total of 2,954 cultured anthers only one gave rise to macroscopic plantlets. Two such plantlets were emerging from the anther, and were transferred to solid MS-0 medium in a 6 oz powder jar. When those anthers which failed to respond were further examined, by gently squeezing the anther contents into a drop of acetocarmine on a

microscope slide, and the anther contents examined microscopically, only dead pollen grains devoid of internal structure were observed. No multinucleate pollen grains, or small developing embryos were detected.

The responding anther was from a bud pre-treated at 4 - 6.5°C for 4 days, and was cultured on the surface of solid NH medium with the addition of 1.0% (w/v) activated charcoal.

3.3.6 Characterisation of the Plantlets Derived from *N. Glutinosa* Anther Culture

The two plantlets transferred to the surface of solid MS-0 medium were examined cytologically. Both were found to possess the expected haploid somatic chromosome number of $n = x = 12$ (see Figure 3.3.10). The plants were multiplied in culture, and eventually transferred to the greenhouse, as described for the *N. tabacum* anther culture derived plants. Two plants derived from each of the original plantlets were grown to flowering. The haploid plants were sterile, failing to set seed or produced viable pollen. The haploid plants differed from diploid *N. glutinosa* plants in a number of ways. The flowering haploid *N. glutinosa* plants measured 28-34 cm compared to 60-68 cm, for the diploid plants. Similarly, the largest leaf of the haploid plant was smaller than that of the diploid plant measuring 6.5 cm long and 5.8 cm wide, as compared to 10.0 cm long and 9.6 cm wide. The leaf width/length ratio was extremely variable, ranging between 0.76 and 1.20 for the diploid and 0.47 - 0.89 for the haploid *N. glutinosa* plants. Flowers were also smaller on the haploid plants, with a maximum length of 2.5 cm compared to 3.8 cm for the diploid plants. The flower width/length ratio was similar, being 0.40 ± 0.03 for the diploid, and 0.45 ± 0.05 for the haploid plants (see Figure 3.3.11).

FIGURE 3.3.1

Meiosis and tetrad formation in *Cyclamen persicum*.

3.3.1 - A-D : Meiosis in *Cyclamen* pollen mother cells.
(X 1300)

3.3.1 - E,F : Tetrads formed as a result of meiosis in the
pollen mother cells. Note the presence of
anomalous structures containing less or more
than 4 spores, presumably resulting from
abnormal reduction divisions.
(X 700)

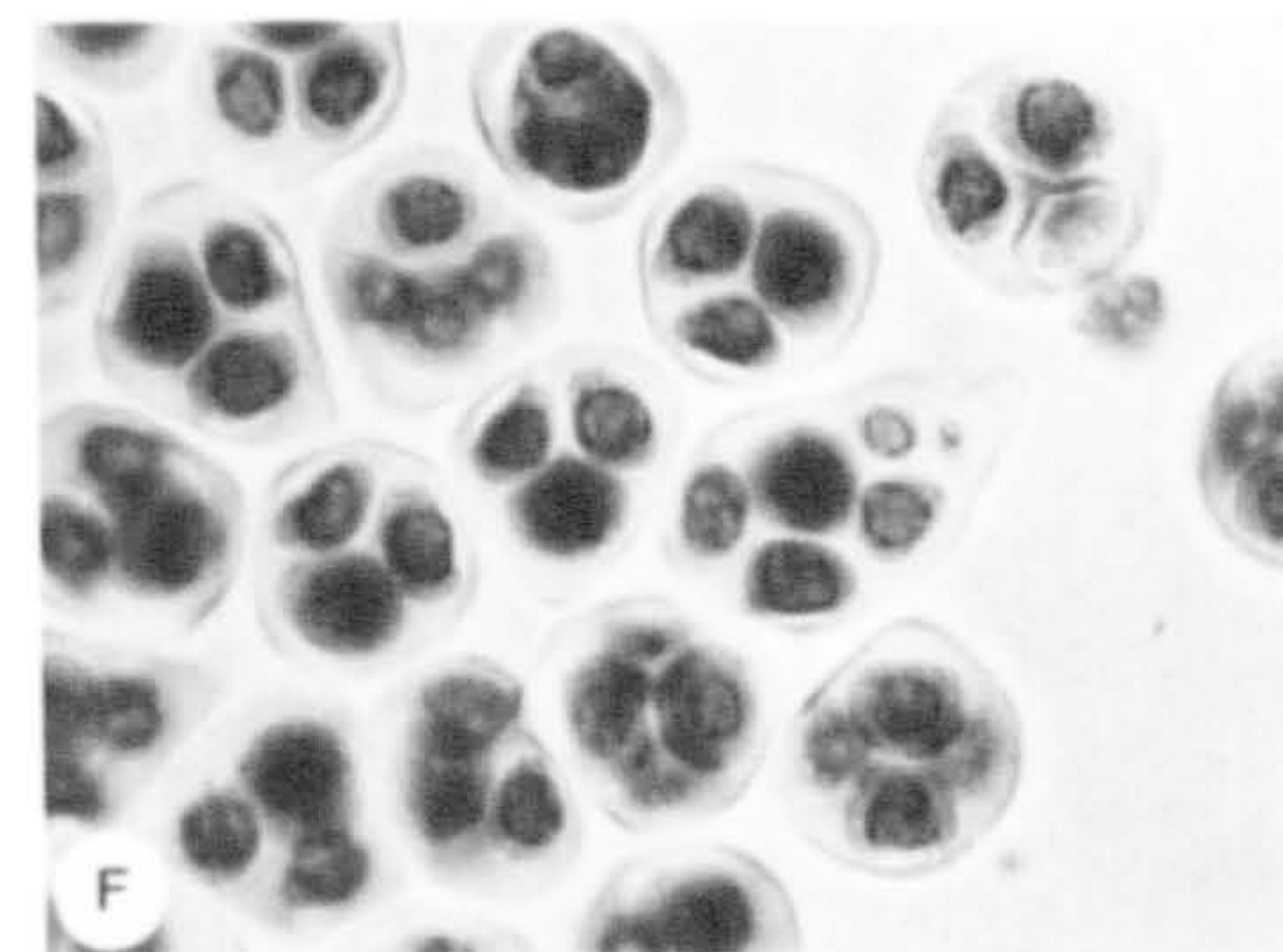
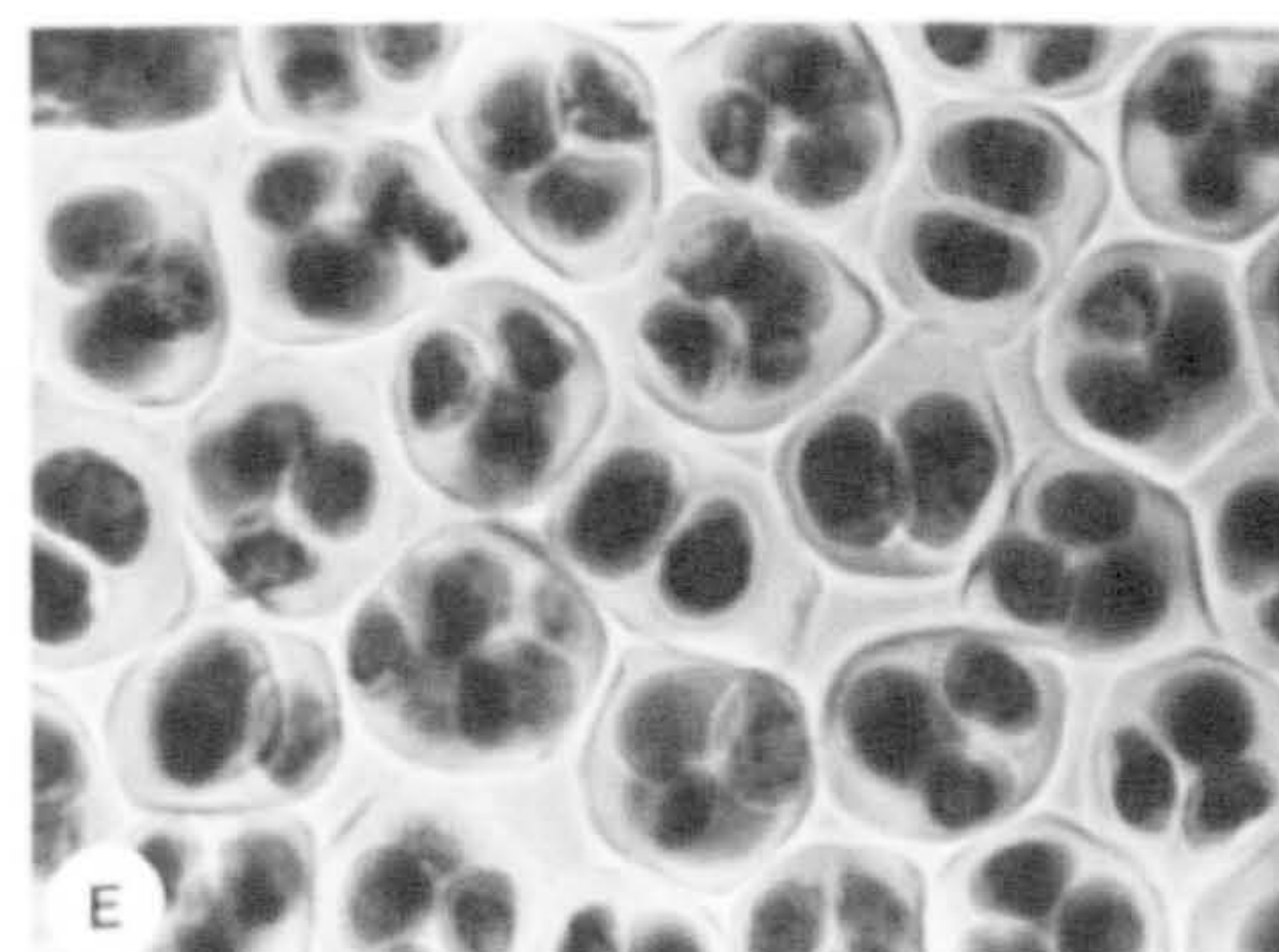
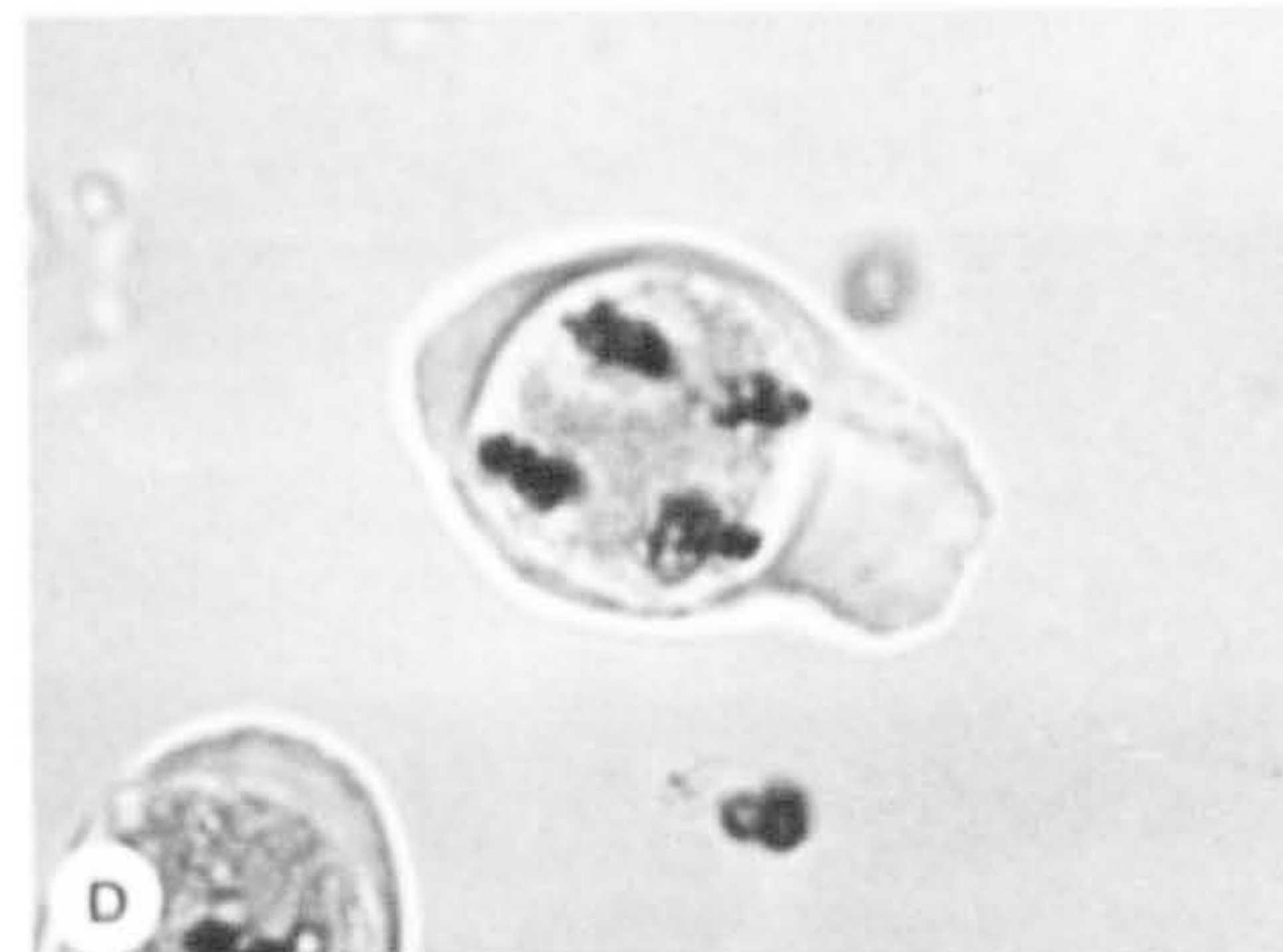
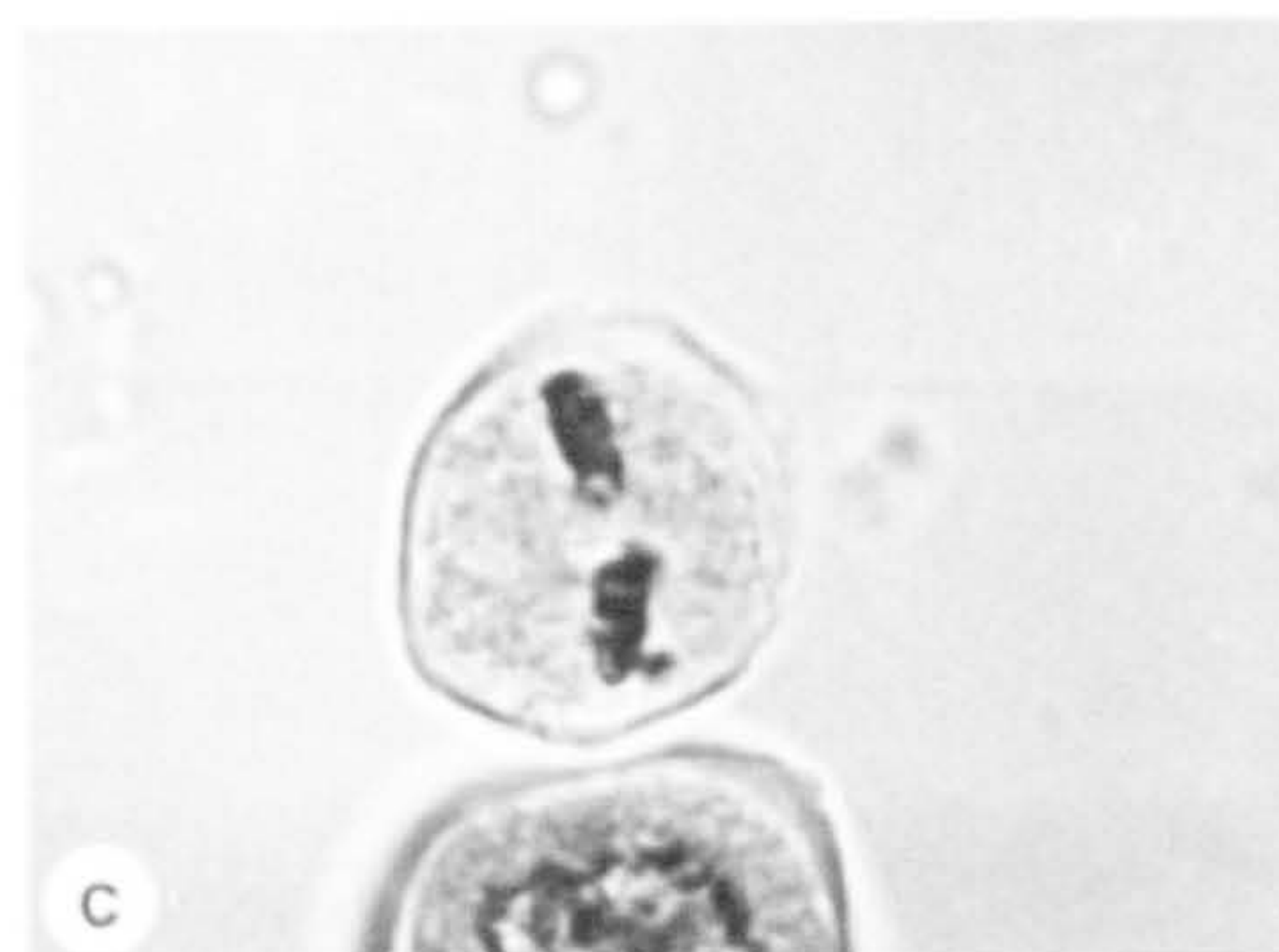
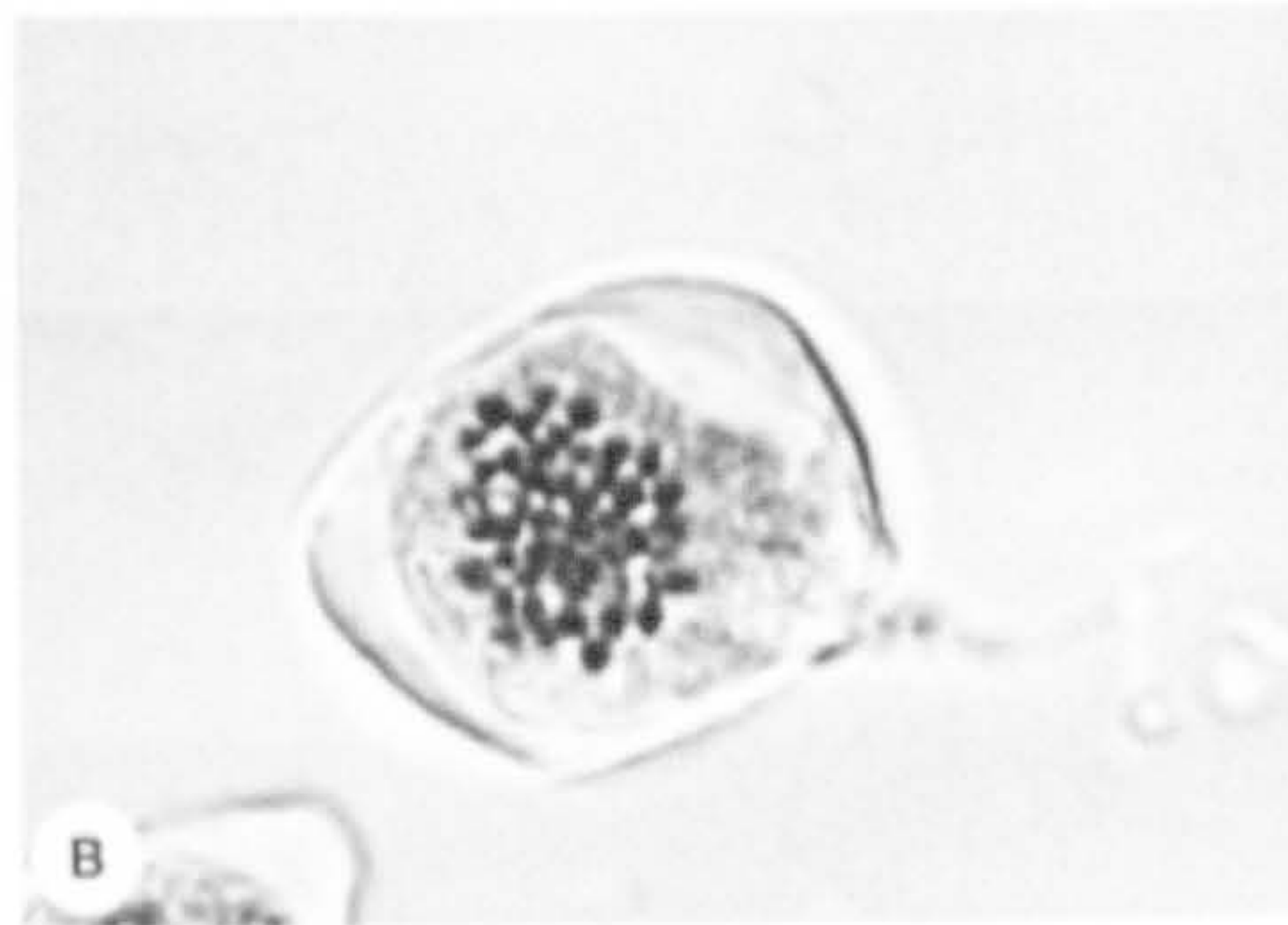


FIGURE 3.3.2

Pollen development in *Cyclamen persicum*.

- 3.3.2 - A : Uninucleate microspores of *Cyclamen* released from the tetrad by gentle pressure on the cover slip of the stained tetrad preparation.
(X 1400)
- 3.3.2 - B : Uninucleate microspores of *Cyclamen* - before vacuole formation. Note the presence of a larger microspore (centre), probably arising from an unreduced gamete (see E and F).
(X 1400)
- 3.3.2 - C : Uninucleate microspore with the formation of a vacuole.
(X 1500)
- 3.3.2 - D : A mixture of uninucleate microspores, and binucleate pollen, resulting from poor synchronisation in the timing of the first pollen mitosis between the uninucleate microspores.
(X 1400)
- 3.3.2 - E and F :
Haploid and diploid metaphase plates at first pollen mitosis. The diploid pollen grain presumably results from unreduced gametes due to abnormalities at meiosis. The haploid ($n = 24$) and diploid ($2n = 48$) chromosome complement is clearly visible.
(X 1600)

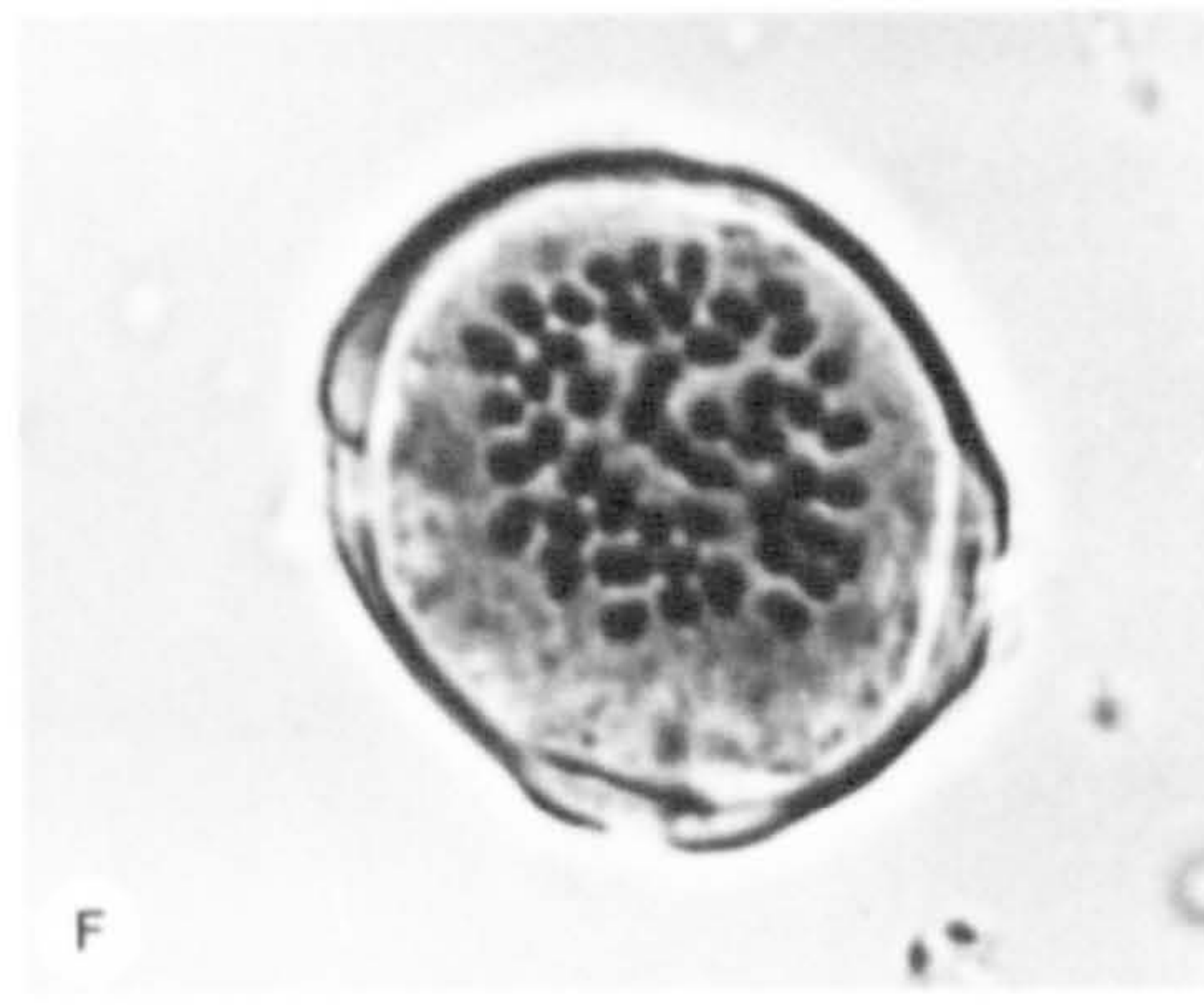
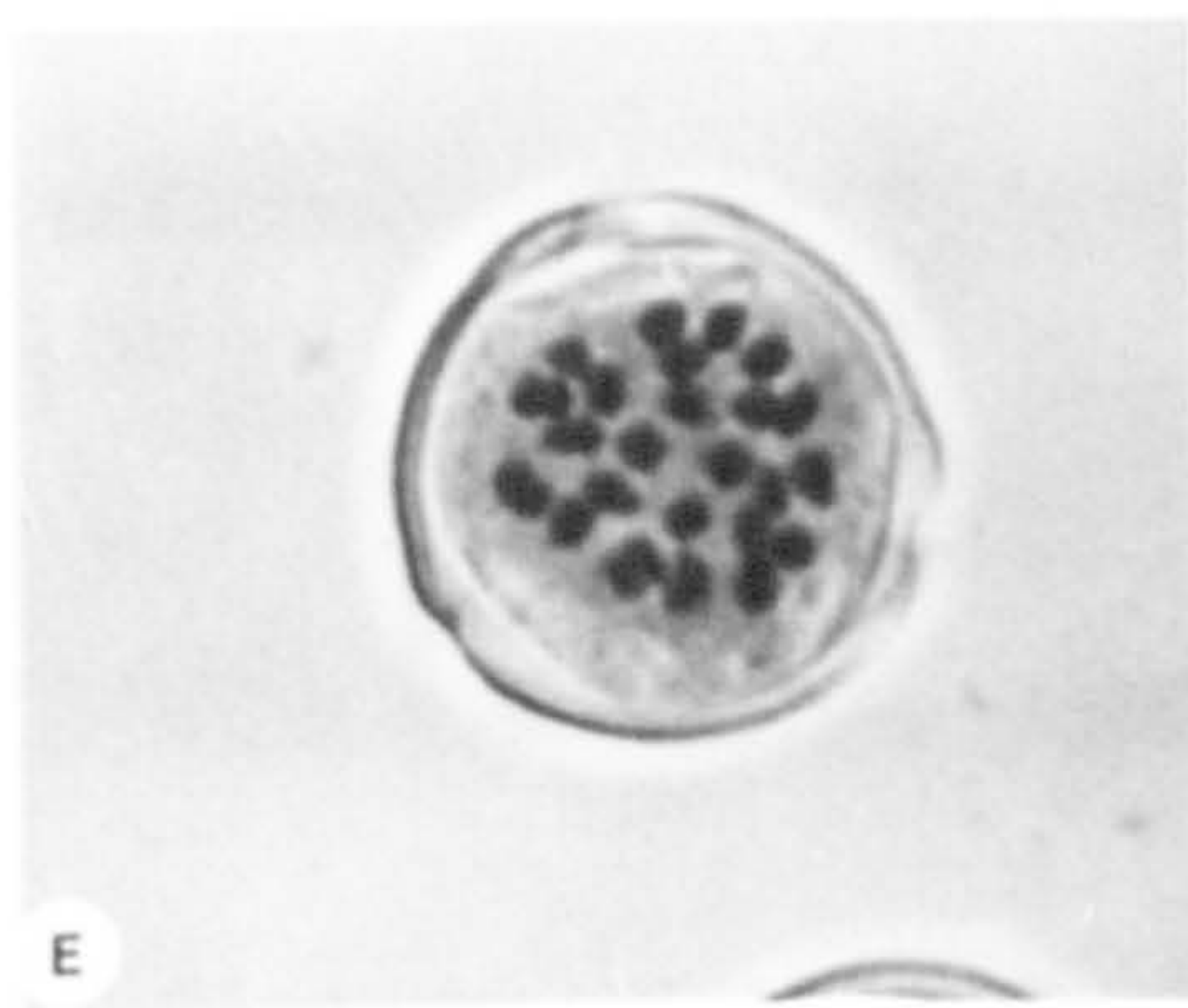
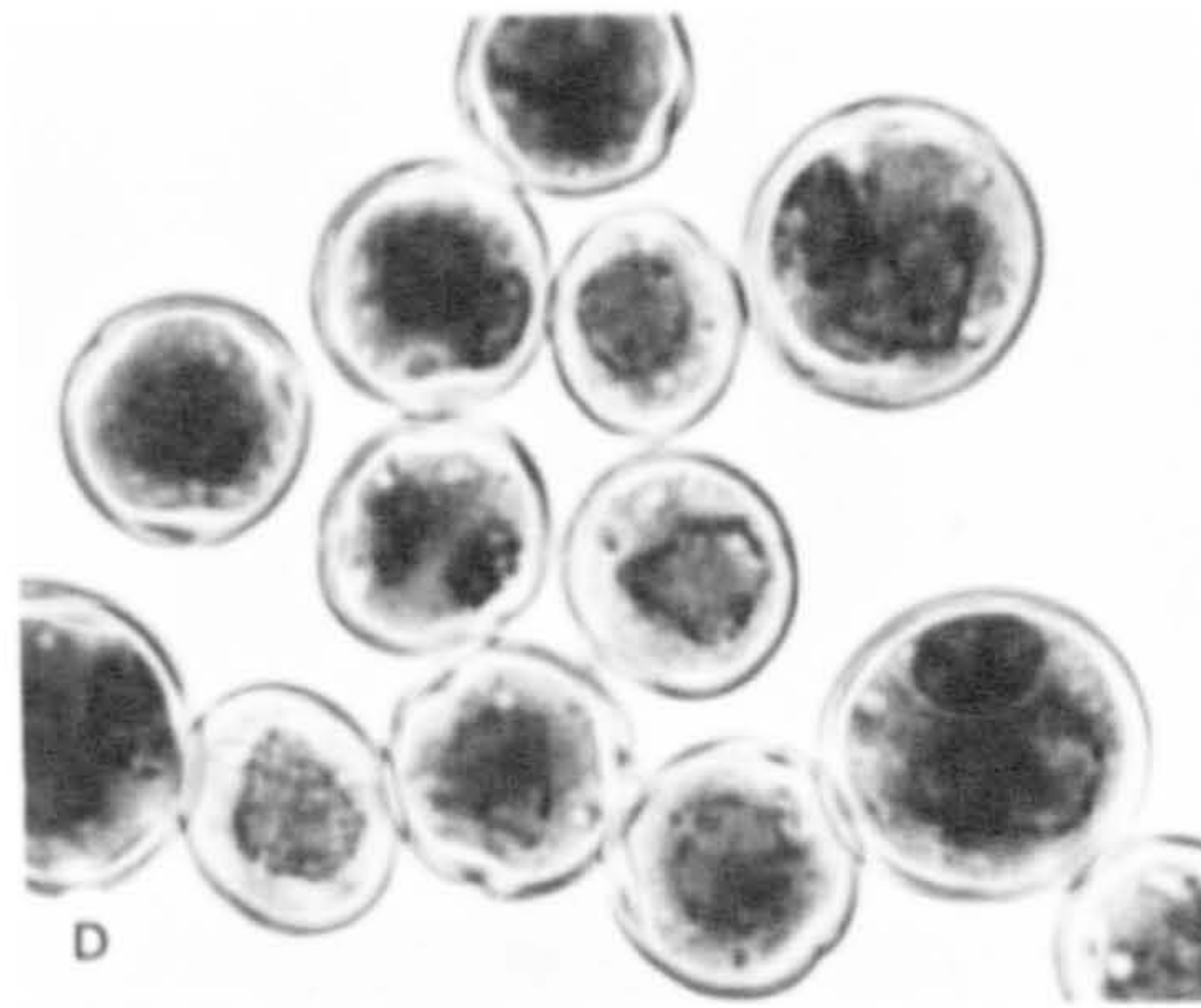
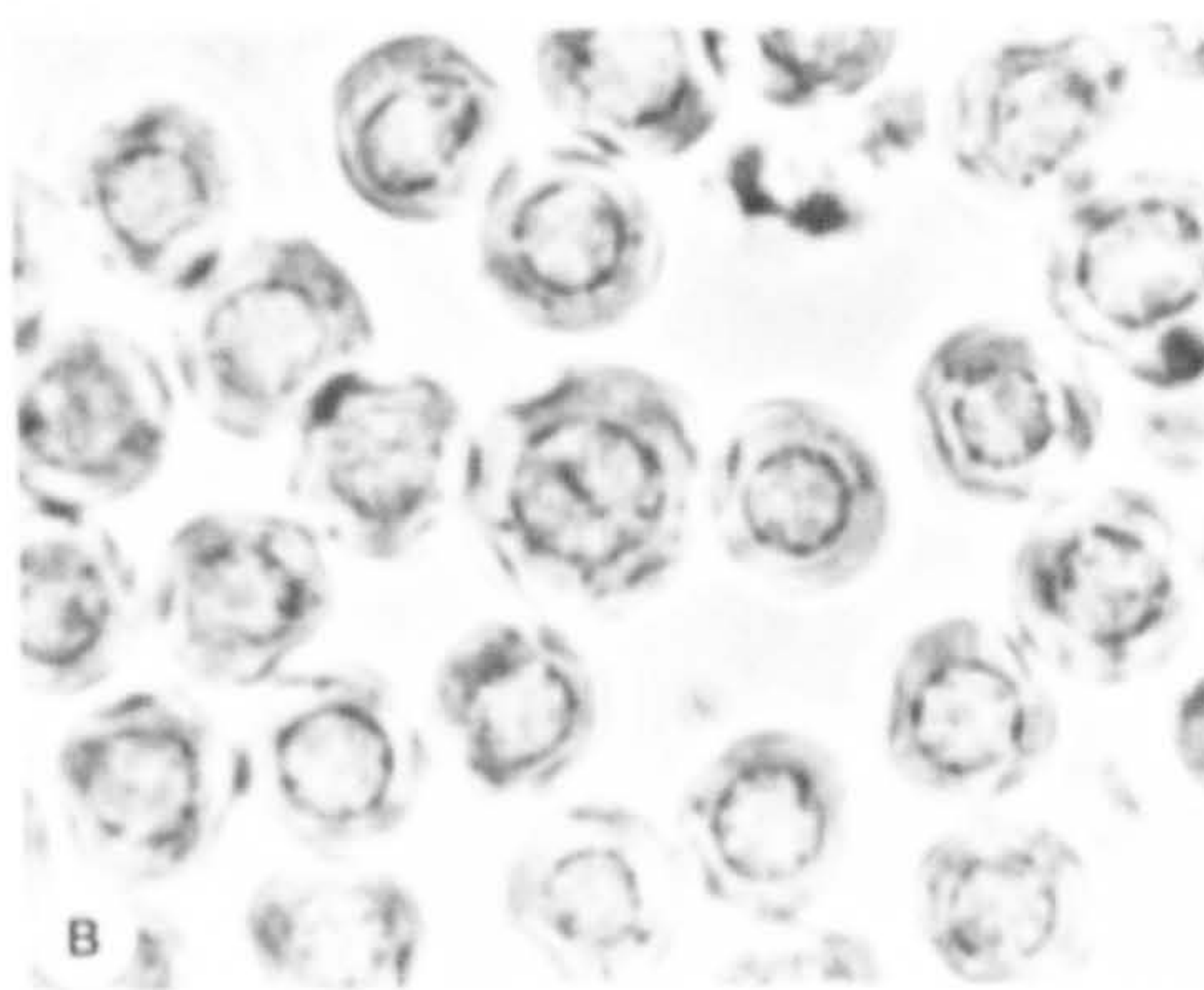
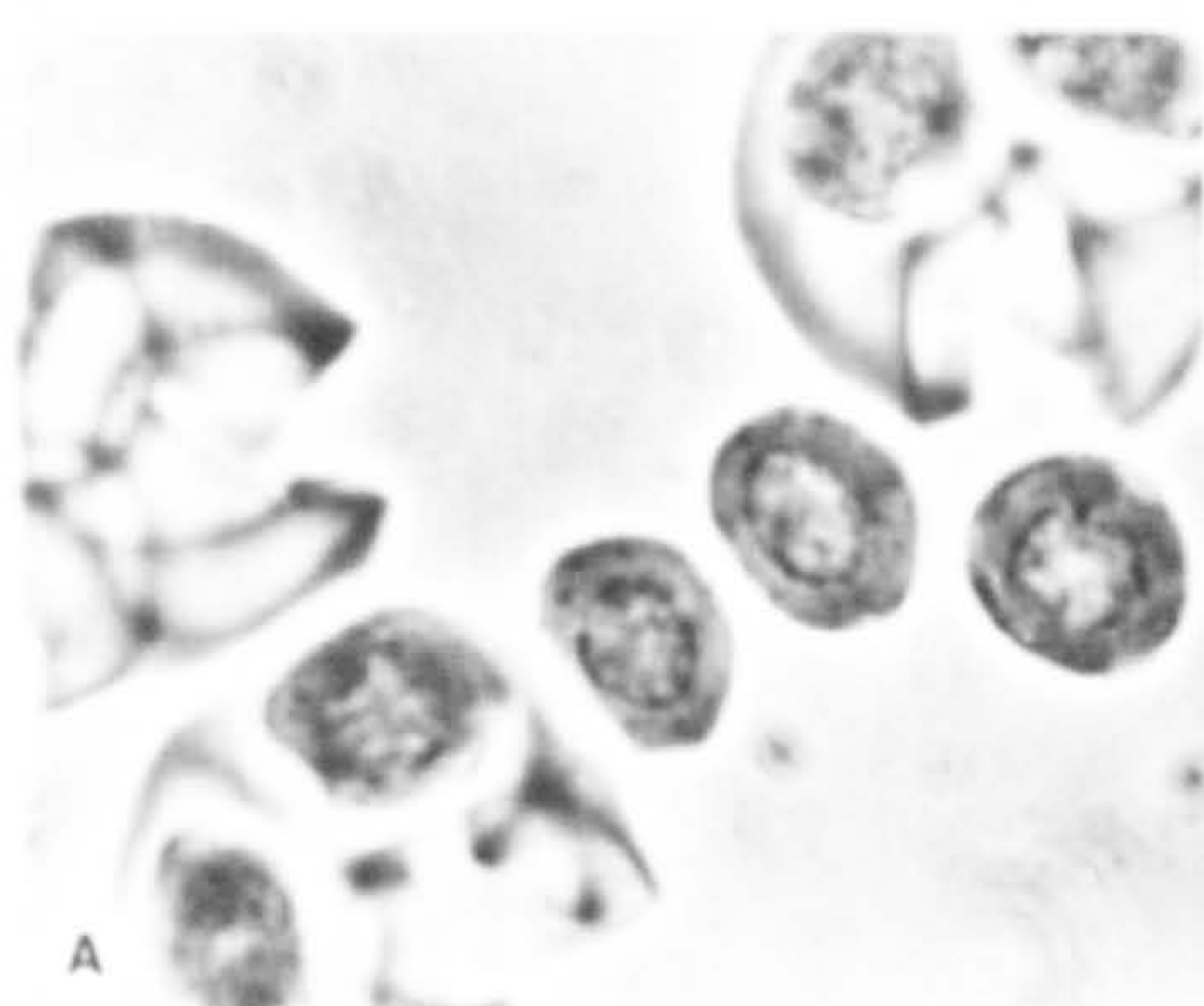


FIGURE 3.3.3

Pollen development in *N. tabacum*.

3.3.3 - A : Tetrad (Stage 1-2).

3.3.3 - B : Uninucleate microspore (Stage 1-2).

3.3.3 - C : Uninucleate microspore following vacuole
formation (Stage 3).

3.3.3 - D : First pollen mitosis (Stage 4).

3.3.3 - E : Early binucleate pollen grain. Note the de-
generating vacuole (Stage 5).

3.3.3 - F : Late binucleate pollen grain. The vacuole is no
longer present (Stage 6).

(all X 1400)

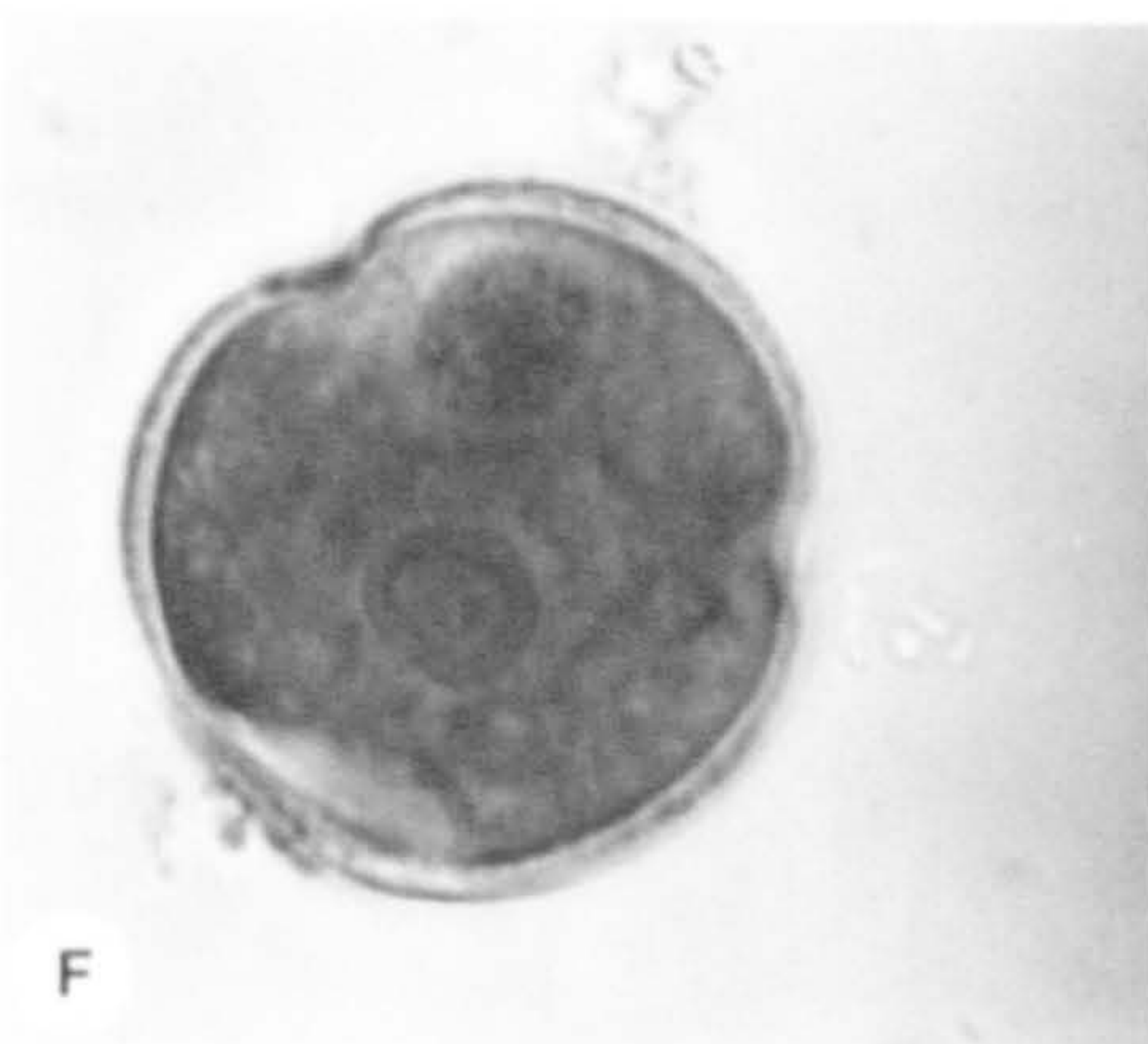
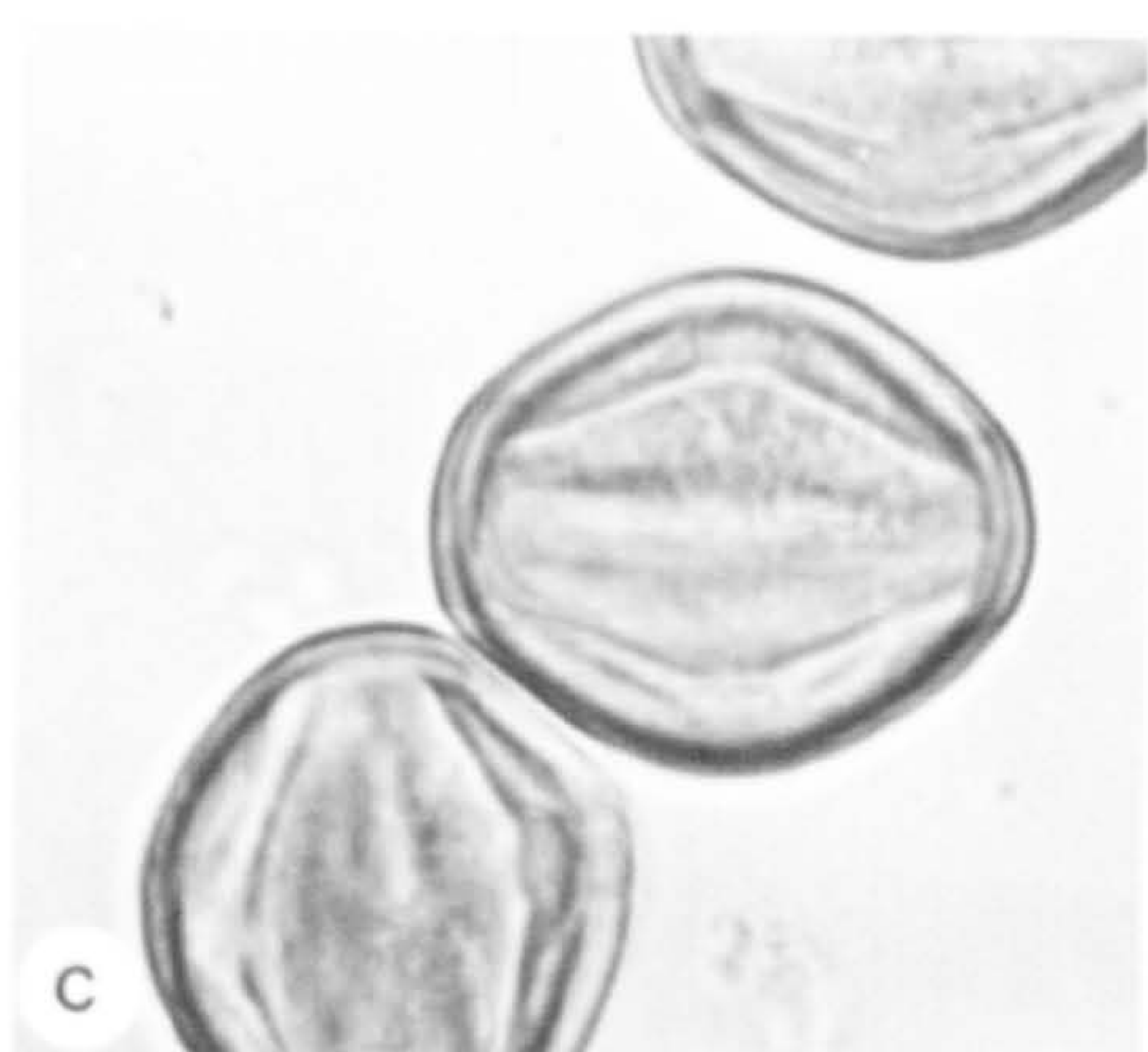
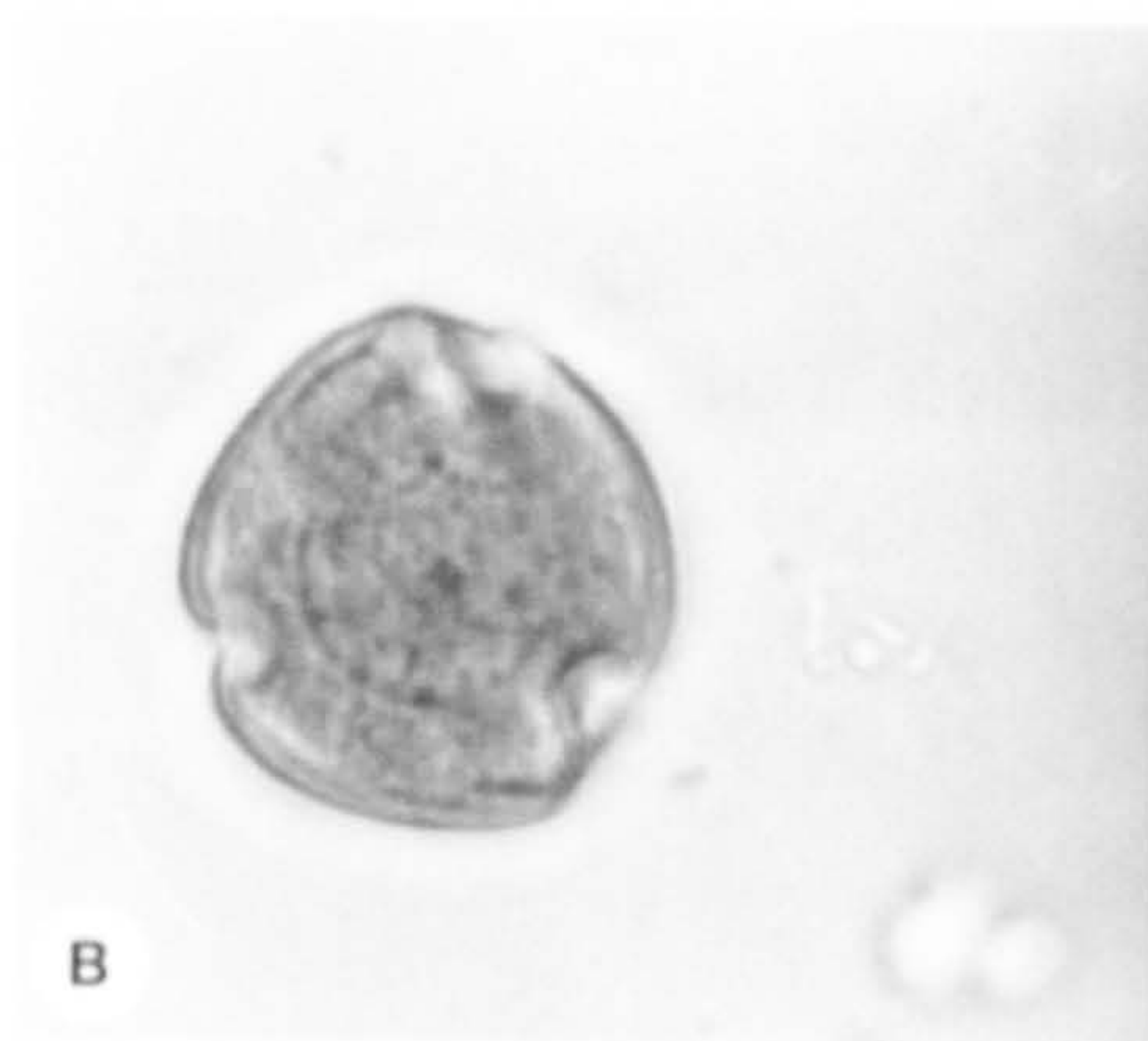
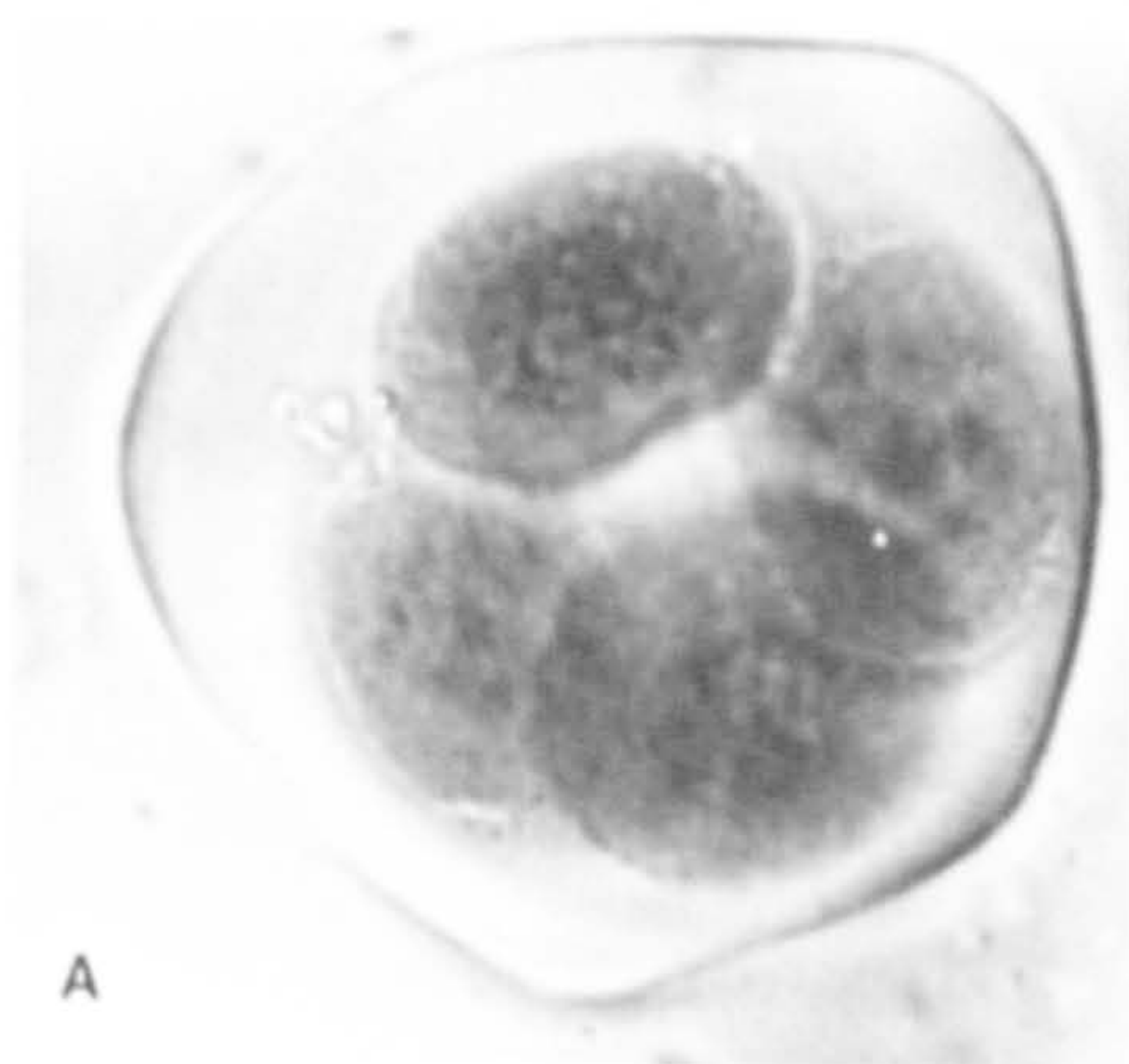


FIGURE 3.3.4

Pollen development in *N. glutinosa*.

3.3.4 - A : Tetrad (Stage 1-2).

3.3.4 - B : Uninucleate microspore (Stage 1-2).

3.3.4 - C : Uninucleate microspore following vacuole formation (Stage 3).

3.3.4 - D : Early binucleate pollen grain. Note the two nuclei and degenerating vacuole (Stage 5).

3.3.4 - E : Late binucleate pollen grain. The vacuole is completely absent (Stage 6).

3.3.4 - F : Mature pollen grain. Note the intense staining of the cytoplasm.

(all X 1400)

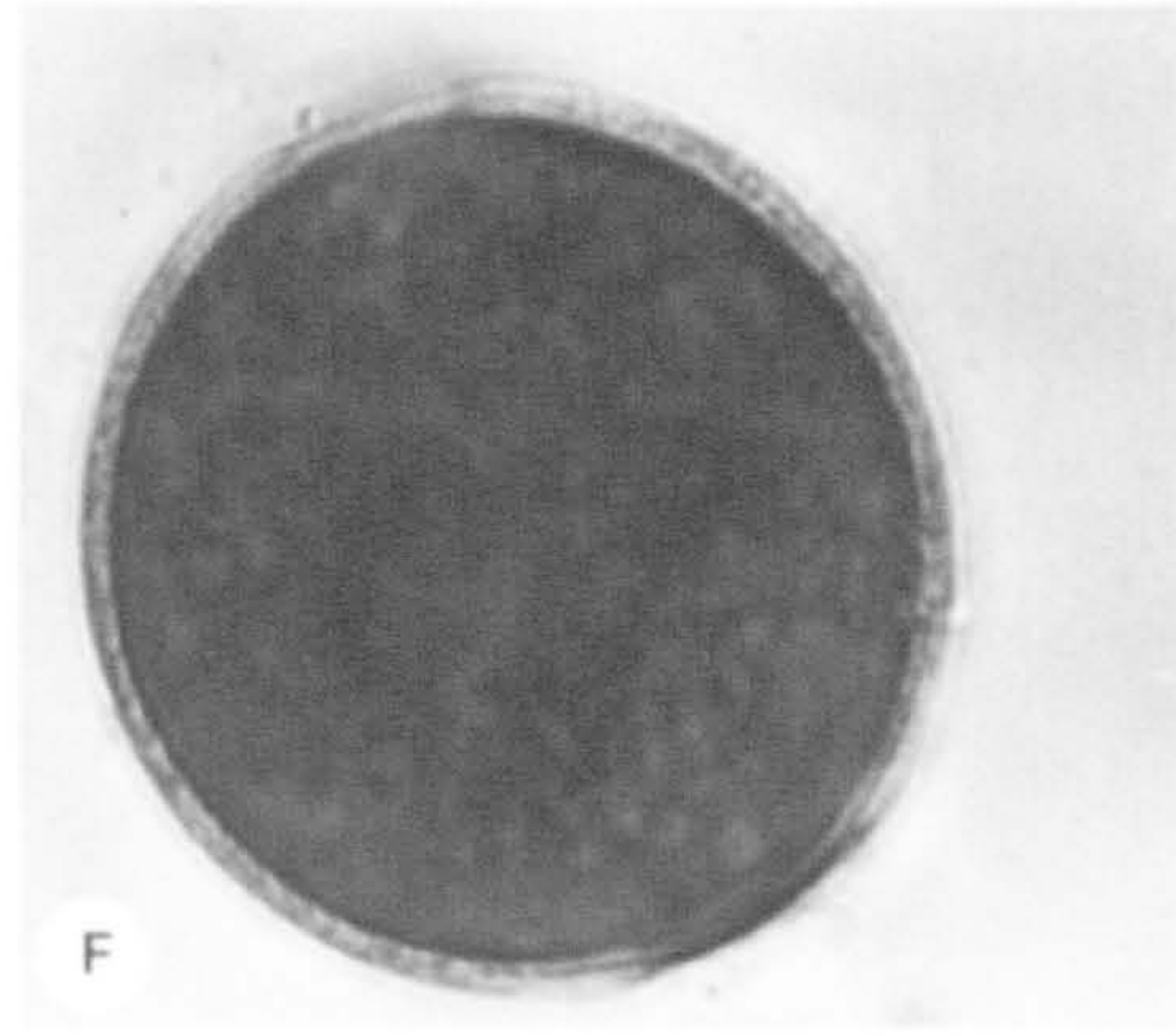
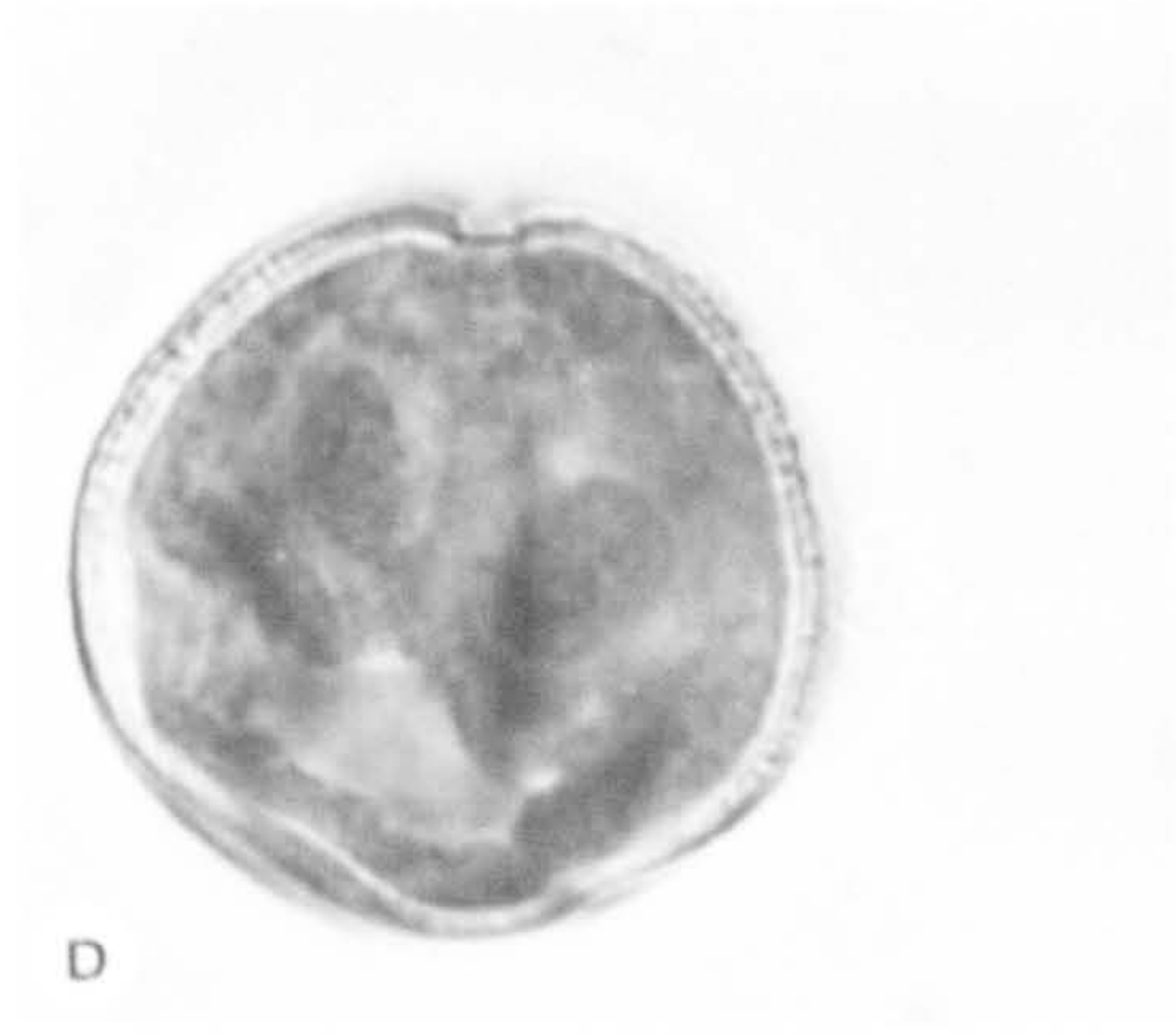
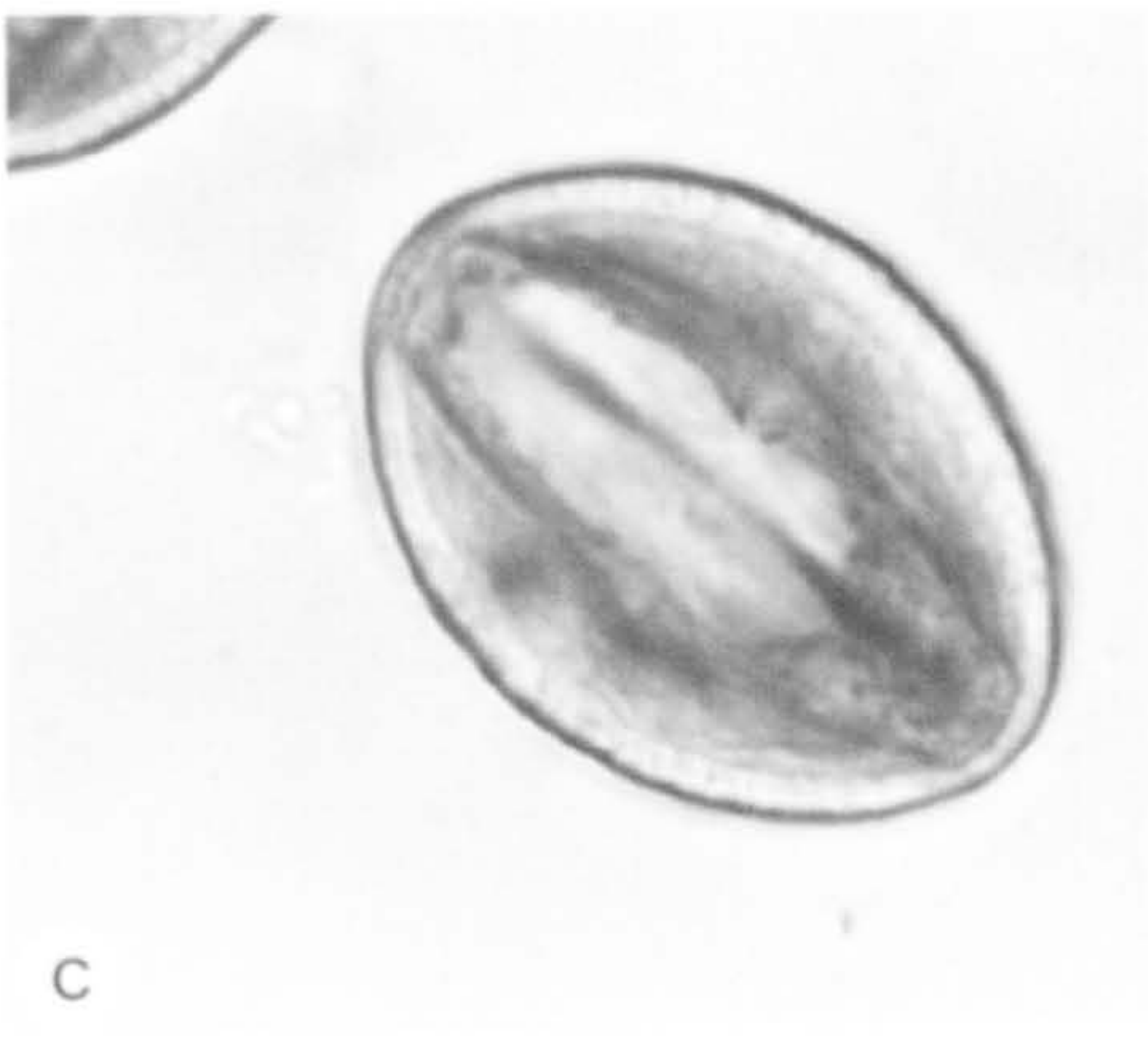
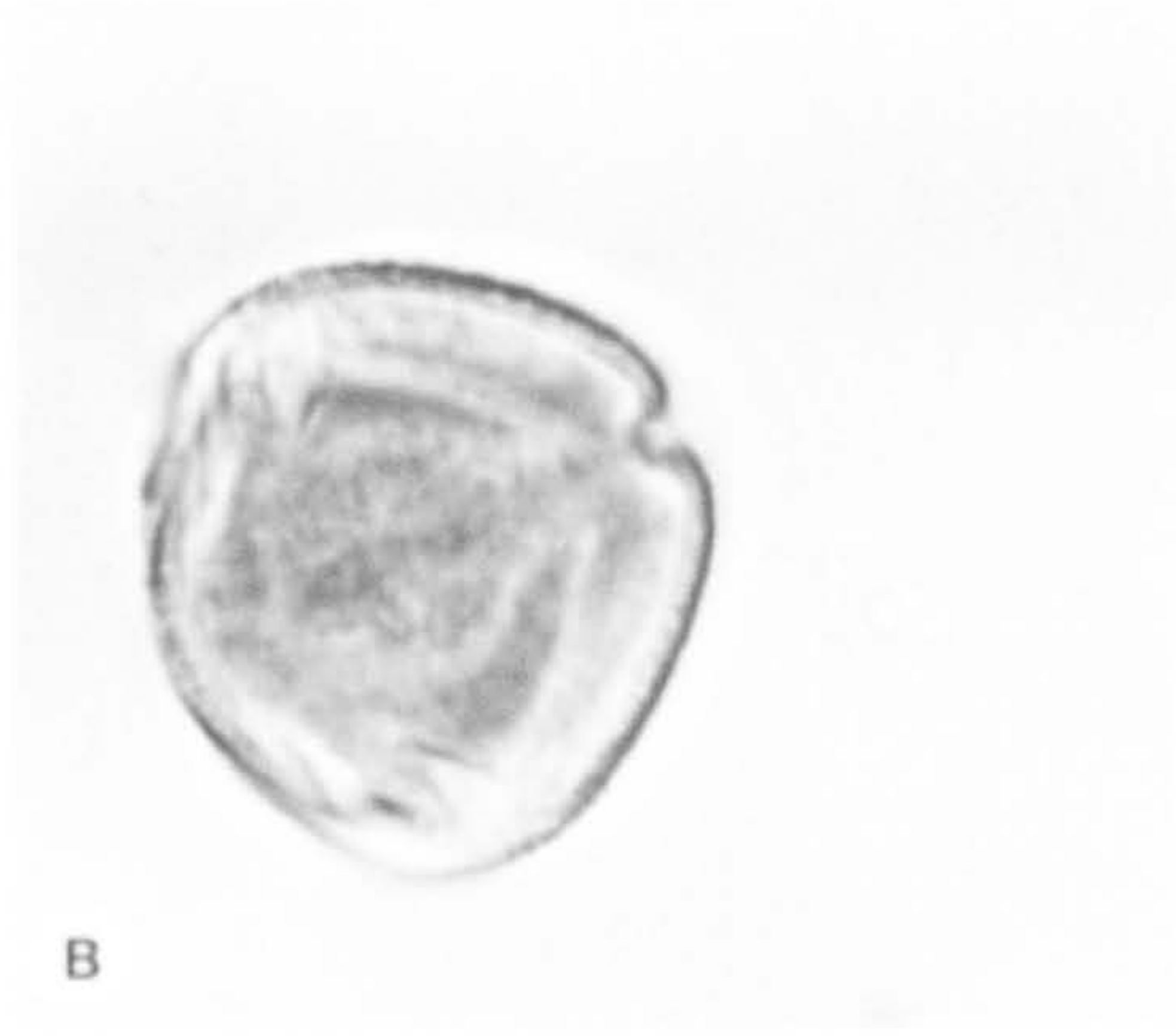
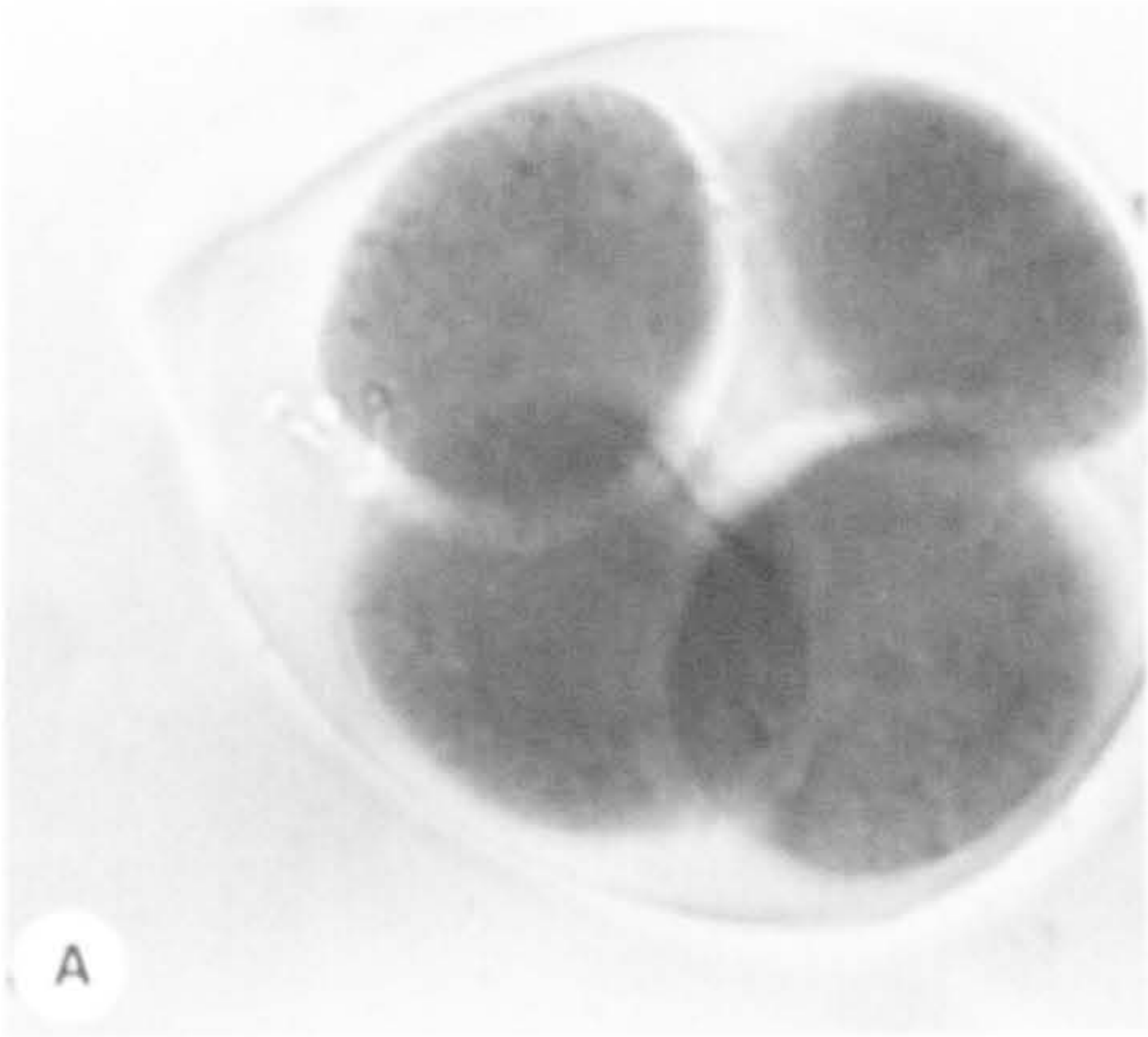


FIGURE 3.3.5

The growth of *N. tabacum*, *N. glutinosa* and *Cyclamen persicum*
buds against time.

The length of a minimum of 8 buds initially judged to contain pollen at the pollen mother cell or tetrad stage of development were measured, and their growth followed over a 12 day period. Bars indicate the range of bud lengths observed.

3.3.5 - A : *N. tabacum*

3.3.5 - B : *N. glutinosa*

3.3.5 - C : *Cyclamen persicum*

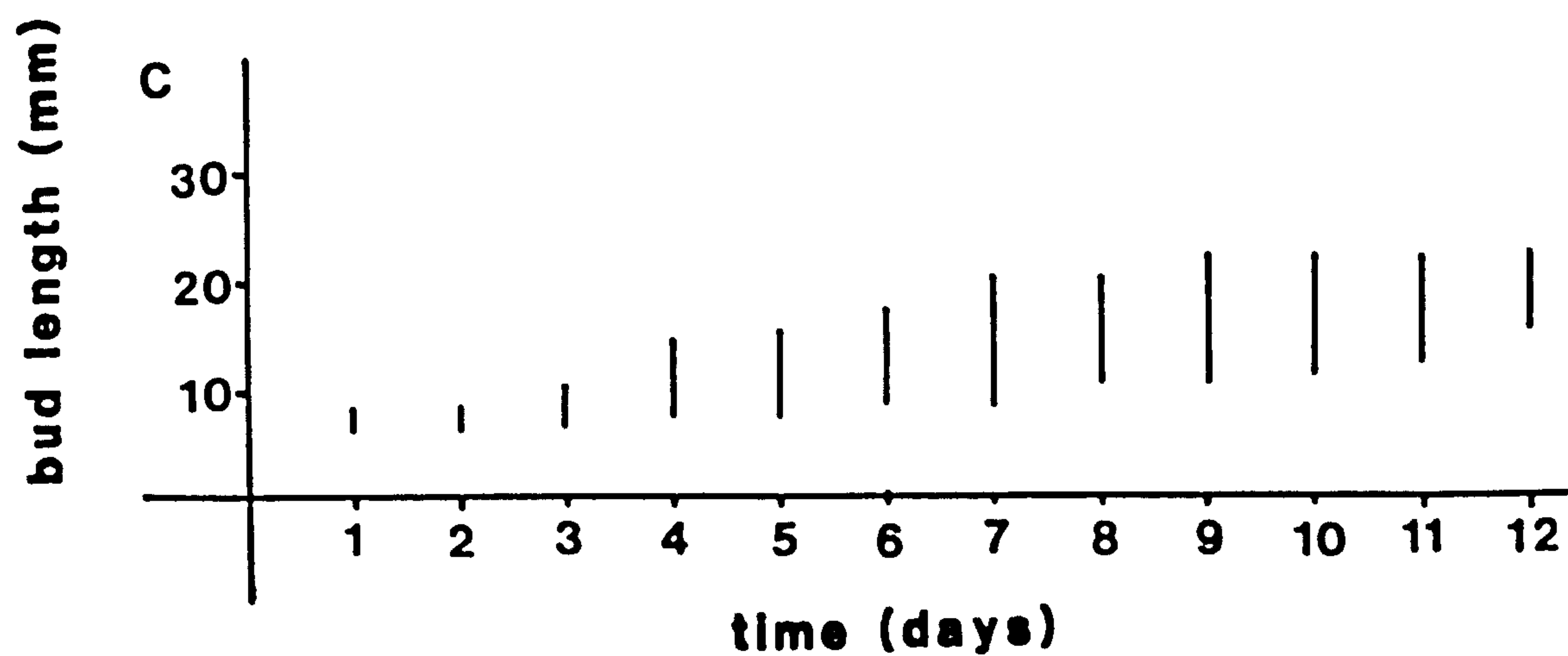
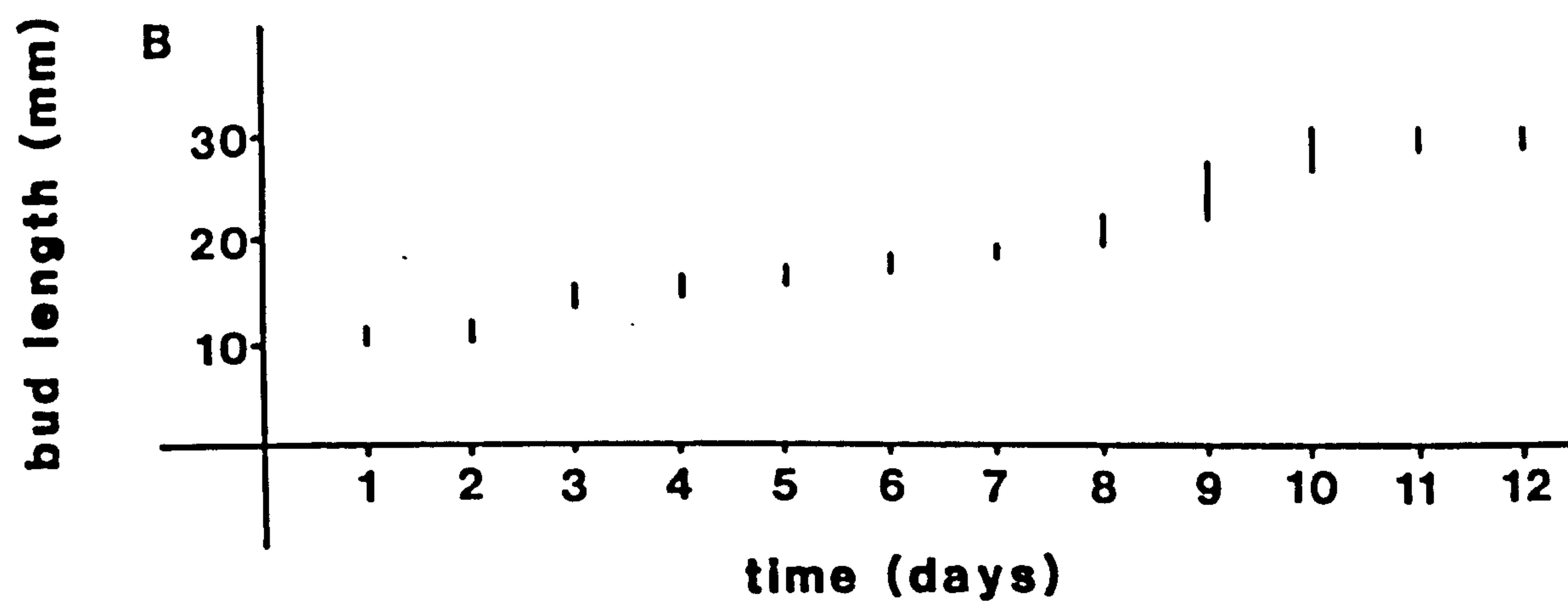
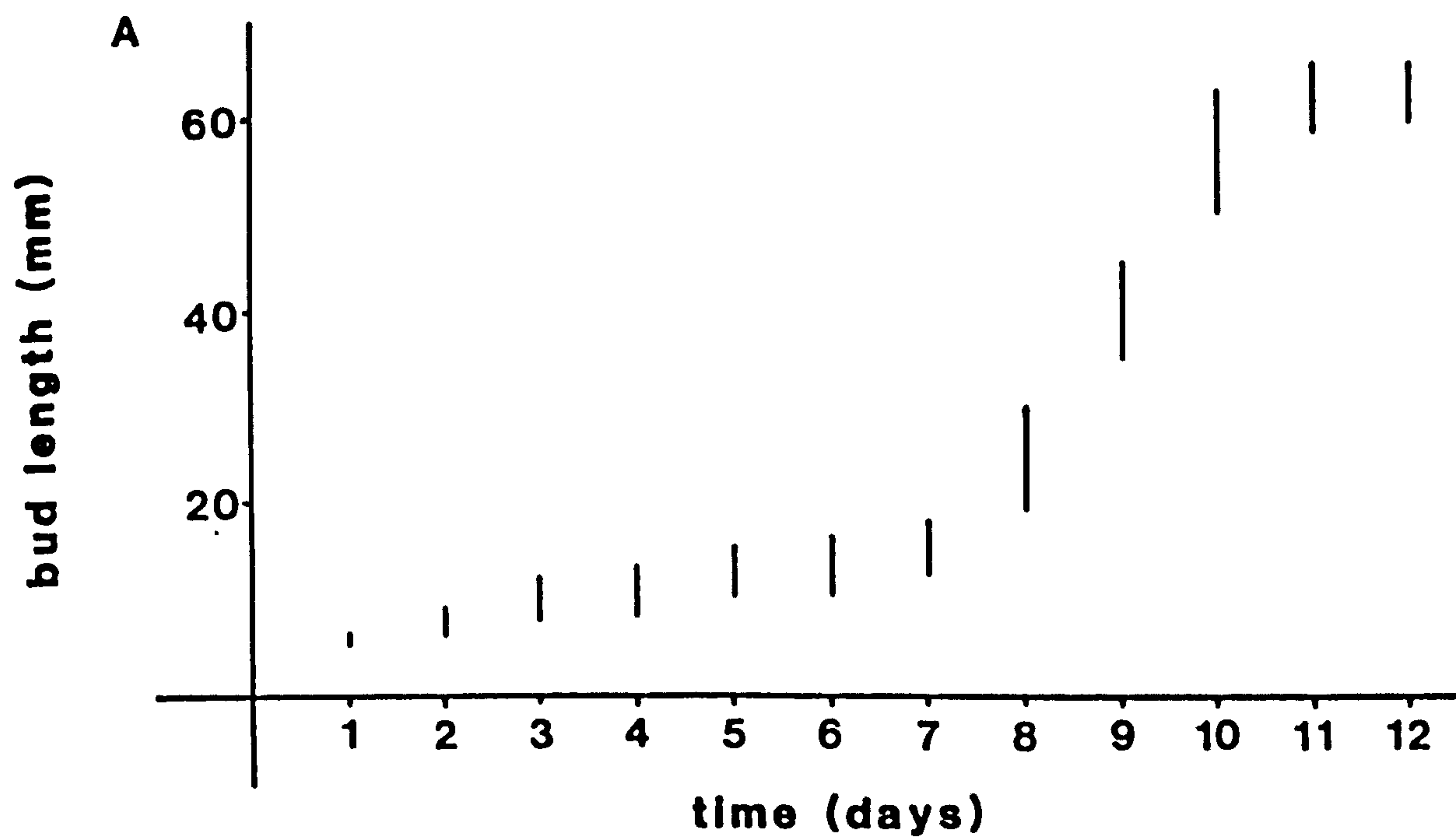


Table 3.3.1 : Anther culture in *Nicotiana tabacum* after bud pre-treatment at 4 - 6.5°C for 0, 4, 8 and 12 days.

a) Uninucleate and mitotic microspores (stages 2-4)

Bud size 17.9 ± 1.0 mm

		T.A.	T.R.	T.E.	A.P.	I.F.	E
0 days	1	109	30	342	11.4	27.5	314
	2	75	11	98	8.9	14.7	131
	3	50	5	23	4.6	10.0	46
	4	50	6	65	10.8	12.0	130
	Total	284	52	528	10.2	18.3	187
4 days	1	-	-	-	-	-	-
	2	55	8	51	6.4	14.5	93
	3	25	1	3	3.0	4.0	12
	4	75	2	17	8.5	2.7	23
	Total	155	11	71	6.5	7.1	46
8 days	1	39	14	142	10.1	35.9	363
	2	49	10	61	6.1	20.4	124
	3	25	5	31	6.2	20.0	124
	4	74	27	345	12.8	36.5	467
	Total	187	56	576	10.3	29.9	308
12 days	1	75	17	127	7.5	22.7	170
	2	75	23	118	5.1	30.7	157
	3	25	12	171	14.3	48.0	686
	4	75	21	151	7.2	28.0	202
	Total	250	73	567	7.8	29.2	228

Table 3.3.1 continued ...

b) Binucleate pollen (stage 5)

Bud size 20.3 ± 1.4 mm

		T.A.	T.R.	T.E.	A.P.	I.F.	E
0 days	1	63	17	103	6.1	27.0	165
	2	75	14	74	5.3	18.7	99
	3	50	1	7	7.0	2.0	14
	4	50	1	10	10.0	2.0	20
	Total	238	33	194	5.9	13.9	82
4 days	1	-	-	-	-	-	-
	2	45	8	64	8.0	17.8	142
	3	48	2	24	12.0	4.2	50
	4	25	6	76	12.7	24.0	305
	Total	118	16	164	10.3	13.6	140
8 days	1	55	31	307	9.9	56.4	558
	2	-	-	-	-	-	-
	3	25	11	239	21.7	44.0	955
	4	25	13	100	7.7	52.0	400
	Total	105	55	646	11.7	52.4	613
12 days	1	-	-	-	-	-	-
	2	-	-	-	-	-	-
	3	25	8	47	5.9	32.0	189
	4	75	19	46	2.4	25.3	61
	Total	100	27	93	3.4	27.0	92

FIGURE 3.3.6

The anther culture response of *N. tabacum* cv. White Burley after 0, 4, 8 and 12 days of bud pretreatment at 4.0 - 6.5°C. Anthers were cultured on solid NH medium.

- 3.3.6 - A : The induction frequency (percentage of cultured anthers responding) against bud pretreatment duration.
- 3.3.6 - B : The anther productivity (mean number of embryos/plantlets per responding anther) against bud pretreatment duration.
- 3.3.6 - C : The anther culture efficiency (the product of the induction frequency and anther productivity) against the pretreatment duration. This indicates the expected recovery of embryos/plantlets from 100 cultured anthers.

- Open circles - uninucleate microspores
- Closed circles - early binucleate microspores

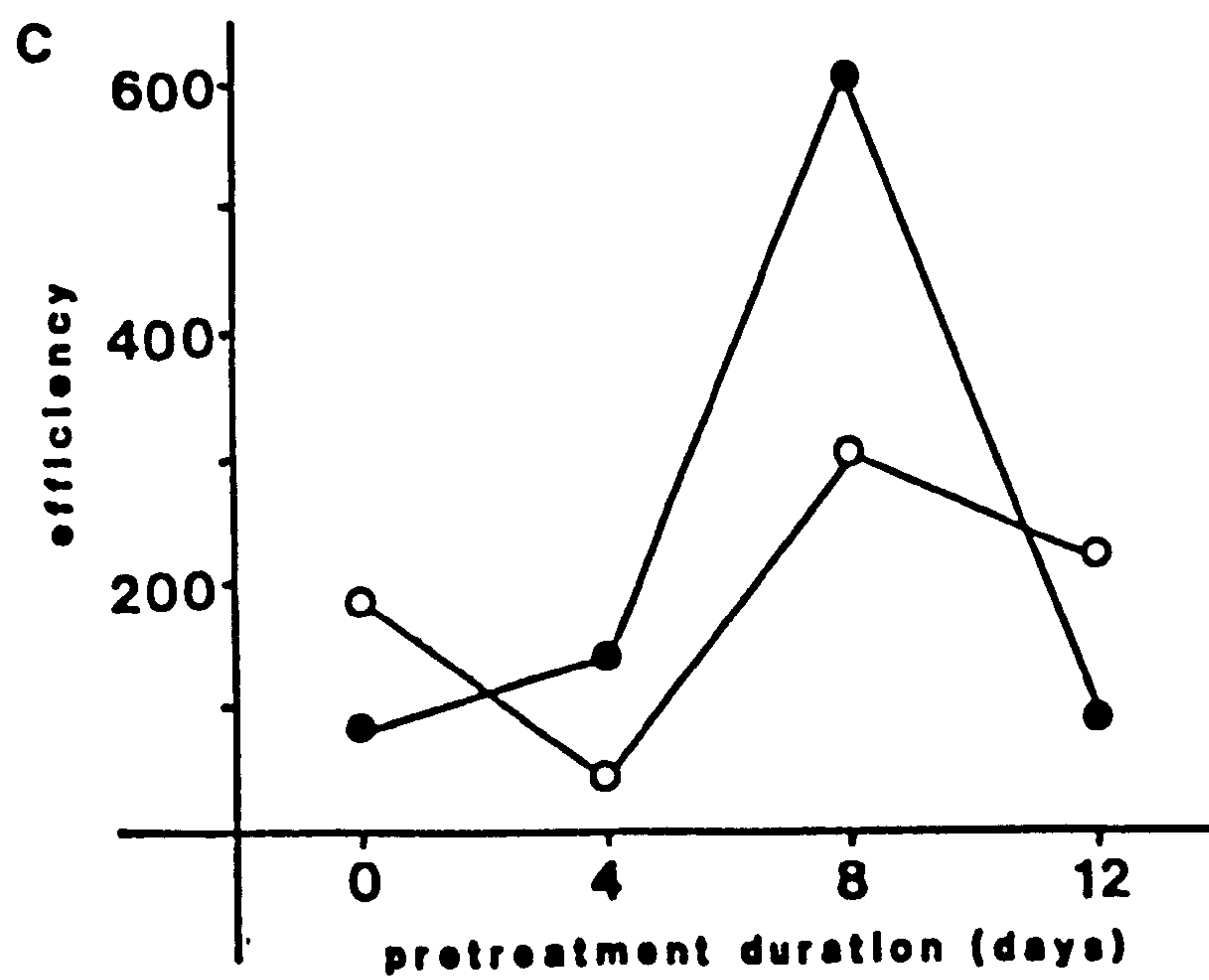
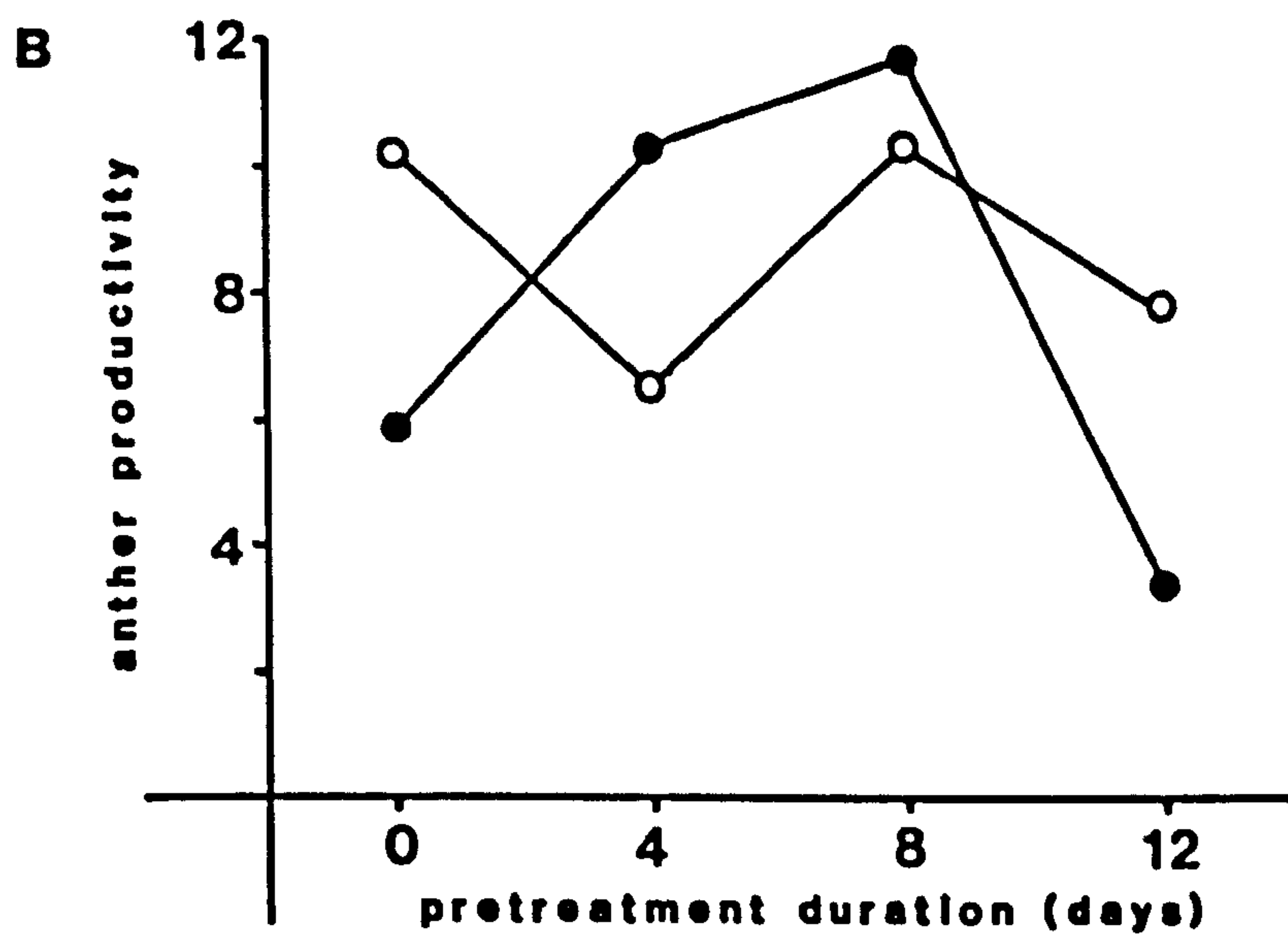
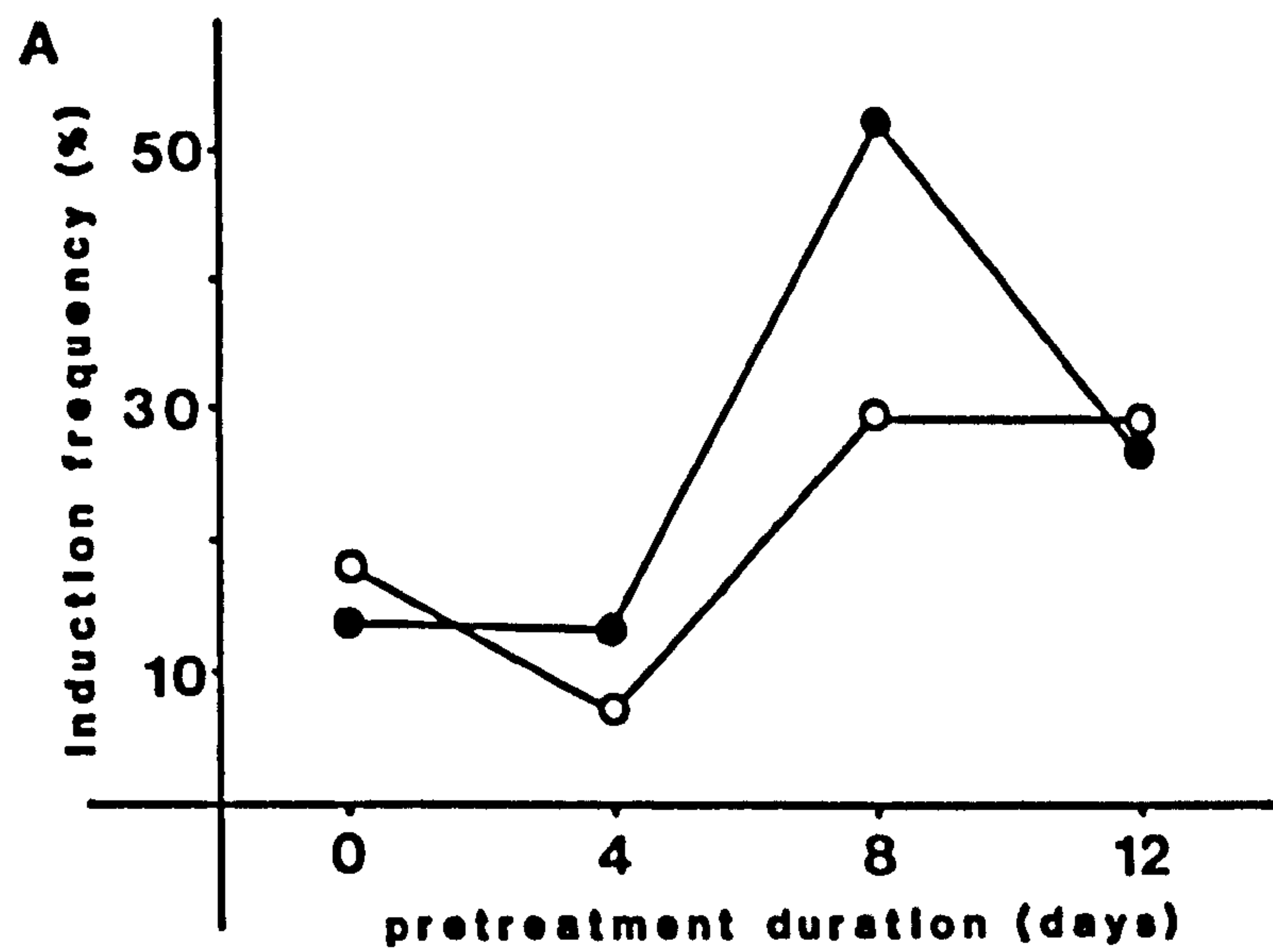


FIGURE 3.3.7

Frequency distribution of anther productivity values obtained following *N. tabacum* cv. White Burley anther culture.

The combined results from anther culture on solid NH medium following bud pretreatment at 4.0 - 6.5°C for 0, 4, 8 and 12 days duration are presented. The frequency histogram indicates the distribution of individual anther productivity values in the size classes indicated. Over 70% of the responding anthers gave rise to only 1-10 embryos/plantlets.

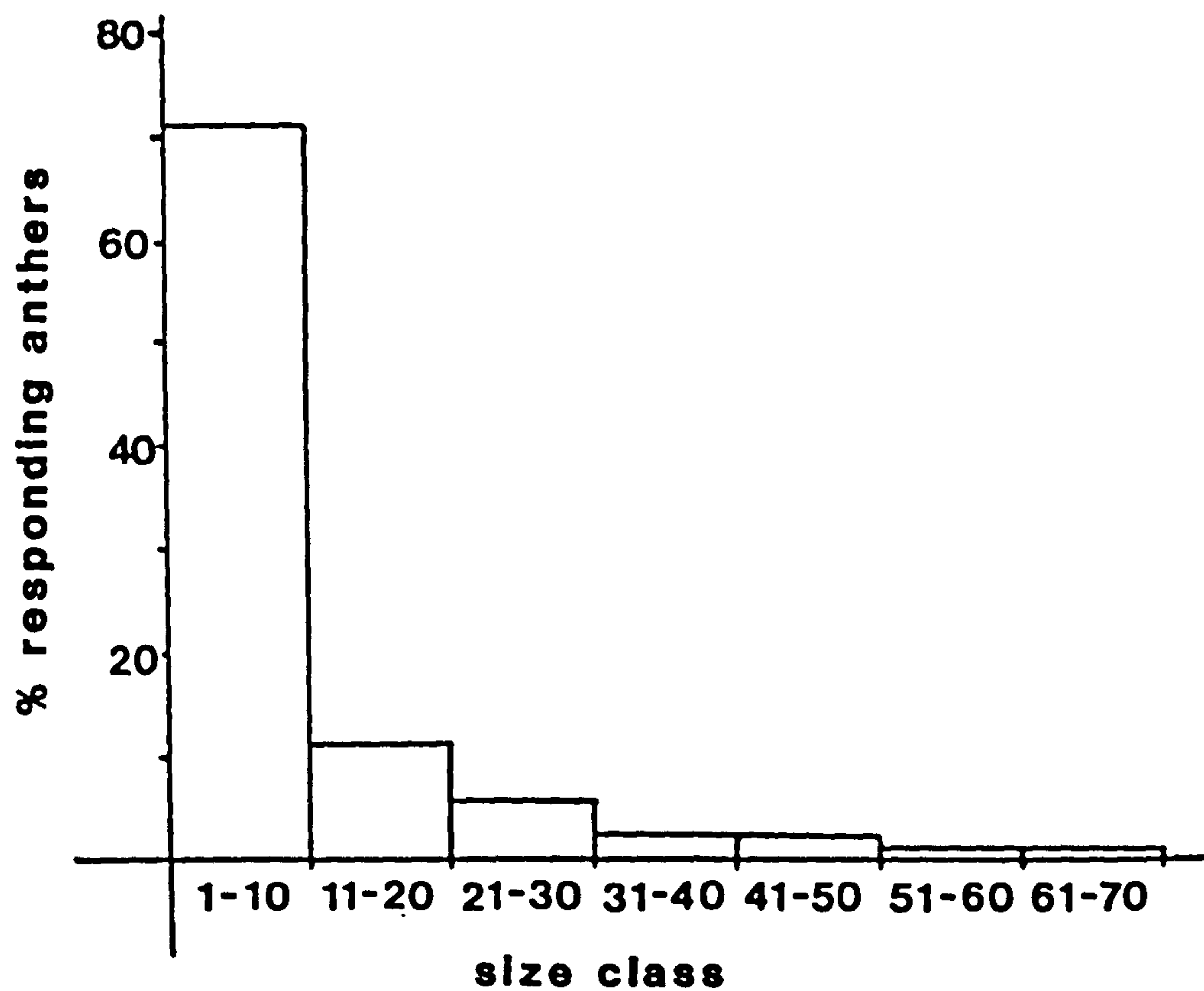
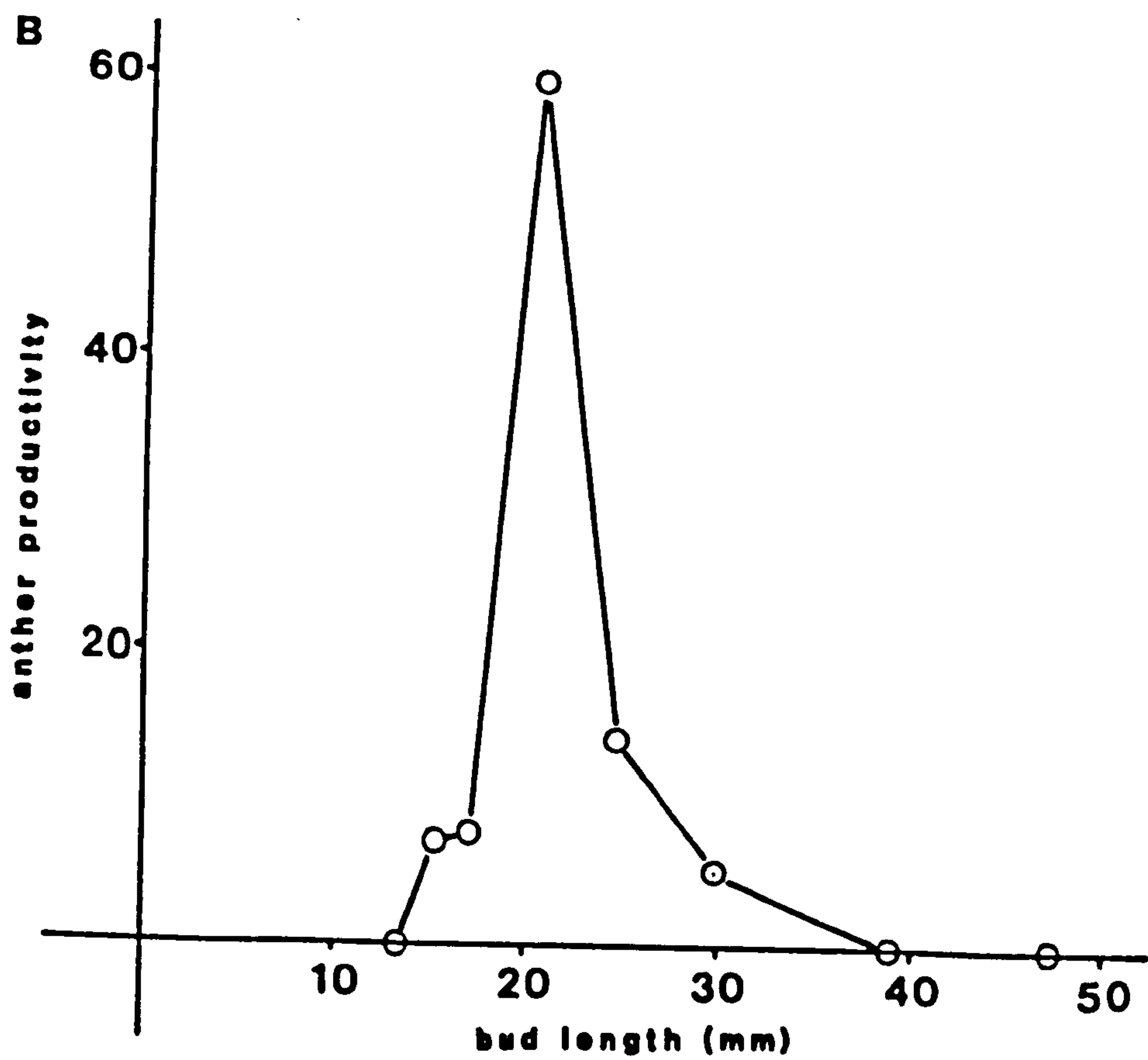
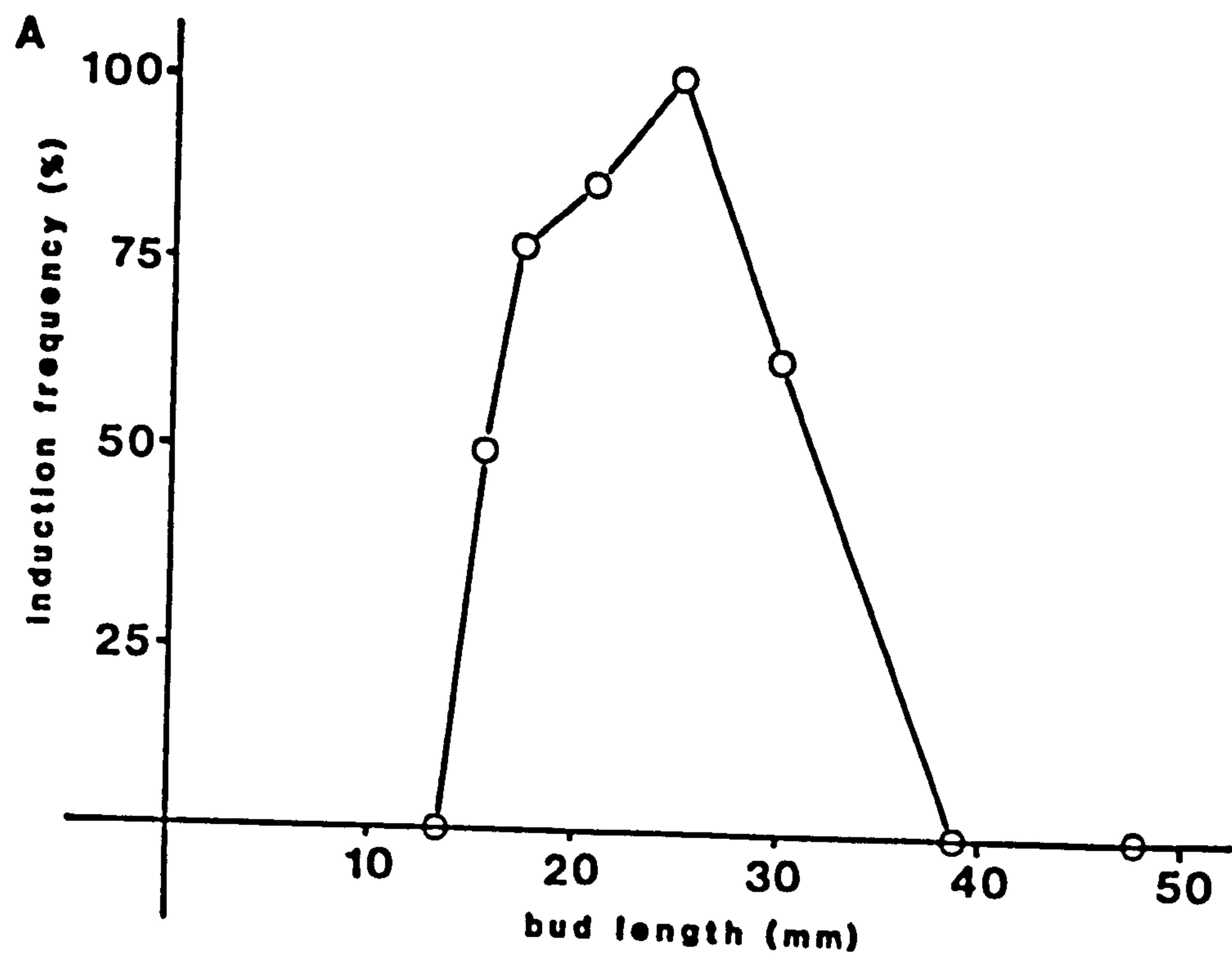


Table 3.3.2 : The effect of initial bud size on the subsequent response of cultured N. tabacum anthers after 8 days bud pre-treatment at 4 - 6.5°C.

Bud Size		I.F.	A.P.	E
Mean	S.D.			
13.6	0.5	0	0	0
15.5	0.5	50	10.4	520
17.3	0.5	78	11.7	889
20.8	1.1	86	88.9	7,645
25.0	1.0	100	21.4	2,140
30.0	1.2	63	7.3	460
39.0	0.8	0	0	0
47.7	3.6	0	0	0



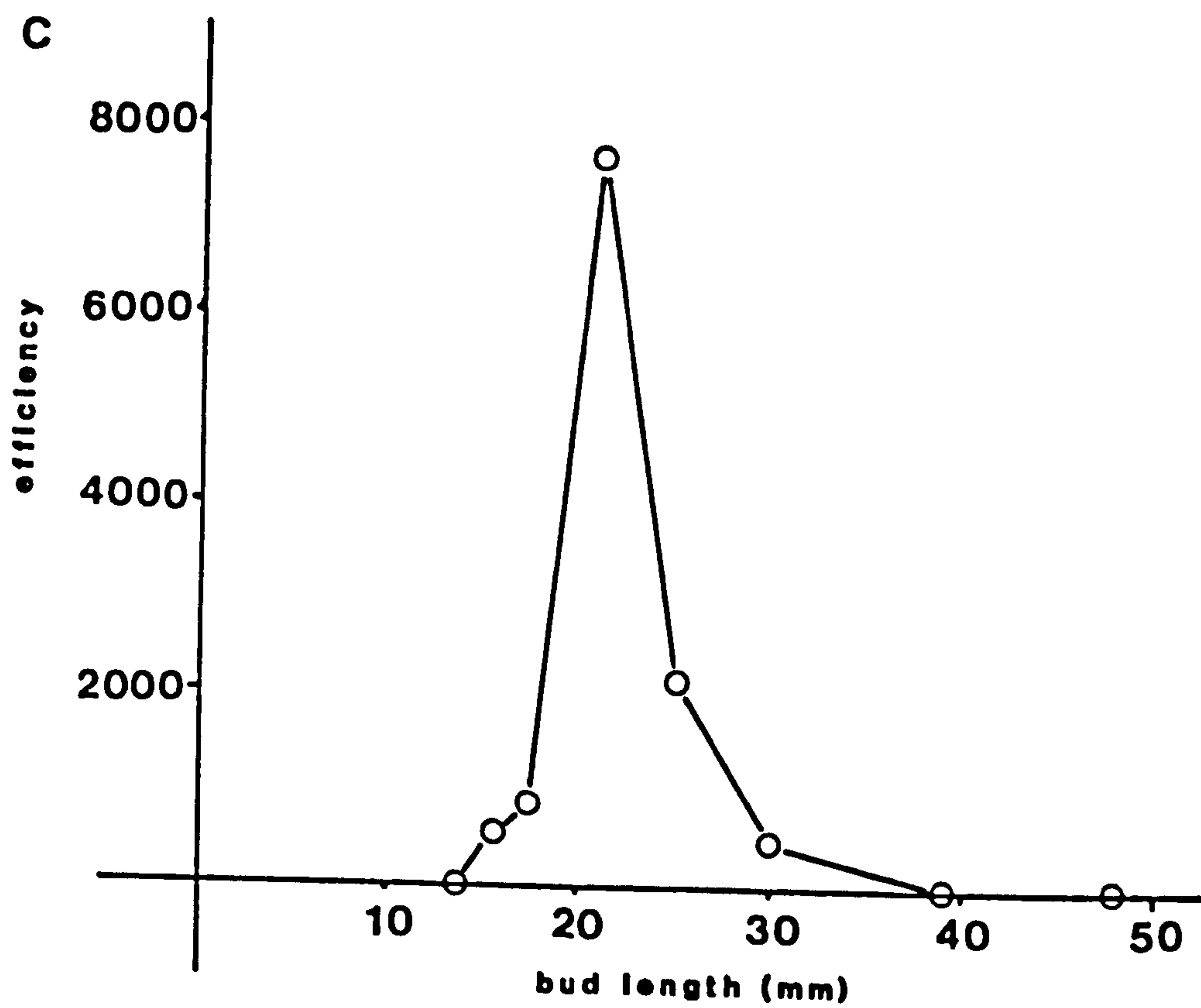


FIGURE 3.3.9

Stages in the recovery of allodihaploid plants following *N. tabacum*
cv. White Burley anther culture.

3.3.9 - A : Pollen derived globular embryo.
(X 20)

3.3.9 - B : Pollen derived torpedo stage embryo.
(X 20)

3.3.9 - C : Macroscopic pollen derived embryo.
(X 20)

3.3.9 - D : Small pollen derived plantlets emerging from
anthers cultured individually in the wells of a
5 x 5 multiwell culture plate. (x 0.5)

3.3.9 - E : Allodihaploid plant (left) derived from anther
culture, and an allotetraploid plant (right) grown
from seed and grown to maturity under the same
conditions.
(X 0.06)

Figures A-C result from anthers floated on liquid NH medium, and
Figure D and the allodihaploid plant in Figure E result from
anthers cultured on solid NH medium.



Table 3.3.3 : Anther culture in *Cyclamen persicum*.

a) After bud pre-treatment at 4 - 6.5°C for 0, 4, 8 and 12 days.

Medium	Pre-treatment duration (days)	T.A.	T.R.	T.E.
NH	0	99	0	0
	4	98	0	0
	8	100	0	0
	12	100	0	0
NH + 1% Activated Charcoal	0	98	0	0
	4	98	0	0
	8	99	0	0
	12	100	0	0
MS-R1	0	105	0	0
	4	100	0	0
	8	97	0	0
	12	99	0	0

b) After bud pre-treatment at 15 ± 2°C for 0, 4, 8 and 12 days.

Medium	Pre-treatment duration (days)	T.A.	T.R.	T.E.
MS-R1	0	100	0	0
	4	99	0	0
	8	92	0	0
	12	95	0	0

c) After bud pre-treatment at 25 ± 2°C for 0, 4, 8 and 12 days.

Medium	Pre-treatment duration (days)	T.A.	T.R.	T.E.
MS-R1	0	88	0	0
	4	105	0	0
	8	110	0	0
	12	94	0	0

Table 3.3.4 : Anther culture in *Nicotiana glutinosa* after bud pre-treatment at 4 - 6.5°C for 0, 4 and 8 days.

a) Anthers cultured on solid NH medium.

Pre-treatment duration (days)	Bud size	T.A.	T.R.	T.E.
0	1	75	0	0
	2	74	0	0
	3	100	0	0
	4	75	0	0
4	1	175	0	0
	2	180	0	0
	3	170	0	0
	4	125	0	0
8	1	120	0	0
	2	155	0	0
	3	135	0	0
	4	65	0	0

b) Anthers cultured on solid NH medium with the addition of 1.0% (w/v) activated charcoal.

Pre-treatment duration (days)	Bud size	T.A.	T.R.	T.E.
0	1	25	0	0
	2	25	0	0
	3	25	0	0
	4	25	0	0
4	1	100	0	0
	2	95	0	0
	3	105	0	0
	4	130	1	2
8	1	25	0	0
	2	25	0	0
	3	25	0	0
	4	25	0	0

Table 3.3.4 continued ...

c) Anthers cultured on solid NH medium with the addition of
0.1 mg/L 2,4-D.

Pre-treatment duration (days)	Bud size	T.A.	T.R.	T.E.
0	1	50	0	0
	2	25	0	0
	3	50	0	0
	4	50	0	0
4	1	50	0	0
	2	75	0	0
	3	75	0	0
	4	50	0	0
8	1	100	0	0
	2	125	0	0
	3	125	0	0
	4	100	0	0

FIGURE 3.3.10

Cytological observations on seed grown and anther culture derived plants of N. tabacum cv. White Burley, and N. glutinosa.

3.3.10 - A : Allotetraploid N. tabacum cv. White Burley
($2n = 4x = 48$) grown from seed.
(X 1000)

3.3.10 - C, and E :
Allodihaploid N. tabacum plants ($n = 2x = 24$)
recovered from anther culture.
(X 1200 and 1000 respectively)

3.3.10 - B : Diploid N. glutinosa ($2n = 2x = 24$) grown from
seed.
(X 1000)

3.3.10 - D, and F :
Haploid N. glutinosa plants ($n = x = 12$) recovered
from anther culture.
(X 12000)

The chromosome preparations are metaphase spreads of actively
dividing root tip cells stained in feulgen.

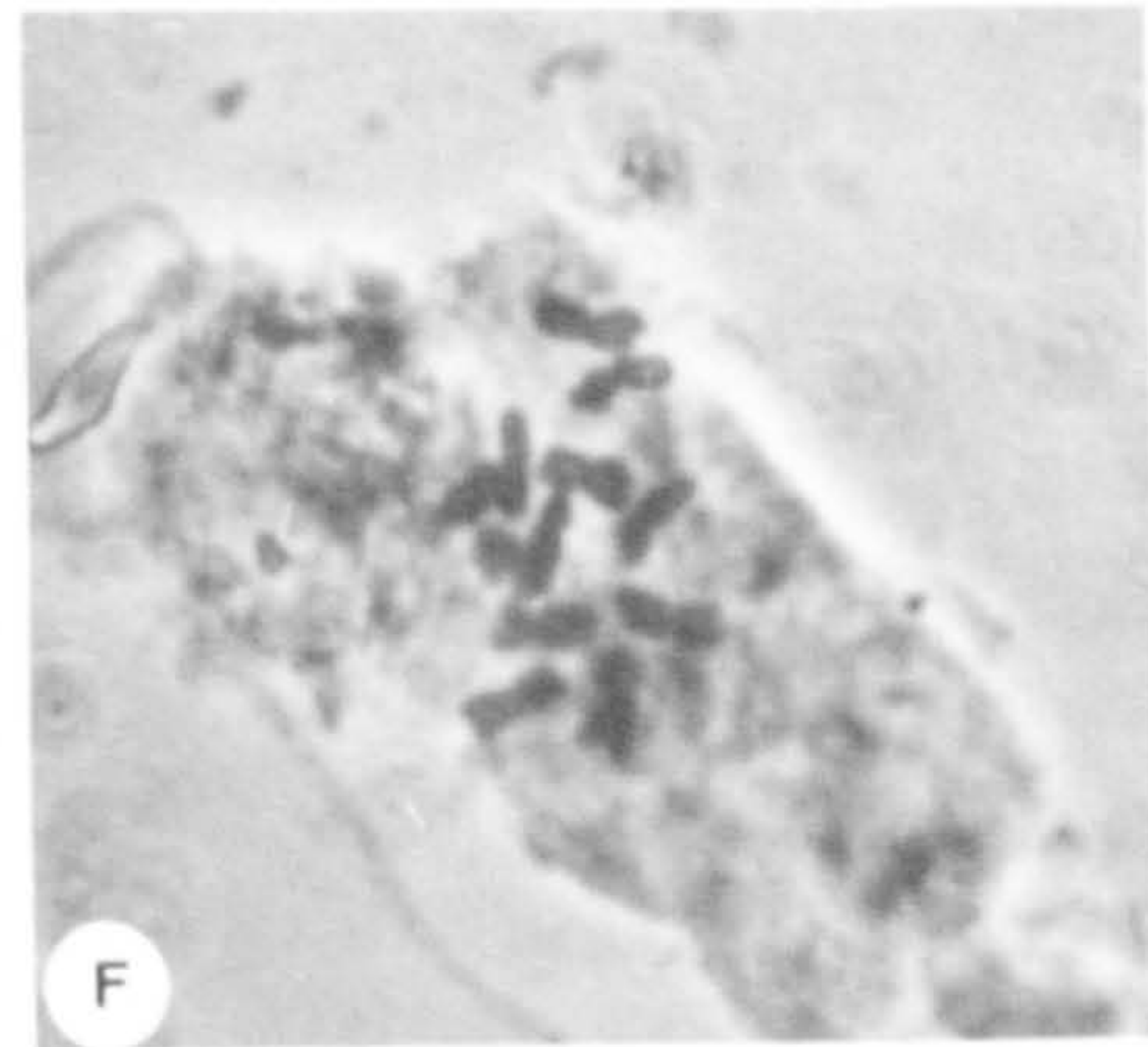
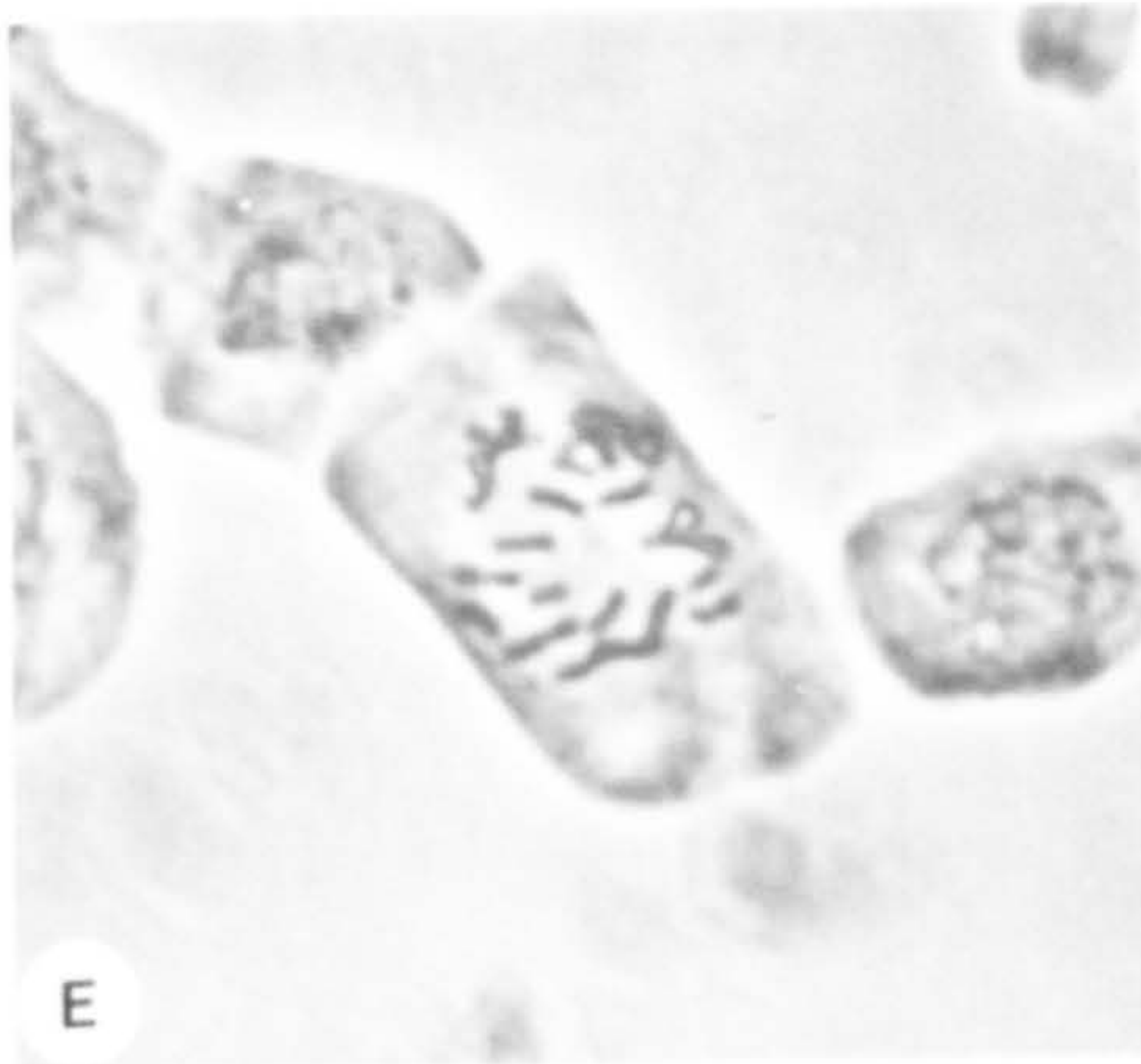
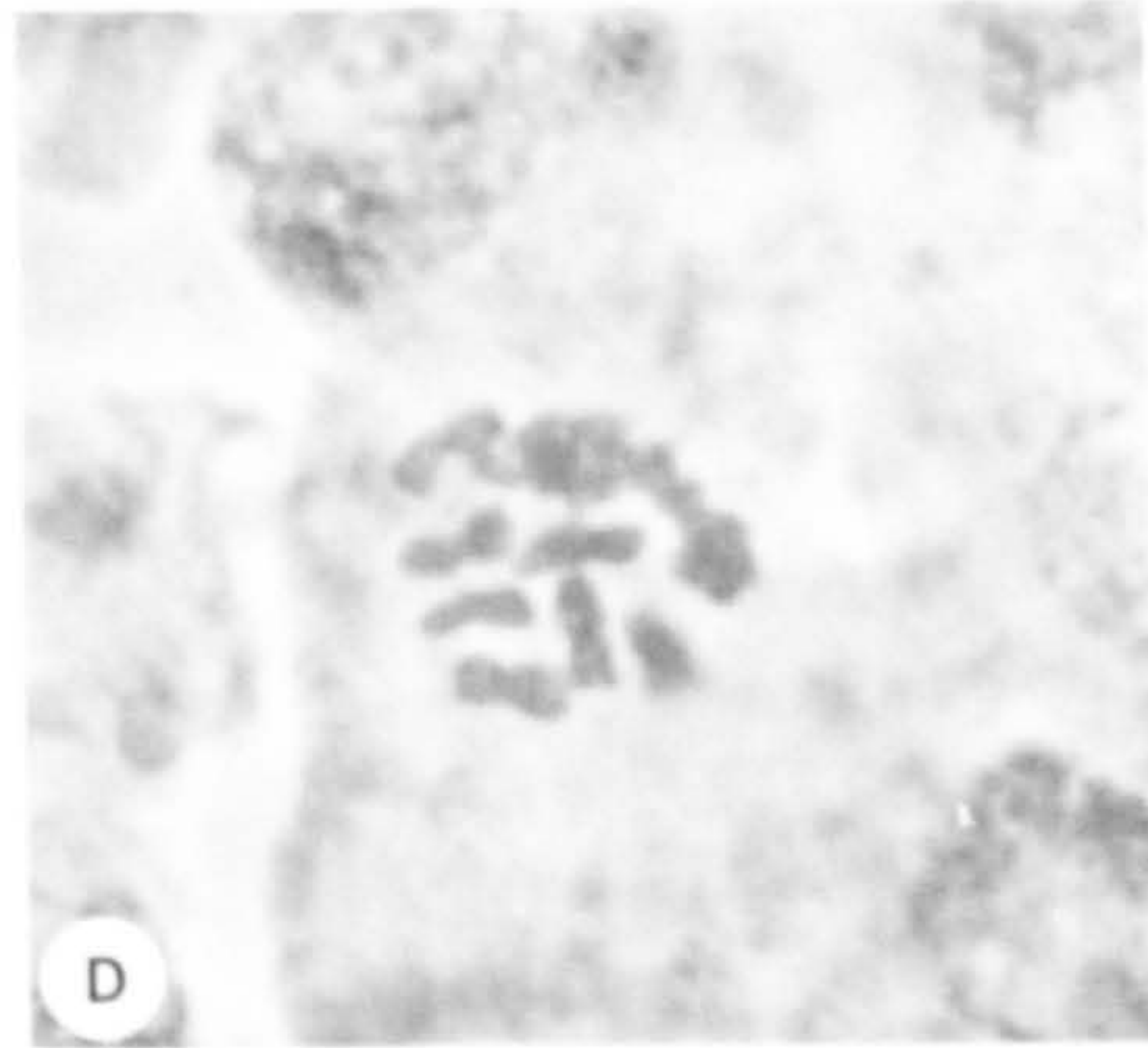
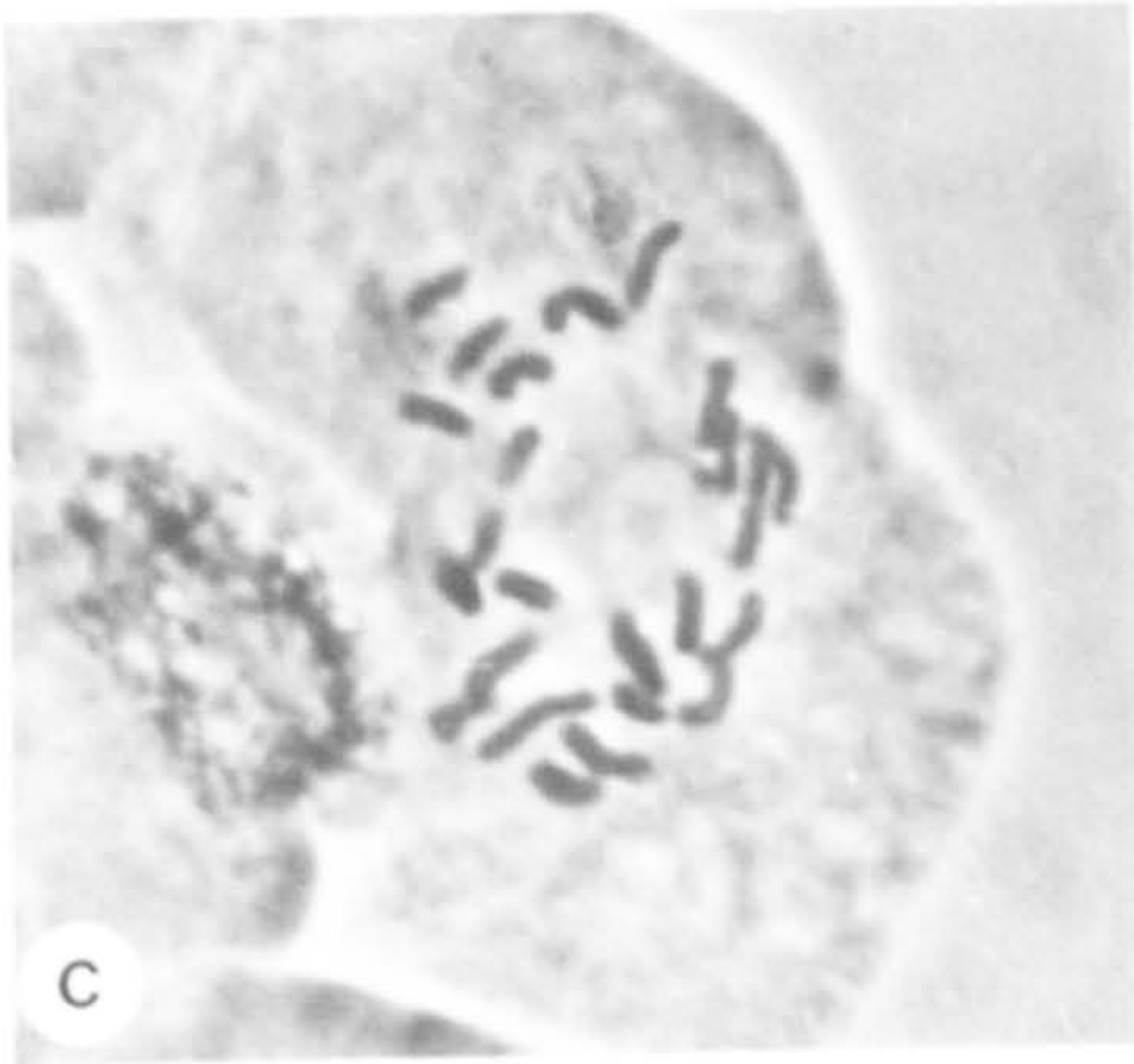
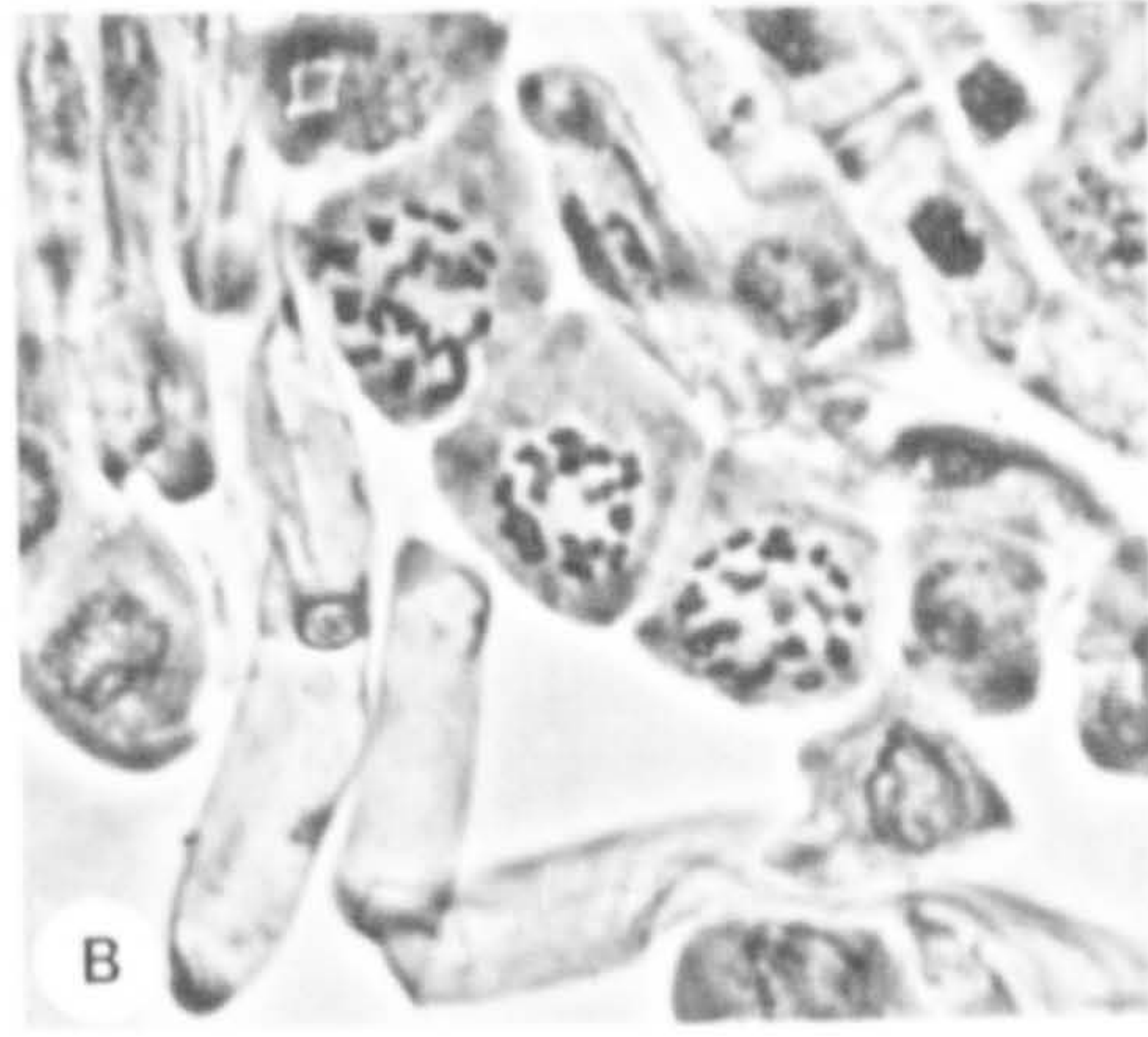
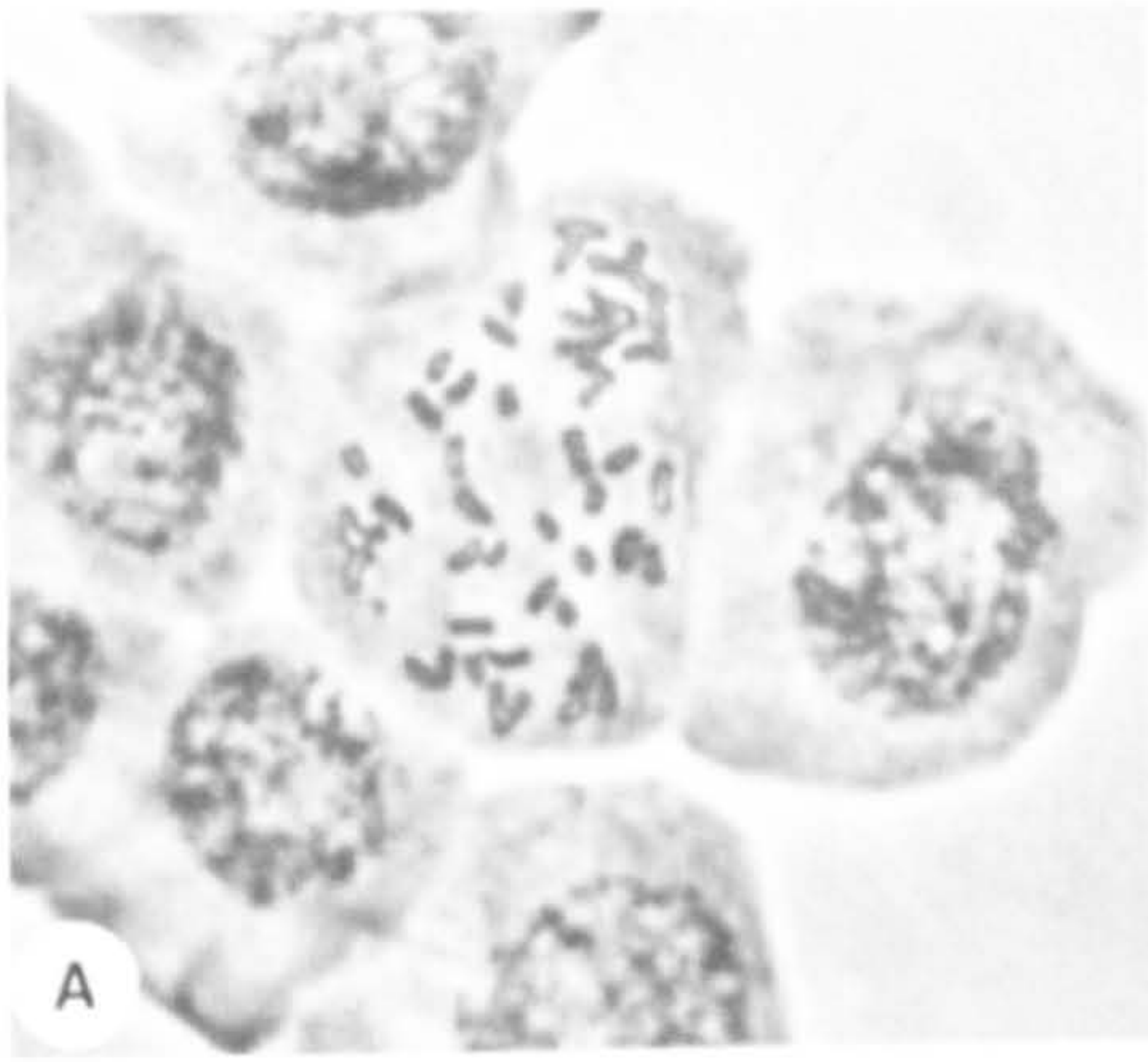


FIGURE 3.3.11

Comparative leaf and floral morphology of haploid and diploid
N. glutinosa plants.

3.3.11 - A : Haploid N. glutinosa plant ($n = x = 12$) recovered
from anther culture.
(X 0.5)

3.3.11 - B : Morphology of flowers from haploid (upper) and
diploid (lower) N. glutinosa plants.
(X 0.5)

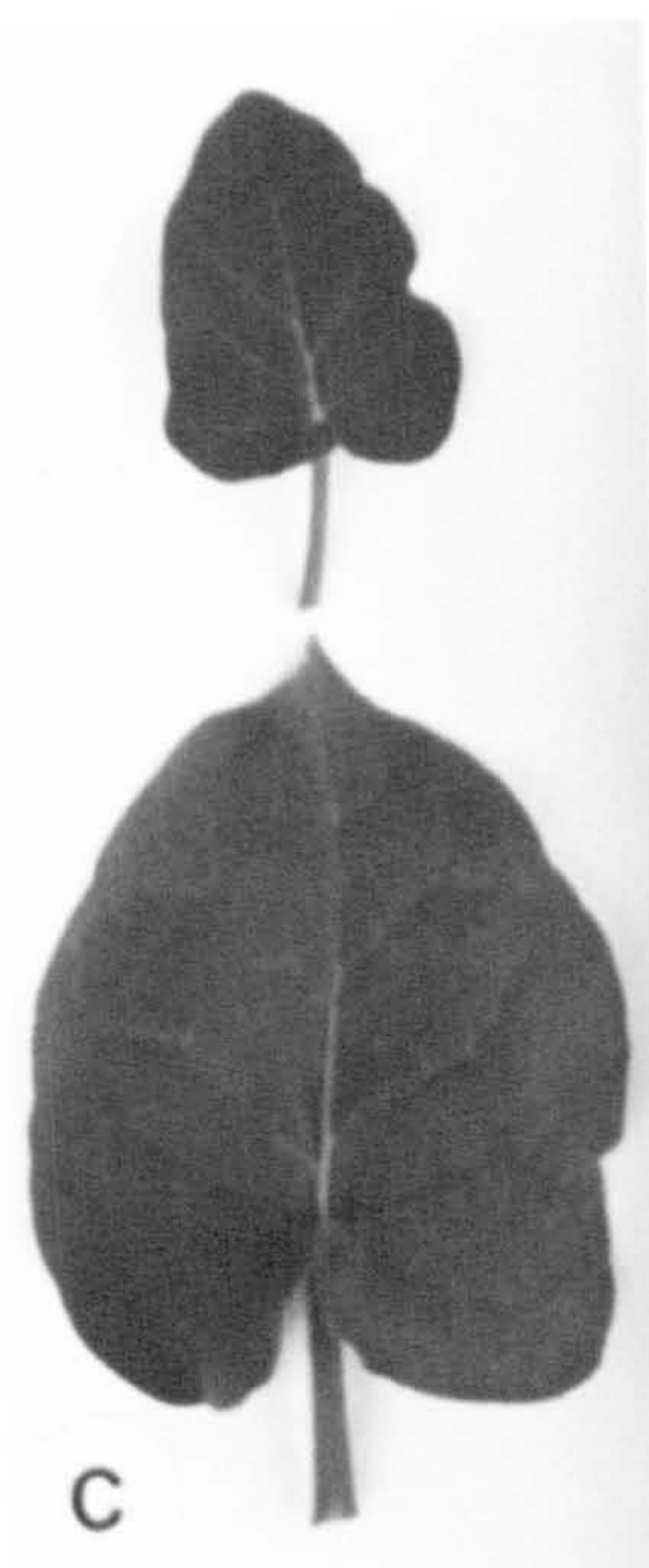
3.3.11 - C : Morphology of leaves from haploid (upper) and
diploid (lower) N. glutinosa plants.
(X 0.5)



A



B



C

3.4 CONCLUSION

The pattern of pollen development was similar in N. tabacum, N. glutinosa and Cyclamen persicum, and followed the standard pattern described in the introduction. No abnormalities were observed in the development of N. tabacum and N. glutinosa pollen. However, tetrads containing additional spores, as well as diads were found in Cyclamen persicum, and presumably resulted from abnormal reduction divisions. This may have accounted for the occurrence of occasional diploid pollen grains.

In N. tabacum cv. White Burley pollen embryogenesis could be induced in the cultured anthers. When directly cultured on solid NH medium anthers containing pollen as the uninucleate stage of development, but possessing a vacuole, gave a response superior to that obtained with anthers containing early binucleate pollen, with respect to the induction frequency and anther productivity. However, the overall efficiency was low, and could be dramatically increased when the buds were pretreated at 4.0 - 6.5°C. The optimum duration of the bud pretreatment was 8 days, and under these conditions anthers containing early binucleate pollen gave the maximum response. The stage of pollen development was found to significantly influence the anther culture response. The culture efficiency was low, or no response was achieved with pollen before the uninucleate vacuolated stage just prior to mitosis, or beyond the early binucleate stage. Most of the plants recovered from N. tabacum anther cultures were found to be allodihaploid ($n = 2x = 24$), and a sample grown to maturity were found to be shorter than their allotetraploid ($2n = 4x = 48$) counterparts. The allodihaploid plants also had smaller, more narrow leaves, and smaller flowers. They were completely sterile.

Under the conditions tested, no macroscopic embryos or emerging plantlets were recovered from cultured Cyclamen anthers. It must be concluded that either conditions under which Cyclamen pollen can be induced to undergo embryogenesis have not been found, or that Cyclamen persicum is entirely recalcitrant to anther culture.

N. glutinosa anther culture was successful, although the recovery of plantlets was very low, only two plantlets were recovered from one responding anther out of a total of 2,954 anthers cultured. The responding anther contained early-late binucleate pollen, and was cultured on solid NH medium containing 1.0% (w/v) activated charcoal following bud pretreatment at 4.0 - 6.5°C for 4 days. The two plantlets were found to possess the haploid chromosome complement ($n = x = 12$). When grown to maturity the plants were found to be shorter than their diploid counterpart, and also possessed narrower leaves and smaller flowers. The plants were sterile.

ISOLATION AND CULTURE OF HAPLOID PROTOPLASTS
SUITABLE FOR SOMATIC HYBRIDISATION STUDIES

4.1 INTRODUCTION

For successful somatic hybridisation a source of large numbers of protoplasts must be provided, and selection schemes designed to eliminate homokaryons or unfused protoplasts permitting the recovery of the small number of heterokaryon derived colonies or plants. The potential advantages of fusing haploid protoplasts of a wild type species with diploid protoplasts of a crop species in facilitating limited gene transfer have been discussed in the General Introduction. The choice of combining diploid protoplasts from nitrate reductase deficient N. tabacum plants with haploid N. glutinosa protoplasts requires the development of a haploid N. glutinosa protoplast system.

Mesophyll protoplasts have previously been isolated from anther culture derived haploid plants, and have proved useful in the recovery of mutant plants and cell lines in several species including N. tabacum (Muller and Grafe, 1978; Caboche, 1980; Muller et al., 1985), N. plumbaginifolia (Negrutui et al., 1985) and Hyoscyamus muticus (Straus et al., 1981; Gebhardt et al., 1981). Protoplasts isolated from non-allelic light sensitive mutant allodihaploid N. tabacum plants have been fused, and intraspecific hybrids recovered which grew dark green (Melchers and Labib, 1974). Haploid plants of N. glutinosa ($n = x = 12$) were recovered from anther culture experiments (Chapter 3). The isolation and culture of mesophyll protoplasts from these plants was assessed to determine their suitability for fusion studies. Protoplasts were also isolated from allodihaploid N. tabacum

cv. White Burley plants, also derived from anther culture experiments (Chapter 3).

An alternative source of haploid protoplasts was also investigated. Tetrads formed as a result of meiosis in the pollen mother cells consist of four haploid spores bound within a thick callose wall. Tetrad protoplast isolation has been reported in a range of species including N. tabacum, N. sylvestris, Petunia hybrida (Bhojwani and Cocking, 1972), Lycopersicon esculentum, Cajanus cajan and Zea mays (Deka et al., 1974). Tetrad protoplasts have been reported to undergo spontaneous fusion (Bhojwani and Cocking, 1972) suggesting that they may be highly fusogenic. It has been suggested that the use of highly fusogenic protoplasts might permit high fusion frequencies to be obtained using fusion inducing treatments which cause less damage to the protoplasts (Boss et al., 1984). Tetrad protoplasts have not been reported to undergo sustained division, eliminating the need for selection schemes to be developed against tetrad protoplasts if used in fusion experiments. The isolation and culture of tetrad protoplasts from N. glutinosa was therefore examined. Tetrad protoplasts were also introduced into nitrate reductase deficient N. tabacum nia-130 and albino Petunia hybrida nurse cultures in attempts to recover tetrad protoplast derived colonies. The nitrate reductase deficient N. tabacum mutant system is discussed in detail in Chapter 5.

4.2 MATERIALS AND METHODS

4.2.1 Source and Growth of Plant Material

Allodihaploid Nicotiana tabacum cv. White Burley ($n = 2x = 24$) and haploid N. glutinosa ($n = x = 12$) plants generated from anther culture experiments (Chapter 3) were transferred from culture to 3½ inch pots containing Levingtons soil-less compost (Fisons, U.K.) and maintained initially in a mist propagator before being transferred to the greenhouse at 18°C (minimum) with a 16 hour photoperiod supplemented with daylight fluorescent tubes (Thorne, U.K.) at an intensity of 4,000 - 5,000 lux (minimum). Diploid N. glutinosa plants ($2n = 2x = 24$) were grown to flowering under the same conditions.

Seeds of Nicotiana tabacum mutant line nia-130 were originally obtained from Dr. A. Muller, Zentralinstitut für Genetic Und Kulturpflanzenforschung, Gatersleben, GDR. Seed was subsequently obtained following self-pollination of bagged flower heads. Small seedlings of nia-130 were pricked out into vacapots (H. Smith Plastics Ltd., Wickford, U.K.) and were maintained in the controlled environment of a growth room (S.B. Refridgeration Ltd., U.K.) at 22°C (day) and 18°C (night) with a 16 hour photoperiod. Light was provided by daylight fluorescent tubes (Thorne, U.K.) at an intensity of 3,000 - 4,000 lux at bench level. Small plants 10-15 cm high were removed from the vacapots and transferred to hydroponic tanks. The hydroponic tanks were constructed using 13L plastic aquaria, painted black to prevent algal growth. The compost was carefully washed from the roots of the plants, and the stem inserted through holes (1.5 cm diameter) in the lid of the hydroponic tank. 4-6 plants were established in each tank. The tanks were filled with tap water supplemented with 5 ml of Bentleys Liquid Growmore compound fertiliser (7-7-7) (J. Bentley Ltd., Barrow-on-Humber, U.K.). 10 ml of a stock solution of 250 g/L

ammonium nitrate was also added to each tank. The nutrient solution was aerated to encourage rooting and prevent decay of the submerged plant parts, and was replaced every 2-3 weeks. The tanks were located in a greenhouse maintained at 20°C (minimum) with a 16 hour photo-period supplemented with daylight fluorescent tubes (Thorne, U.K.) at 5,000 lux (minimum).

An albino cell suspension of Petunia hybrida cv. Comanche was provided by Dr. J.B. Power, who isolated this mutant in this laboratory several years ago.

4.2.2 Contamination Control

Routine greenhouse contamination control was carried out as described in Chapter 2.2.3.

4.2.3 Media and Enzyme Preparation, Sterilisation and Storage

Media and enzyme solutions were prepared, sterilised and stored as described in Chapter 2.2.4 and 2.2.5. Full details of media composition are given in Appendix 1.

4.2.4 Mesophyll Protoplast Isolation

Young fully expanded leaves were removed from greenhouse grown haploid N. glutinosa, allodihaploid N. tabacum cv. White Burley and also from N. tabacum nia-130 plants grown in hydroponics. The leaves were surface sterilised by immersion in 7.5% (v/v) Domestos (Lever Bros. Ltd., U.K.), followed by four washes in sterile tap water. When possible the lower epidermis of the leaves was removed by peeling with fine forceps, and the peeled leaf fragments floated on the surface of 10 ml CPW 13M in a 9 cm petri dish (Sterilin Ltd., U.K.). When the leaves proved difficult to peel, they were chopped into thin slices and also placed into 10 ml CPW 13M solution. After 1-2 hours the CPW 13M

was removed and replaced with 10 ml of enzyme solution containing 1.5% (w.v) meicelase P (Meiji Seika Kaisha Ltd., Japan) and 0.5% (w.v) macerozyme R10 (Yakult Honsha Co. Ltd., Japan). The leaf pieces were incubated in enzyme solution overnight (13-16 hours) at $22 \pm 2^{\circ}\text{C}$ on a rotary shaker (30 cycles/minute) in the dark. Following incubation the protoplasts were released into the enzyme solution and recovered following flotation on CPW 21S as described in Chapter 2.2.10.

4.2.5 Albino *Petunia hybrida* Protoplast Isolation

An albino *Petunia hybrida* cv. Comanche cell suspension was maintained in liquid UM medium on a rotary shaker (80 cycles/minute) at $22 \pm 2^{\circ}\text{C}$ in the light (1,300-2,300 lux). Suspensions were sub-cultured every 7 days by transferring 10 ml of cell suspension to 70 ml of fresh medium in a 250 ml erlenmeyer flask. Protoplasts were isolated 3 or 4 days after subculture. The cells were allowed to settle and the medium removed with a pasteur pipette. 20 ml of enzyme solution containing 2% (w/v) rhozyme HP 150 (Rohm and Haas Co., U.S.A.) 2% (w/v) meicelaseP (Meiji Seika Kaisha Ltd., Japan) and 0.3% (w/v) macerozyme R10 (Yakult Honsha Co. Ltd., Japan) was added, and the cells incubated at $22 \pm 2^{\circ}\text{C}$ overnight (13-16 hours) on a rotary shaker (30 cycles/minute). Subsequently the enzyme solution was passed through a 64 μm sieve, and the protoplasts recovered following flotation on CPW 21S as described in Chapter 2.2.10.

4.2.6 *N. glutinosa* Tetrad Protoplast Isolation

Buds were selected on the basis of length so as to contain pollen at the tetrad stage of development. Based on the results of Chapter 3, buds measuring 14 mm in length were selected. The buds were surface sterilised in 10% (v/v) Domestos (Lever Bros. Ltd., U.K.) followed by four washes in sterile tap water. 25 buds were placed

individually in the wells of a sterile multiwell dish (100 mm square with 25 wells) (Sterilin Ltd., U.K.) and one anther from each bud removed to determine the stage of pollen development. The remaining anthers in those buds determined to contain tetrads were removed, and divided into six groups. The anthers were crushed in 2 ml of one of six enzyme solutions tested, 1 or 2% helicase (Reactifs IBF, France), 1 or 2% cellulase R10 (Yakult Honsha Co. Ltd., Japan) or 1 or 2% driselase (Kyowa - Hakko Kogyo Co. Ltd., Japan) in CPW 9M. The remaining anther debris was removed and the tetrads incubated in 3 cm petri dishes (A/C Nunc, Kamstrup, Denmark) at 25°C for two hours, in the dark.

Tetrad protoplasts were subsequently isolated in 2% (w/v) driselase. Protoplasts were centrifuged at 80 x g for 5 minutes, and resuspended in CPW 9M.

4.2.7 Viability Determination

Protoplast viability was determined on the basis of fluorescence following treatment with fluorescein diacetate, as described in Chapter 2.2.11.

4.2.8 Leaf Mesophyll Protoplast Culture

Leaf mesophyll protoplasts isolated from haploid N. glutinosa and allodihaploid N. tabacum plants were cultured in liquid MS-P19M KMP8 and AAP 19M media. The protoplasts were suspended at 2.5×10^4 , 5.0×10^4 and 1.0×10^5 protoplasts/ml of medium, and cultured in 5 cm petri dishes (A/S Nunc, Kamstrup, Denmark), 4 ml medium/dish at $25 \pm 2^\circ\text{C}$ in the dark, or with continuous illumination at 2,100-2,300 lux provided by cool white fluorescent tubes (Thorne, U.K.).

4.2.9 N. glutinosa Tetrad Protoplast Cultures

N. glutinosa tetrad protoplasts were also cultured as described in 4.2.8, except 1.5 ml of medium was cultured in 3 cm petri dishes.

N. glutinosa tetrad protoplasts were also introduced to albino Petunia hybrida cv. Comanche and N. tabacum nia-130 nurse protoplast cultures. Albino P. hybrida cell suspension protoplasts were cultured at a density of 5.0×10^4 protoplasts/ml KMP8 media, 8 ml per 9 cm petri dish and were maintained at $25 \pm 2^\circ\text{C}$ in the dark for 1 or 2 days before $1 - 2 \times 10^5$ N. glutinosa tetrad protoplasts in 0.5 ml CPW 9M were introduced. The cultures were maintained for a further 14 days, and the osmoticum reduced by dilution of the colonies with KM8 medium. After a further 28 days growth at $25 \pm 2^\circ\text{C}$ with continuous illumination (2,100 - 2,300 lux). The cultures were examined for green or pale green colonies.

N. tabacum nia-130 leaf mesophyll protoplasts were cultured at a density of 5.0×10^4 /ml liquid AA-P19M medium at $25 \pm 2^\circ\text{C}$ in the dark for two days before $1 - 2 \times 10^5$ tetrad protoplasts were introduced into the culture as for P. hybrida nurse cultures. After a further 21 days the colonies were transferred to selection medium, MSN0₃4.5 M, and cultured at $25 \pm 2^\circ\text{C}$ with continuous illumination for a further 4-6 weeks. The cultures were examined for actively growing green or pale green colonies.

All protoplast isolation and culture experiments were performed a minimum of four times.

4.3 RESULTS

4.3.1 Isolation and Culture of Haploid N. glutinosa, and Allodihaploid N. tabacum Mesophyll Protoplasts

Mesophyll protoplasts were successfully isolated from the leaves of haploid N. glutinosa, and allodihaploid N. tabacum plants, derived from anther culture (see Figure 4.3.1 A and B). In the case of N. tabacum, the lower epidermis was readily removed by peeling, and the protoplast yield varied between 2.7 and 4.5×10^5 protoplast/g leaf tissue. The viability of the protoplasts as determined by fluorescence following treatment with F.D.A. was between 38 and 72%. When cultured, protoplast division was infrequent. Occasional divisions were observed after 12 days of culture in MS-P19M, KMP8 and AA-P19M media, when the protoplasts were suspended at a density of 5×10^4 or 1×10^5 protoplasts/ml, maintained at $25 \pm 2^\circ\text{C}$ in the dark.

The lower epidermis of leaves from haploid N. glutinosa plants proved difficult to remove by peeling, and these leaves were usually cut into fine slices. The protoplast yield was lower than that obtained for allodihaploid N. tabacum leaves, ranging between 5.8×10^4 and 1.4×10^5 protoplasts/g. The sliced leaf pieces were poorly digested in the enzyme solution leaf tissue. The viability as determined by fluorescence following treatment with F.D.A. was however similar, being between 34 and 76%. When haploid N. glutinosa leaf mesophyll protoplasts were cultured in MS-P19M, AA-P19M and KMP8 media, no protoplast division was observed. The protoplasts remained spherical after 2 days, but subsequently lost their shape, and degenerated.

4.3.2 Isolation and Culture of N. glutinosa Tetrad Protoplasts

When buds measuring 14 mm in length were selected, and one anther from each of 25 buds examined to determine the stage of pollen

development, usually between 6 and 12/25 buds were found to contain tetrads. When the tetrads were released into the enzymes tested, and observed after 2 hours incubation at 25°C in the dark tetrad protoplasts were observed in 1 and 2% driselase, and 1 and 2% helicase (see Figure 4.1 C). Only partial dissolution of the tetrad occurred in 1 and 2% cellulase R10, resulting in the occurrence of some tetrad protoplasts, and some partly digested or wholly complete tetrads. Using driselase and helicase, 90-100% conversion of tetrads to isolated tetrad protoplasts occurred. Tetrad protoplasts were small (approximately 12 μ in diameter) and spherical. Each anther gave rise to approximately 4×10^4 tetrad protoplasts, and 20 anthers from a total of five buds gave a yield of over 5×10^5 tetrad protoplasts.

Tetrad protoplasts isolated with 2% driselase were cultured in MS-P19M, KMP8 and AA-P19M media. No division was observed, although the protoplasts remained spherical after 6 days in culture. However, the protoplasts were observed to agglutinate into small clumps.

Tetrad protoplasts isolated with 2% driselase were also introduced into albino Petunia hybrida cv. Comanche, and nitrate reductase deficient N. tabacum nia-130 mutant nurse cultures. The cultures were maintained as described, and later examined for the growth of either green or pale green colonies amongst the pale albino P. hybrida protoplasts, or for actively growing colonies among those small colonies incapable of growth on a medium containing nitrate as sole nitrogen source, in the case of the N. tabacum nia-130 nurse cultures. Following this procedure no colonies were recovered.

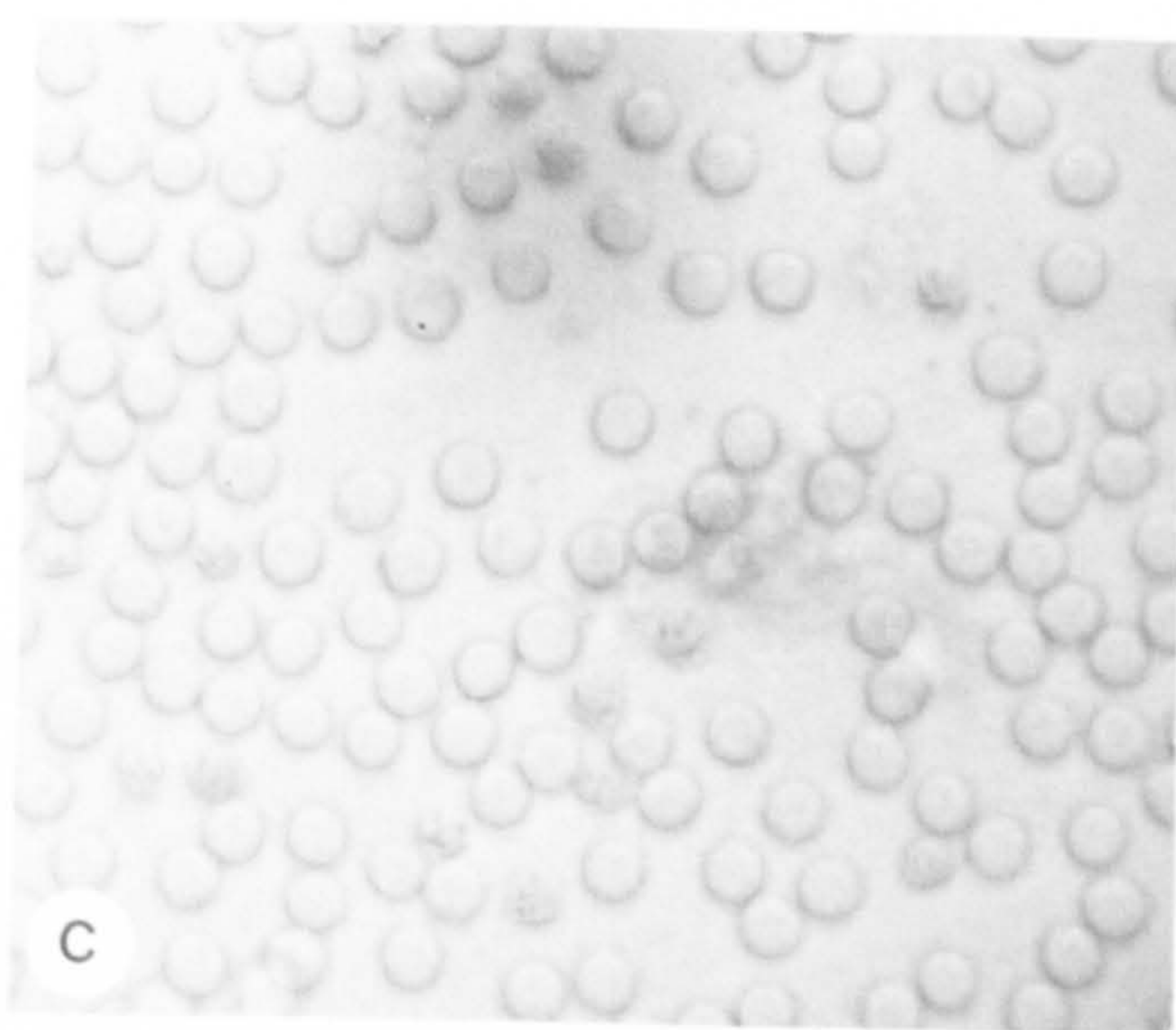
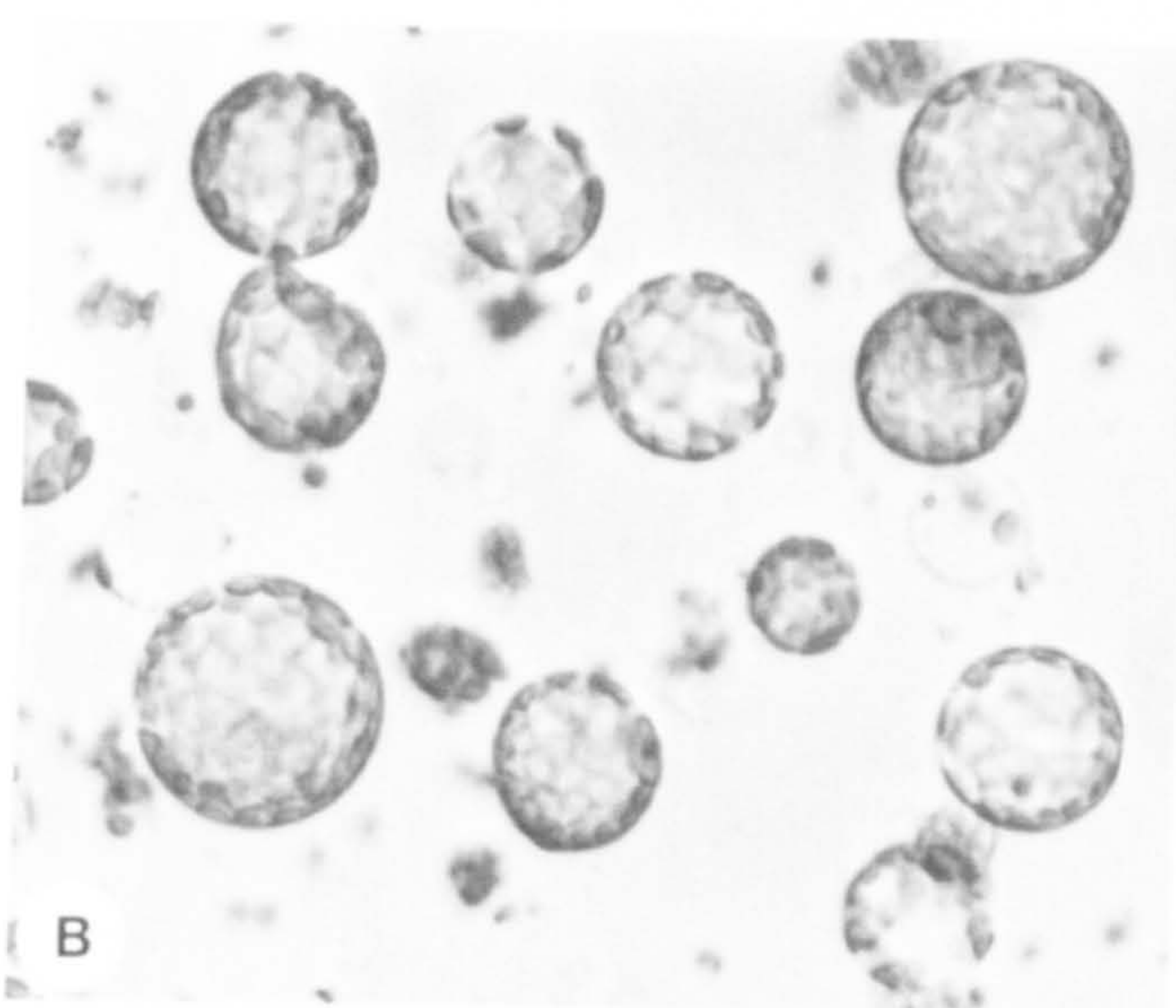
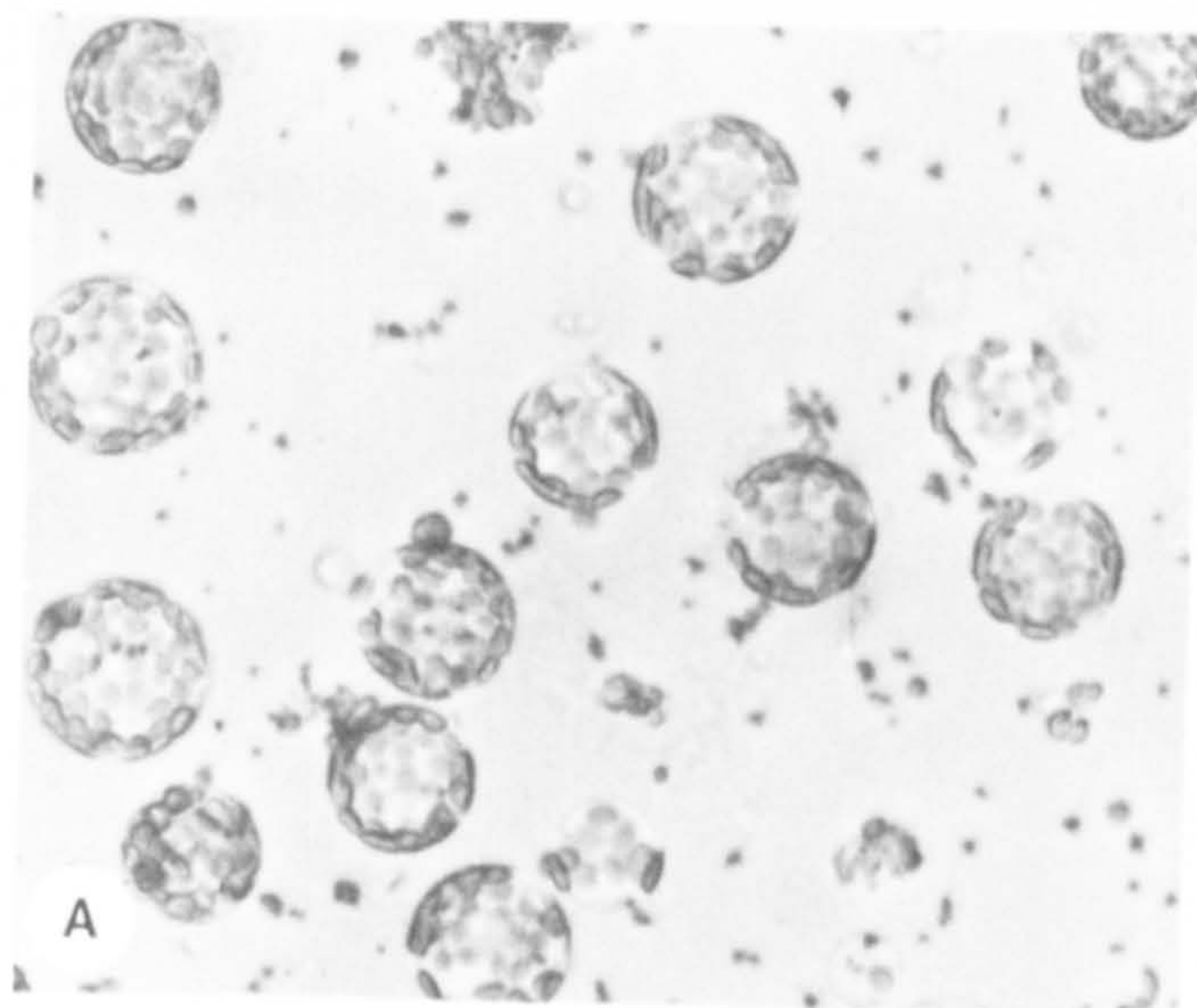
FIGURE 4.3.1

Mesophyll protoplasts isolated from allodihaploid N. tabacum cv. White Burley, and haploid N. glutinosa plants, and haploid tetrad protoplasts isolated from diploid N. glutinosa plants.

4.3.1 - A : Mesophyll protoplasts isolated from allodihaploid
N. tabacum plants.
(X 300)

4.3.1 - B : Mesophyll protoplasts isolated from haploid
N. glutinosa plants.
(X 300)

4.3.1 - C : Tetrad protoplasts isolated from diploid
N. glutinosa plants.
(X 300)



4.4 CONCLUSION

Leaf mesophyll protoplasts can be isolated from haploid N. glutinosa and allodihaploid N. tabacum plants obtained following anther culture. The yield of protoplasts from allodihaploid N. tabacum leaves is similar to that reported for normal allotetraploid N. tabacum plants (Pental et al., 1982). The yield of protoplasts from haploid N. glutinosa leaves was low, however the leaves were difficult to peel, and the chopped leaf slices were poorly digested in the enzyme solution tested.

Protoplasts isolated from leaves of allodihaploid N. tabacum plants divided infrequently in the media tested, and no protoplast division was observed among cultured haploid N. glutinosa protoplasts.

N. glutinosa tetrad protoplasts were readily isolated. Sufficient tetrad containing anthers for the isolation of over 5×10^5 tetrad protoplasts were generally obtained following the selection of 25 buds measuring approximately 14 mm in length. Tetrad protoplasts were readily released from the callose wall of the tetrad following incubation in either driselase or helicase, at a concentration of 1 or 2%. 2% driselase was selected for further tetrad protoplast isolation experiments. Tetrad protoplasts were cultured in MS-P19M, KM8P and AA-P19M media, but were not observed to undergo division. The protoplasts were frequently clumped in masses after 4-6 days of culture.

When introduced to nurse cultures of albino Petunia hybrida and nitrate reductase deficient N. tabacum protoplasts, which had been cultured for 1 or 2 days, no colonies derived from tetrad protoplasts were recovered. Tetrad protoplasts can therefore be isolated from N. glutinosa plants in suitable numbers for somatic hybridisation attempts.

SOMATIC HYBRIDISATION BETWEEN NITRATE REDUCTASE DEFICIENT

NICOTIANA TABACUM LEAF MESOPHYLL PROTOPLASTS (2n)

AND N. GLUTINOSA TETRAD PROTOPLASTS (n)

5.1 INTRODUCTION

Tetrad protoplasts can be isolated from a wide range of species, and the potential advantages of using tetrad protoplasts in fusion studies have been discussed in the General Introduction. As has been noted, tetrad protoplast fusion can be brought about (Deka et al., 1977), but no somatic hybrid plants or tissues obtained. This may reflect a lack of research activity in this area, a lack of suitable systems with which to fully test the potential of tetrad protoplasts, or some biological reason may exist, preventing the formation and/or the development of heterokaryons formed following fusions involving tetrad protoplasts. The isolation of N. glutinosa tetrad protoplasts can readily be achieved, as detailed in Chapter 4.

Nitrate reductase deficient mutants of Nicotiana tabacum have been recovered following selection for resistance to chlorate. Chlorate an analogue of nitrate, is reduced to chlorite by the nitrate reductase enzyme. Chlorite is toxic to plant cells (Muller and Grafe, 1978). Plant regeneration was subsequently reported from the nitrate reductase deficient cell lines, and the mutant plants well characterised genetically (Muller, 1983). The nia-130 mutant plant line is homozygous for two unlinked recessive nuclear mutations, which are structural loci for the nitrate reductase apoprotein. The plants were found to possess the normal somatic chromosome complement of $2n = 4x = 48$, and set seed following self-pollination. The growth of

the nia-130 mutant plants, and isolation and culture of leaf mesophyll protoplasts from these plants has been studied in detail (Pental et al., 1982). Leaf mesophyll protoplasts isolated from the nia-130 mutant were developed for somatic hybridisation and transformation studies. The yield of nia-130 protoplasts is high, close to that obtained for wild type N. tabacum plants, and the cultured protoplasts have a high plating efficiency and grow rapidly in a medium containing an organic nitrogen source. Colonies derived from dividing nia-130 protoplasts have a high regeneration capacity. In reconstruction experiments designed to mimick the result of somatic hybridisation, it was found to be possible to recover small numbers of protoplast derived colonies of wild type origin following the introduction of a small number of wild type protoplasts, and selection of the colonies on a medium containing nitrate as sole source of nitrogen. In control experiments no revertant colonies were obtained when nia-130 colonies alone were placed on selection medium. The nitrate reductase deficient N. tabacum nia-130 mutant is therefore ideal for use as half selection in somatic hybridisation studies.

Somatic hybrids have previously been recovered between N. tabacum nia-130 leaf mesophyll protoplasts, and N. glutinosa cell suspension protoplasts (Cooper-Bland et al., 1985a). Selection of hybrids was based on nitrate reductase proficiency, and the green colour and regeneration potential of the recovered colonies. Colonies derived from unfused or homokaryon N. glutinosa cell suspension protoplasts were pale green and failed to regenerate on a medium which permitted the regeneration of nia-130 protoplast derived colonies. This species combination was chosen to determine if N. glutinosa tetrad protoplasts could be fused with nia-130 leaf mesophyll protoplasts, and somatic hybrid colonies and plants recovered. Such hybrids might be expected to possess a

functionally triploid chromosome number of $3n = 5x = 60$ and may therefore be useful in studies designed to achieve limited gene transfer from N. glutinosa into N. tabacum preceding N. glutinosa chromosome elimination as discussed in the General Introduction.

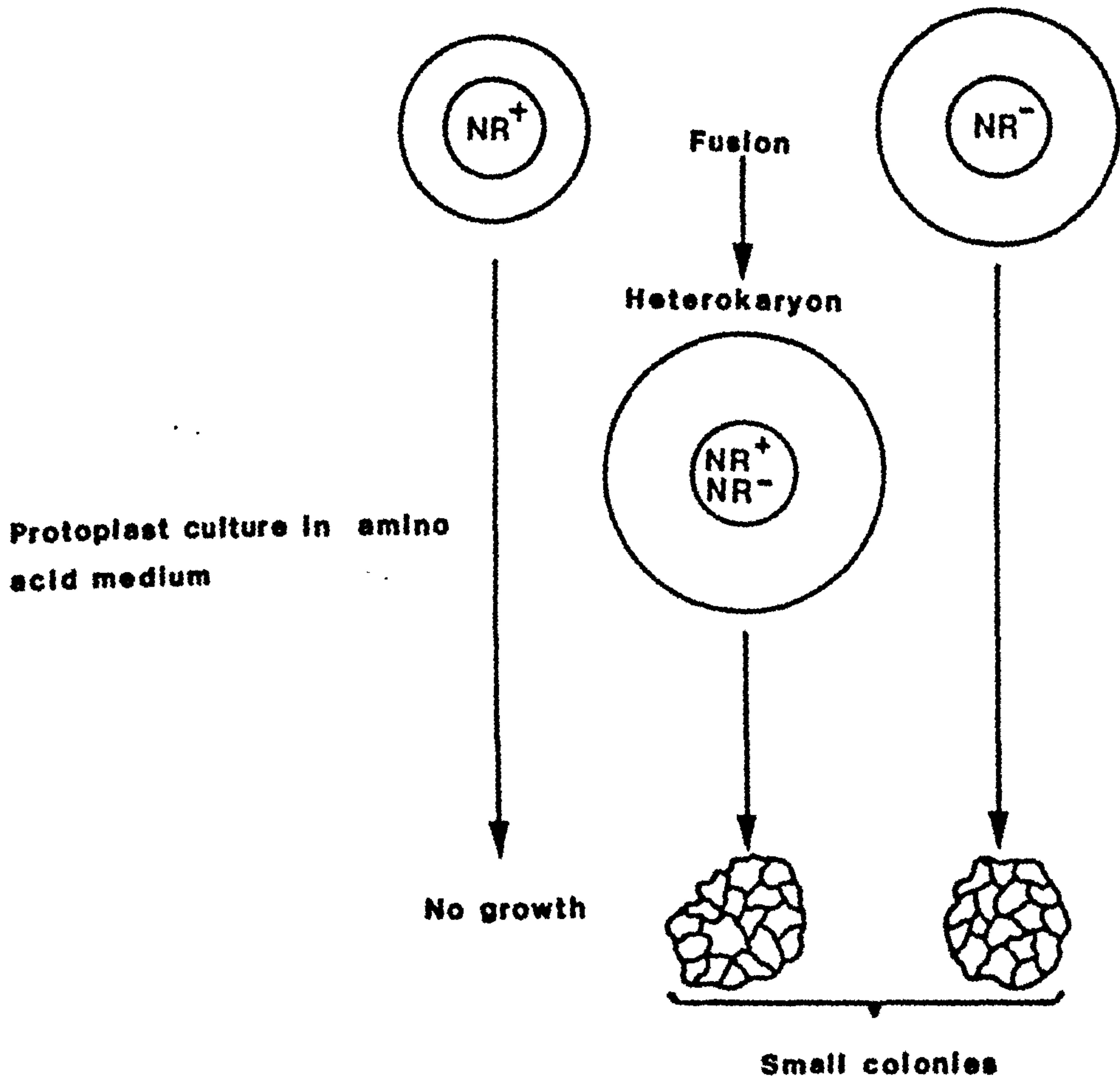
The selection scheme is detailed in Figure 5.1.1, and relies on the observed lack of division of tetrad protoplasts, when cultured alone, or in nia-130 nurse cultures (Chapter 4). Following fusion it is anticipated that only unfused and homokaryon nia-130 protoplasts, and heterokaryons formed between nia-130 protoplasts and N. glutinosa tetrad protoplasts will be capable of division in a medium supplemented with a reduced nitrogen source (AA-P19M). Subsequently only colonies derived from heterokaryons will be capable of continued growth when small colonies derived from fusion treated protoplasts are transferred to a selection medium containing nitrate as sole nitrogen source.

FIGURE 5.1.1

Diagram explaining the selection scheme designed to recover
somatic hybrids between *N. tabacum* *nia*-130 leaf mesophyll
protoplasts, and *N. glutinosa* tetrad protoplasts.

N. glutinosa
tetrad protoplasts

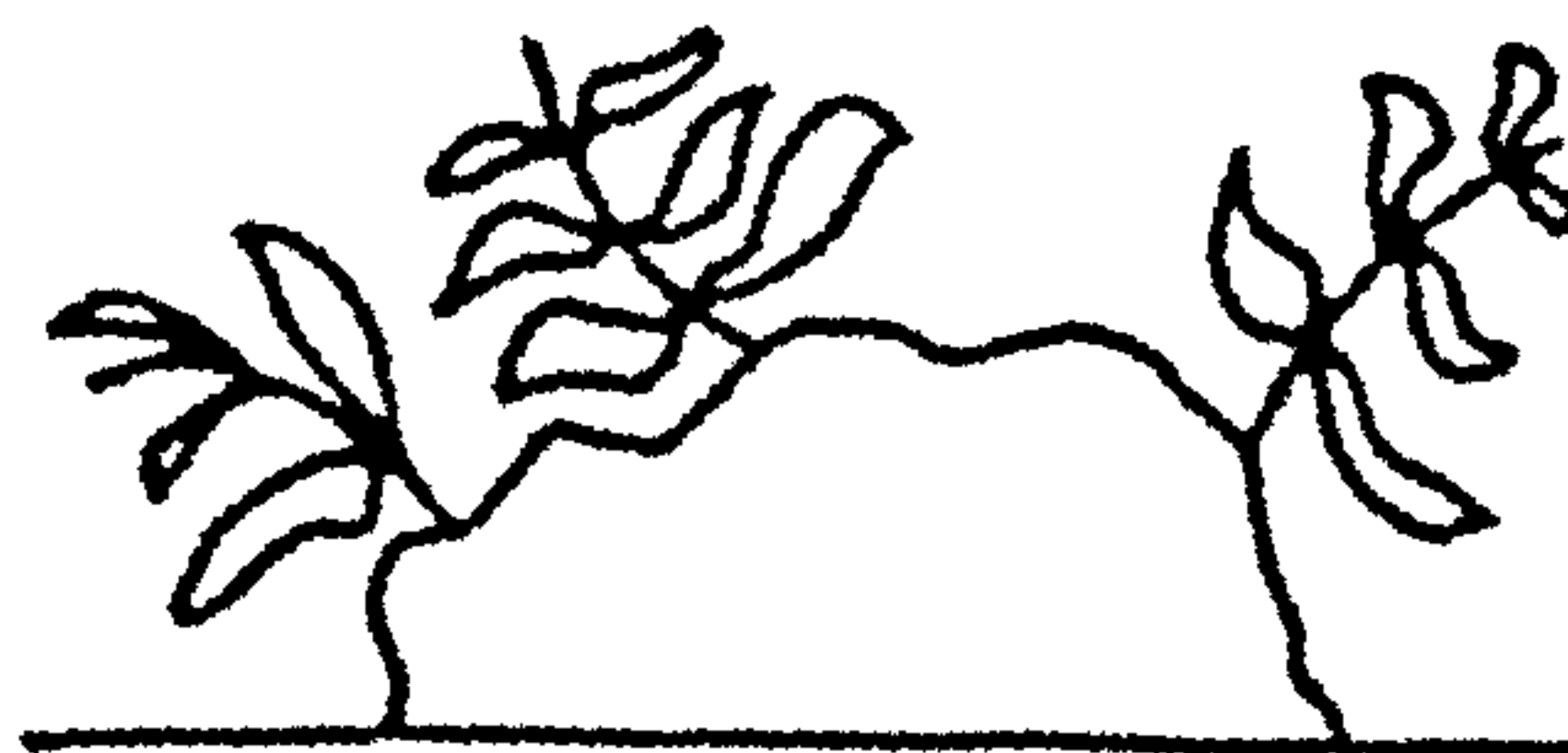
N. tabacum nia 130
mesophyll protoplasts



colonies transferred to selection medium

Sustained division No growth

Transfer to regeneration medium



Putative somatic hybrids grown to maturity for analysis

5.2 MATERIALS AND METHODS

5.2.1 Source and Growth of Plant Material

Seeds of Nicotiana tabacum cv. Gatersleben, and the nitrate reductase deficient mutant line nia-130 ($2n = 4x = 48$) were originally obtained from Dr. A. Muller, Zentralinstitut für Genetik und Kulturpflanzenforschung, Gatersleben, G.D.R. Seed was subsequently obtained following self-pollination of bagged flower heads. The nia-130 mutant plants were grown as described in Chapter 4.2.1.

N. tabacum cv. Gatersleben and N. glutinosa plants were grown as previously described for N. glutinosa in Chapters 2 and 3. Routine greenhouse pest control was performed as described in Chapter 2.

5.2.2 Media and Enzyme Preparation, Sterilisation and Storage

Media and enzyme solutions were prepared, sterilised and stored as described in Chapter 2. Full details of media composition are given in Appendix 1.

5.2.3 N. tabacum nia-130 Mesophyll Protoplast Isolation

Young fully expanded leaves were removed from nia-130 plants established in hydroponics. Leaves were surface sterilised in 7.5% (v/v) Domestos (Lever Bros. Ltd., U.K.) followed by four washes in sterile tap water. The lower epidermis was frequently difficult to remove by peeling with fine forceps, so the leaves were usually cut into fine slices 1-2 mm thick, and leaf pieces floated on the surface of 10 ml CPW 13M (see Appendix 1) in 9 cm petri dishes (Sterilin Ltd., U.K.). After 1-2 hours the CPW 13M was removed and replaced with 10 ml of enzyme solution containing 1.5% (w/v) meicelase P (Meiji Seika Kaisha Ltd., Japan) and 0.5% (w/v) macerozyme R10 (Yakult Honsha Co. Ltd., Japan). The leaf pieces were incubated in enzyme solution

overnight (13-16 hours) at $22 \pm 2^{\circ}\text{C}$ on a rotary shaker (30 cycles/minute) in the dark. Following incubation the protoplasts were released into the enzyme solution and recovered following flotation on CPW 21S as described in Chapter 2.2.10.

5.2.4 N. glutinosa Tetrad Protoplast Isolation

Buds were selected on the basis of length so as to contain pollen at the tetrad stage of development. Based on the results of Chapter 3, buds measuring approximately 14 mm in length were selected. The buds were surface sterilised for 20 minutes in 10% (v/v) Domestos (Lever Bros. Ltd., U.K.) followed by four washes in sterile tap water. 25 buds were placed individually in the wells of a sterile plastic 25 well grid (100 mm square) (Sterilin Ltd., U.K.), and one anther from each bud removed and examined to determine the stage of pollen development. The remaining anthers in those buds determined to contain tetrads were gently crushed in 4 ml of 2% (w/v) driselase (Kyowa-Hakko Kogyo Co. Ltd., Japan) in CPW 9M. The tetrad suspension was passed through a $64\text{ }\mu\text{m}$ sieve to remove debris, and incubated at 25°C in darkness for 2 hours.

5.2.5 Protoplast Fusion and Culture

5×10^5 N. tabacum n1a-130 leaf mesophyll protoplasts in 6 ml of CPW 13M were mixed with an equal number of N. glutinosa tetrad protoplasts still in 4 ml of enzyme solution. After centrifugation ($80 \times g$, 5 minutes), the supernatant was removed and replaced with 10 ml of high pH/ Ca^{2+} fusogen without disturbing the pellet. High pH/ Ca^{2+} fusogen was prepared by dissolving 0.05 M $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 0.05 M glycine - NaOH buffer pH 10.4, with the addition of 10% (w/v) mannitol. After incubation at 30°C for 30 minutes the supernatant was removed and replaced with 10 ml CPW 13M without disturbing the pellet. The

CPW 13M was removed and the pellet finally resuspended in AA-P19M to give a final density of 5×10^4 mesophyll protoplasts/ml medium. Fusion treated protoplasts were cultured in 5 cm petri dishes (A/S Nunc, Kamstrup, Denmark), 4 ml medium/dish, and maintained at $25 \pm 2^\circ\text{C}$ in the dark. After 21 days small colonies were transferred to selection medium MS NO₃ 4.5 M by gently pelleting the colonies in a centrifuge tube (80 x g, 3 minutes), removing the supernatant, and re-suspending the colonies in selection medium at a density of approximately 1×10^3 colonies/ml. The colonies were cultured in 9 cm petri dishes (Sterilin Ltd., U.K.), 8 ml medium/dish at $25 \pm 2^\circ\text{C}$ with continuous illumination provided by cool white fluorescent tubes (Thorn, U.K.) at an intensity of 2,100-2,300 lux. After a further 6-8 weeks any actively growing green colonies were transferred to regeneration medium (MS-D3) and regenerated shoots subsequently rooted in MS-0 medium. Putative somatic hybrids were transferred to 3½ inch pots containing Levingtons soil-less compost (Fisons Ltd., U.K.) in a mist propagator for 7 days, before being grown to maturity in the greenhouse.

5.2.6 Morphological and Cytological Analysis of Putative Somatic Hybrid Plants

Putative somatic hybrid plants were grown to maturity together with N. glutinosa and N. tabacum cv. Gatersleben. All comparisons between the putative somatic hybrids, and their fusion partners were carried out when the plants were flowering. The plant height, dimensions of the largest leaves and flowers, and also the mean value for leaf width/length ratio and flower width/length ratio (based on a minimum of six measurements) were recorded. The flower colour was also noted based on the Royal Horticultural Societies flower colour charts.

The somatic chromosome number of the putative somatic hybrids and of N. tabacum and N. glutinosa were determined following the methods in Chapter 3.2.7.

5.2.7 Biochemical Analysis of Putative Somatic Hybrid Plants

a) Nitrate Reductase Assay

Nitrate reductase activity was measured following the methods of Jaworski (1971). This method consists of incubating a known amount of tissue sample in a buffer consisting of 0.1 M sodium phosphate buffer, pH 7.5 containing 40 mM KNO_3 , 4% (v/v) n-propanol, 1 mg/ml streptomycin sulphate (Sigma) and 50 $\mu\text{g/ml}$ Cycloheximide (Sigma). A callus sample of 200 mg was incubated in 2 ml of buffer for 90 minutes at 27°C in the dark. Nitrite was detected in the buffer solution following incubation by the addition of 0.5 ml of 1% (w/v) sulphanilic acid and 0.5 ml of 0.3% (w/v) α -naphthylamine, both dissolved in 30% (v/v) acetic acid. After 30 minutes the samples were centrifuged (2,000 x g, 10 minutes) and absorbance measured at 540 nm using a Unicam SP 600 spectrophotometer. Readings were calibrated against a standard curve of absorbance against nitrite concentration (see Appendix 2). Activity was expressed as N Moles NO_2^- /100 mg tissue/hour.

b) Extraction of Soluble Proteins from Leaves, and Leaf Callus for Isoenzyme and Fraction 1 Protein Analysis

Soluble proteins were extracted from fully expanded leaves from the upper part of the plant, ground in an ice cold mortar in ice cold extraction buffer (2 ml extraction buffer/g fresh weight of leaf tissue). A little sand was added to aid homogenisation. The slurry was passed through two layers of gauze and 0.1 g/ml sephadex G25-80 added. The extracts were kept on ice, and then centrifuged at

11,500 x g for five minutes in an M.S.E. micro centaur centrifuge. The supernatant was used as a source of soluble proteins.

Soluble proteins were also extracted from callus in the same way except 1 ml extraction buffer/g callus tissue was used. Callus was grown on MSP1 medium in the case of N. tabacum cv. Gatersleben, N. glutinosa, and the putative somatic hybrids, and AA medium in the case of nia-130 callus. Cultures were maintained at $25 \pm 2^{\circ}\text{C}$ with constant illumination of 2,100-2,300 lux.

For isoenzyme analysis the extraction buffer consisted of 0.1 M Tris-HCl, pH 6.8, 30% (v/v) glycerol, 40 mM dithiothreitol, 4.0 mM MgCl_2 and 20 μM leupeptin (Sigma). This was used at half strength for leaf extractions, and at full strength for leaf callus extractions.

For Fraction 1 protein analysis the extraction buffer consisted of 0.05M Tris-HCl, pH 6.8, 18% (w/v) glycerol, 0.5% (v/v) β - mercaptoethanol, 2.0 mM MgCl_2 and 10 μM leupeptin. Fraction 1 protein was only extracted from leaf tissue.

Extracts for isoenzyme analysis were either used immediately, or stored at -20°C until use. Extracts for Fraction 1 protein analysis were freshly prepared.

c) Protein Assay

The soluble protein content of leaf and callus extracts was determined spectrophotometrically by measuring the level of absorption of light at 500 nm following the complexing of alkaline copper treated protein with Folin phenol reagent (Lowry et al., 1951).

The protein standard, or sample to be tested was made up to 1.2 ml with distilled water. 6 ml of freshly prepared alkaline copper solution was added with immediate mixing. After 10 minutes at room temperature 0.3 ml of Folin and Ciocalteus phenol reagent (2 normal

stock, Sigma) was added, with immediate mixing. Transmission at 500 nm was measured after a further 30 minutes using a Unicam SP 600 spectrophotometer. Bovine serum albumen (Sigma) was used as a standard. The calibration curve is given in Appendix 2.

Alkaline copper solution was prepared by adding, in order, 1 ml 1% (w/v) copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) and 1 ml 2% (w/v) sodium tartrate solution to 98 ml of a freshly prepared solution of 2% (w/v) sodium carbonate (Na_2CO_3) in 0.1 M sodium hydroxide.

d) Isoenzyme Analysis Following Isoelectric Focusing of Soluble Protein Extracts

Isoelectric focusing (IEF) of soluble proteins was carried out using pre-cast LKB Ampholine PAG plates, on an LKB multiphor flat bed electrophoresis unit, model 2117. The unit was connected to the water pump of a Gallenkamp water bath fitted with a Grant cooling unit maintaining the flat bed temperature at 10°C. It was not necessary to pre-focus the IEF gels prior to the application of the protein samples. The gels were run at a constant current of 25 milliamps until the power rose to 25 watts, and subsequently at a constant power of 25 watts until a running voltage of 1,400 volts was attained. The gels were run at a constant 1,400 volts for 1½ - 2 hours. Power was provided by a Camlab EC 500 power pack (E.C. apparatus, U.S.A.).

20-40 µl of soluble protein extract from the leaves of N. tabacum cv. Gatersleben, N. glutinosa and putative somatic hybrids was loaded onto filter paper wicks 2 cm from the cathode using a pH range 3.5 - 9.5 LKB PAG-plate. The anode was 1M H_3PO_4 and cathode 1M NaOH. Following isoelectric focusing, leaf esterases were visualised by staining the gel in 100 ml of a solution consisting of 0.05 M Tris HCl pH 7.0 and 0.2% (w/v) Fast Blue RR salt (Sigma) to which was added 3 ml of 2% (w/v) α naphthyl acetate in 60% (v/v)

acetone solution, immediately prior to use (Smith et al., 1970). The gel was incubated at room temperature for 40-60 minutes, after which time the reactions were terminated by transferring the gel to 8% (v/v) acetic acid, which also served as a destaining solution.

15-30 µl of soluble proteins extracted from leaf callus tissue was also loaded onto paper wicks 2 cm from the cathode, but using a pH range 4.0 - 6.5 LKB PAG-plate. The Anode was 0.5 M H₃PO₄ and cathode 0.1 M β alanine. Leaf callus peroxidases were visualised following isoelectric focusing by staining the gel in 100 ml of a solution consisting of 0.05 M Tris-HCl pH 7.2, 0.02 M NaCl, 0.05% (w/v) 4-chloro-1-Naphthol (Sigma) in 20% (v/v) ethanol. 3 ml of H₂O₂ was added immediately before use (Pental et al., 1985). The gel was incubated at room temperature for 20 minutes, and then the reactions terminated by transferring the gel to 8% (v/v) acetic acid.

The isoelectric focusing point (pI) of prominent bands specific to either N. tabacum or N. glutinosa was noted, based on information provided by the manufacturers of the PAG-plates (LKB) (see Appendix 2).

e) Fraction 1 Protein Analysis

Isoelectric focusing of the subunit polypeptides of Fraction 1 protein (ribulose biphosphate carboxylase/oxygenase, E.C. 41139) was performed following a method based on that of Cammeart and Jacobs (1980).

Fraction 1 protein was first purified following discontinuous polyacrylamide gel electrophoresis (Disc-PAGE) (Davis, 1964) in which a separating gel, 10 cm deep was overlayed by a stacking gel 5 cm deep. A vertical slab gel electrophoresis unit (LKB model number 2001) was used, cooled by circulating tap water (8-14°C). A stock solution of 30% (w/v) acrylamide (BDH-electran) and 0.8% (w/v) N N methylene

bisacrylamide (BDH - electran) was prepared, and filtered through Whatman No. 1 filter paper. This solution was stored over 1% amberlite MB-3 ion exchange resin (to remove acrylic acid) at 4°C in the dark for up to 8 weeks. By suitable dilutions, gels of different acrylamide concentrations could be prepared from this stock solution.

The separation gel consisted of 6% (w/v) acrylamide, 0.16 (w/v) N N methylene bisacrylamide, 0.075 M Tris-HCl pH 8.4 and 10% (v/v) glycerol. The stacking gel consisted of 3% (w/v) acrylamide, 0.017 M Tris-HCl pH 6.8 and 10% (v/v) glycerol. This was layered above the polymerised separating gel. A plastic well former was inserted into the stacking gel before it polymerised, giving 10 wells of approximately 500 µl volume.

The gels were de-aerated under vacuum and polymerised by the addition of 1 µl/ml TEMED (N,N,N,'N' - tetramethylethylene diamine) and 0.02% (w/v) ammonium persulphate (from a freshly made stock solution of 140 mg ammonium persulphate in 5 ml H₂O). Polymerisation took 40-50 minutes at room temperature.

The gels were loaded with 100 µl of soluble protein extract per well, and were run for 19 hours at a constant current of 10 milli-amps. Power was supplied by a coral instruments power pack (F. Copley and Co. Ltd., U.K.). The anode and cathode running buffers consisted of 0.025 M Tris-HCl pH 8.3 with 0.129 M glycine.

Fraction 1 protein was identified following staining of the gels for total protein for 2-3 minutes in 0.1% (w/v) PAGE Blue '83 (BDH) 50% (v/v) methanol, and 10% (v/v) acetic acid, followed by destaining in a solution of 5% (v/v) methanol and 10% (v/v) acetic acid, for 2-3 minutes. The position of Fraction 1 protein was assumed on the basis of its slow migration and high staining intensity. Once located the Fraction 1 protein bands were cut out in acrylamide blocks

6 mm x 3 mm x 1.4 mm and placed individually in eppendorph tubes containing 1 ml of a freshly prepared solution of 1.5% (v/v) Ampholine pH range 5-8 (L.K.B.), 5% sucrose, 8 M urea and 0.1% (w/v) dithiothreitol (DTT). Nitrogen gas was passed through this solution to de-oxygenate it prior to use. The eppendorph tubes were sealed and kept at 4°C until the blocks sank, usually 20-25 minutes. The blocks were used immediately.

Isoelectric focusing of the Fraction 1 protein subunit polypeptides was performed using 1.5 mm thick slab gels on an LKB multiphor flat bed electrophoresis unit as described for isoenzyme analysis. Gels were prepared from a stock solution of 24.25% (w/v) acrylamide, and 0.75% (w/v) N N methylene bisacrylamide prepared and stored as described for Disc.PAGE. The gel contained a final concentration of 5% (w/v) acrylamide, 0.15% (w/v) N N methylene bisacrylamide, 8 M urea and 1.5% Ampholine (LKB) pH range 3-10 (prepared by mixing one part LKB Ampholine pH range 3-10 with two parts LKB Ampholine pH range 5-8). The gel was polymerised as described for Disc.PAGE, and was cast between glass and perspex plates with 1.5 mm thick spacers, and with a polyester backing film covering the glass plate. Once polymerisation was complete the gel was transferred to the multiphor unit, and pre-focused at a constant current of 15 milliamps for two hours. The flat bed temperature was maintained at 15°C. Once pre-focused, the gel was loaded 2 cm from the cathode with the acrylamide blocks containing the dissociated Fraction 1 protein. The gel was run at a constant current of 15 milli-amps until a running voltage of 1,200 volts was attained. The gel was run for five hours at a constant 1,400 volts.

The polypeptides of Fraction 1 protein subunits were visualised following overnight fixation of the gel in 12% (w/v) trichloro acetic acid and 3.5% (w/v) sulphasalicylic acid by staining in 0.15% (w/v)

PAGE Blue (83 (BDH) in 2.5% (v/v) ethanol and 8% acetic acid at 60°C for 10 minutes. The gels were destained over several days in several changes of 25% (v/v) ethanol and 8% (v/v) acetic acid.

5.3 RESULTS

5.3.1 Protoplast Isolation and Culture

Young fully expanded leaves from N. tabacum nia-130 plants grown in hydroponics yielded between 2.2 and 5.6×10^5 mesophyll protoplasts/g leaf tissue. Sustained protoplast division in AA-P19M medium (diluted to AA-P14.5 M after 14 days) gave rise to colonies 46/50 of which regenerated shoots when transferred to solid AA-D3 medium (see Figure 5.3 A, C and E).

Tetrad protoplasts were readily obtained following incubation of N. glutinosa tetrads in 2% (w/v) driselase (see Figure 5.3 B). A 90-100% conversion of tetrads to tetrad protoplasts occurred, and as detailed in Chapter 4, 20 anthers from five buds were sufficient for the isolation of over 5.0×10^5 tetrad protoplasts. The lack of N. glutinosa tetrad division in AA-P19M, and in nia-130 nurse protoplast cultures was reported in Chapter 4.

5.3.2 Protoplast Fusion, Culture and Selection of Putative Somatic Hybrids

In preliminary fusion experiments it was not possible to visualise heterokaryons between nia-130 leaf mesophyll protoplasts, and N. glutinosa tetrad protoplasts, based on the merging of the chloroplast containing mesophyll protoplast cytoplasm, with the chloroplast free tetrad protoplast cytoplasm. The use of fluorescent marker dyes such as fluorescein isothiocyanate (FITC) to label the tetrad protoplast cytoplasm also failed to permit the unequivocal detection of heterokaryons visually.

A fusion experiment was performed between 5×10^5 nia-130 leaf mesophyll protoplasts, and approximately 5×10^5 N. glutinosa tetrad protoplasts. Following fusion the mesophyll protoplasts were observed

to undergo division in AA-P19M medium giving rise to small colonies. After 21 days the colonies were transferred to selection medium (MS NO₃ 4.5 M). Following selection, two actively growing green colonies were detected in the fusion treated protoplast derived colonies (see Fig.5.3.2 D). No green colonies were observed in an unfused nia-130 control.

The two colonies were transferred to regeneration medium (MSD-3). One colony regenerated six shoots, five of which were successfully established in the greenhouse. The second colony was lost due to bacterial contamination.

5.3.3 Morphological and Cytological Examination of the Putative Somatic Hybrids and their Fusion Partners

The five putative somatic hybrids, N. tabacum cv. Gatersleben, diploid N. glutinosa and haploid N. glutinosa plant (derived from anther culture, Chapter 3) were grown to maturity (see Fig.5.3.2) and the morphology of the plants examined as described in the Materials and Methods. The results are presented in Table 5.3.1.

The flowering putative somatic hybrids measured between 57 and 62 cm in height, compared with 113 cm for N. tabacum, 62 cm for the diploid and 32 cm for the haploid N. tabacum plants. The leaf and floral morphology of N. tabacum and N. glutinosa differed quite considerably. N. glutinosa leaves were petiolate, and almost as wide as their length. N. tabacum leaves were larger, lacked a petiole and were twice as long as their width. The putative somatic hybrid leaves were similar to N. tabacum leaves in shape, but were slightly petiolate, and were smaller than either N. tabacum or diploid N. glutinosa leaves. The putative somatic hybrid leaves were however larger than those of the haploid N. glutinosa plant (see Figure 5.3.3 A).

N. glutinosa flowers possessed a distinctive bilateral symmetry; and were more intensively coloured than the larger N. tabacum flowers. The flowers of N. glutinosa had a wider corolla tube, reflected in the flower width/length ratio. The putative somatic hybrids possessed flowers which were larger than those of haploid and diploid N. glutinosa plants, but smaller than those of N. tabacum. The leaf width/length ratio of the putative somatic hybrid flowers was intermediate between that of the two fusion partners (See Table 5.3.1 and Figure 5.3.3 B).

The five putative somatic hybrids were found to possess the expected allopolyploid but functionally triploid somatic chromosome complement of $3n = 5x = 60$, which is the summation of that of N. tabacum ($2n = 4x = 48$) and the haploid N. glutinosa chromosome complement ($n = x = 12$) (see Figure 5.3.4).

5.3.4 Biochemical Characterisation of the Putative Somatic Hybrids, and their Fusion Partners

a) Nitrate Reductase Activity

The nitrate reductase activity of callus of the five putative somatic hybrids, and of diploid N. glutinosa grown on solid MS N0₃ medium, and of nia-130 callus grown on solid AA medium was measured as described in the Materials and Methods. Although the value measured was found to vary considerably for callus samples from the same culture, the five putative somatic hybrids were found to possess a nitrate reductase activity of between 350-580 n M NO₂⁻ produced/hour/100 mg callus. N. glutinosa was found to possess between 420 and 560 n M NO₂⁻ produced/hour/100 mg callus, and nia-130 callus between 0 and 15 n M NO₂⁻ produced/hour/100 mg callus. The nitrate reductase proficiency of the five putative somatic hybrids was confirmed by the ability of callus cultures initiated from leaf explants to proliferate

MS NO₃ medium in which nitrate is the sole nitrogen source.

b) Isoenzyme Analysis

Soluble protein extracts from leaves of the five putative somatic hybrids, and their fusion partners were found to contain between 7.0 - 12.5 µg protein/µl. 20-40 µl of soluble protein extract was applied to each channel on an LKB pH 3.5 - 9.5 PAGE plate, and the proteins separated by isoelectric focusing. Leaf esterases were visualised as described in the Materials and Methods. The leaf esterase zymogram is presented in Fig.5.3.5 A. Two prominent N. tabacum specific esterase bands at pI 6.0 and 6.9, and three prominent N. glutinosa specific esterase bands at pI 5.1, 5.6 and 7.2 are indicated. The putative somatic hybrids were found to possess all five esterase bands.

Soluble protein extracts from leaf callus of the putative somatic hybrids and their fusion partners were found to contain 3.9 - 7.4 µg protein/µl. 15-30 µl of soluble protein extract was applied to each channel on an LKB pH 4.0 - 6.5 PAG plate, and the proteins separated by isoelectric focusing. Leaf callus peroxidases were visualised as described in the Materials and Methods. The leaf callus peroxidase zymogram is presented in Fig. 5.3.5 B. Three prominent N. tabacum specific peroxidase bands, at pI 3.9, 4.3 and 5.0, and one prominent N. glutinosa specific peroxidase band at pI 4.2 were observed. The putative somatic hybrids were found to possess all four prominent peroxidase bands.

No prominent esterase or peroxidase bands were present in the putative somatic hybrids which did not correspond to bands present in either one or both of the fusion partners.

c) Fraction 1 Protein Analysis

Fraction 1 protein was initially purified following Disc PAGE of total soluble protein extracts from leaves of the five putative somatic hybrids and their fusion partners. 100 µl of soluble protein extract containing 390-740 µg protein was loaded per channel. The Fraction 1 protein bands were visualised as described in the Materials and Methods, and acrylamide blocks containing Fraction 1 protein placed in a solution containing 8 M urea, dissociating the Fraction 1 protein subunit polypeptides. These were subsequently separated by isoelectric focusing, and visualised as described in the Materials and Methods. The result is presented in Fig. 5.3.6. The cytoplasmically encoded large subunit polypeptides located in the alkaline region of the gel, and nuclear encoded small subunit polypeptides located in the acid region of the gel were positioned differently for N. tabacum and N. glutinosa. The five putative somatic hybrids possessed the cytoplasmically encoded large subunit polypeptide pattern of N. tabacum, and a nuclear encoded small subunit polypeptide pattern which was the summation of that found for both N. tabacum and N. glutinosa.

FIGURE 5.3.1

N. tabacum nia-130 leaf mesophyll, and N. glutinosa tetrad proto-
plasts, protoplast division, selection and regeneration from a
protoplast derived colony.

5.3.1A Leaf mesophyll protoplasts from N. tabacum nia-130 plants
grown in hydroponics (X 250).

5.3.1B N. glutinosa tetrad protoplasts (X 250).

5.3.1C Dividing N. tabacum protoplast derived cell (X 250).

5.3.1D Selection for putative somatic hybrids on a medium
containing nitrate as sole nitrogen source.

N. tabacum nia-130 protoplast derived colonies (left)
are brown and fail to proliferate. Two actively
growing green colonies are indicated in the N. tabacum
nia-130 and N. glutinosa tetrad protoplast fusion dish
(right) (X 0.47).

5.3.1E Shoot regeneration from an N. tabacum nia-130 protoplast
derived colony on solid AA-D3 medium (X 1.4).

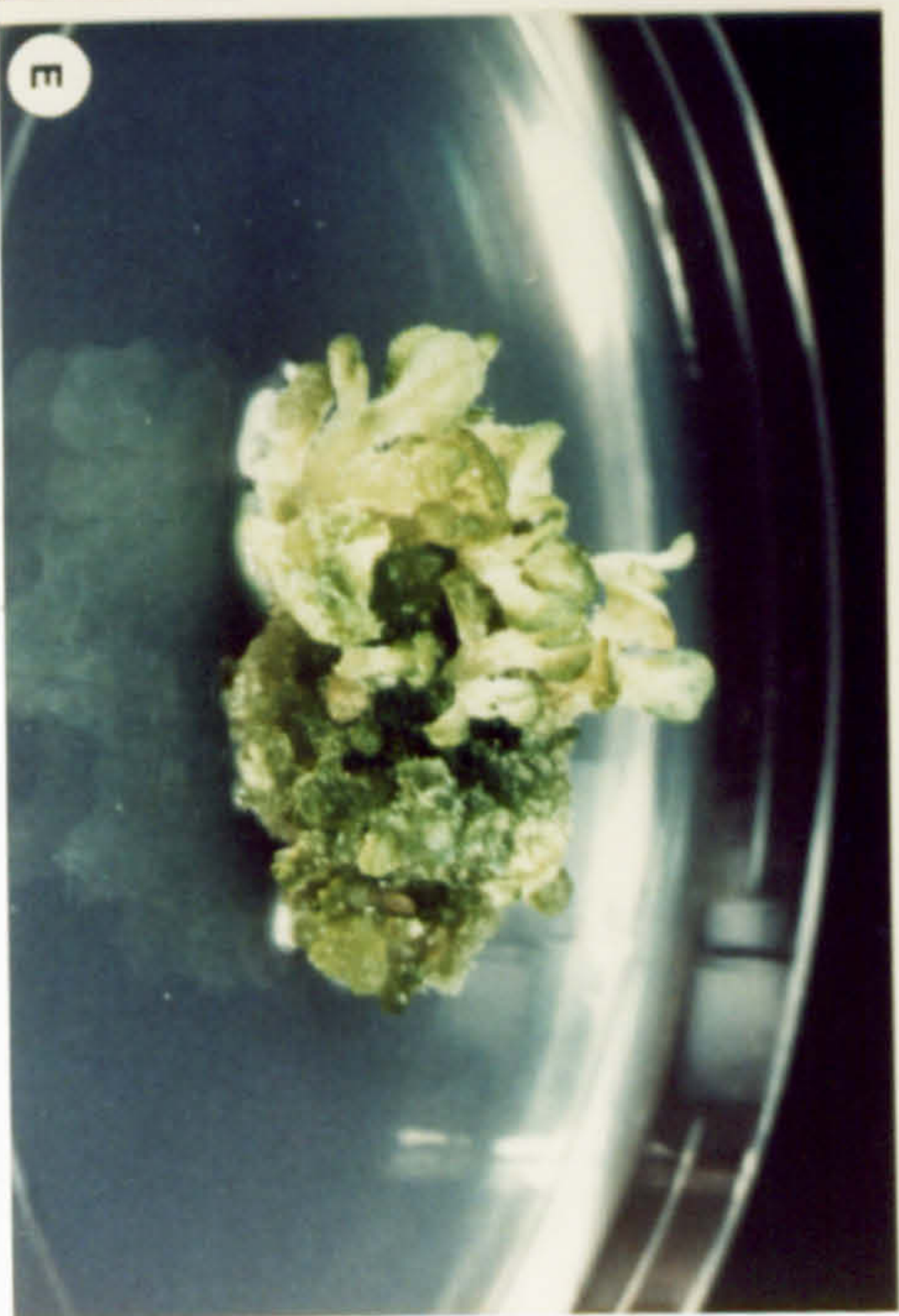
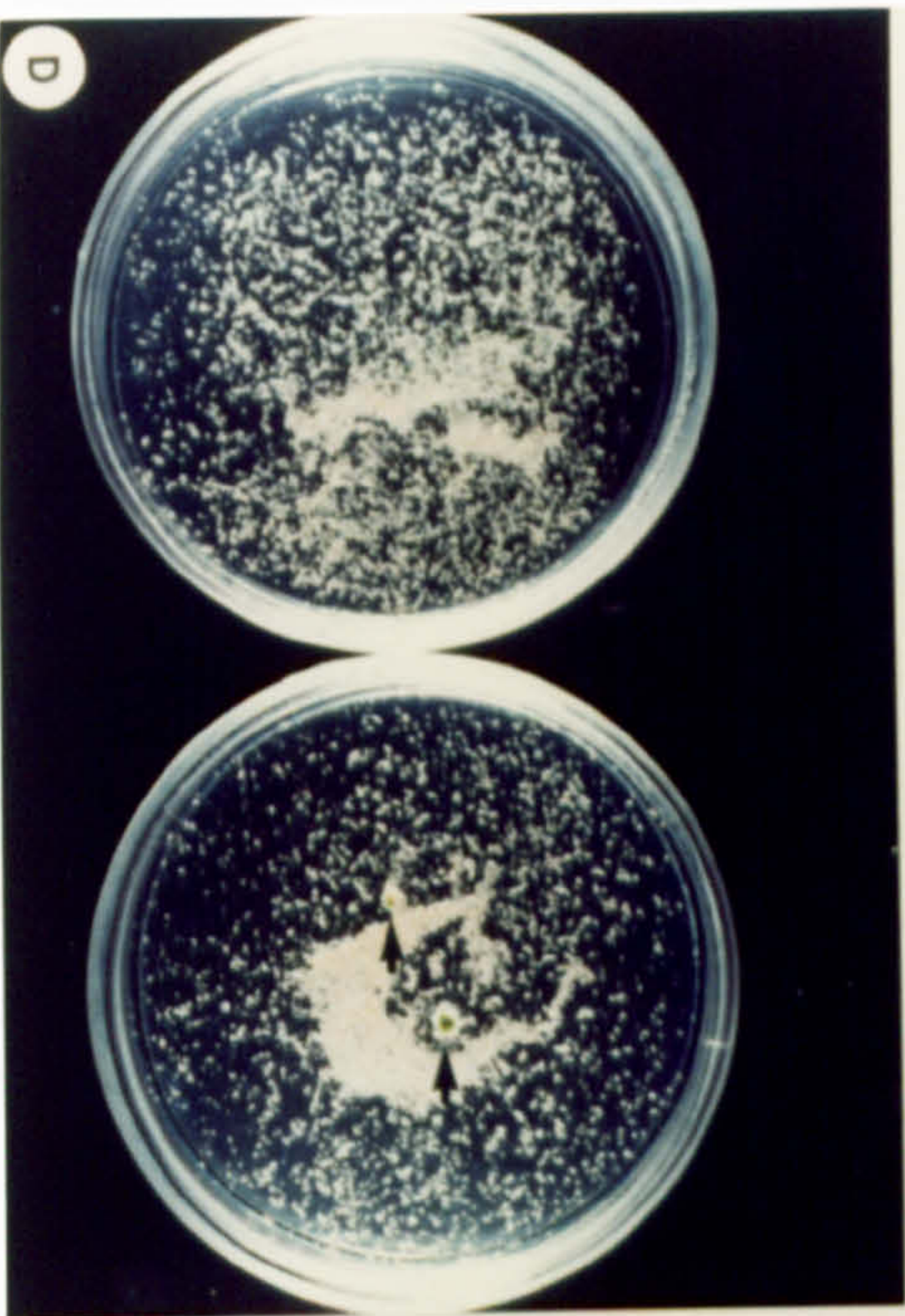
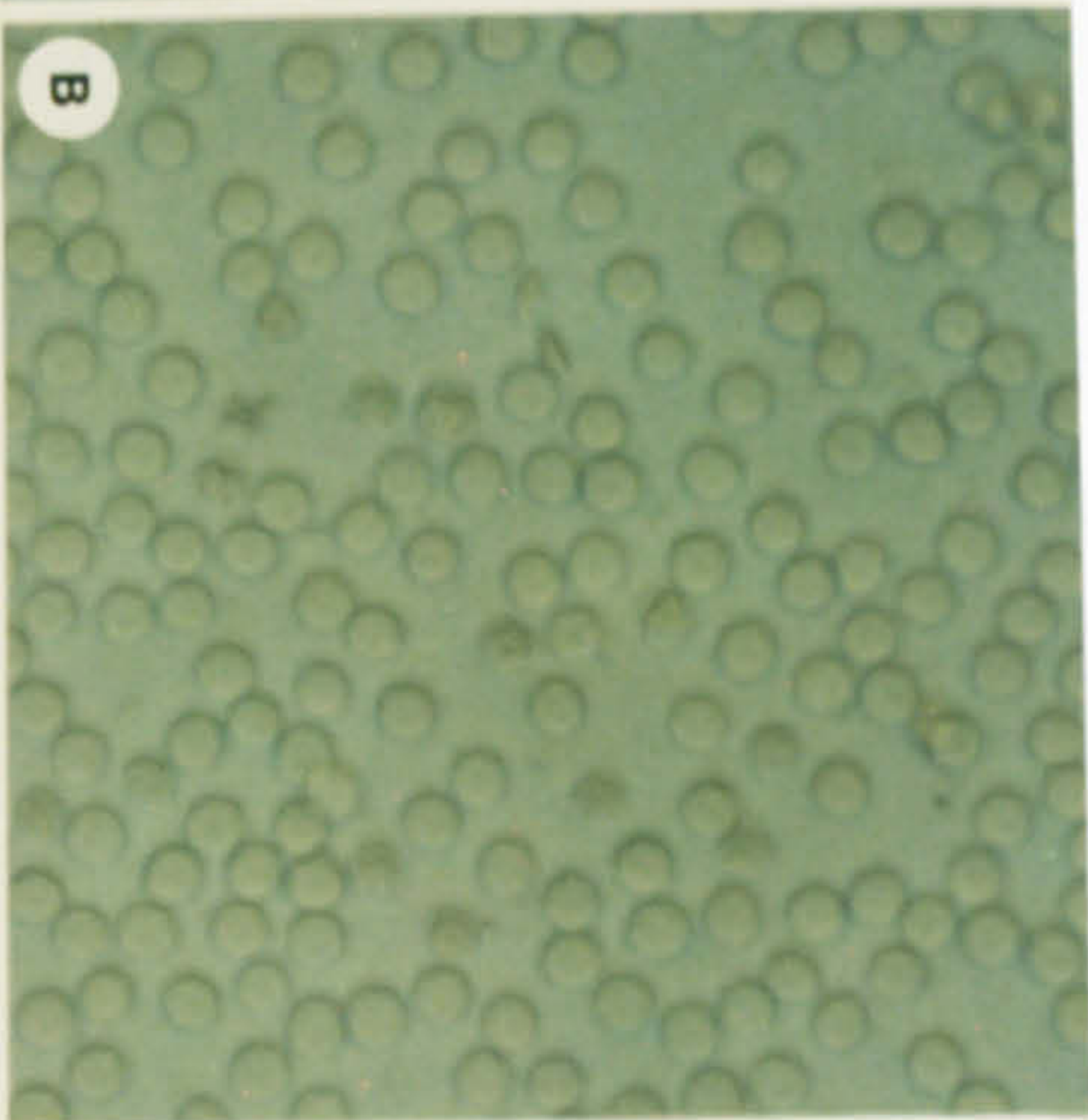
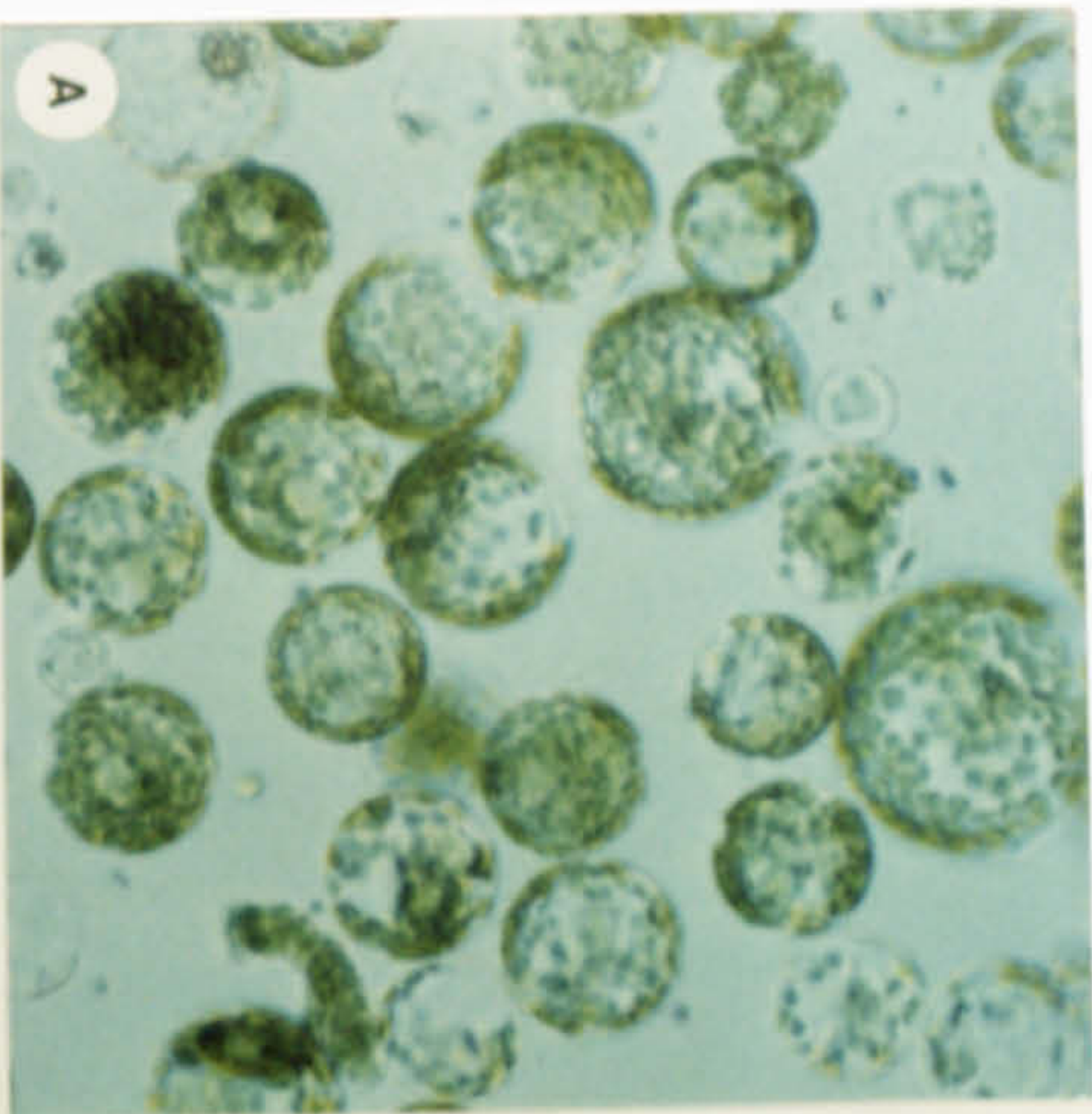


FIGURE 5.3.2

Flowering putative somatic hybrid plants, and their fusion partners.

5.3.2A Diploid N. glutinosa (left), a putative somatic hybrid (centre) and N. tabacum cv. Gatersleben (right) (X 0.13).

5.3.2B All five putative somatic hybrid plants successfully transferred to the greenhouse (X 0.13).

One plant (second from left) failed to grow normally initially, but subsequently flowered and was morphologically the same as the other four putative somatic hybrids (X 0.13).



FIGURE 5.3.3

Leaf and floral morphology of a putative somatic hybrid, and its fusion partners.

5.3.3A Leaf morphology. N. tabacum cv. Gatersleben (left), putative somatic hybrid (centre), haploid N. glutinosa (right upper) and diploid N. glutinosa (right lower) (X 0.28).

5.3.3B Flower morphology. N. tabacum cv. Gatersleben (left), putative somatic hybrid (centre), haploid N. glutinosa (right upper) and diploid N. glutinosa (right lower) (X 0.73).



TABLE 5.3.1 Morphological characterisation of N. tabacum, N. glutinosa (haploid and diploid plants), and the five putative somatic hybrids,NGT 1, 2, 4, 5 and 6.

Plant	Plant Height cm	Flower Colour	Largest Leaf (L x W) mm	Leaf W/L Ratio	Largest Flower (L x W) mm	Flower W/L Ratio
<u>N. tabacum</u> cv. Gatersleben	110 - 118	52C	266 x 175	0.66	56 x 10	0.18
<u>N. glutinosa</u> (diploid)	60 - 65	35B	100 x 96	0.96	38 x 14	0.37
<u>N. glutinosa</u> (haploid)	28 - 34	37D	65 x 58	0.89	25 x 10	0.40
NGT 1	60	48A	145 x 103	0.71	49 x 12	0.24
NGT 2	57	48C	145 x 104	0.72	48 x 12	0.25
NGT 4	61	38D	160 x 142	0.89	48 x 12	0.25
NGT 5	62	48D	185 x 110	0.59	46 x 10	0.22
NGT 6	58	47C	162 x 136	0.84	48 x 13	0.27

FIGURE 5.3.4

Somatic chromosome complement of *N. tabacum* cv. Gatersleben,
and four of the five putative somatic hybrids, and meiosis in a
pollen mother cell of *N. glutinosa*.

5.3.4A Metaphase spread of *N. tabacum* cv. Gatersleben

($2n = 4x = 48$).

(X 1,200)

5.3.4B Meiosis in a pollen mother cell of *N. glutinosa*, showing

the haploid chromosome complement ($n = x = 12$). (X 900)

5.3.4C - F Metaphase spread of four of the five putative
somatic hybrids, showing a somatic chromosome complement
of 60. (X 1,200)

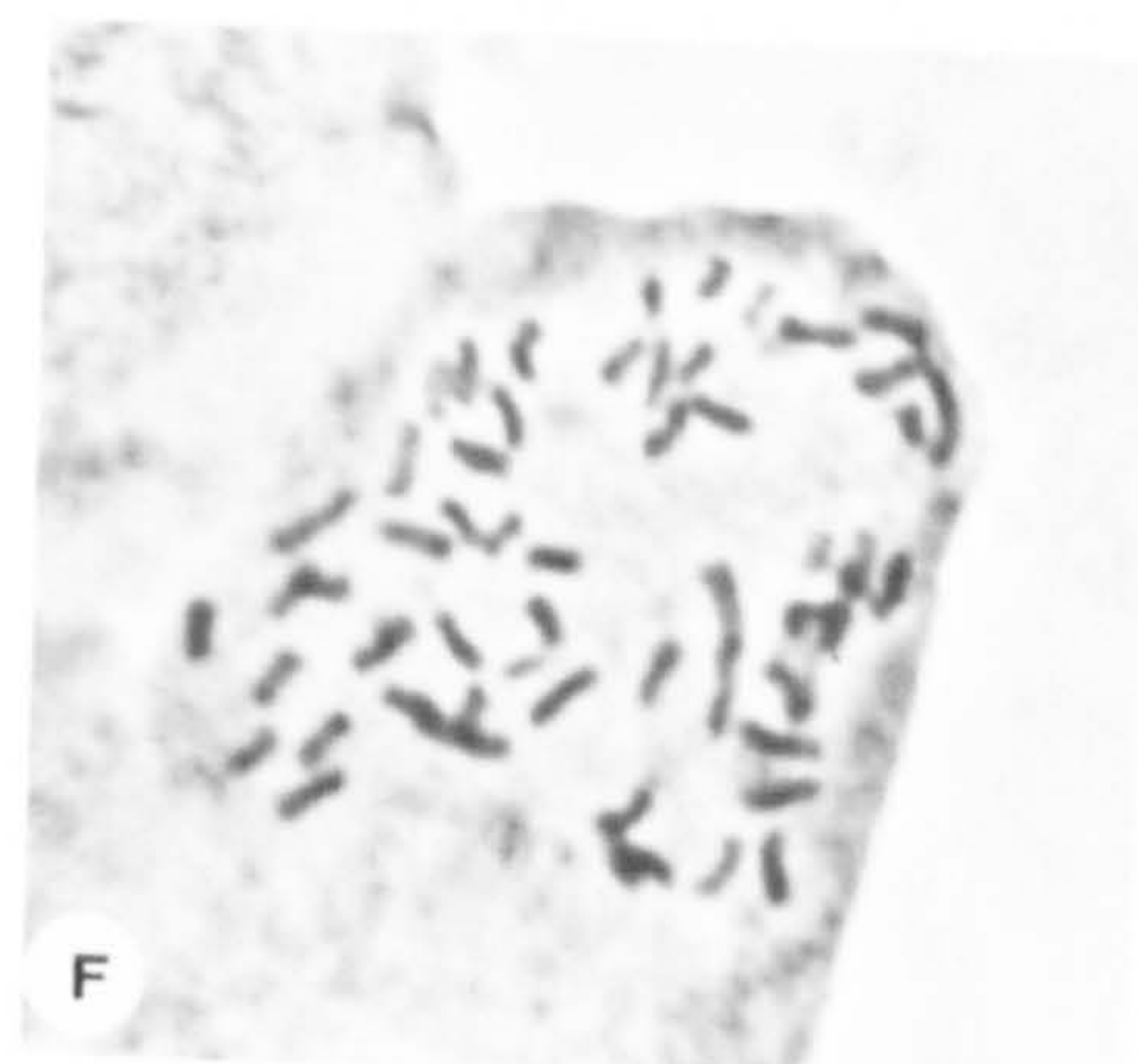
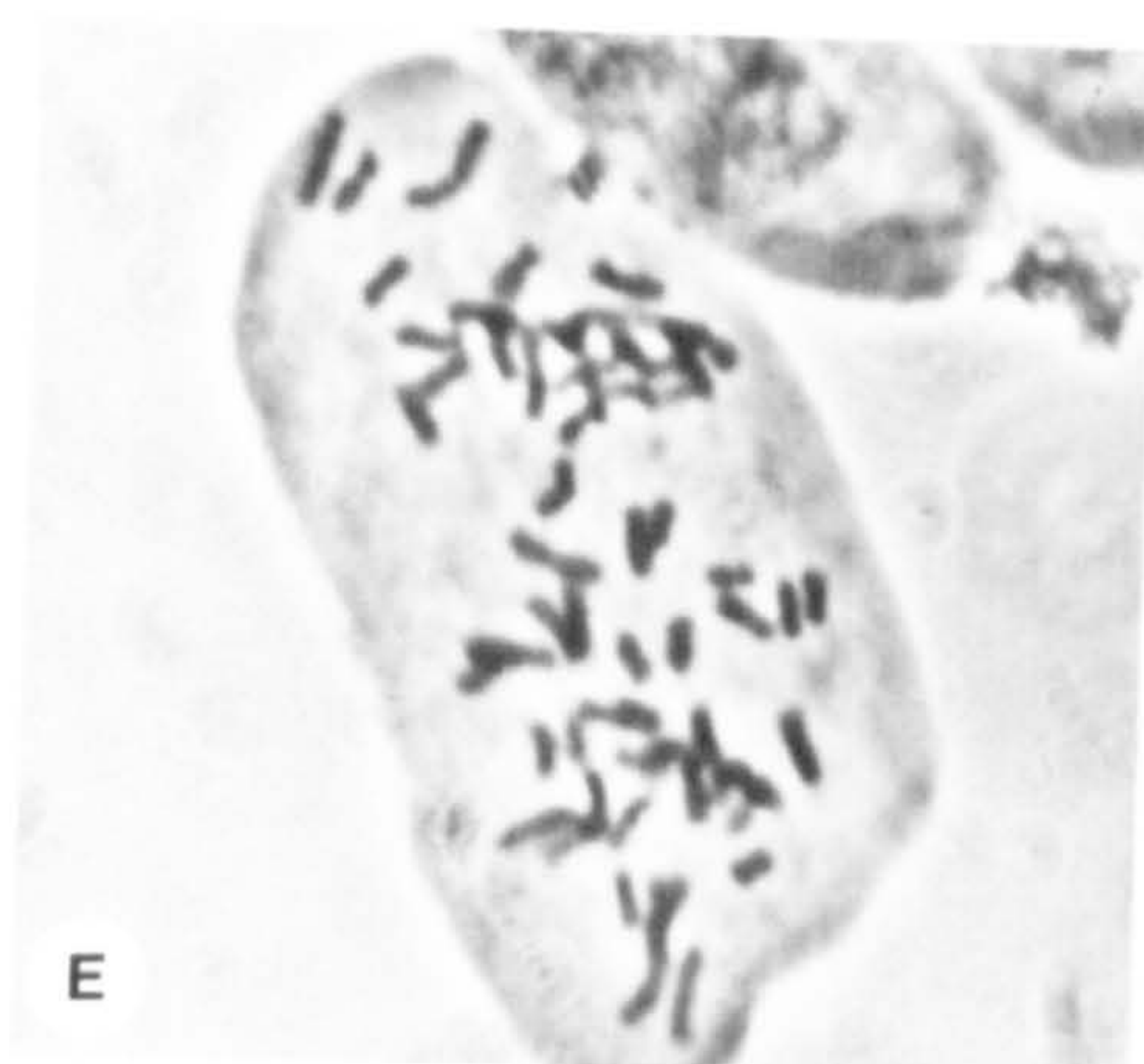
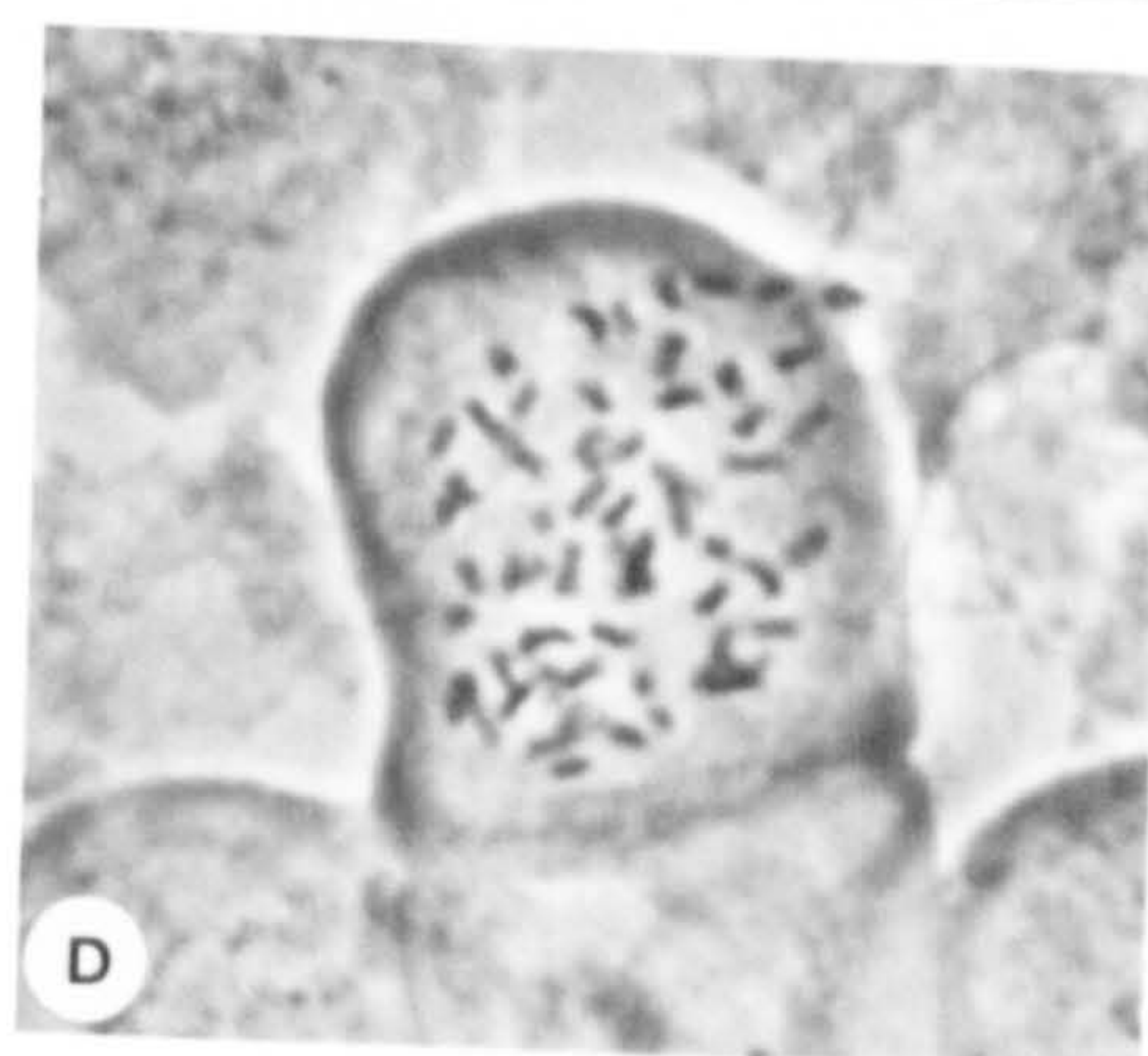
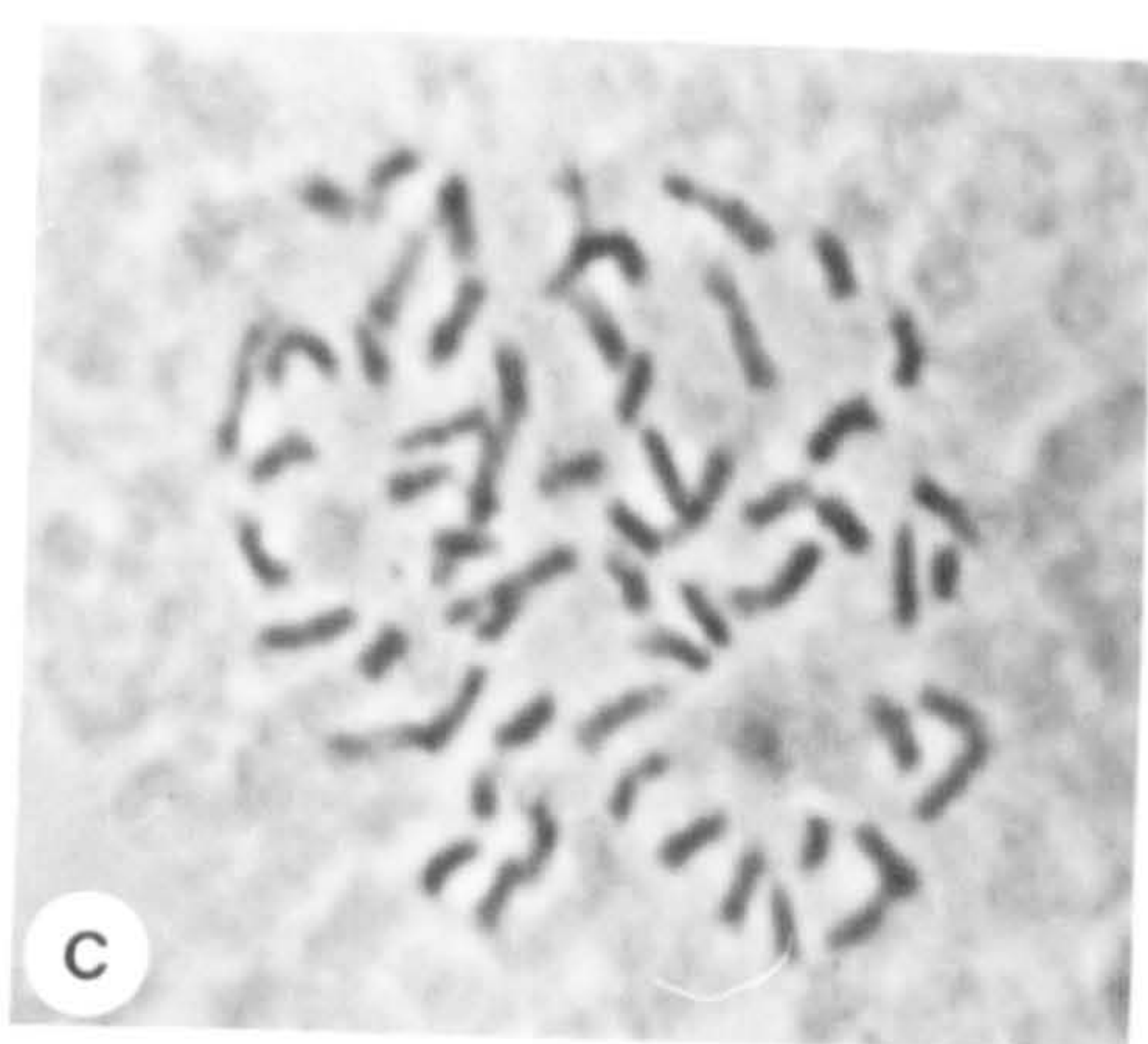
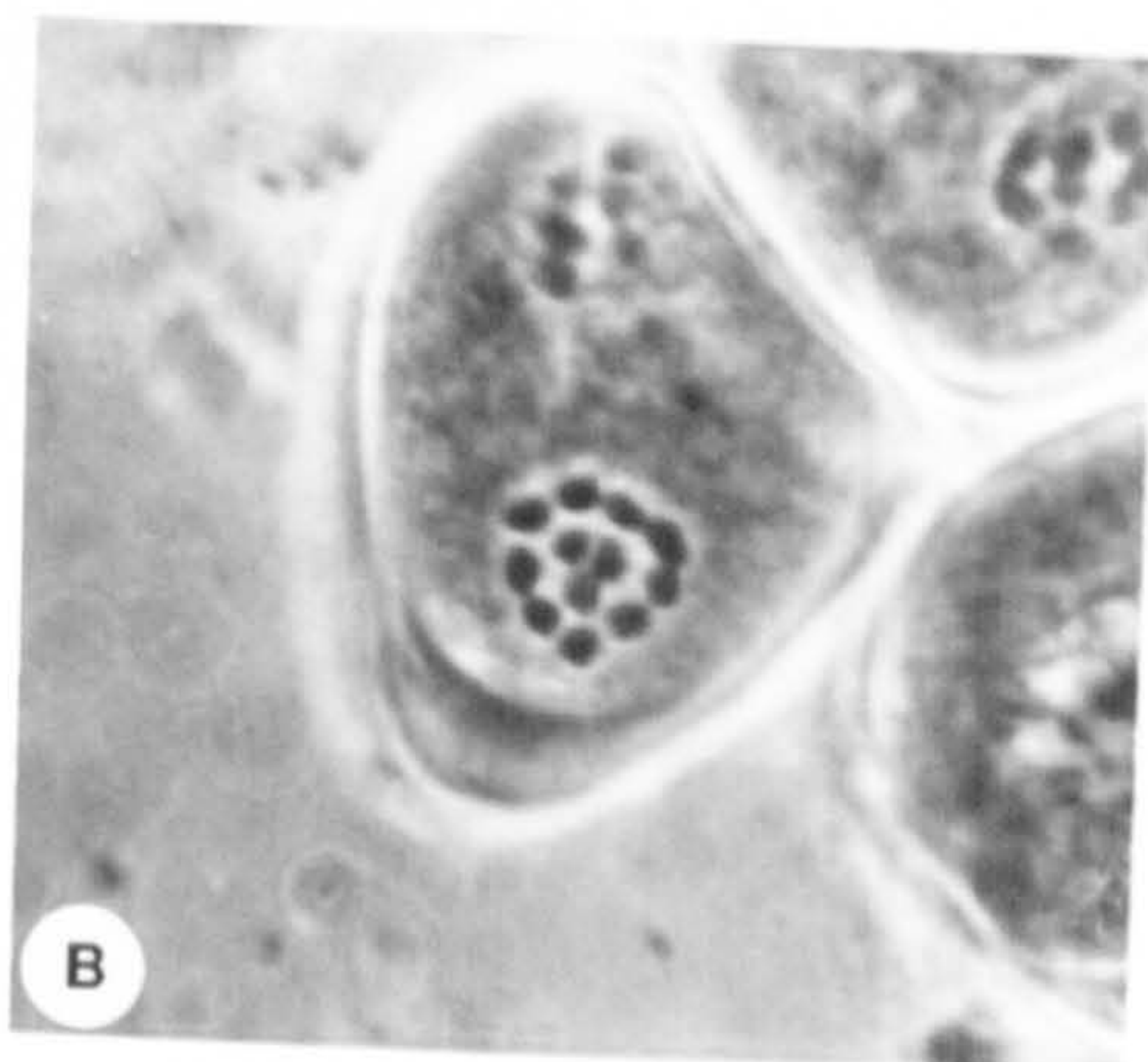
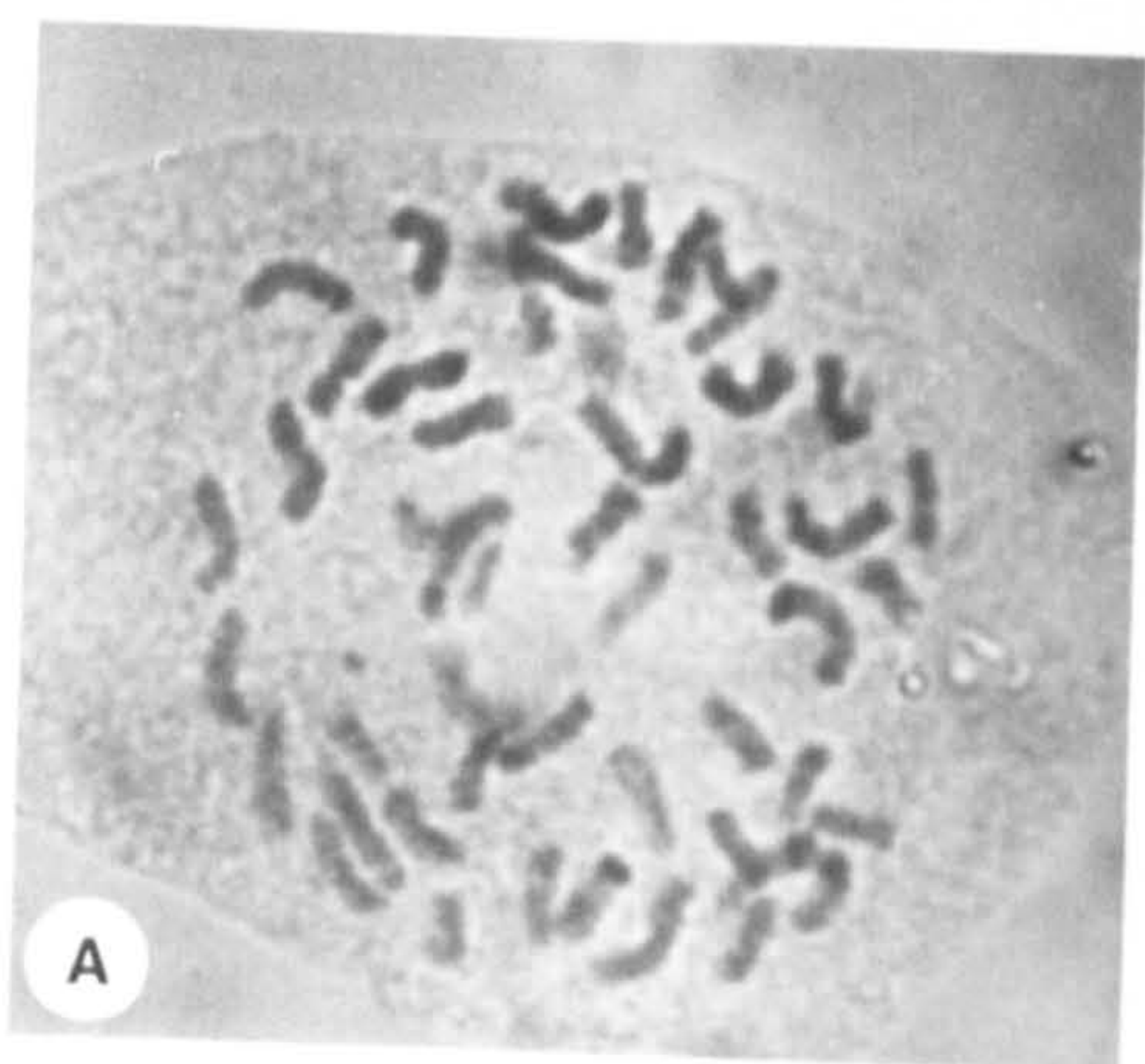


FIGURE 5.3.5

Isoelectric focusing of leaf esterases, and leaf callus peroxidases, of the five putative somatic hybrids, and their fusion partners.

5.3.5A Leaf esterase zymogram. Channel 1, physical mix (1:1) of proteins from N. tabacum nia-130, and N. glutinosa; Channel 3 and 9, N. tabacum nia-130; Channels 4-8, putative somatic hybrids.

Prominent N. glutinosa specific bands are indicated at pI 5.1, 5.6 and 7.2, and N. tabacum specific bands at pI 6.0 and 6.9.

5.3.5B Leaf callus peroxidase zymogram. Channel 1, physical mix (1:1) of proteins from N. tabacum nia-130 and N. glutinosa; Channel 2 and 10, N. glutinosa; Channels 3 and 9, N. tabacum nia-130; Channels 4-8, putative somatic hybrids.

Prominent N. tabacum specific bands are indicated at pI 3.9, 4.3 and 5.0, and on N. glutinosa specific band at pI 4.2.

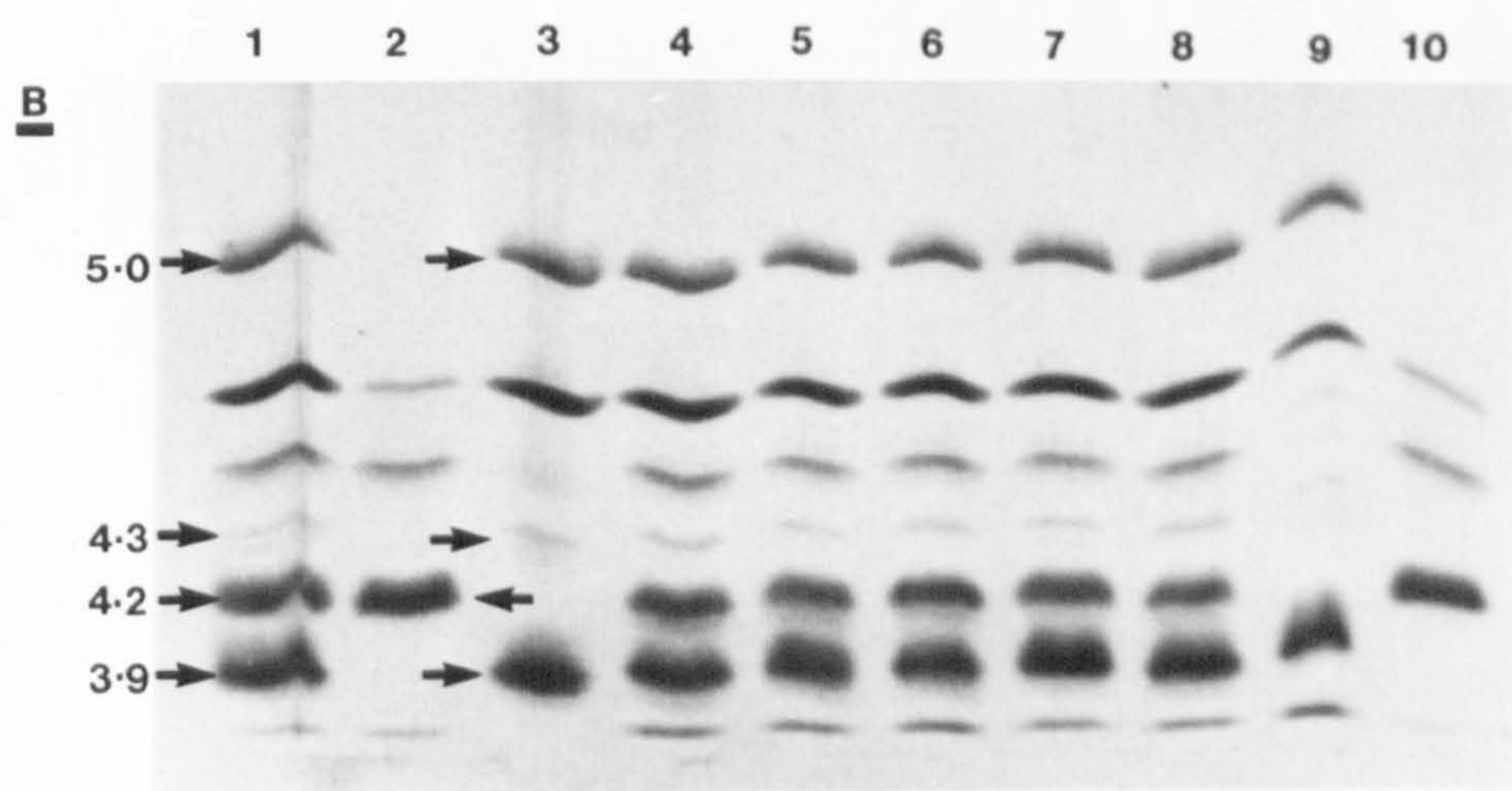
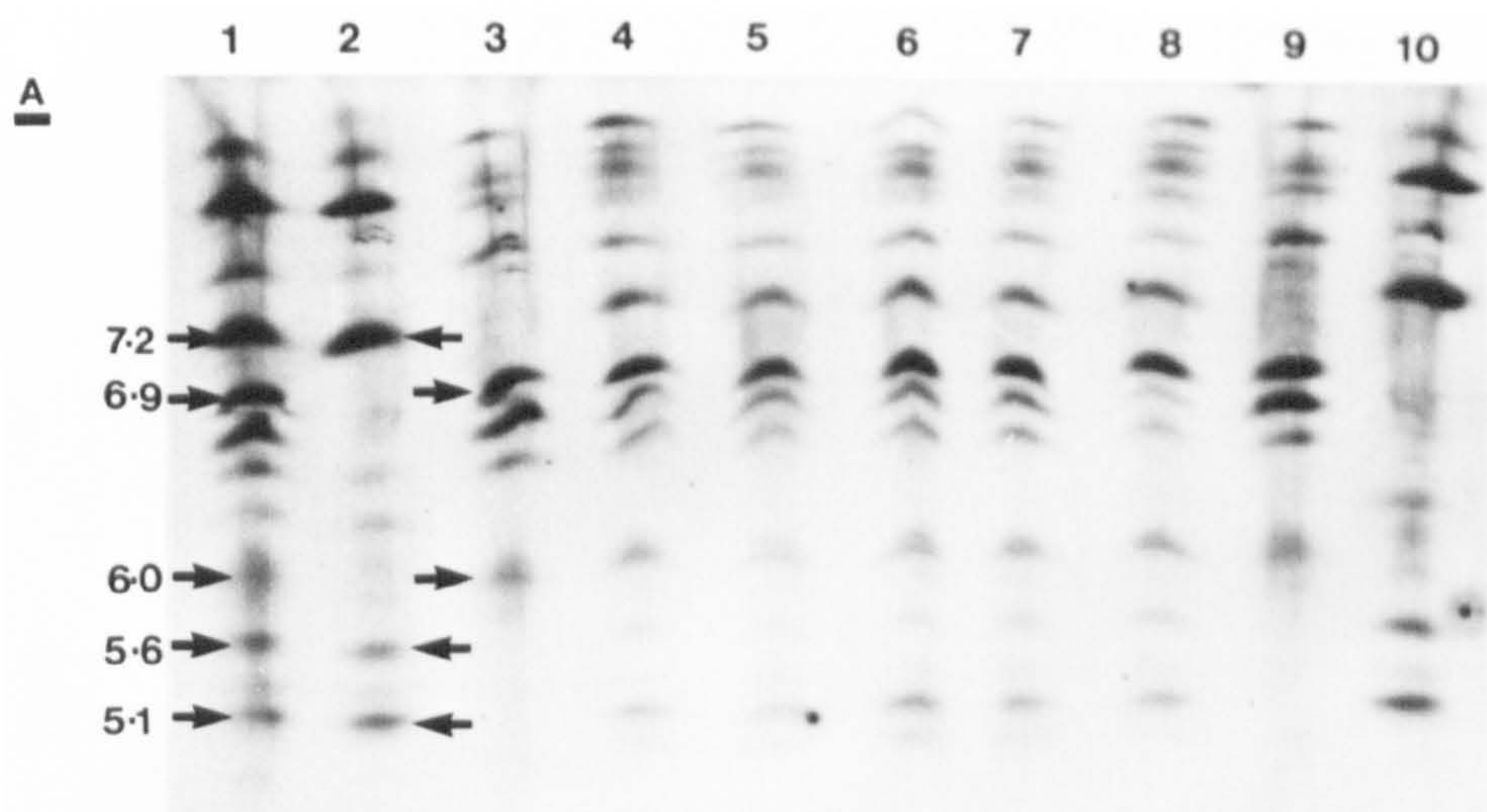
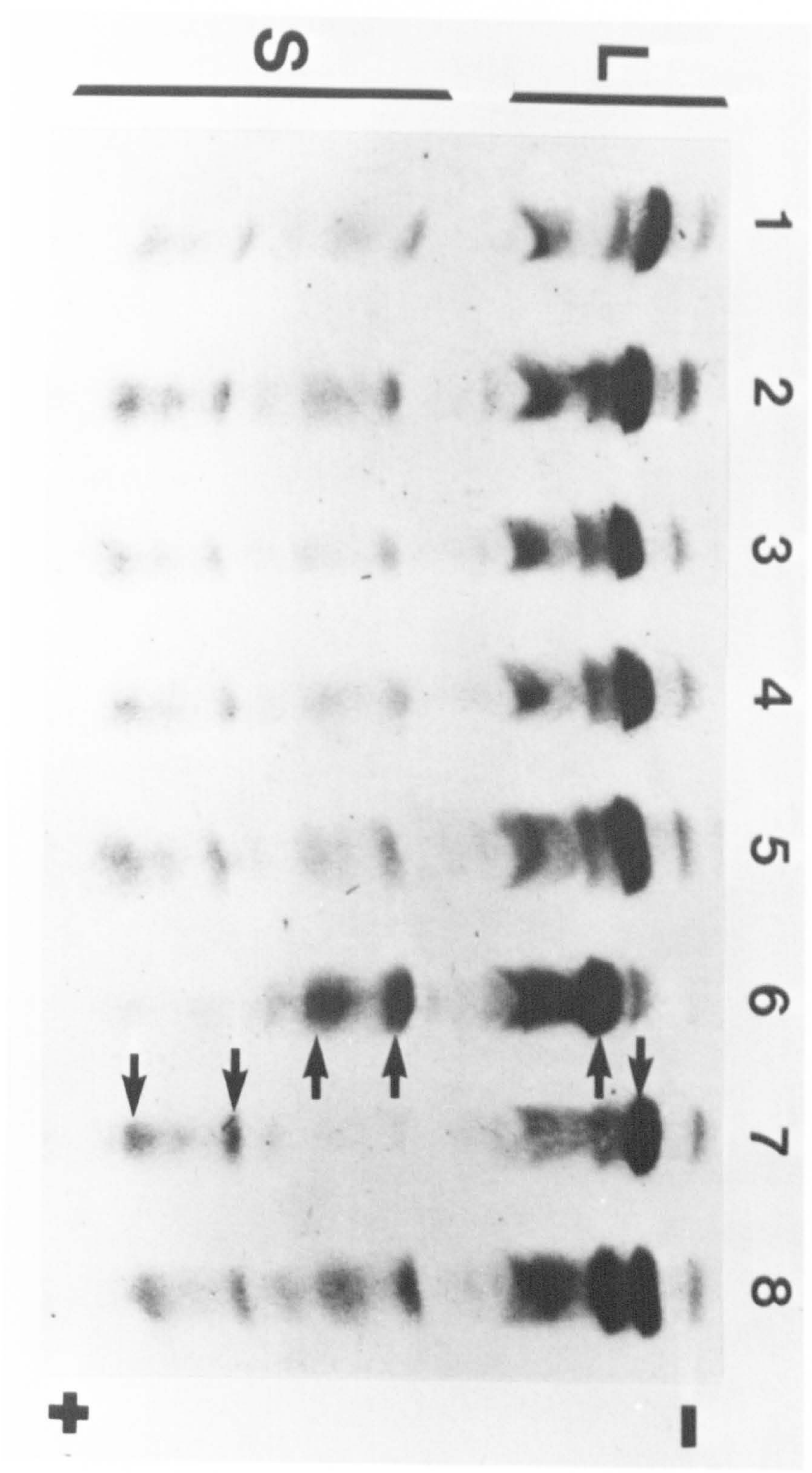


FIGURE 5.3.6

Isoelectric focusing of the Fraction 1 protein subunit
polypeptides of the five putative somatic hybrids, and their
fusion partners.

Channels 1-5, putative somatic hybrids; Channel 6, N. glutinosa;
Channel 7 N. tabacum cv. Gatersleben, and Channel 8, physical
mix of Fraction 1 protein from N. tabacum and N. glutinosa (1:1).

The position of the small subunit (nuclear encoded) polypeptides
(S) and large subunit polypeptides (L) is indicated.



5.4 CONCLUSION

Heterokaryons could not be unequivocally identified visually among fusion treated N. tabacum nia-130 leaf mesophyll protoplasts and N. glutinosa tetrad protoplasts. Following fusion between 5×10^5 nia-130 protoplasts, and an equal number of N. glutinosa tetrad protoplasts, the mesophyll protoplasts underwent division giving rise to small colonies in AA-P19M medium. When these were transferred to a selection medium containing nitrate as sole nitrogen source, two actively growing green colonies were recovered, one of which regenerated giving rise to six shoots, five of which were successfully established in the greenhouse. The second colony was lost due to bacterial contamination. The five putative somatic hybrids possessed a number of morphological features characteristic of either N. tabacum or N. glutinosa. The putative somatic hybrids possess a somatic chromosome complement of 60, which is the summation of that of N. tabacum ($2n = 4x = 48$) and the haploid chromosome complement of N. glutinosa ($n = x = 12$).

Biochemical characterisation of the five putative somatic hybrids revealed that they possess a leaf esterase and leaf callus peroxidase zymogram which is the summation of that found for N. tabacum and N. glutinosa. In both cases, the putative somatic hybrids were found to possess bands unique to both N. tabacum and N. glutinosa. Fraction 1 protein analysis revealed that the five putative somatic hybrids possess the cytoplasmically encoded large subunit polypeptide pattern of N. tabacum, and nuclear encoded small subunit polypeptides of both N. tabacum and N. glutinosa.

Based on this evidence the five putative somatic hybrids were established to be true somatic hybrids between Nicotiana tabacum and N. glutinosa. The hybrids appear to contain the expected pentaploid,

but functionally triploid somatic chromosome complement of
 $3n = 5x = 60$. This result demonstrates that tetrad protoplasts,
unable to undergo sustained division can be induced to fuse with
leaf mesophyll protoplasts, and somatic hybrid plants recovered.

CHAPTER 6

EXAMINATION OF THE FERTILITY OF THE FIVE TRIPLOID SOMATIC HYBRIDS BETWEEN *N. TABACUM* (2n) AND *N. GLUTINOSA* (n) AND CHARACTERISATION OF THE FIRST BACKCROSS PROGENY BETWEEN THE SOMATIC HYBRIDS, AND *N. TABACUM*

6.1 INTRODUCTION

For somatic hybrid plants to be of value in the improvement of crop plants propagated by seed, the somatic hybrids must possess some degree of fertility (Gleba and Evans, 1983). The self fertility of somatic hybrid plants can be assessed over a number of generations. Hybrids between *N. tabacum* and *N. rustica* were found to increase in fertility over two generations of inbreeding, selecting for the most fertile individuals in the progeny (Hamill et al., 1985). However, it is in backcrossing to the crop plant that gene flow between a wild type species and a crop species might be brought about, following somatic hybridisation. The predicted behaviour of a triploid somatic hybrid between diploid protoplasts of a crop species, and haploid protoplasts of a wild type species has been discussed in the Introduction (see also Pental and Cocking, 1985). Random and uneven segregation of the haploid set of chromosomes at meiosis may result in progeny possessing a variable number of chromosomes from the haploid chromosome set following backcrosses of the triploid somatic hybrid with its diploid fusion partner. However, for this to be achieved the somatic hybrid must be fertile.

Somatic hybrids between *N. tabacum* (2n) and *N. glutinosa* (2n) have varied in their fertility, from being completely sterile (Horn

et al., 1983) to being cross compatible with both N. tabacum and N. glutinosa (Uchimaya et al., 1984). In this Chapter, the fertility of the triploid somatic hybrids between N. tabacum (2n) and N. glutinosa (n) will be assessed, and the progeny resulting from the first backcross between the somatic hybrid, and N. tabacum characterised morphologically and biochemically.

6.2 MATERIALS AND METHODS

6.2.1 Source and Growth of Plant Material

N. tabacum cv. Gatersleben, and N. glutinosa were obtained and maintained as described previously (see Chapter 5). The five somatic hybrids labelled NGT1, 2, 4, 5 and 6 were recovered following fusions between N. tabacum leaf mesophyll protoplasts (2n) and N. glutinosa tetrad protoplasts (n). They all possess the expected pentaploid, but functionally triploid chromosome complement of $3n = 5x = 60$. The somatic hybrids, their progeny and the fusion partners, N. tabacum and N. glutinosa were grown together under the same conditions as described in Chapter 5.2.1.

6.2.2 Pollen Development and Viability Determination

Anthers containing pollen mother cells undergoing meiosis were squashed in acetocarmine on a slide and were examined microscopically after five minutes.

Pollen viability was determined by placing freshly dehisced pollen from anthers of a number of different flowers in acetocarmine solution. After five minutes the pollen was observed microscopically. Viable pollen grains were deeply stained with acetocarmine, whereas non viable pollen grains were not. The validity of this procedure was verified by comparing the germination of pollen grains in 10% (w/v) sucrose solution after 2 hours at 27°C with the viability as determined by staining in acetocarmine. A minimum of 300-350 pollen grains were examined.

The size of the pollen grains was also measured using an eye piece graticule. A minimum of 20 viable pollen grains were measured.

6.2.3 Sexual Crosses

Sexual crosses were performed by introducing pollen of the desired parent to the Stigma of an emasculated flower. Flowers were carefully emasculated before they opened, and before the anthers had dehisced. The emasculated flowers were individually bagged with self sealing polythene bags (90 x 57 mm), and labelled. The stigma was usually receptive 2-3 days after emasculation, indicated by a sticky surface secretion. Following pollination the bage was replaced until the stigma withered (usually 5-6 days) when the bags were removed. Labelled seed pods were harvested when ripe, and the seed stored dry at room temperature.

6.2.4 Seed Set and Viability Determinations

Seed set following crosses involving the five somatic hybrids, was compared to that obtained following self pollination of N. tabacum and N. glutinosa.

Viability was determined following germination on solid MS-0 medium at $25 \pm 2^{\circ}\text{C}$ with constant illumination (2,100-2,300 lux). A minimum of 200 seeds were tested for each cross, and germination was assessed after 14 days.

6.2.5 Tentoxin Sensitivity Tests

The sensitivity of germinating seedlings to the fungal toxin tentoxin was assessed following the methods Durbin and Uchytíl (1977). Single 5.5 cm filter papers (Whatman No. 1) in 5 cm petri dishes (A/C Nunc, Kamstrup, Denmark) were soaked with either 0.8 ml of a solution containing 20 µg/ml tentoxin (Sigma) or distilled water as a control. A minimum of 100 seeds were sprinkled onto the filter paper and germinated at $25 \pm 2^{\circ}\text{C}$ with constant illumination (2,100-2,300 lux).

After 10-14 days the seedlings were examined and scored as being either green (tentoxin resistant) or bleached (tentoxin sensitive).

6.2.6 Morphological Characterisation of the Progeny of Backcrosses Between the Somatic Hybrid Plants, and *N. tabacum*

The progeny of reciprocal crosses between two of the five somatic hybrid plants (NGT 2 and 6) and *N. tabacum* cv. Gatersleben were grown to maturity as described previously (6.2.1). The progeny of NGT 2 ♀ x *N. tabacum* ♂ and NGT 6 ♀ x *N. tabacum* ♂ were studied in detail, and the following morphological features recorded :

- 1 - Days from germination to opening of the first flower.
- 2 - Plant height at flowering.
- 3 - Flower colour (based on the Royal Horticultural Society flower charts).
- 4 - Flower length and corolla tube diameter.
- 5 - Mean flower corolla tube diameter/flower length ratio (based on a minimum of 3 flower measurements).
- 6 - Stigma length (longer or shorter than the corolla tube).
- 7 - Largest leaf dimensions, and leaf width/length ratio based on the largest leaf dimension.

6.2.7 Biochemical Characterisation of the Progeny of Backcrosses Between the Somatic Hybrids and *N. tabacum*

Five of the progeny of crosses between NGT 2 ♀ x *N. tabacum* ♂ and NGT 6 ♀ x *N. tabacum* ♂ were further characterised for their leaf esterase zymogram.

Soluble protein extracts (20-40 µl) were applied to LKB pH

range 3.5 - 9.5 PAG plates, and proteins separated by isoelectric focusing. Leaf esterases were visualised by staining the gel as described in Chapter 5.

To achieve a better separation of the prominent esterase bands characteristic of either N. tabacum or N. glutinosa, leaf esterases were also visualised following isoelectric focusing of soluble leaf proteins on an LKB pH range 5.5 - 8.5 PAG plate. The anode for this gel was 0.4 M HEPES, and cathode 0.1 M NaOH. All other conditions were as described previously for pH range 4 - 6.5 and 3.5 - 9.5 gels.

The pI of prominent N. tabacum or N. glutinosa specific bands was estimated from information supplied by the manufacturer of the gels (LKB) (see Appendix 2).

6.3 RESULTS

6.3.1 Pollen Development, Viability and Size

At meiosis the reduction division in the somatic hybrid plants was found to be atypical, in that at telophase II chromosomes not associated with the newly forming haploid nuclei were observed (see Figure 6.3.1). Accurate chromosome counts were only occasionally possible and 27, 28 and 30 chromosomes were observed in newly forming haploid nuclei at telophase II, in separate pollen mother cells.

Pollen viability varied between 45 and 75% in the somatic hybrids as compared to 99 and 95% respectively for N. glutinosa and N. tabacum. The pollen from the somatic hybrids was found to be slightly larger and more variable in size than that from either of the fusion partners (see Table 6.3.1).

6.3.2 Seed Set and Viability Following Reciprocal Crosses Between the Five Somatic Hybrids, and N. tabacum

Viable seed was recovered following reciprocal crosses between the five somatic hybrids, and N. tabacum cv. Gatersleben. Following self pollination N. tabacum seed pods contained approximately 1,150 - 1,750 seeds. N. glutinosa seed pods were somewhat smaller, containing approximately 850-1,050 seeds. Seed pods collected from the five somatic hybrids following pollination with N. tabacum, contained approximately 150-350 seeds, and in the reciprocal crosses approximately 450-600 seeds.

The viability of the seed recovered from self pollination of N. tabacum and N. glutinosa was very high, being 98-99%. The viability of seed recovered following reciprocal crosses between the five somatic hybrids, and N. tabacum ranged between 33 and 66% (see Table 6.3.1).

Pods containing approximately 850-1,050 seeds were recovered following pollination of N. tabacum cv. Gatersleben with N. glutinosa. The viability was found to be 17%.

6.3.3 Tentoxin Sensibility Tests

Tentoxin sensitivity tests were performed on germinating seeds as described in the Materials and Methods. N. tabacum cv. Gatersleben was found to be resistant to tentoxin. All the germinating seedlings were green. N. glutinosa was found to be tentoxin sensitive, and all the germinating seedlings were bleached in the presence of tentoxin, but grew green in controls in the absence of tentoxin. All the germinating seedlings resulting from reciprocal crosses between the somatic hybrids and N. tabacum were also found to be tentoxin resistant (see Figure 6.3.2).

6.3.4 Morphology of the Backcross Progeny between Somatic Hybrids NGT 2 and NGT 6 ♀ and N. tabacum ♂

A random sample of 25 plants from crosses between somatic hybrids NGT 2 and NGT 6 (♀) and N. tabacum (♂) were grown to maturity and examined as described in the Materials and Methods. The results are presented in Tables 6.3.2 and 6.3.3, and illustrated in Fig. 6.3.3.

Overall considerable variation was observed among the backcrossed progeny of the two somatic hybrid plants. The height of the flowering plants ranged between 78 and 135 cm. The original hybrids were more uniform in their height, as were N. tabacum and N. glutinosa. The offspring of the somatic hybrids were all taller than the somatic hybrids themselves. The time from germination to flowering was also more variable among the hybrid progeny than was found for either N. tabacum or N. glutinosa.

The flowers of the hybrid progeny were generally longer than the original hybrids, being more like N. tabacum flowers in shape. The flower colour varied, with a wide range of different flower colours being observed (see Fig.6.3.3). Overall, flower colours were more similar to N. tabacum than the original hybrids, or N. glutinosa. The leaves of the hybrid offspring were larger than the original hybrids, and frequently lacked the small winged petiole found on the original hybrids.

6.3.5 Morphology of the Sexual Hybrid between N. tabacum (♀) and N. glutinosa (♂)

The sexual hybrids between N. tabacum and N. glutinosa were found to be similar in many ways to the somatic hybrids. The flowering plants measured 92-112 cm in height, and all possessed flowers which were identical in colour (51 C of the Royal Horticultural Society's colour chart). The flowers were similar in size and shape to those found on the somatic hybrids, having maximum dimensions of 47 x 12 mm (a flower width/length ratio of 0.26). The leaves of the sexual hybrids were larger than those of the somatic hybrids, having a maximum size of 200 x 150 mm (a leaf width/length ratio of 0.75). The sexual hybrids between N. tabacum and N. glutinosa were completely sterile.

6.3.6 Biochemical Characterisation of the Backcross Progeny between NGT 2 and NGT 6, and N. tabacum

Leaf esterase zymograms for N. tabacum, N. glutinosa, the parent somatic hybrid plants and the examples of the first backcross progeny, are presented in Figures 6.3.4 and 6.3.5.

Originally the leaf esterase zymogram was visualised following

separation of soluble leaf proteins on an LKB pH range 3.5 - 9.5 PAG plate (Fig. 6.3.4). A prominent N. glutinosa specific band at pI 7.2 was identified in the somatic hybrid plants, but only in five of the ten backcross progeny (plants 2-1, 2-4, 2-5, 6-1 and 6-5). Two other prominent N. glutinosa bands at pI 5.1 and 5.6 were not clearly resolved on this gel, but the presence of the N. glutinosa specific band at pI 5.1 appeared to coincide with the presence of the N. glutinosa specific band at pI 7.2.

In an attempt to further clarify the leaf esterase zymogram, soluble leaf protein extracts were also separated on an LKB pH range 5.5 - 8.5 PAG plate and leaf esterases visualised as described previously. The zymogram is presented in Fig. 6.3.5. As found previously an N. glutinosa specific esterase band at pI 7.2 was found in the somatic hybrids, and five of the ten backcross progeny (plants 2-1, 2-4, 2-5, 6-1 and 6-5). The same backcross progeny plants were also found to possess a second prominent N. glutinosa specific band at pI 5.6.

In all cases, the somatic hybrids and the progeny of crosses between the somatic hybrids and N. tabacum possessed two prominent N. tabacum specific bands at pI 6.0 and 6.9.

No difference was found in the leaf esterase zymogram between haploid and diploid N. glutinosa plants. The sexual hybrid between N. tabacum and N. glutinosa possessed a leaf esterase pattern which was the summation of the parental leaf esterase zymogram patterns.

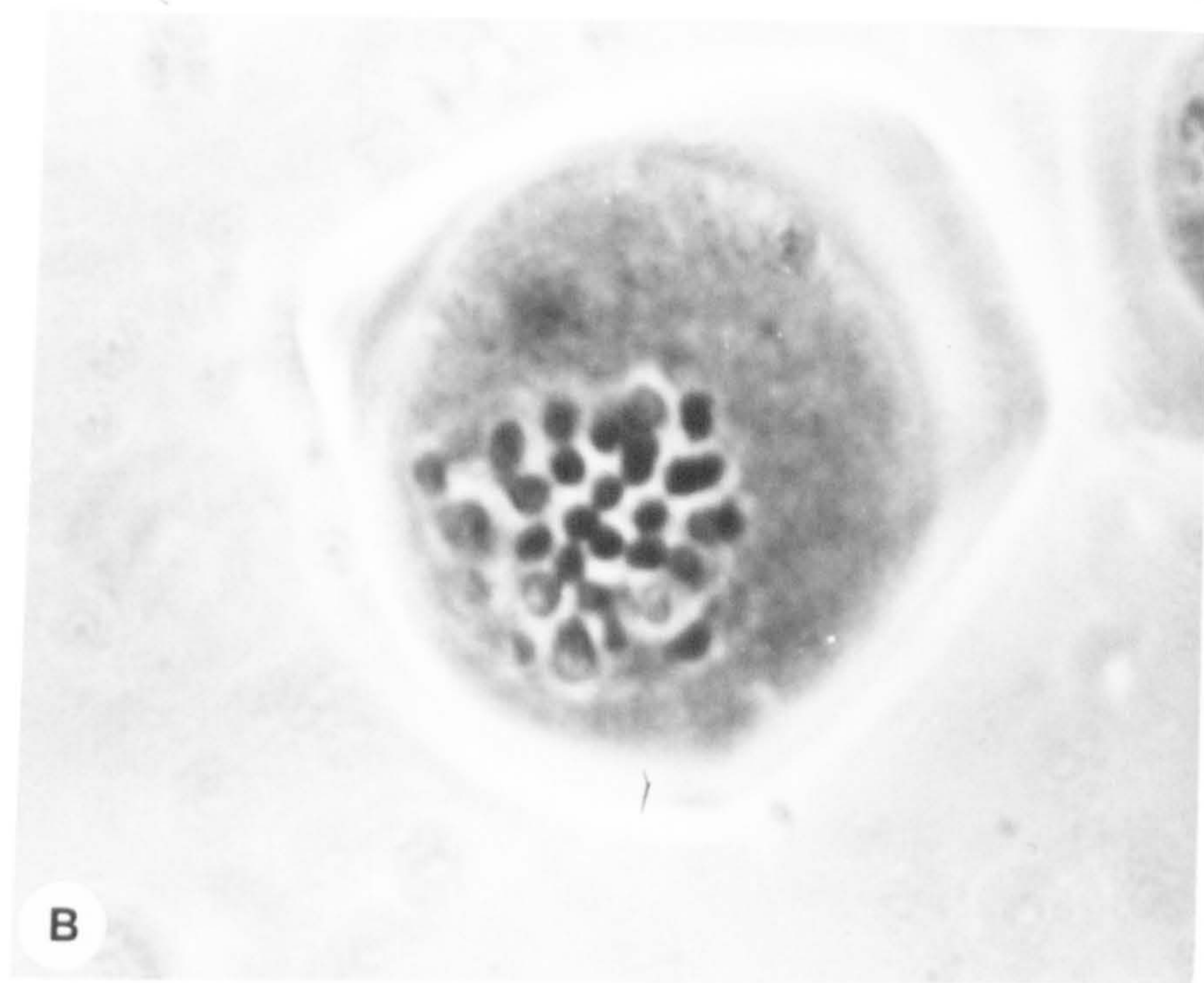
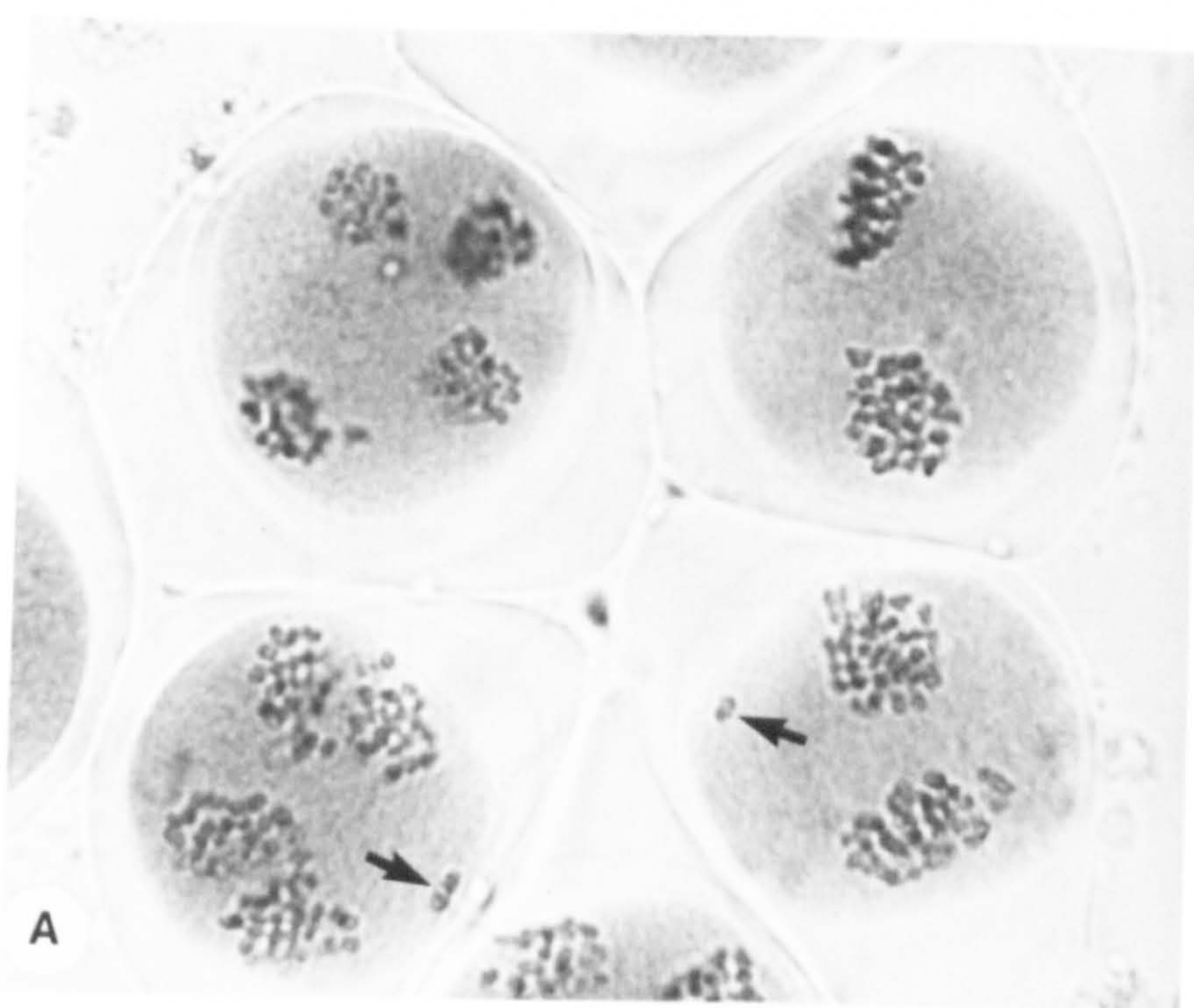


TABLE 6.3.1

Pollen viability and size, and seed viability of *N. tabacum*,
N. glutinosa, and the five somatic hybrids

Plant	Pollen Viability %	Pollen Diameter μm		Seed Viability %*	
		Mean	S.D.	Nt ♀	Nt ♂
<u><i>N. tabacum</i></u> cv. Gatersleben	95	31.9	0.9	98	98
<u><i>N. glutinosa</i></u>	99	32.8	0.9	17	-
NGT 1	74	33.1	2.0	66	54
NGT 2	69	35.0	3.0	52	48
NGT 4	45	34.3	3.5	53	-
NGT 5	64	35.1	2.2	60	47
NGT 6	50	36.3	3.0	66	33

* Seed viability following crosses involving *N. tabacum* cv. Gatersleben as maternal partner (Nt ♀) or paternal partner (Nt ♂)

N. glutinosa seed recovered following self pollination had a viability of 99-100%.

FIGURE 6.3.2

Tentoxin sensitivity tests on germinating seeds of N. tabacum
cv. Gatersleben, N. glutinosa and somatic hybrid NGT 2

- Top - N. tabacum cv. Gatersleben seeds recovered following self pollination and germinated in the presence (left) and absence (right) of tentoxin.
- Centre - N. glutinosa seeds recovered following self pollination and germinated in the presence (left) and absence (right) of tentoxin.
- Bottom - Seeds recovered following crosses between somatic hybrid NGT 2 (♀) and N. tabacum cv. Gatersleben (♂) germinated in the presence (left) and absence (right) of tentoxin.

N. tabacum and all five hybrids were found to be resistant to tentoxin, whereas N. glutinosa was found to be sensitive to tentoxin.



FIGURE 6.3.3

Morphological characterisation of the progeny obtained
following crosses between somatic hybrid NGT 2 (♀) and
N. tabacum cv. Gatersleben (♂)

6.3.3A - The range of flower colours obtained. Each flower is from a different plant, and the flowers on a given plant were all similar in colour.

Centre top is most like the original somatic hybrids, and centre bottom like N. tabacum (X 1.0).

6.3.3B - Examples of the first backcross progeny showing the wide range of growth habit and plant height. (X 0.1).



TABLES 6.3.2 and 6.3.3

Morphological characterisation of the progeny obtained
following crosses between somatic hybrids NGT 2 and NGT 6 (♀),
and N. tabacum cv. Gatersleben (♂).

Table 6.3.2

Plant	Height (cm)	Days to Flowering	Flower Colour	Largest Flower (L x W) (mm)	Flower W/L Ratio	Largest Leaf (L x W) (mm)	Leaf W/L Ratio	Stigma + or -
<u>N. tabacum</u>	110-118	107-112	52C	56 x 10	0.17	266 x 175	0.66	-
<u>N. glutinosa</u> (n)	30- 35	N.D.	37D	25 x 10	0.45	65 x 58	0.89	-
<u>N. glutinosa</u> (2n)	60- 65	86- 94	35B	38 x 14	0.40	100 x 96	0.96	-
NCT 2	57	N.D.	48C	48 x 12	0.24	145 x 104	0.72	
2- 1	111	95	51C	50 x 13	0.25	230 x 155	0.67	-
2- 2	100	105	38B	56 x 12	0.21	270 x 150	0.56	-
2- 3	110	106	52C	55 x 14	0.25	270 x 180	0.67	+
2- 4	115	106	55D	50 x 12	0.23	260 x 175	0.67	-
2- 5	106	105	55D	56 x 11	0.20	260 x 160	0.62	-
2- 6	89	95	48D	53 x 12	0.23	250 x 165	0.65	+
2- 7	130	98	49B	64 x 12	0.28	270 x 200	0.74	+
2- 8	124	101	51C	54 x 11	0.20	210 x 160	0.76	-
2- 9	101	100	55C	55 x 13	0.24	260 x 175	0.67	+
2-10	100	100	55D	54 x 11	0.20	250 x 150	0.60	-
2-11	120	104	54D	54 x 14	0.25	270 x 100	0.67	+
2-12	103	108	56B	53 x 13	0.25	270 x 175	0.65	-
2-13	118	108	55B	50 x 13	0.25	270 x 165	0.61	+
2-14	112	107	54C	50 x 11	0.21	290 x 205	0.71	+
2-15	106	109	55B	49 x 13	0.27	275 x 160	0.58	+
2-16	113	108	54C	47 x 11	0.24	265 x 170	0.64	-
2-17	115	107	55D	50 x 12	0.23	310 x 150	0.48	-
2-18	112	117	54D	53 x 11	0.21	285 x 180	0.63	+
2-19	103	128	55C	48 x 11	0.23	265 x 180	0.68	-
2-20	108	125	54D	48 x 11	0.22	245 x 150	0.61	-
2-21	107	120	54D	53 x 10	0.19	260 x 130	0.50	-
2-22	95	127	54C	49 x 11	0.22	270 x 170	0.63	-
2-23	129	124	55B	58 x 10	0.17	230 x 125	0.54	-
2-24	135	118	56B	52 x 11	0.21	230 x 135	0.54	+
2-25	111	130	55C	53 x 11	0.21	255 x 155	0.62	-

Table 0.3.3

Plant	Height (cm)	Days to Flowering	Flower Colour	Largest Flower (L x W) (mm)	Flower W/L Ratio	Largest Leaf (L x W) (mm)	Leaf W/L Ratio	Stigma + or -
<u>N. tabacum</u>	110-118	107-112	52C	56 x 10	0.17	266 x 175	0.66	-
<u>N. glutinosa</u> (n)	30- 35	N.D.	37D	25 x 10	0.45	65 x 58	0.89	-
<u>N. glutinosa</u> (2n)	60- 65	86- 94	35B	38 x 14	0.40	100 x 96	0.96	-
NCT 6	58	N.D.	47C	48 x 13	0.26	162 x 136	0.84	-
6- 1	85	93	51A	58 x 13	0.23	230 x 140	0.61	-
6- 2	78	92	54C	55 x 12	0.21	250 x 180	0.72	+
6- 3	94	100	55B	50 x 12	0.24	270 x 190	0.70	+
6- 4	105	96	52A	48 x 13	0.27	280 x 200	0.71	+
6- 5	120	95	55B	56 x 11	0.20	220 x 160	0.73	+
6- 6	94	95	56D	53 x 12	0.22	230 x 165	0.72	-
6- 7	130	103	55B	49 x 13	0.26	270 x 180	0.67	-
6- 8	102	103	56D	55 x 12	0.22	265 x 190	0.72	-
6- 9	101	99	56D	49 x 13	0.27	250 x 190	0.76	+
6-10	117	103	55C	55 x 13	0.23	295 x 220	0.76	-
6-11	88	101	55A	53 x 14	0.26	240 x 200	0.83	+
6-12	78	100	51C	58 x 12	0.21	230 x 170	0.74	-
6-13	95	103	54D	52 x 12	0.23	290 x 190	0.66	-
6-14	106	109	56B	52 x 12	0.23	205 x 145	0.71	-
6-15	115	107	54C	52 x 12	0.24	285 x 165	0.58	-
6-16	90	108	54B	45 x 13	0.29	255 x 200	0.78	+
6-17	92	109	56A	50 x 13	0.25	240 x 170	0.71	-
6-18	105	106	55B	53 x 12	0.23	265 x 200	0.75	-
6-19	106	109	55B	48 x 12	0.25	290 x 180	0.62	+
6-20	111	110	55C	54 x 11	0.21	220 x 155	0.70	+
6-21	94	117	55B	58 x 11	0.19	260 x 135	0.52	+
6-22	118	115	55B	58 x 12	0.19	225 x 135	0.60	-
6-23	108	113	56C	47 x 12	0.20	260 x 170	0.65	-
6-24	117	112	55C	52 x 12	0.25	275 x 170	0.62	+
6-25	104	>130	N.D.	N.D.	N.D.	225 x 165	0.73	N.D.

FIGURE 6.3.4

Leaf esterase zymogram visualised following separation of soluble leaf proteins by isoelectric focusing on an LKB pH range 3.5 - 9.5 PAG plate

1	6.5	}	backcross progeny
2	6.4		
3	6.3		
4	6.2		
5	6.1		
6	NGT 6		
7	2.5	}	backcross progeny
8	2.4		
9	2.3		
10	2.2		
11	2.1		
12	NGT 2		
13	<u>N. tabacum</u> x <u>N. glutinosa</u> F1 (sexual hybrid)		
14	<u>N. glutinosa</u> (diploid)		
15	<u>N. glutinosa</u> (haploid)		
16	physical mix (1:1) of proteins from <u>N. tabacum</u> and <u>N. glutinosa</u>		
17	<u>N. tabacum</u> cv. Gatersleben		
18	Somatic hybrid 10a x <u>N. tabacum</u> (sexual hybrid)		
19	Somatic hybrid 10a		
20	Somatic hybrid A1 x <u>N. tabacum</u> (sexual hybrid)		
21	Somatic hybrid A1		

Prominent N. glutinosa specific esterase bands at pI 5.1 5.6 and 7.2 and N. tabacum specific bands at pI 6.0 and 6.9 are indicated.

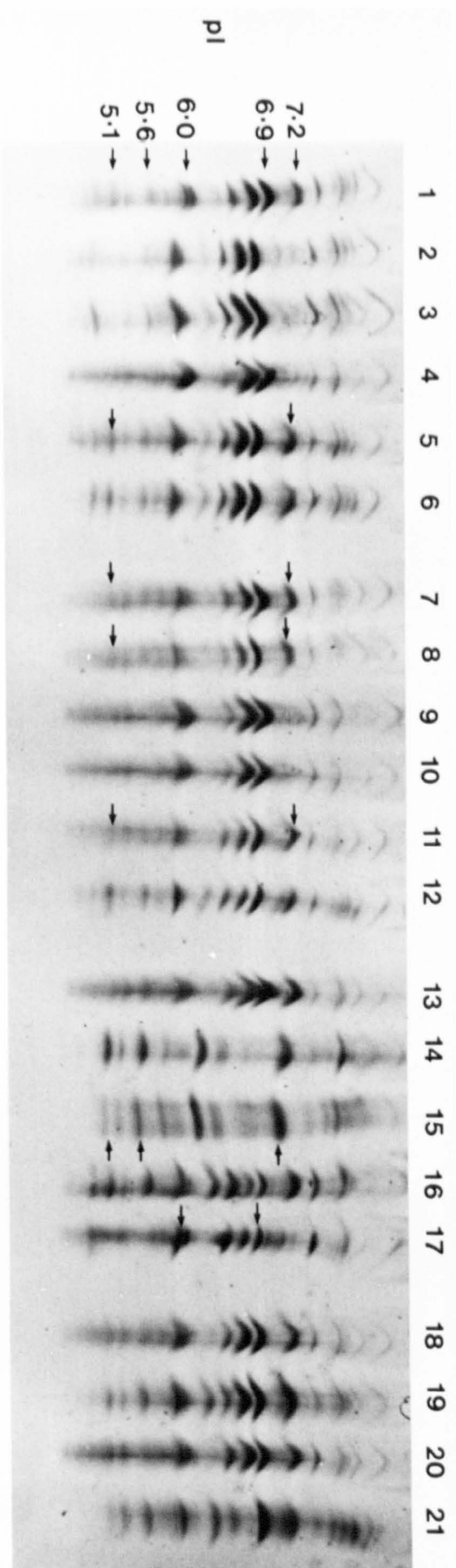
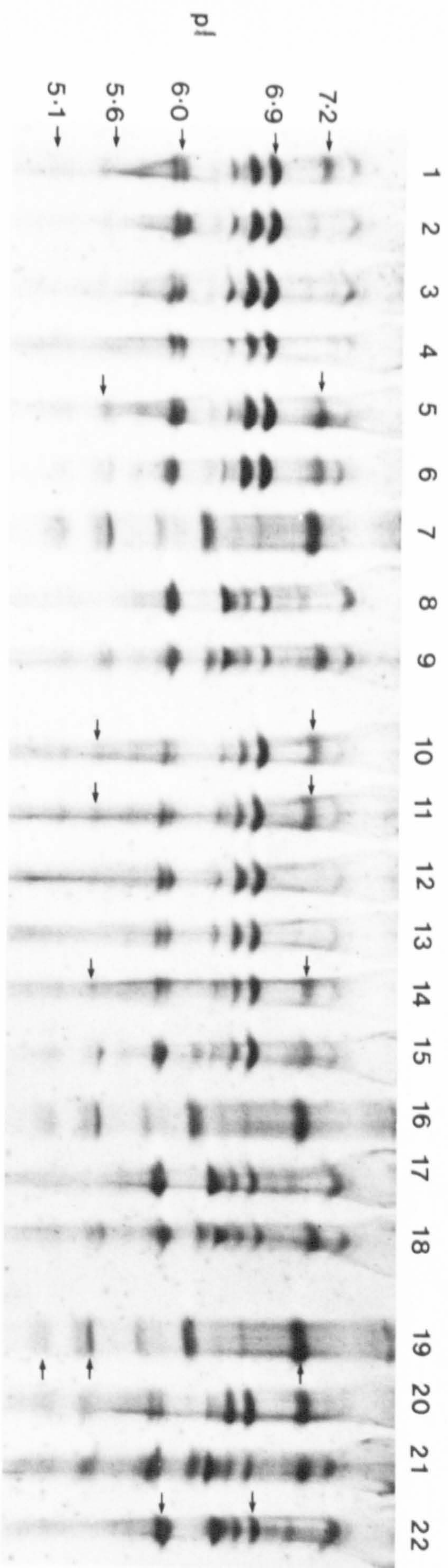


FIGURE 6.3.5

Leaf esterase zymogram visualised following separation of soluble leaf proteins by isoelectric focusing on an LKB pH range 5.5 - 8.5 PAG plate

- 1 6.5
- 2 6.4
- 3 6.3
- 4 6.2
- 5 6.1
- 6 NGT 6
- 7 N. glutinosa (haploid)
- 8 N. tabacum
- 9 physical mix (1:1) of proteins from N. tabacum and N. glutinosa
- 10 2.5
- 11 2.4
- 12 2.3
- 13 2.2
- 14 2.1
- 15 NGT 2
- 16 N. glutinosa (haploid)
- 17 N. tabacum
- 18 physical mix (1:1) of proteins from N. tabacum and N. glutinosa
- 19 N. glutinosa (diploid)
- 20 N. tabacum x N. glutinosa F1 (sexual hybrid)
- 21 physical mix (1:1) of proteins from N. tabacum and N. glutinosa
- 22 N. tabacum

Prominent N. glutinosa specific esterase bands at pI 5.1, 5.6 and 7.2 and N. tabacum specific bands at pI 6.0 and 6.9 are indicated.



6.4 CONCLUSION

The five allopolyploid (functionally triploid) somatic hybrids between N. tabacum (2n) and N. glutinosa (n) produced viable pollen, although chromosomes not associated with the newly forming haploid nuclei were observed during meiosis in the pollen mother cells. The somatic hybrids were found to be fertile and set seed following reciprocal crosses with N. tabacum cv. Gatersleben. The results of tentoxin sensitivity tests performed on germinating seed from crosses between the somatic hybrids (♀) and N. tabacum (♂) confirm previous findings of Fraction 1 protein analysis (Chapter 5), that the hybrids possess the large subunit (chloroplast encoded) of N. tabacum (see Discussion).

Considerable morphological variation was observed among the first backcross progeny of the somatic hybrids to N. tabacum. Flower colours and flower shape varied from being much the same as the original somatic hybrids, to being similar to N. tabacum. The plants were variable in height but mostly taller than the original somatic hybrids, and also possessed longer, wider leaves.

Analysis of the leaf esterase zymogram of a sample of the first backcross progeny indicated the loss of N. glutinosa specific bands in some of the plants. No N. tabacum specific isoenzyme bands were missing.

Based on the cytological observations of chromosome behaviour at meiosis, and the morphological and biochemical analysis of the backcross progeny, it would appear that random segregation or elimination of chromosomes of the haploid N. glutinosa set at meiosis has resulted in the somatic hybrids possessing gametes with a reduced complement of N. glutinosa chromosomes. The progeny resulting from backcrosses to N. tabacum therefore contain a reduced, and variable

number of N. glutinosa chromosomes which may contribute to the observed morphological variation.

CHAPTER 7

GENERAL DISCUSSION

Since the experimental results in the different chapters are interrelated, a detailed discussion is not presented at the end of each chapter. Instead the experimental results have been briefly summarised to highlight the conclusions of each chapter.

7.1 TISSUE CULTURE OF CYCLAMEN PERSICUM

Tissue culture studies were carried out, for two reasons, on somatic tissues of Cyclamen persicum cv. T.R. "mini". Firstly, it was desirable to obtain a better understanding of the culture requirements of Cyclamen, since this information may be of direct relevance in the development of suitable media and cultural conditions for the maintenance of pollen derived embryos, or calli, following anther culture. Secondly, should anther culture generate haploid calli, regeneration of plants from these calli would be necessary. A knowledge of the conditions under which regeneration can be brought about in somatic tissue cultures may be applicable to the regeneration of haploid plants from haploid tissue cultures.

The earliest studies performed on Cyclamen tissue cultures used explants from the corm (Mayer, 1956; Stichel, 1959). However, problems were encountered due to systemic microbial contamination of the corm tissue. This was to some extent overcome by the use of antibiotics (Stichel, 1959; Geier, 1977), or by complicated procedures involving curing (drying in sterile air) the explants prior to culture (Okumoto and Takabayashi, 1969). However, the problem of contamination plant material was avoided by Morel (1975) who used petiole explants,

and achieved shoot regeneration. The shoots were subsequently transferred successfully to the greenhouse. Similarly Geier (1977), and Geier et al. (1979) obtained shoot regeneration from leaf explants, but in contrast to the results of Morel (1975), petiole explants gave rise to callus which did not give rise to shoot regeneration.

In the present study the response of cultured leaf and petiole explants taken from mature Cyclamen plants was assessed. The plants were maintained in the controlled environment of a growth cabinet, and explants cultured on a variety of different media and hormone regimes, coupled with different culture environments. A range of media based on full strength M.S. salts (Murashige and Skoog, 1962), which have previously been found to support callus initiation, growth and morphogenesis in many plant species, failed to support the growth of callus tissue from cultured Cyclamen explants. However, consistent with the findings of Leowenberg (1969), Cyclamen callus tissue proliferated from leaf and petiole explants cultured on a medium based on M.S. salts at reduced strength. When compared to members of the Nicotiana, which are well known to be highly responsive to tissue culture, the frequency with which successful callus initiation occurred was very low. At best, less than half of the cultured explants gave rise to callus, whereas all the cultured N. tabacum and N. glutinosa explants cultured in this study gave rise to callus growth on suitable media. Failed Cyclamen explants were usually blackened, possibly as a result of the action of copper containing oxidase enzymes such as polyphenol oxidase, and tyrosinase, which are released or synthesised, and presented with oxidative conditions when tissues are wounded (Lerch, 1981). The polyphenols released are known to be toxic to plant cells, possibly due to binding with proteins by hydrogen bonds. In addition to blackened explants, severe browning of sectors of the callus initiated was also noted.

The type of callus proliferating from the explants was variable. In some cases friable callus was produced. This callus failed to give rise to adventitious roots or shoots. In contrast extremely compact, dense callus which could only be cut with a scalpel upon subculturing was capable of giving rise to occasional adventitious roots and shoots, and leaf like structures. No apparent correlation was noted between the culture medium on which the callus was obtained, and the frequency or type of regeneration observed.

In the present study the time between subculture and shoot or root regeneration was usually 6-8 weeks. A similar response has been noted in long term callus cultures, derived from corm explants. Sporadic shoot regeneration was observed 4-6 months after subculturing the callus on a medium which supported its growth (Leowenberg, 1969). Similarly, Okumoto and Takabayashi (1969) maintained corm derived callus explants on the same medium for over 3 months before occasional adventitious root and shoot regeneration occurred.

The apparent lack of control over the pattern of development in Cyclamen callus cultures has also been noted previously. Wicart et al. (1984) described the regeneration of unipolar and bipolar corm line structures bearing either roots, or shoots, or both, as occurring in a random fashion. Similar results led to Geier et al. (1979) to conclude that it is not possible to direct regeneration in Cyclamen callus cultures by altering the hormone regime under which they are maintained. The reason for some species being recalcitrant in culture when others are not is not fully understood. The Cyclamen callus tissue may not be receptive to the plant hormones tested, or may contain endogenous hormone levels which mask the effect of the in vitro culture environment. Limitations may exist on the degree of totipotency possessed by the explant material. The culture response of recalcitrant species

is usually investigated by empirical research methods, and until a better understanding of the molecular events underlying the differentiation of disorganised callus cells into organised shoot meristems is achieved. This will remain the only approach.

While greenhouse grown plant material is most frequently utilised in tissue culture studies, aseptically germinated seedlings have considerable advantages over such material. Once established, they provide a source of explants free from microbial contamination. Microbial contamination is known to be a problem in Cyclamen tissue culture, especially when corm explants are to be cultured. In addition a seasonal variation in the response of Cyclamen explants in tissue culture has been reported (Geier et al., 1979). Although the experiments reported in Chapter 2 did not allow this to be assessed in the present study, the use of aseptically germinated seedlings would overcome any seasonal variation since the seedlings could be germinated and maintained under controlled conditions.

The callusing response of leaf and petiole explants from aseptically germinated Cyclamen seedlings was superior to that obtained from leaf and petiole explants from mature Cyclamen plants. Up to 100% of cultured explants gave rise to a proliferation of callus tissue. The browning observed in callus tissue derived from leaf and petiole explants from mature plants, was much reduced or absent in callus obtained from leaf and petiole explants from aseptically germinated Cyclamen seedlings. Similarly Pistacia shoot tips derived from young aseptically germinated plants were also found to be less susceptible to browning than greenhouse grown material (Alderson and Borghchi, 1982). In contrast to leaf and petiole explants, corm explants from aseptically germinated Cyclamen seedlings responded poorly in culture, with only 30% of explants giving rise to some

callus growth.

In subsequent subculture passages on media containing a range of hormone regimes designed to induce adventitious root or shoot regeneration, callus initiated from the four tissue types was found to respond in different ways. Root explants initially gave rise to friable callus, which remained as such in subsequent transfers. Similar to the results obtained from friable leaf and petiole callus derived from mature Cyclamen plants, this friable callus failed to regenerate any adventitious roots or shoots under the conditions tested. Corm explants initially gave rise to a limited proliferation of friable callus which was severely blackened, possibly due to polyphenols, discussed previously. On subsequent transfers, the callus failed to grow. The prolific callus growth from petiole and leaf explants consisted of friable and compact dense sectors. In subsequent transfers, friable callus remained as such, and no adventitious roots or shoots were recovered. However, the dense compact callus proliferated and gave rise to some friable callus sectors, from which regeneration was never observed, as well as dense callus sectors. The dense callus occasionally gave rise to apparently organised nodular tissue (see Figure 2.3.2, Chapter 2). The occurrence of nodular protocorm-like structures has been observed by Wicart et al. (1984). Histological structures revealed that the protocorm-like structures had an organisation almost identical to that of seed grown corm tissue (Wicart et al., 1984). Although the nodular tissue observed in the present study would appear to closely resemble the protocorm-like structures, histological examination would be necessary to confirm this.

Occasional adventitious roots and shoots regenerated from sectors of dense, or nodular callus tissue. Occasional isolated leaf-

like structures were also recovered. Although shoots and roots occasionally arose from the same explant, shoots were never found to be directly associated with a root system, and attempts were made to root the shoots, to permit their transfer and further development in the greenhouse. The procedures tested to stimulate rooting of the shoots were limited by the in vitro tissue supply. Shoots obtained from leaf and petiole of both mature plants and aseptically germinated Cyclamen seedlings were utilised. When successful, rooting was achieved, but this was at the expense of the shoots, which either began to re-callus or senesce. Hence no shoots were successfully transferred to the greenhouse. A similar problem was reported by Geier et al. (1979) who suggested that the conditions necessary for root initiation and shoot survival were mutually exclusive. Adventitious shoots directly associated with a root system have been regenerated from corm callus and can be readily transferred to the greenhouse (Geier, 1977; Geier et al., 1979). However, in the present study, the corm explants did not give rise to callus capable of growth and morphogenesis, and this approach could not therefore be tested.

During the production of this thesis, Wainwright and Harwood (1985) have reported on the tissue culture of explants from aseptically germinated Cyclamen seedlings. Shoot regeneration was reported from cotyledon, petiole, corm and root explants, with cotyledons giving rise to the highest recovery of adventitious shoots (75% of explants). This is in contrast to the infrequent shoot regeneration reported in the present study. Cyclamen is a pseudomonocot, for which only one of the cotyledons develops in the embryo. The single cotyledon strongly resembles a true leaf, (Widmer, 1980) and for this reason no attempt was made to distinguish between the cotyledon

and five or six true leaves present on the Cyclamen seedlings used for culture. Following the findings of Harwood and Wainwright (1985), of the superior frequency of shoot regeneration obtained from cotyledon explants, it may be advantageous to make the distinction between the true leaves, and the cotyledon in future studies.

In conclusion, media based on M.S. salts (Murashige and Skoog, 1962) and containing NAA and BAP were found most suitable for the maintenance of growth of somatic Cyclamen tissues, and such media may be useful in anther culture studies. The failure to obtain reliable and high frequency shoot regeneration from Cyclamen somatic tissues suggests that similar problems may be encountered with haploid tissues which may be derived from anther culture experiments. It would therefore be advantageous if regeneration could be avoided in anther culture studies, by encouraging direct pollen embryogenesis.

7.2 CYCLAMEN ANTHER CULTURE

Cyclamen persicum is propagated commercially by seed. Seed production is usually brought about following open cross pollination, resulting in a highly heterozygous condition. There is a commercial need therefore, to improve the degree of uniformity found within the Cyclamen crop. Although the production of almost genetically uniform F1 hybrids has been achieved (Asma, 1973), the seed is expensive and germination slow and non-uniform (Heydecker and Wainwright, 1976). In addition, the genetic uniformity of the F1 hybrids depends on the parents homozygosity, and presumably the hybrids result from crosses between inbred Cyclamen plants. Inbreeding depression prevents the direct exploitation of inbred lines of Cyclamen, and may limit the availability of homozygous parents for F1 hybrid seed production. However, the use of anther culture may permit the production of true breeding lines of Cyclamen which may be used directly, or contribute to F1 hybrid seed production. It was this possibility which initiated the Cyclamen anther culture work presented in Chapter 3.

Anther culture in Cyclamen persicum has been attempted previously (Geier, 1977), but without success. In the previous study, emphasis was placed on optimising the culture media, and 32 different media compositions were assessed (Geier, 1977).

Anther culture can be divided into two distinct phases. Induction and maintenance. In the induction phase, pollen grains are switched from their normal sporophytic path of development, and induced to become embryogenically competent. In the maintenance phase, conditions must be such that the pollen grains can express their embryogenic competence (Sunderland, 1980). Since no success was achieved by Geier (1977) in manipulating the maintenance phase, an alternative approach was assessed in this study. Bud stress pre-

treatments designed to induce embryogenically competent pollen grains were applied.

Embryogenically competent pollen grains can be identified at an early stage of anther culture in N. tabacum being slightly smaller, and staining less intensively with acetocarmine (Sunderland and Wicks, 1971; Wernick et al., 1978). Pollen dimorphism in vivo clearly parallels the formation of embryogenically competent pollen grains in cultured anthers, and are visually distinct from normal pollen grains (Dale, 1975; Horner and Street, 1978). A strong correlation exists between the degree of pollen dimorphism, and the anther culture response (Sunderland, 1980). However, not all species which respond to anther culture exhibit pollen dimorphism and apparently normal pollen grains have been observed to undergo additional divisions which may lead to pollen derived embryos (Sunderland and Evans, 1980). It would appear that while some embryogenically competent pollen grains are morphologically distinct from normal pollen grains, this is not exclusively the case.

Cyclamen pollen is relatively small, measuring less than 20 μm in diameter, less than half the size of N. tabacum and N. glutinosa pollen. The pollen grains are also numerous, up to 1.2×10^6 pollen grains per anther. No pollen dimorphism of the type described by Dale (1975) or Horner and Street (1978) was observed in Cyclamen pollen, although anomalous pollen grains resulting from abnormal reduction divisions were occasionally observed. Such abnormalities are common, particularly in the genus Datura, where the frequency of occurrence is known to increase with plant age (Collins et al., 1974).

For these reasons, the pollen was not examined cytologically after bud stress pretreatments and/or anther culture, since no morphological feature may exist in Cyclamen pollen enabling the detection

of a successful anther culture response. Even if embryogenically competent pollen grains were visually distinct they may be so infrequent, that, among the large number of small pollen grains they may go undetected. For these reasons, anthers were cultured and examined for the emergence of macroscopic calli, embryos or plantlets.

The culture medium used was based on M.S. salts at reduced strength, a modification known to permit the proliferation of somatic callus tissue from previous reports (Leowenberg, 1969) as well as this study. The regeneration of Cyclamen plants from somatic tissue cultures proved unreliable as was discussed earlier. The addition of hormones to the anther culture medium is known to cause somatic callus to proliferate from the anther wall in Cyclamen anther cultures, (Geier, 1977). Such callus may compete with and mask the emergence of pollen derived calli or embryos. In Nicotiana anther cultures pollen derived embryos are frequently lost due to callusing in an anther culture medium containing hormones, whereas in a hormone free medium normal embryo development can often continue. For these reasons, hormones were excluded from the Cyclamen anther culture media, in an attempt to achieve direct pollen embryogenesis. Activated charcoal was also added to the culture medium, since this has been reported to enhance the anther culture response (Wernick and Kohlenbach, 1976; Johansson, 1983).

No response was observed from Cyclamen anthers in culture indicating a successful anther culture response. No macroscopic structures were obtained, and the anthers were frequently blackened after a few weeks in culture, possibly resulting from copper containing oxidases liberated as a result of anther excision or senescence, and discussed earlier. The pollen within the anthers was found to be non-viable as determined by its poor staining with acetocarmine, and

lack of internal organisation.

Failure to achieve an anther culture response in Cyclamen may be due to a number of factors. Throughout these experiments, the plant material used was Cyclamen persicum cv. T.R. "mini". This is highly heterozygous plant material, and each plant can effectively be regarded as an individual genotype. The genotype is known to be one of the prime factors influencing the response of cultured anthers (Feroughi-Weir et al., 1982; Bullock et al., 1982; Lazar et al., 1984). It is possible that only a few individuals among the Cyclamen population possess a genotype which would permit a successful anther culture response under the appropriate conditions. Given that only a limited number of buds were available, it was not possible to assess the effect of bad pretreatments on the subsequent anther culture response for each individual plant. Instead, the anthers from several plants were pooled, and this may well have reduced the chances of success in the anther culture experiments. The number of buds available from a given genotype could have been increased by vegetatively propagating the Cyclamen plants, but the number of individuals obtainable within the time of this study would still be low, and this was not attempted. F1 hybrid seed, if produced from homozygous parents would be genetically uniform, and has recently been used in Cyclamen tissue culture studies (Wainwright and Harwood, 1985). However, within the constraints of commercially sponsored research this possibility could not be investigated.

The poor correlation between the bud size, and the stage of pollen development may have further reduced the potential pool of embryogenically competent pollen grains exposed to anther culture. A very low frequency of responding anthers may not have been detected given the number of anthers cultured in the present investigation.

Cyclamen pollen is small and numerous, preventing cytological observation through the stages of bud pretreatment, and anther culture. A successful initiation of pollen embryogenesis which failed to give rise to developing macroscopic embryos due to deficiencies in the culture media or conditions employed may have occurred, and gone undetected.

In conclusion, failure to obtain an anther culture response in Cyclamen persicum was due to two factors. Firstly, we lack adequate knowledge of the basic requirements for a successful anther culture response. There is no valid explanation at present to explain the lack of response in some species, and high frequency of pollen embryogenesis achieved in others. Secondly, the Cyclamen plant material was poor for anther culture studies, for several reasons. The material was genetically heterozygous, and the size and development of the pollen unsuitable for systematic studies to be performed. In addition, the apparent recalcitrance of the somatic Cyclamen tissues in vitro suggests that Cyclamen persicum may not be responsive to other tissue culture manipulations, including anther culture.

7.3 ALTERNATIVE APPROACHES TO UNIFORMITY IN CYCLAMEN

Anther culture although being the most widely used method, is not the only method by which haploid plants can be generated. The culture of unpollinated ovaries or ovules was first demonstrated to give rise to maternal haploids by San Noeum (1976) in Hordeum vulgare. Subsequently haploids have been obtained, using this method, in a number of other species, including Triticum aestivum and N. tabacum (Zhu and Wu, 1979) Oryza sativa (de Beaville, 1980; Zhou and Yang, 1980) and Petunia axillaris (de Verna and Collins, 1985). The induction of plants from unpollinated ovaries or ovules in vitro is even less well understood than anther culture (Yang and Zhou, 1982).

Large numbers of haploids have also been recovered following interspecific hybridisation between Hordeum species. Following the cross between Hordeum vulgare and Hordeum bulbosum, specific chromosome elimination occurs, resulting in the loss of the complete Hordeum bulbosum chromosome complement (Symko, 1969; Kasha and Kao, 1970; Subrahmanyam and Kasha, 1973).

Both of these techniques were considered to be outside the scope of this thesis.

Clonal propagation of Cyclamen through tissue culture has been proposed as an alternative approach to uniformity (Okumoto and Takabayashi, 1969; Morel, 1975 and Fersing et al., 1982; Wainwright and Harwood, 1985). However the conclusion of Geier et al. (1979) that mass clonal propagation of Cyclamen is not likely to be achieved, at least in the near future, would appear to remain valid, since as yet, no reliable method for the in vitro propagation of large numbers of Cyclamen plants within a short time span exists.

The production of F1 hybrid seed (Asma, 1973) remains the

most reliable method by which more uniform Cyclamen plants can be obtained, although the degree of uniformity will depend on the homozygosity of the parents involved, and problems still occur due to the slow non-uniform germination of the seed, and its high production cost (Hedecker and Wainwright, 1976).

7.4 ANTHR CULTURE IN NICOTIANA SPECIES

Anther culture experiments were carried out on N. tabacum cv. White Burley, and N. glutinosa. N. tabacum cv. White Burley anther culture was chosen as a model system with which to assess the effectiveness of the culture methods and conditions used in the attempts at inducing an anther-culture response in Cyclamen. The effect of bud pretreatments and the stage of pollen development prior to bud pretreatments was assessed using this system. Failure to obtain an anther culture response for such a responsive species would have indicated a fault in the basic culture protocol. However, no such fault was detected.

The culture response of N. tabacum cv. White Burley has been well characterised in previous reports (Dunwell, 1976; Sunderland and Roberts, 1979), and can be quantified according to three parameters :

- 1) Induction Frequency (I.F.). The frequency with which anthers gave rise to a culture response, expressed as a percentage of plated anthers.
- 2) Anther Productivity (A.P.). The mean number of embryos /plantlets emerging from each responding anther.
- 3) Efficiency (E). The product of the induction frequency and anther productivity; this gives an indication of the expected recovery of anther culture derived embryos/plantlets for 100 cultured anthers.

Under the conditions used, the maximum induction frequency and anther productivity obtained for direct anther culture was 13.9% and 5.9 respectively. This compares very favourably with the value of 22% and 16.4 obtained by Dunwell (1979) using similar culture conditions. However, it was previously found that a significant im-

provement could be achieved in both parameters, by increasing the volume of the culture vessel while maintaining the same volume of culture medium (Dunwell, 1979). In the present study, the culture conditions were not altered, however a very significant increase in the induction frequency up to 52.4% was achieved when the buds were pretreated at 4 - 6.5°C for 8 days prior to anther culture. Under these conditions, the anther productivity was also increased to 11.7. When the buds were pretreated at 4-6.5°C, the greatest anther culture response was achieved when the pollen was at the early binucleate stage (stage 5 - Sunderland (1974)) of pollen development at the start of the experiment. A similar finding was reported by Sunderland and Roberts (1979). The results presented in Chapter 3 indicate a very sharp increase in the induction frequency and anther productivity around this stage of development, falling off very sharply for pollen at an earlier or later stage of development. This is most clearly reflected in the results of the culture efficiency (E) presented in Figure 3.3.8. These results although based on a single experiment do indicate the importance of the stage of pollen development prior to bud pretreatment and anther culture.

Extreme variation was observed between replicates of experimental treatments, preventing meaningful statistical analysis of the results. Such extreme batch to batch variation in the anther culture response has also been noted by Sunderland and Roberts (1979) who ascribed it to ever changing growth conditions of plants maintained in the greenhouse. It seems likely that the excision of buds over the 16-day harvesting period used in the present study may also influence the physiological status of the donor plant further contributing the variation observed. For this reason, the data for the replicate experiments was finally combined as was the data reported previously

by Sunderland and Roberts (1979).

In contrast to N. tabacum anther cultures from which large numbers of anther culture derived plants could readily be generated, N. glutinosa anther cultures were much less successful. Only two plantlets were recovered from a total of 2,954 cultured N. glutinosa anthers. N. glutinosa anther culture was attempted in order that haploid plants might be generated and would be available for the isolation of haploid protoplasts for subsequent fusion studies using haploid systems (see 7.7).

The production of haploid N. glutinosa plants has been reported previously (Nitsche 1972; Nakamura and Itagaki, 1973; Nakamura et al., 1974; Tomes and Collins, 1976). Response has ranged from complete failure (Nitsch 1969; Hlasnikova, 1977) to, in a few cases, the production of a few plants, some of which were haploid, and some diploid (Tomes and Collins, 1976). Reproducible plant production with an induction frequency of 11.9% and anther productivity of 2.4 can also be achieved (Nakamura et al., 1974).

The two plants recovered in the present study resulted from anthers cultured on a medium containing 1.0% activated charcoal, following bud pretreatment at 4.5 - 6.0°C for 4 days. The buds contained pollen at the early to late binucleate stage of development (stages 5-6). Successful anther culture in N. glutinosa as reported by Nakamura et al. (1974) was also achieved on a medium containing activated charcoal. Activated charcoal may be effective in stimulating an anther culture response either by removing substances contained within the culture medium which are inhibitory to a culture response (Johansson, 1983), or by removing substances emanating from the anther itself, which may also prevent a culture response (Tyagi et al., 1980; Johansson, 1983).

The switch from a gametophytic to a sporophytic path of development is not clearly understood. Two distinct possibilities exist. A pre-existing pool of embryogenically competent pollen may exist within the anther, and the bud pretreatments, and anther culture conditions may permit the expression of this latent potential. In support of this, it has been shown that the conditions under which the donor plants are maintained, even before meiosis can influence the subsequent anther culture response (Heberle-Bors, 1982b). Sunderland and Roberts (1979) have suggested that the role of the cold pretreatment of the bud prior to anther culture is to delay the senescence of the somatic anther tissues, while enabling the embryogenically competent pollen grains to express their full potential.

Alternatively, it has been suggested that the bud stress pretreatments redirect the course of development of previously normal pollen grains such that they are capable of undergoing embryogenesis. Although a larger number of equal first pollen mitoses have been observed following temperature stress bud pretreatments (Nitsch, 1974), the effect must also extend beyond the first pollen mitosis since in the present study temperature stress bud pretreatments were effective at increasing the induction frequency and anther productivity even when N. tabacum buds containing early binucleate pollen were selected. These results confirm the findings of Sunderland and Roberts (1979).

A random selection of the N. tabacum cv. White Burley plantlets obtained from anther culture in this study were examined cytologically, and 98% of the plants were found to possess the allodihaploid somatic chromosome complement of $n = 2x = 24$. Both of the N. glutinosa plants obtained from anther culture were found to possess the haploid chromosome complement of $n = x = 12$. These results are consistent with previous findings indicating that the majority of plants derived

from anther culture within the Nicotiana are haploid (Sunderland, 1971; Sharma et al., 1983).

When grown to maturity the allodihaploid N. tabacum and haploid N. glutinosa plants resembled their parents closely, but were between $1/3$ and $1/2$ the size of their allotetraploid and diploid parents. The leaves were more narrow for the allodihaploid N. tabacum plants. All the haploid plants were found to be sterile, as would be expected. However, after several months growth, and following cutting back of the allodihaploid N. tabacum plants one fertile shoot was observed, presumably the result of endomitosis causing a doubling of the chromosome complement. Doubling the chromosome number of haploid plants to achieve fertile homozygous plants would have been necessary if haploid Cyclamen plants had been generated. This can be readily achieved in Nicotiana species using colchicine treatments either of the developing inflorescence (Nakamura et al., 1974) or young anther derived plantlets (Chowdhary, 1984).

Failure to obtain a high frequency of haploid plants from N. glutinosa anthers may reflect differences in the culture requirements of N. tabacum and N. glutinosa specifically for the maintenance of embryogenic pollen grains. It may also be due to a failure to induce a fraction of embryogenically competent pollen grains. N. glutinosa anther culture was attempted to generate haploid plants for the isolation of haploid protoplasts. Large numbers of individual haploid plants were not necessary, since the two haploid plantlets recovered could be rapidly multiplied in vitro before transfer to the greenhouse.

7.5 HAPLOID PROTOPLAST ISOLATION AND CULTURE

Allodihaploid N. tabacum cv. White Burley and haploid N. glutinosa plants produced via anther culture were grown in the greenhouse, and leaves removed for mesophyll protoplast isolation experiments. Mesophyll protoplasts were successfully isolated from allodihaploid N. tabacum plants, and occasionally underwent division. Haploid N. glutinosa mesophyll protoplasts failed to divide. Conditions have previously been described which enable the culture of allodihaploid N. tabacum mesophyll protoplasts (Caboche, 1982). The low frequency of division in cultured allodihaploid N. tabacum mesophyll protoplasts, and complete lack of division in haploid N. glutinosa mesophyll protoplasts observed in this study, may be due to the growth condition of the plant material. In both cases, the original plant material was close to flowering at the beginning of the protoplast isolation experiments. The physiological status of the donor plant is known to influence the subsequent yield and viability of protoplasts (Cassels and Cocker, 1982; Davey, 1983). A more reliable source of plant material for protoplast isolation might be shoot cultures, as used by Caboche, (1982). Alternatively, cell suspensions or callus cultures initiated from haploid plant tissues could be used, although care would be necessary to ensure the maintenance of a predominantly haploid population of cells.

Anther culture is only successful in a limited number of plant species. Tetrads formed as a result of meiosis in the pollen mother cells, and consisting of four haploid spores bound within a thick callose matrix, are found throughout fertile flowering plants. N. glutinosa tetrad protoplasts were readily obtained, following incubation of the tetrads in 2% Driselase. The tetrad protoplasts were small, measuring approximately 12 μm in diameter, compared with

40-50 μm diameter for N. tabacum leaf mesophyll protoplasts. The tetrad protoplasts did not possess a vacuole, and failed to divide in any of the culture media, or under any of the culture conditions tested. Tetrad protoplast isolation has been reported in a wide range of species, including Cajanus cajan, Lycopersicon esculentum, Nicotiana tabacum, N. sylvestris, Petunia hybrida, Triticum aestivum and Zea mays (Bhojwani and Cocking, 1972; Bajaj, 1974; Deka et al., 1977). In all previous studies, tetrad protoplasts failed to undergo sustained division.

In most previous reports concerning the isolation and culture of haploid protoplasts, the systems have primarily been developed for the ultimate recovery of mutant cell lines and plants. For example, using such systems naphthaleneacetic acid tolerant mutants of N. tabacum (Muller et al., 1985), amino acid auxotrophs of N. plumbaginifolia (Negrutiu et al., 1985), and nitrate reductase deficient auxotrophs (Straus et al., 1981) and temperature sensitive mutants of Hyocyanus muticus (Gebhardt et al., 1981) have been recovered. Such studies require a system in which protoplast division and plant regeneration occurs at high frequency. In contrast, in the present study haploid protoplasts were investigated as a delivery system for the haploid genome of a wild type species in somatic hybridisation with diploid protoplasts of a crop species. The observed lack of division in N. glutinosa haploid leaf mesophyll protoplasts, and tetrad protoplasts could be advantageous in that counter selection against the haploid protoplasts is not necessary following protoplast fusion. This simplifies the subsequent recovery of heterokaryon derived cell colonies.

Lack of division has been extensively exploited in somatic hybridisation studies involving diploid protoplasts of cultured cell

or leaf mesophyll origin. Somatic hybrid cell lines have been recovered following fusion between Petunia hybrida and Parthenocissus tricuspidata protoplasts. The Parthenocissus tricuspidata protoplasts were derived from a hormone independant tumorous cell line, and only occasionally underwent one or two divisions. Somatic hybrid colonies were recovered on the basis of hormone independant growth of the heterokaryon derived colonies (Power et al., 1976). Similarly, the knowledge that protoplasts from the sexual hybrid between Petunia hybrida and Petunia parodii were capable of sustained division on a medium which only permitted limited development of the P. parodii protoplasts, and which failed to support P. hybrida protoplast division has been used in the recovery of somatic hybrids between these two species (Power et al., 1977). The observed lack of division in some wild type protoplast systems has been exploited in combination with albino mutant systems capable of sustained division, enabling green somatic hybrid colonies to be selected as a result of complementation between the normal pigmentation of the wild type protoplast system and division capability of the albino protoplast system. In this way, somatic hybrids have been recovered between a nuclear albino Daucus carota mutant and a wild type Aegipodium podagraria (Dudits et al., 1979) and between a nuclear albino N. tabacum, and wild type N. glauca (Evans et al., 1980).

Where the protoplast systems naturally undergo wall regeneration and cell division under the chosen culture conditions, it has been suggested that differences in the sensitivity of the fusion partners to amino acid analogues, growth substances or other drugs might be employed in selection schemes (Cocking et al., 1974). The inhibition of division in cultured Petunia hybrida protoplasts by actinomycin D at a concentration which does not affect the growth of P. parodii

protoplasts has enabled the recovery of somatic hybrids between these two species (Power et al., 1977). However, a naturally occurring differential sensitivity to such compounds cannot always be detected, and a number of alternative treatments have been developed which specifically inhibit division in one or both of the fusion partners. For example, Petunia hybrida protoplasts treated with iodoacetate, and Solanum nigrum protoplasts treated with diethyl pyrocarbonate have been fused, and a somatic hybrid cell line recovered. The fusion partners were incapable of division (Nehls, 1978). Similarly iodoacetate treated protoplasts of a streptomycin resistant N. tabacum mutant have been fused with N. sylvestris protoplasts, and somatic hybrids recovered on the basis of sustained cell division, and resistance to streptomycin (Medgyesy et al., 1980).

In addition to chemical treatments which prevent subsequent cell division, x or γ irradiation has also been used at a level which causes complete inhibition of cell division. Somatic hybrids have been recovered between iodoacetate treated N. tabacum protoplasts, and x irradiated N. plumbaginifolia protoplasts. The inactivation of cell division in a wild type fusion partner by irradiation has been used in combination with albino, or auxotrophic mutant systems. Thus somatic hybrids have been recovered following protoplast fusion between a nuclear albino Daucus carota, and x irradiated Petroselinum hortense, (Dudits et al., 1980). Similarly somatic hybrids have been recovered following fusion between nitrate reductase deficient N. tabacum protoplasts, and x irradiated Datura innoxia, and also x irradiated Physalis minima protoplasts. In this case selection was based on nitrate reductase proficiency and sustained cell division (Gupta et al., 1983). Using the same selection scheme, somatic hybrids have also been recovered between a nitrate reductase deficient N. tabacum and wild type N. glutinosa following irradiation of the

N. glutinosa protoplasts prior to fusion (Cooper-Bland et al., 1985b). Irradiation may cause fragmentation of the chromosomes resulting in the loss of some of the genetic material from the irradiated fusion partner (Itoh and Futsuhara, 1983). Strong selection for cytoplasmically encoded characters of the irradiated protoplast has enabled the recovery of cybrids which do not possess any of the nuclear encoded characteristics of the irradiated fusion partner (Fluhr et al., 1984). The use of irradiation as a means of preventing cell division of one of the fusion partners in attempts at producing a balanced somatic hybrid possessing the amphiploid chromosome number, would therefore appear to be unsuitable.

The lack of division observed in cultured tetrad protoplasts in this study, and reported previously would not therefore exclude such protoplasts from use in attempts at somatic hybridisation. It is clear from the preceding discussion that non-dividing protoplasts can form viable heterokaryons which are capable of sustained division. In contrast to the difficulty encountered with recovering haploid N. glutinosa plants via anther culture, from which haploid leaf mesophyll protoplasts could be isolated, tetrads were readily available, and tetrad protoplasts easily liberated. In principle tetrad protoplast isolation should be possible for all fertile flowering plant species. This wide availability clearly makes tetrad protoplasts superior to haploid protoplasts from anther culture derived haploid plants.

Tetrad protoplasts differ from normal somatic protoplasts in a number of ways. Tetrad protoplasts are smaller, and do not possess a vacuole. During meiosis cytoplasmic changes occur which result in the dedifferentiation of the chloroplasts and mitochondria. This may influence the retention or segregation of these organelles during the

subsequent development of heterokaryon derived colonies. Tetrad protoplasts isolated from homozygous species would be genetically identical. However, random segregation and recombination during meiosis in a heterozygous species will result in a genetically heterogeneous population of tetrad protoplasts. A range of phenotypes might therefore be expected among somatic hybrids recovered following fusions involving tetrad protoplasts from heterozygous species.

Somatic hybridisation describes the recovery of novel hybrid plants following fusions between protoplasts isolated from somatic cells. Tetrads are not somatic cells, since they consist of four haploid spores. We have proposed that hybrids generated as a result of fusions between somatic protoplasts and tetrad (gametic) protoplasts should be described as gametosomatic hybrids to indicate their true origin (Pirrie and Power, 1985). This terminology will be used in the following discussion.

Tetrad protoplasts have not previously been used in the recovery of gametosomatic hybrid plants or cell lines. In an attempt to generate novel triploid gametosomatic hybrids between N. tabacum (2n) and N. glutinosa (n), N. glutinosa tetrad protoplasts were chosen for gametosomatic hybridisation experiments.

7.6 GAMETOSOMATIC HYBRIDISATION BETWEEN N. TABACUM (2n) AND
N. GLUTINOSA (n) : PROTOPLAST FUSION AND THE RECOVERY OF
PUTATIVE GAMETOSOMATIC HYBRID PLANTS

Gametosomal hybrids have not been reported previously. This may reflect a lack of research activity into the use of tetrad protoplasts, or a lack of suitable systems with which to fully test the potential of tetrad protoplasts in attempts at gametosomatic hybridisation. Alternatively some biological incompatibility may exist preventing the formation and/or development of heterokaryons following fusions involving tetrad protoplasts.

In the present study fusion experiments were performed between leaf mesophyll protoplasts from nitrate reductase deficient N. tabacum plants, and tetrad protoplasts from N. glutinosa plants. The nitrate reductase deficient mutant was recovered following selection for resistance to chlorate in allodihaploid N. tabacum cell suspensions (Muller and Grafe, 1978). Chlorate, an analogue of nitrate is reduced to chlorite by the nitrate reductase enzyme. Chlorite is toxic to plant cells. The mutant plant used in this study (nia-130) has been well characterised genetically, and is homozygous for two unlinked recessive nuclear mutations, which are structural loci for the nitrate reductase apoprotein. The plants were found to possess the normal allotetraploid somatic chromosome complement of $2n = 4x = 48$, and set seed following self pollination. The growth of the nia-130 mutant plants and isolation and culture of leaf mesophyll protoplasts has been reported in detail (Pental et al., 1982). The yield of nia-130 leaf mesophyll protoplasts is close to that of wild type tobacco plants, and the cultured protoplasts have a high plating efficiency, and grow rapidly in a medium containing an organic nitrogen source. Colonies derived from dividing nia-130 protoplasts have a

high regeneration capacity. In reconstruction experiments designed to mimic the result of somatic hybridisation, it was found to be possible to recover small numbers of protoplast derived colonies of wild type origin following the introduction of a small number of wild type protoplasts, and selection of the colonies on a medium containing nitrate as sole source of nitrogen. In control experiments no revertant colonies were obtained when nia-130 colonies alone were placed on selection medium. The nitrate reductase deficient N. tabacum nia-130 mutant is therefore ideal for use as half selection in somatic hybridisation studies.

Somatic hybrids have been recovered between the nitrate reductase deficient N. tabacum nia-130 mutant and N. glutinosa. (Cooper-Bland et al., 1985a). Selection was based on nitrate reductase proficiency, green colour and regeneration capacity of the heterokaryon derived calli. In the present study the lack of division will further simplify the selection of putative somatic hybrid colonies, since only heterokaryon derived colonies will be capable of sustained growth on a medium containing nitrate as sole nitrogen source.

Somatic hybrids have previously been recovered between N. tabacum and N. glutinosa using a number of different methods. Nagao (1979) observed that calli derived from N. tabacum protoplasts were bright green whereas calli derived from N. glutinosa protoplasts were colourless. The somatic hybrid calli were selected on the basis of their intermediate pale green colour. Using an alternative approach Uchimaya (1982) screened a number of plants regenerated following fusions between N. tabacum and N. glutinosa suspension cell protoplasts, analysing their Fraction 1 protein profiles, and detected a somatic hybrid. Horn et al. (1984) used protoplasts isolated from a 5-methyltryptophan resistant non-regenerable cell suspension of

N. tabacum in fusions with N. glutinosa mesophyll protoplasts, and recovered somatic hybrids based on selection for 5-methyltryptophan resistance, and regeneration potential. The somatic hybrids recovered previously have been found to possess either the chloroplasts of N. glutinosa (Uchimaya, 1982; Horn et al., 1984; Cooper-Bland et al., 1985a) or the chloroplasts of N. tabacum (Cooper-Bland et al., 1985a), suggesting that no incompatibilities exist which exclude either nuclear cytoplasmic combination between these two species.

Protoplast fusion experiments were performed between N. tabacum nia-130 leaf mesophyll protoplasts, and N. glutinosa tetrad protoplasts using a modified high pH/Ca²⁺ fusion protocol (Keller and Melchers, 1973) adopted following the observations of Deka et al., (1977). Mesophyll protoplasts have been reported to be sensitive to fusion treatments (Kao and Michayluk, 1974) and the survival of only heterokaryons, and protoplasts from cell suspension cells has been reported following fusions between leaf mesophyll protoplasts, and cell suspension protoplasts (Hamill, 1983). A balance must exist between the severity of the fusion treatment employed, which may increase the number of heterokaryons, but reduce the subsequent post fusion viability of the heterokaryons (Keller and Melchers, 1973; Wallin et al., 1974; Kao and Michayluk, 1974; Ward et al., 1979). It has recently been proposed that higher fusion frequencies could be obtained if naturally fusogenic protoplasts were used in fusion experiments (Boss et al., 1984). Tetrad protoplasts have been reported to undergo spontaneous fusion (Bhojwani and Cocking, 1972; Ito and Maeda, 1973) and may well be naturally fusogenic. However, following fusion between N. tabacum nia-130 leaf mesophyll protoplasts and N. glutinosa tetrad protoplasts heterokaryons could not be unequivocally identified by visual means. Previously heterokaryons

have been detected by the merging of the chloroplasts of the mesophyll protoplast component, with the chloroplast free, dense cytoplasm of the cell suspension protoplast component in heterokaryons (Kao and Michayluk, 1974; Cocking et al., 1977). Heterokaryon detection has been aided by labelling the suspension cell cytoplasm with non toxic fluorescence agents such as fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RITC) (Galbraith and Mauch, 1980). Fluorescein and chlorophyll B share the same wavelength of excitation, but fluorescein emits green light, and chlorophyll B red light. Thus heterokaryons between mesophyll protoplasts and cell suspension protoplasts labelled with FITC fluoresce both red and green under appropriate U.V. excitation, facilitating their identification (Patnaik et al., 1982). In the present study, neither the merging of the mesophyll and tetrad protoplast cytoplasm, nor labelling the tetrad protoplast cytoplasm with FITC permitted the unequivocal identification of heterokaryons, possibly due to the relatively small volume of the tetrad protoplasts. For this reason, fusion frequencies could not be directly assessed. However, because of the efficiency of the selection procedure employed for the recovery of putative gametosomatic hybrid colonies, this was not necessary. Assuming that no biological incompatibilities exist preventing their further development, heterokaryons formed as a result of fusion between the mesophyll and tetrad protoplasts should give rise to colonies which would be recovered following selection of nitrate reductase proficient colonies.

Following selection, two putative gametosomatic hybrid colonies were recovered, one of which gave rise to six shoots, five of which were successfully transferred to the greenhouse. The five gametosomatic hybrids were therefore the result of the same hybrid event.

7.7 GAMETOSOMATIC HYBRIDISATION BETWEEN N. TABACUM (2n) AND
N. GLUTINOSA (n) : MORPHOLOGICAL, CYTOLOGICAL AND BIOCHEMICAL
CHARACTERISATION OF THE FIVE PUTATIVE GAMETOSOMATIC HYBRID
PLANTS

The five putative gametosomatic hybrid plants were grown to maturity in the greenhouse, together with N. tabacum cv. Gatersleben, haploid and diploid N. glutinosa and the sexual hybrid between N. tabacum (♀) and N. glutinosa (♂). N. tabacum cv. Gatersleben was used throughout the comparative studies since this represents the wild type of the nia-130 mutant.

The five putative gametosomatic hybrids were found to possess morphological features present in both fusion partners. The intermediate leaf and floral morphology, and flower colour were similar to that found in the sexual hybrid, and previously reported somatic hybrids between N. tabacum and N. glutinosa (Nagao, 1979; Uchimaya, 1982; Horn et al., 1984; Cooper-Bland et al., 1985a).

The somatic chromosome complement of the five gametosomatic hybrids was found to be the expected pentaploid, but functionally triploid complement of $3n = 5x = 60$, which is the summation of the N. tabacum chromosome complement of $2n = 4x = 48$ and the haploid N. glutinosa chromosome complement of $n = x = 12$. Considerable variation in the chromosome complement of somatic hybrids between N. tabacum and N. glutinosa has been reported. Nagao (1979) found five somatic hybrids which possessed the expected amphiploid complement of 72, whereas the remaining 28 somatic hybrids were aneuploids, possessing 50-88 chromosomes. The single somatic hybrid reported by Uchimaya (1982) also possessed 72 chromosomes. The four somatic hybrids reported by Horn et al. (1984) were found to be aneuploids ranging between 30 and 60 chromosomes.

Variations from the expected amphiploid somatic chromosome complement resulting from the addition of the chromosome complements of the two fusion partners can occur for a variety of different reasons. Variations may arise prior to fusion treatments if cultured cells are used as a source of protoplasts. Most long-term cell cultures contain a mixture of diploid, polyploid and aneuploid cells (Balyss, 1980). The aneuploid somatic hybrids between N. tabacum and N. glutinosa reported by Horn et al. (1984) resulted from fusions in which the N. tabacum protoplasts were isolated from a long term suspension culture. This culture was found to be aneuploid with a mean chromosome number of 47 and standard deviation of 7.9 (based on 25 chromosome counts). It is likely that the use of protoplasts isolated from this culture contributed to the variation in chromosome numbers observed in the resulting somatic hybrids. However, in the present study the protoplasts used in fusions resulting in the production of the gametosomatic hybrids were isolated directly from the plant, and were not therefore subject to any tissue culture effect prior to fusion. Multiple fusions may occur and these would result in a higher than expected chromosome complement. Following fusion variation may arise during the tissue culture phase prior to the regeneration of the somatic or gametosomatic hybrid plants, in much the same way as variation occurs in other cell cultures. During the tissue culture phase chromosome elimination may occur. Such eliminations may occur at random, or may be unidirectional. Unidirectional chromosome elimination has been reported for somatic hybrids between Datura innoxia and Atropa belladonna, which only possess a few chromosomes from Atropa belladonna, but a full complement of Datura innoxia chromosomes (Krumbiegel and Schieder, 1981). Similarly, the gradual loss of N. glauca chromosomes from somatic hybrid cell lines between Glycine max and N. glauca (Kao, 1977; Wetter and Kao, 1980)

and also of N. tabacum chromosomes from somatic hybrid cell lines between Glycine max and N. tabacum (Chien et al., 1982) has been reported. Leaf mesophyll protoplasts are usually in a pre-replicative stage (G1 or G0) of the cell cycle (Galbraith et al., 1981) and protoplasts isolated from actively growing cell suspensions may be at any stage of the cell cycle. It has been reported that fusions between protoplasts at different stages of the cell cycle can result in premature chromosome condensation, breakage and ultimately elimination (Szabados and Dudits, 1980). Again, this may have contributed to the variation observed in the somatic hybrids between N. tabacum cell suspension protoplasts and N. glutinosa mesophyll protoplasts reported by Horn et al. (1983). However, in the present study tetrad protoplasts also in a pre-replicative stage of the cell cycle (G0 or G1) (Sunderland, 1974) were fused with leaf mesophyll protoplasts, and therefore chromosome elimination, for this reason, would not be expected. Unidirectional chromosome elimination may occur as a result of inherent biological incompatibilities between the fusion partners. Chromosome instability and unidirectional elimination is known to occur in certain interspecific crosses between *Nicotiana* species (Gengedeví et al., 1982) and between Hordeum bulbosum and H. vulgaris discussed earlier. In the combination of N. tabacum and N. glutinosa previous reports of somatic hybrids which possess the expected amphiploid chromosome complement of 72, and also of stable sexual hybrids suggest that inherent biological incompatibilities causing chromosome instability and loss are not present in this species combination. The recovery of amphipentaploid, but functionally triploid gametosomatic hybrids in this study may well be due therefore to a combination of factors. Inherent biological incompatibilities do not exclude the recovery of stable amphiploid somatic, gametosomatic and sexual hybrids in this species combination, and the use of protoplasts

directly isolated from the plant, and being in the same stage of the cell cycle may have contributed to the recovery of the amphipentaploid gametosomatic hybrids. However, since the five plants originate from the same hybrid event, further studies would need to be performed to see to what extent these factors would result in the production of stable amphiploids among a population of gametosomatic hybrids.

Although the gametosomatic hybrids possessed an apparently intermediate morphology between that of their fusion partners, and had the expected chromosome complement resulting from the summation of the allotetraploid N. tabacum and haploid N. glutinosa chromosome complements, the N. tabacum and N. glutinosa chromosomes were indistinguishable from each other, and so further evidence of hybridity was sought.

Although the gametosomatic hybrids were selected on a medium containing nitrate as sole nitrogen source, and that leaf callus from the five gametosomatic hybrids was capable of growing on the same medium, the nitrate reductase enzyme activity of the gametosomatic hybrids was also measured. The gametosomatic hybrids were found to possess a nitrate reductase enzyme activity close to that of diploid N. glutinosa callus, despite possessing a single nitrate reductase enzyme gene. This would be expected, since the level of nitrate reductase activity has previously been found to be independent of the number of genes present, indicating complete compensation for gene dosage effects by regulatory mechanisms (Muller, 1983).

The hybrid nature of cell lines and plants has frequently been confirmed by isoenzyme analysis. Isoenzymes are multiple molecular forms of an enzyme which exhibit similar substrate specificities (Scandalios, 1969). This does not, however, imply that the enzymes share the same physiological function (Smith et al., 1970). Species

specific banding patterns are often present following electrophoretic separation of the isoenzymes in polyacrylamide or starch gels, and visualisation of the position of the functional isoenzymes by appropriate staining techniques. Somatic hybrids may be represented by the summation of the parental banding patterns (Carlson et al., 1972; Power et al., 1976), or as a summation of the parental banding patterns with addition of novel hybrid bands not present in either parent (Maliga et al., 1978; Gleba and Hoffman, 1978, 1979). Chromosome loss may be indicated by the loss of specific isoenzyme bands.

Leaf esterase and leaf callus peroxidase isoenzyme patterns visualised after isoelectric focusing of soluble protein extracts, displayed both N. tabacum and N. glutinosa specific bands. The putative gametosomatic hybrids were found to possess a banding pattern which was the summation of the parental banding patterns, confirming the hybridity of the gametosomatic hybrids. A similar hybrid isoenzyme pattern was found for leaf esterases of a sexual hybrid between N. tabacum (♀) and N. glutinosa (♂). No difference was found between the leaf esterase zymogram of haploid and diploid N. glutinosa plants, as would be expected for a homozygous inbred line.

Fraction 1 protein (ribulose biphosphate carboxylase/oxygenase E.C. 4 11 39) is the major soluble protein in the leaves of higher plants, and has a molecular weight of approximately 520,000 (Kawashima and Wildman, 1970). The protein is composed of a large subunit, encoded by the chloroplast genome, maternally inherited in the genus *Nicotiana* (Chan and Wildman, 1972), and a small subunit coded for by nuclear genes, and inherited in a mendelian fusion (Kawashima and Wildman, 1972). The polypeptide composition of the subunits of purified carboxymethylated Fraction 1 protein has been determined by isoelectric focusing in 8 M urea (Kung et al., 1974). The large sub-

unit characteristically possesses three polypeptides which have their isoelectric focusing point in the alkaline region of the gel. The position of the polypeptides is frequently obscured by artifact bands which are largely due to oxidation of thiol groups during dissociation of the protein in 8 M urea, and also to cyanate (derived from urea) interacting with amino acid side chains of the polypeptides. Gels are normally interpreted by locating the most densely stained band and regarding it, and the bands immediately above and below as being the characteristic pattern of the chloroplast encoded large subunit polypeptides (Kung et al., 1974; Kumar et al., 1981; Li et al., 1983). The small subunit may possess a number of polypeptides which have their isoelectric focusing points in the acid region of the gel, and is not subject to as many artifact bands as found with the large subunit polypeptides. Species specific differences in the location of the large and small subunit polypeptides makes the analysis of the Fraction 1 protein subunit polypeptides useful in determining the nuclear and cytoplasmic composition of putative somatic and gameto-somatic hybrids.

Analysis of Fraction 1 protein isolated from somatic hybrids between N. glauca and N. langsdorffii (Carlson et al., 1972) indicated the presence of the small subunit polypeptides (nuclear encoded) from both species, and large subunit polypeptides (chloroplast encoded) from N. langsdorffii (Kung et al., 1975). The segregation of chloroplast types indicated by Fraction 1 protein analysis has been reported for a number of somatic hybrid combinations, including Petunia parodii and P. parviflora (Power et al., 1980), N. tabacum and N. rustica (Douglas et al., 1980; Hamill et al., 1984) and N. tabacum and N. glutinosa (Uchimaya, 1982; Cooper-Bland et al., 1985a). Fraction 1 protein analysis relies on gene expression, and may not

detect a mixed chloroplast condition if one chloroplast type only represents 10-15% of the total population(Fluhr, 1983). Chloroplast DNA restriction analysis provides a muchmore precise tool for the analysis of cytoplasmic inheritance following somatic hybridisation. Chloroplast DNA restriction analysis has confirmed the segregation of chloroplast types in somatic hybrids, indicated by Fraction 1 protein analysis in a number of combinations, including Petunia parodii and P. parviflora (Kumar et al., 1981, 1982), and N. tabacum and N. glutinosa (Uchimaya, 1982; Uchimaya et al., 1984).

Somatic hybrids recovered between N. tabacum and N. glutinosa have been found to possess either the N. tabacum chloroplast type (Cooper-Bland et al., 1985a) or the N. glutinosa chloroplast type (Uchimaya 1982; Horn et al., 1983; Cooper-Bland et al., 1985a). The results of Fraction 1 protein analysis indicate that the five gametosomatic hybrids recovered in this study possess the chloroplasts of N. tabacum. Many factors are thought to influence the sorting out of chloroplast types following protoplast fusion, including the physiological status of the donor protoplasts, the relative contribution from each protoplast type, as well as inherent biological incompatibilities which may favour the retention of one chloroplast type (Fluhr, 1983). Since somatic hybrids between N. tabacum and N. glutinosa have been recovered which contain either chloroplast type, biological incompatibilities would not appear to limit the nuclear cytoplasmic combinations that can result from somatic hybridisation. Cytoplasmic changes occurring during meiosis result in the dedifferentiation of both mitochondria and chloroplasts in the pollen mother cells (Heslop-Harrison, 1977). It might have been predicted that this would favour the retention of the chloroplasts donated from the tetrad protoplast in the gametosomatic hybrids re-

ported in this study. However, this was not the case. Tetrad protoplasts are considerably smaller in diameter than N. tabacum mesophyll protoplasts. Based on electron microscopical studies of sections of plant cells and protoplasts, and on measurements of tetrad protoplasts, it would appear that the tetrad protoplast contains only $\frac{1}{4}$ - $\frac{1}{2}$ the volume of cytoplasm of the mesophyll protoplast (Ms. A. Gowland, Pers. Comm.). The relatively smaller cytoplasmic contribution from the tetrad protoplast may therefore influence the subsequent pattern of chloroplast segregation. However, no data is available on the relative number of proplastids present in the cytoplasm of the tetrad protoplast, and this may also be an important factor when considering the subsequent chloroplast segregation pattern. It should be emphasised that the five gametosomatic hybrids were derived from a single fusion event, and it remains to be seen what pattern of chloroplast segregation will be obtained in a large population of gametosomatic hybrids.

The five gametosomatic hybrids were found to possess the small subunits (nuclear encoded) of both N. tabacum and N. glutinosa, consistent with their hybrid nature.

Based on the morphological, cytological and biochemical analysis the five plants recovered following fusions between N. tabacum nia-130 leaf mesophyll protoplasts (2n) and N. glutinosa tetrad protoplasts (n) were confirmed to be gametosomatic hybrids.

7.8 GAMETOSOMATIC HYBRIDISATION BETWEEN N. TABACUM (2n) AND
N. GLUTINOSA (n) : ANALYSIS OF THE FERTILITY AND PROGENY OF
THE GAMETOSOMATIC HYBRID PLANTS

N. tabacum and N. glutinosa are sexually compatible species, although some difficulty may be encountered in sexual hybridisation with particular N. tabacum cultivars. The diploid F1 ($2n = 3x = 36$) is completely sterile, and fertility is restored only upon chromosome doubling to give the amphiploid $4n = 6x = 72$ (Clausen and Goodspeed, 1925). By backcrossing the amphiploid sexual hybrid ($4n = 6x = 72$) between N. tabacum and N. glutinosa to N. tabacum, it is possible to generate allopolyploid but functionally triploid plants which possess a chromosome complement corresponding to that of the gametosomatic hybrids. The fertility of the sexually produced allopolyploid (but functionally triploid) hybrids has been studied. At meiosis normal pairing of the 24 pairs of N. tabacum chromosomes occurred. The 12 N. glutinosa chromosomes did not appear to associate with the N. tabacum chromosomes although limited trivalent formation may have occurred. In subsequent backcrosses to N. tabacum the N. glutinosa chromosome complement was rapidly eliminated (Clausen and Cameron, 1957).

It has recently been proposed that a similar situation may arise at meiosis in a triploid somatic or gametosomatic hybrid between diploid protoplasts of a crop species, and haploid protoplasts of a wild type species (see General Introduction, and Pental and Cocking, 1985). The random segregation of the unpaired chromosomes from the haploid set would eventually result in their complete elimination following backcrosses to the diploid fusion partner. The resulting progeny would need to be assessed for the introgression of characteristics from the wild type (haploid) chromosome set.

As a first step towards testing this, allopolyploid (but functionally triploid) gametosomatic hybrids were generated between N. tabacum (2n) and N. glutinosa (n). For such gametosomatic hybrids to be of value in studies designed to examine the introgression of characteristics from the eliminated haploid chromosome set, the gametosomatic hybrids must be fertile.

During meiosis in the pollen mother cells of the gametosomatic hybrids, chromosomes not associated with the newly forming haploid nuclei were observed at telophase II. However, viable pollen grains were formed, and these were found to be slightly larger than those of either fusion partner, which may be significant since it has been observed that the pollen size of closely related species correlates directly to the nuclear DNA content. The gametosomatic hybrids set seed in reciprocal crosses with N. tabacum cv. Gatersleben. Seed set and viability was slightly lower than that of either fusion partner, but was still sufficient to generate many hundreds of viable seeds.

Tentoxin sensitivity tests were performed on germinating seed obtained following crosses between the gametosomatic hybrids (♀) and N. Tabacum (♂). All the progeny were found to be insensitive to tentoxin. Tentoxin is a cyclic tetrapeptide [cyclo - (L leucyl - N - methyl - (Z) - dehydro - phenyl - alanyl - glycyl - N - methyl - L - alanyl -)] produced by the fungus Alternaria tenuis. It results in chlorosis in many plant species due to a disruption of chloroplast development (Schadler et al., 1976). Some members of the Nicotiana, including N. tabacum are insensitive to tentoxin, whereas others, including N. glutinosa are sensitive to tentoxin (Durbin and Uchytel, 1977). Tentoxin sensitivity is therefore a useful indication of the chloroplast type present in the progeny of somatic or gametosomatic hybrids between tentoxin sensitive and insensitive species (Aviv et

al., 1980; Flick and Evans, 1982). The insensitivity to tentoxin of seedlings recovered from the crosses between the gametosomatic hybrids (♀) and N. tabacum (♂) confirmed the earlier results of Fraction 1 protein analysis, which indicated that the gametosomatic hybrids possess the N. tabacum chloroplast type.

A sample of 50 plants derived from backcrosses between two of the gametosomatic hybrids (♀) and N. tabacum were grown to maturity, and the morphology of these plants was examined. The height, leaf and flower shape and flower colour varied from being similar to that of the original gametosomatic hybrids, to being more similar to N. tabacum. Since N. tabacum and N. glutinosa chromosomes could not be visually distinguished from each other, cytological analysis of these plants was not performed. However, analysis of the leaf esterase zymogram clearly indicated the loss of N. glutinosa specific isoenzyme bands in some of the gametosomatic hybrid backcross progeny. No N. tabacum specific bands were lost. This evidence combined with the morphological study, and the observed chromosome behaviour at meiosis suggests the loss of N. glutinosa chromosomes in the first backcross progeny of the gametosomatic hybrids. Further backcrosses would be necessary to completely eliminate the remaining N. glutinosa chromosomes.

Morphological variation has previously been reported among the progeny of a somatic hybrid between N. tabacum and N. glutinosa backcrossed twice to N. glutinosa. The chromosome number was found to vary between 34 and 38, and the morphological variation was attributed to the plants possessing a variable number of N. tabacum chromosomes. Elimination of N. glutinosa specific nuclear markers was also observed after backcrossing the same somatic hybrid to N. tabacum (Uchimaya et al., 1984).

Similar findings have been reported following self-pollination of allotriploid plants generated between wheat and rye (O'Mara, 1940). The allotriploid plants had a complete wheat diploid chromosome set, but only a haploid chromosome set from rye. The progeny were found to contain a variable number of rye chromosomes. After several generations of self pollination a series of alien monosomic addition lines were created, which differed from each other for many morphological features. Unidirectional chromosome elimination and the creation of alien addition lines may be useful in studies designed to assign characteristics to specific chromosomes. In human cell biology, specific chromosome elimination in human and mouse somatic hybrid cell lines has proved invaluable for mapping human chromosomes (Ruddle, 1972; Goss, 1978).

Monosomic addition lines have also proved useful in plant improvement. Rust resistance was incorporated into wheat following irradiation of an alien monosomic addition line of wheat, containing a single Aegilops umbellata chromosome carrying the rust resistance factor (Sears, 1956). Similar transfers have been made from Agropyron elongatum to wheat (Knott, 1961; Sharma and Knott, 1966), from Agropyron intermedium to wheat (Wienhues, 1966), and from rye into wheat (Driscoll and Jenson, 1964).

In conclusion the fertile gametosomatic hybrids between N. tabacum (2n) and N. glutinosa (n) would appear to be ideal starting material for the development of monosomic addition lines, as well as for studies which necessitate the complete elimination of all the chromosomes of the haploid fusion partner.

7.9 POSSIBLE LIMITATIONS TO THE APPLICATION OF SOMATIC AND GAMETOSOMATIC HYBRIDISATION TO PLANT BREEDING

In the present study gametosomatic hybrids were recovered between N. tabacum and N. glutinosa, two sexually compatible species. This species combination was chosen as a model system, with which to resolve the use of tetrad protoplasts in cell fusion and ultimately gametosomatic hybridisation. Somatic hybrids have previously been recovered between these species, using a similar selection scheme. Failure to recover gametosomatic hybrids, in this study, would therefore have been almost certainly due to the use of tetrad protoplasts, instead of somatic cell protoplasts, and not the parent species.

The demonstration that somatic hybrid plants can be recovered between sexually incompatible species (Melchers et al., 1978) led to speculation that somatic hybridisation would significantly contribute to traditional plant breeding techniques by permitting a free exchange of genetic information between crop species, unhindered by sexual incompatibility considerations. However, this has so far failed to be achieved.

For somatic hybridisation to be attempted, at least one of the fusion partners must be capable of efficient and reproducible plant regeneration from protoplasts. This requirement has only been met for a limited number of crop species, and their wild relatives, and currently this may significantly limit the potential application of somatic and gametosomatic hybridisation.

Heterokaryons can be formed by protoplast fusion between widely divergent species. For example, heterokaryon formation and nuclear fusion has been observed between Daucus carota and Hordeum vulgare (Dudits et al., 1976). Despite this, and reports of heterokaryon derived cell division following protoplast fusion between widely

divergent species, few interspecific somatic hybrids between sexually incompatible species have been reported. This apparent discrepancy has led to speculation that mechanisms may act during the development of the somatic hybrid cell line to prevent the recovery of some somatic hybrid plants. Such somatic incompatibility mechanisms (Harms, 1983b) could operate at a number of levels. Somatic and gametosomatic hybrids are likely to be subject to similar somatic incompatibility mechanisms, which may be active processes requiring intracellular recognition mechanisms, or passive processes resulting from basic differences in the physiology of the fusion partners.

Constabel et al. (1980) have reported the precipitation of granular material in fused cell sap vacuoles within heterokaryons, which may affect the viability of these heterokaryons. Following nuclear fusion, which has been observed to occur prior to mitosis in plant heterokaryons, there must be a synchronisation between the cell cycles of the two contributing chromosome sets. As already discussed, premature condensation may occur when nuclei at different stages of the cell cycle are brought together in heterokaryons. This may lead to chromosome fragmentation and elimination (Szabados and Dudits, 1980).

Chromosome instability has been reported in vertebrate somatic hybrid cell lines. In fusion products between closely related rodent species, chromosome elimination occurs in an apparently random fusion. However, human-rodent cell lines tend to preferentially lose human chromosomes, and this has been extensively exploited in studies to assign human characters to specific chromosomes (Ruddle, 1972; Goss, 1978). The mechanism for chromosome elimination is not fully understood, but would appear to reflect a somatic incompatibility mechanism (Harms, 1983b).

Similarly, in plants, chromosome eliminations possibly the

result of somatic incompatibility are known to occur. Interspecific hybrids between Hordeum vulgare and H. bulbosum exhibit a preferential loss of the H. bulbosum chromosome complement (Symko, 1969; Kasha and Kao, 1970); a similar situation occurring in some interspecific Nicotiana crosses (Gengeverdi et al., 1982). The reported aneuploid chromosome complement of many somatic hybrids may also be the result of somatic incompatibility. However, a number of other factors could contribute to the observed variations from the expected additive chromosome number. As already discussed, the use of protoplasts isolated from long-term cell cultures known to contain diploid, polyploid and aneuploid cells (Bayliss, 1980), may contribute to the observed variation in chromosome numbers of somatic hybrids. In addition, variation could arise during the tissue culture phase of the developing somatic hybrid.

Chromosome elimination, whether due to somatic incompatibility or not, may well be advantageous. Many of the characters which a given crop plant or wild type species might possess will be undesirable, and may be lost due to chromosome elimination. The ability to direct chromosome elimination by irradiating one of the fusion partners (Harms, 1983a) may be useful in generating asymmetric or partial hybrids, which would need to be assessed to determine exactly what contribution the irradiated fusion partner had made to the hybrid plant (Pental and Cocking, 1985).

Irradiation has also been used to overcome somatic incompatibility Gupta et al. (1984) found that somatic hybridisation between Datura innoxia and irradiated Physalis minima was successful whereas sexual hybridisation, and somatic hybridisation without irradiating either fusion partner failed. Chromosome elimination may also be necessary before plant regeneration can occur in some distant interspecific somatic hybrids. The somatic hybrid cell line between N. tabacum and

Petunia hybrida only underwent shoot regeneration after the progressive loss of N. tabacum specific peroxidase isoenzyme bands. The regenerated shoots also lacked N. tabacum genes for ribosomal RNA and the small subunit of Fraction 1 protein, indicating extensive unilateral chromosome elimination (Pental et al., 1985).

The apparent chromosome restructuring observed in tissue culture cells (Murata and Orton, 1983) may also prove advantageous in somatic and gametosomatic hybridisation. Interchange between sections of Petunia parodii and P. parviflora chromosomes has been reported in the somatic hybrid between these two species (White and Rees, 1985). In addition to large changes in chromosome structure, less dramatic structural changes may occur (Evans et al., 1984) and such changes may include recombination between the chromosomes of the fusion partners in somatic and gametosomatic hybrid cell lines. By passing an allotriploid hybrid embryo, produced by sexually crossing diploid Lolium perenne with tetraploid L. multiflorum, through a tissue culture phase, and regenerating over 2,000 plants from this material, a wide range of different phenotypes were obtained, and this variation was perpetuated through seed progeny. Some of the variants represented combinations of characteristics which were agronomically important, and had not been obtained in hybrids not passed through such a tissue culture phase (Larkin and Scowcroft, 1981). The tissue culture phase, an integral part of somatic and gametosomatic hybridisation, may therefore facilitate a greater degree of genetic recombination than otherwise might be obtained through conventional sexual hybridisation, even if it were possible.

Fertile amphiploid sexual hybrids between N. tabacum and N. glutinosa possessing $4n = 6x = 72$ chromosomes, can be backcrossed to N. tabacum, generating amphipentaploid (but functionally triploid)

hybrids similar to the gametosomatic hybrids reported in this study. This system may therefore provide an ideal opportunity to compare the introgression of N. glutinosa traits into N. tabacum with and without the intervention of a tissue culture phase.

Somatic incompatibility does not prevent the recovery of amphiploid somatic hybrids in all species combinations; such hybrids have been recovered in the interspecific sexually incompatible combination of Petunia parodii and P. parviflora (Power et al., 1980).

Somatic hybrids between sexually incompatible species have so far proved sterile, and this may reflect a final effect of somatic incompatibility, a failure to coordinate reproductive development in the somatic hybrid plant. The value of protoplast fusion for the improvement of seed propagated species will not be realised until a large population of fertile somatic or gametosomatic hybrid plants possessing agronomically useful traits are integrated into conventional breeding programs and the progeny evaluated under field conditions (Evans et al., 1983). It will not be until a large number of somatic and gametosomatic hybrids are produced and between a large range of sexually compatible and incompatible species that the full effect of somatic incompatibilities can be fully assessed. This in turn will only be possible if progress in regenerating protoplast systems for a wide range of species is forthcoming.

7.10 FUTURE RESEARCH AREAS ARISING FROM THIS PRESENT STUDY

This study is the first to show that tetrad protoplasts can be successfully used in protoplast fusion experiments, and that gameto-somatic hybrid plants be recovered. It proved impossible to assess fusion frequency since heterokaryons could not be visually identified. It would be interesting to develop methods by which heterokaryons between leaf mesophyll and tetrad protoplasts could be visualised, so as to compare the throughput from heterokaryon to somatic and gameto-somatic hybrid plant in this species combination. The ability to closely follow electro-fusion as it occurs may permit the eventual identification of such heterokaryons facilitating such a study.

Tetrad protoplasts differ from normal somatic protoplasts in that during meiosis changes in the cytoplasm have resulted in the de-differentiation of the chloroplasts and mitochondria. In addition the volume of the tetrad cytoplasm is less than that of a typical leaf mesophyll protoplast, and will therefore be a minor component in the heterokaryon. Both considerations may influence the pattern of cytoplasmic inheritance in the resulting gametosomatic hybrid plants. Evidence obtained in this study showed that the chloroplasts of the diploid leaf mesophyll protoplast fusion partner were retained in the gametosomatic hybrids and this may indicate a general trend. However, since all five gametosomatic hybrids were derived from the same fusion event, it would be unwise to draw any conclusion. In the future it would be of interest to assess the distribution of chloroplast types among a large number of gametosomatic hybrids. Such a study will be possible since the recovery of gametosomatic hybrids between N. tabacum and N. glutinosa has proved reproducible following chemical and electro-fusion (R. Sotak, pers. comm.). In addition, mitochondrial DNA restriction analysis may reveal differences in the mitochondrial re-

combination events which occur in somatic hybrids, and may also occur in gametosomatic hybrids.

Tetrad protoplasts have not been shown to undergo sustained division, therefore there is no need to develop counter-selection against the tetrad protoplast parent species. It is therefore possible to immediately attempt gametosomatic hybridisation using any auxotrophic mutant such as the nitrate reductase deficient N. tabacum used in this study and any other species as tetrad protoplast donor. In this way it should be possible to rapidly assess the limitations of gametosomatic hybridisation due to somatic incompatibility.

In this study the morphological and biochemical analysis of the first backcross progeny between the gametosomatic hybrids and N. tabacum suggested incomplete elimination of the N. glutinosa genome. Further backcrosses must be attempted and these would almost certainly result in the complete elimination of the N. glutinosa chromosome set. The resulting plants would need to be assessed cytologically, morphologically and biochemically for the introgression of N. glutinosa characters. In addition self-pollination of the gametosomatic hybrids may result in the recovery of a range of alien monosomic addition lines. Twelve such lines should in theory be recovered, responding to the addition of each of the twelve chromosomes of the haploid N. glutinosa chromosome set. By combining cytological, morphological and biochemical studies on these addition lines it should also be possible to assign N. glutinosa characters to a particular chromosome, in much the same way as has been achieved in human cell genetics.

Even though tetrad protoplasts have not been shown to be capable of sustained division, the nucleus must be totipotent since it contributes to the zygote and results in the development of a full organism. In addition pollen embryogenesis clearly demonstrates the

potential totipotency of the tetrad protoplast. While the observed lack of division in tetrad protoplasts has proved useful in this study, in simplifying the recovery of putative gametosomatic hybrids, it seems likely that conditions will be found in which tetrad protoplasts will undergo sustained division and plant regeneration in their own right. In monocotyledonous species where the totipotency of somatic cells would appear to be severely limited, future research into tetrad protoplast culture might be rewarding.

In conclusion it would appear that the potential advantages of tetrad protoplasts have only just begun to be realised. There is, however, a growing body of opinion of the view that somatic hybridisation of plants can not be extended beyond sexually incompatible species because of insurmountable problems of infertility. It is only by thorough investigation of a large number of species combinations that the limitations of somatic and gametosomatic hybridisation can be assessed, and tetrad protoplasts may provide a means by which such an assessment can be performed.

It therefore remains to be seen to what extent cell fusion will contribute to plant breeding programmes.

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APPENDIX 1

MEDIA FORMULATIONS

1.1.1 Media based on Murashige and Skoog (1962) salts, and modified M.S. salts at reduced strength.

Nutrient	Concentration (mg/L)	
	MS Salts	Modified MS Salts
KNO ₃	1900	950
CaCl ₂ ·2H ₂ O	440	220
MgSO ₄ ·7H ₂ O	370	785
NH ₄ NO ₃	1650	825
KH ₂ PO ₄	170	85
KI	0.83	0.83
CoCl ₂ ·6H ₂ O	0.025	0.025
H ₃ BO ₃	6.2	0.62
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.25
Mn SO ₄ ·4H ₂ O	22.3	22.3
Cu SO ₄ ·5H ₂ O	0.025	0.025
Zn SO ₄ ·5H ₂ O	8.6	8.6
Fe SO ₄ ·7H ₂ O	27.85	27.85
Na EDTA	37.25	37.25
Glycine	2.0	2.0
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Thiamine HCl	0.1	5.0
Myo - inositol	100.0	100.0
Sucrose	30000	30000

The components of full strength M.S. salts (without sucrose) can be obtained commercially in dried packet form (Flow Labs. Ltd.).

1.1.2 Additions to media based on full strength M.S. salts.

<u>Medium Code</u>	<u>Additional Components *</u>	
MS-0	-	
MS-P1	2.0	NAA
	0.5	BAP
MS-P19M	2.0	NAA
	0.5	BAP
	9.0% w/v	Mannitol
MS-P2	0.1	NAA
MS-D3	2.0	IAA
	1.0	BAP
MS-D4	0.05	NAA
	0.5	BAP
MS-Z	1.0	Zeatin
UM	2.0	2,4-D
	0.25	Kinetin
	9.9	Thiamine HCl
	9.5	Pyridoxine HCl
	4.5	Nicotinic acid
	2.0 g/L	Casein hydrolysate

* mg/L unless otherwise indicated.

For all solid media 0.8% (w/v) agar (Sigma type IV) was melted into the medium. All media were adjusted to pH 5.8.

1.1.3 Additions to media based on modified M.S. salts at reduced strength.

<u>Medium Code</u>	<u>Additional Components *</u>	
MS-A1	2.0	NAA
	1.0	BAP
	75.0	Adenine Sulphate
MS-A19M	2.0	NAA
	1.0	BAP
	75.0	Adenine Sulphate
	9.0% (w/v)	Mannitol)
MS-A2	2.0	NAA
	1.0	BAP
MS-B	0.2	NAA
	0.1	BAP
MS-C	2.0	NAA
	0.1	BAP
MS-D	0.2	NAA
	1.0	BAP
MS-E	0.5	NAA
	2.5	BAP
MS-F	0.1	NAA
	2.5	BAP
MS-R1		-
MS-R2	0.1	NAA

* mg/L unless otherwise stated.

1.2.1 Media based on a modified Whites medium (MW) (White, 1954).

Nutrient	Concentration (mg/L)
Ca(NO ₃) ₂ ·4H ₂ O	208.5
MgSO ₄ ·7H ₂ O	720
Na ₂ SO ₄	200
KNO ₃	80
Cu SO ₄ ·5H ₂ O	0.001
Fe Na EDTA	4.59
H ₃ Bo ₃	1.5
KCl	65.0
KI	0.75
Mn SO ₄ ·4H ₂ O	7.0
Mo O ₃	0.0001
Na H ₂ PO ₄ ·2H ₂ O	18.7
Zn SO ₄ ·7H ₂ O	3.0
Nicotinic acid	0.5
Thiamine HCl	0.1
Pyridoxine HCl	0.1
Glycine	3.0
Sucrose	30,000

1.2.2 Additions to media based on modified Whites medium.

<u>Medium Code</u>	<u>Additional Component (mg/L)</u>
MW-A2	2.0 NAA
	1.0 BAP
MW-D	0.2 NAA
	1.0 BAP

1.3.1 Media based on Gamborgs B5 medium (Gamborg et al., 1968).

Nutrient	Concentration (mg/L)
KNO ₃	2,500
CaCl ₂ .2H ₂ O	150
Mg SO ₄ .7H ₂ O	250
(NH ₄) ₂ SO ₄	134
Na H ₂ PO ₄ .H ₂ O	150
KI	0.75
H ₃ BO ₃	3.0
Mn SO ₄	10.0
Zn SO ₄ .7H ₂ O	2.0
Na ₂ MoO ₄ .2H ₂ O	0.25
Cu SO ₄ .5H ₂ O	0.025
CoCl ₂ .6H ₂ O	0.025
Na ₂ EDTA	37.3
Fe SO ₄ .7H ₂ O	27.8
Inositol	100
Pyridoxin HCl	1.0
Thiamine HCl	10.0
Nicotinic acid	1.0
Sucrose	30,000

1.3.2 Additions to media based on Gamborgs B5 medium.

<u>Medium Code</u>	<u>Additional Component (mg/L)</u>
B5-A2	2.0 NAA
	1.0 BAP
B5-D	0.2 NAA
	1.0 BAP

1.4.1 Media based on Nitsch's medium N69 from Nitzsche and Wenzel (1978).

Nutrient	Concentration (mg/L)
KNO ₃	1,000
KH ₂ PO ₄	300
NH ₄ NO ₃	1,000
Ca(NO ₃) ₂ .4H ₂ O	499
KCl	65
Mg SO ₄ .7H ₂ O	71.6
Mn SO ₄ 4H ₂ O	6.5
Zn SO ₄ .7H ₂ O	2.75
Na Fe EDTA	32
KI	0.83
H ₃ BO ₃	1.6
Glycine	2.0
Thiamine HCl	0.1
Pyridoxine HCl	0.1
Nicotinic acid	0.5
Sucrose	30,000

1.4.2 Additions to media based on Nitsch's N69 medium.

<u>Medium Code</u>	<u>Additional Component (mg/L)</u>	
N69-P1	2.0	NAA
	0.5	BAP
N69-D3	2.0	IAA
	0.5	BAP
N69-A2	2.0	NAA
	1.0	BAP
N69-D	0.2	NAA
	1.0	BAP

1.5.1 Media based on that of Leowenberg (1969).

Nutrient	Concentration (mg/L)
Ca(NO ₃) ₂ ·4H ₂ O	216
NH ₄ NO ₃	600
KNO ₃	120
Mg SO ₄ ·7H ₂ O	72
KH ₂ PO ₄	38
KCl	65
KI	0.75
Mn SO ₄ ·4H ₂ O	6.5
Zn SO ₄ ·7H ₂ O	2.7
H ₃ BO ₃	1.6
(NH ₄) ₁₀ Mo ₇ O ₂₄ ·4H ₂ O	0.006
Na Fe EDTA	25
L-asparagine	66
L-glutamic acid	73.5
Myo inositol	100
Calcium pantothenate	0.5
Choline chloride	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.2
Nicotinic acid	0.5

1.5.2 Additions to media based on that of Leowenberg (1969).

<u>Medium Code</u>	<u>Additional Component (mg/L)</u>
L-AZ	2.0 NAA
	1.0 BAP
L-D	0.2 NAA
	1.0 BAP

1.6.1 Media based on that of Kao and Michayluk (1975).

Nutrient	Concentration (mg/L)
NH ₄ NO ₃	600
KNO ₃	1,900
Ca Cl ₂ .2H ₂ O	600
Mg SO ₄ .7H ₂ O	300
KH ₂ PO ₄	170
KCl	300
Sequestrene	28
KI	0.75
H ₃ BO ₃	3.0
Mn SO ₄ .H ₂ O	10.0
Zn SO ₄ .7H ₂ O	2.0
Ma Mo O ₄ .5H ₂ O	0.25
Co Cl ₂ 6H ₂ O	0.025
Cu SO ₄ .5H ₂ O	0.025

1.6.2 Supplement to KM-8P, and K-P8.

Component	Concentration (mg/L)	
	K-P8	KM-8P
Inositol	100	100
Nicotinamide	1.0	1.0
Pyrodixine HCl	1.0	1.0
Thiamine HCl	1.0	1.0
D-Ca Pantothenate	0.5	1.0
Folic acid	0.2	0.4
P-ABA	0.01	0.02
Biotin	0.005	0.01
Choline chloride	0.5	1.0
Riboflavin	0.1	0.2
Ascorbic acid	1.0	2.0
Vitamin A	0.005	0.01
Vitamin D ₃	0.005	0.01
Vitamin B ₁₂	0.01	0.02
Na Pyruvate	5.0	20.0
Citric acid	10.0	40.0
Malic acid	10.0	40.0
Fumaric acid	10.0	40.0
Fructose	125	250
Ribose	125	250
Xylose	125	250
Mannose	125	250
Rhamnose	125	250
Cellobiose	125	250
Sorbitol	125	250
Mannitol	125	250
Vitamin free casamino acids	125	250
Coconut milk	10 ml/L	20 ml/L
2,4-D	0.2	0.2
Zeatin	0.5	0.5
NAA	1.0	1.0
Sucrose	250	250
Glucose	100,000	100,000

1.7 Composition of Caboche's medium (Ca-9M) (Caboche, 1980).

Nutrient	Concentration (mg/L)
NH ₄ NO ₃	400
Ca Cl ₂ .2H ₂ O	293
Mg SO ₄ .7H ₂ O	246
KH ₂ PO ₄	68
Fe SO ₄ .7H ₂ O	27
Na ₂ EDTA	37
H ₃ BO ₃	6.2
Mn SO ₄ .H ₂ O	0.17
Zn SO ₄ .7H ₂ O	0.28
Co Cl ₂ .6H ₂ O	0.024
Cu SO ₄ .5H ₂ O	0.025
Na ₂ Mo O ₄ .2H ₂ O	0.024
Inositol	180
Pyridoxine	0.5
NAA	0.1
BAP	1.0
Sucrose	30,000
Mannitol	90,000

2 mM Glutamine was added to the medium immediately before use.

1.8.1 Amino acid media for nitrate reductase deficient cells and tissues (Muller and Grafe, 1978).

Nutrient	Concentration (mg/L)
KCl	2,940
Ca Cl ₂ .2H ₂ O	440
KH ₂ PO ₄	170
Mg SO ₄ .7H ₂ O	370
KI	0.83
Co Cl ₂ .6H ₂ O	0.025
H ₃ BO ₃	6.2
Na ₂ Mo O ₄ .2H ₂ O	0.25
Mn SO ₄ .4H ₂ O	22.3
Cu SO ₄ .5H ₂ O	0.025
Zn SO ₄ .7H ₂ O	8.6
Fe SO ₄ .7H ₂ O	27.85
Na ₂ EDTA	37.25
Myo Inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.1
Thiamine HCl	0.5
L-glutamine	877
L-glycine	75
L-aspartic acid	266
L-arganine	228

1.8.2 Additions to amino acid media.

<u>Medium Code</u>	<u>Additional Component (mg/L)</u>	
AA-P19M	2.0	NAA
	0.5	BAP
AA	1.0	2,4-D
	0.2	Kinetin
	0.1	Ga ₃

1.9 Selection medium for the recovery of colonies able to utilize nitrate as sole nitrogen source (MS-NO₃)
(Pental et al., 1982).

Nutrient	Concentration (mg/L)
KNO ₃	3,970
Ca Cl ₂ .2H ₂ O	440
KH ₂ PO ₄	170
Mg SO ₄ .7H ₂ O	370
KI	0.83
Co Cl ₂ .6H ₂ O	0.025
H ₃ BO ₃	6.2
Na ₂ Mo O ₄ .2H ₂ O	0.25
Mn SO ₄ .4H ₂ O	22.3
Cu SO ₄ .5H ₂ O	0.025
Zn SO ₄ .7H ₂ O	8.6
Fe SO ₄ .7H ₂ O	27.85
Na ₂ EDTA	37.25
Inositol	100
Nicotinic acid	0.5
Thiamine HCl	0.5
Pyridoxine HCl	0.1
Sucrose	30,000
NAA	0.1
BAP	0.5

Mannitol was also added at 9.0% (w/v) (MS-NO₃ 9M) or 4.5% (w/v) (MS-NO₃ 4.5M).

1.10 Cell and protoplast washing medium (CPW)
(Frearson et al., 1973).

Component	Concentration (mg/L)
KH ₂ PO ₄	27.2
KNO ₃	101
Ca Cl ₂ .2H ₂ O	1,480
Mg SO ₄ .7H ₂ O	246
KI	0.16
Cu SO ₄ .5H ₂ O	0.025

The following additions were made :

CPW 9M	9% w/v	mannitol
CPW 13M	13% w/v	mannitol
CPW 21S	21% w/v	sucrose

1.11 Nitsches medium 'H' (Nitsch, 1969).

Component	Concentration (mg/L)
KNO ₃	950
NH ₄ NO ₃	720
Mg SO ₄ .7H ₂ O	185
Ca Cl ₂	166
KH ₂ PO ₄	68
Mn SO ₄ .4H ₂ O	25
H ₃ BO ₃	10
Zn SO ₄ .7H ₂ O	10
Na MoO ₄ .2H ₂ O	0.25
Cu SO ₄ .5H ₂ O	0.025
Na ₂ EDTA	37.25
Fe SO ₄ .7H ₂ O	2.8
Myo Inositol	100
Glycine	2
Nicotinic acid	5
Pyridoxine HCl	0.5
Thiamine HCl	0.5
Follic acid	0.5
Biotin	0.05
Sucrose	20,000

APPENDIX 2

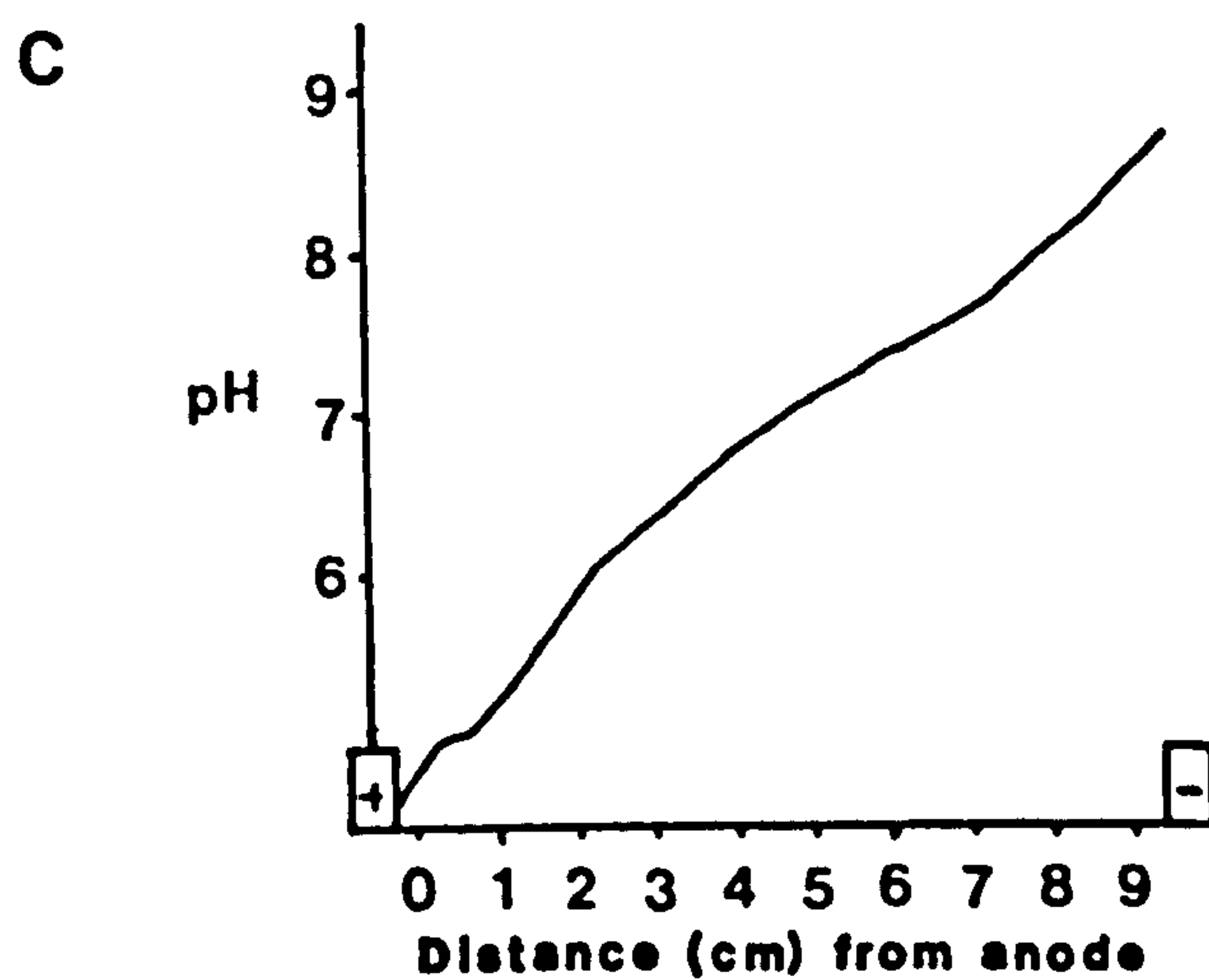
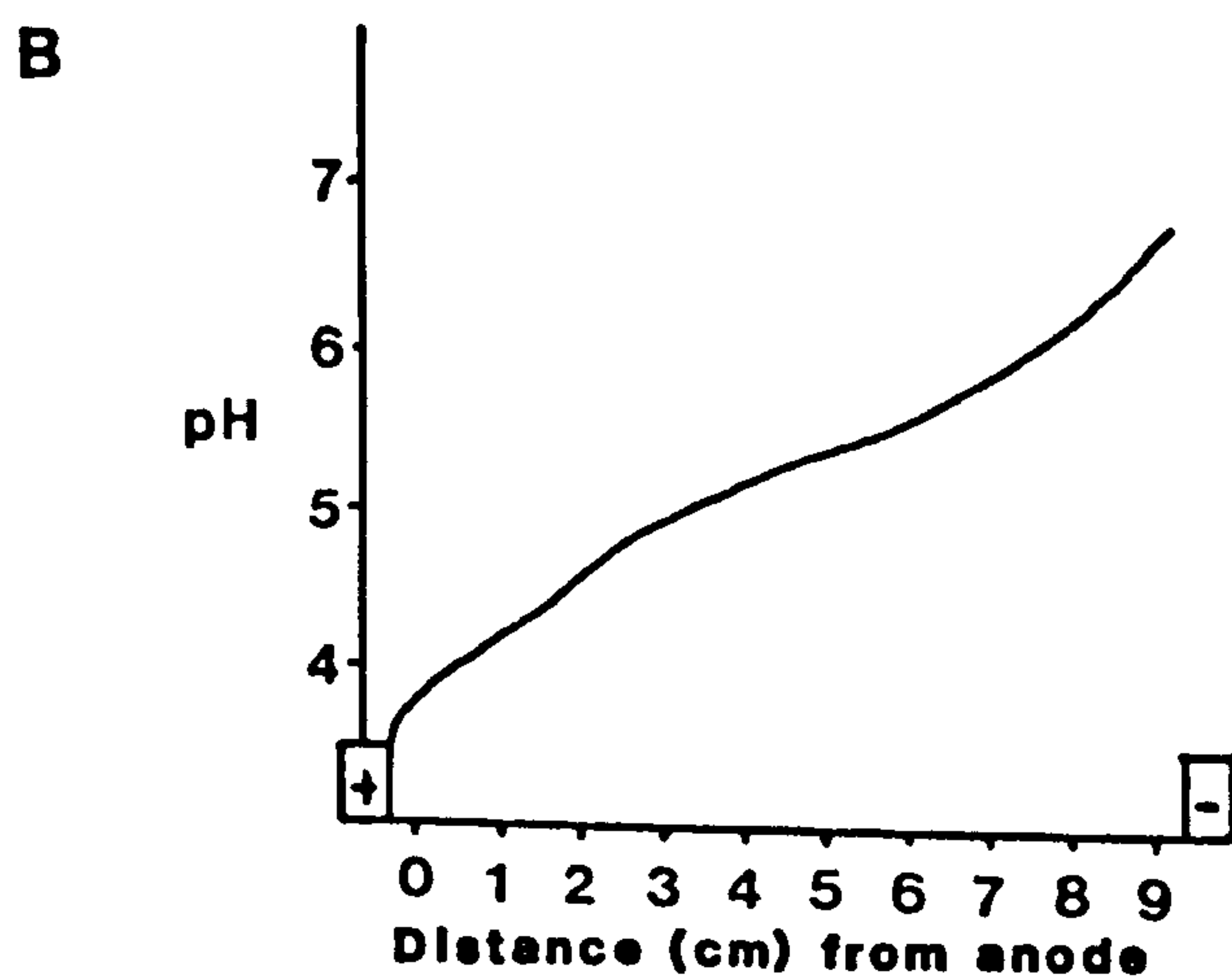
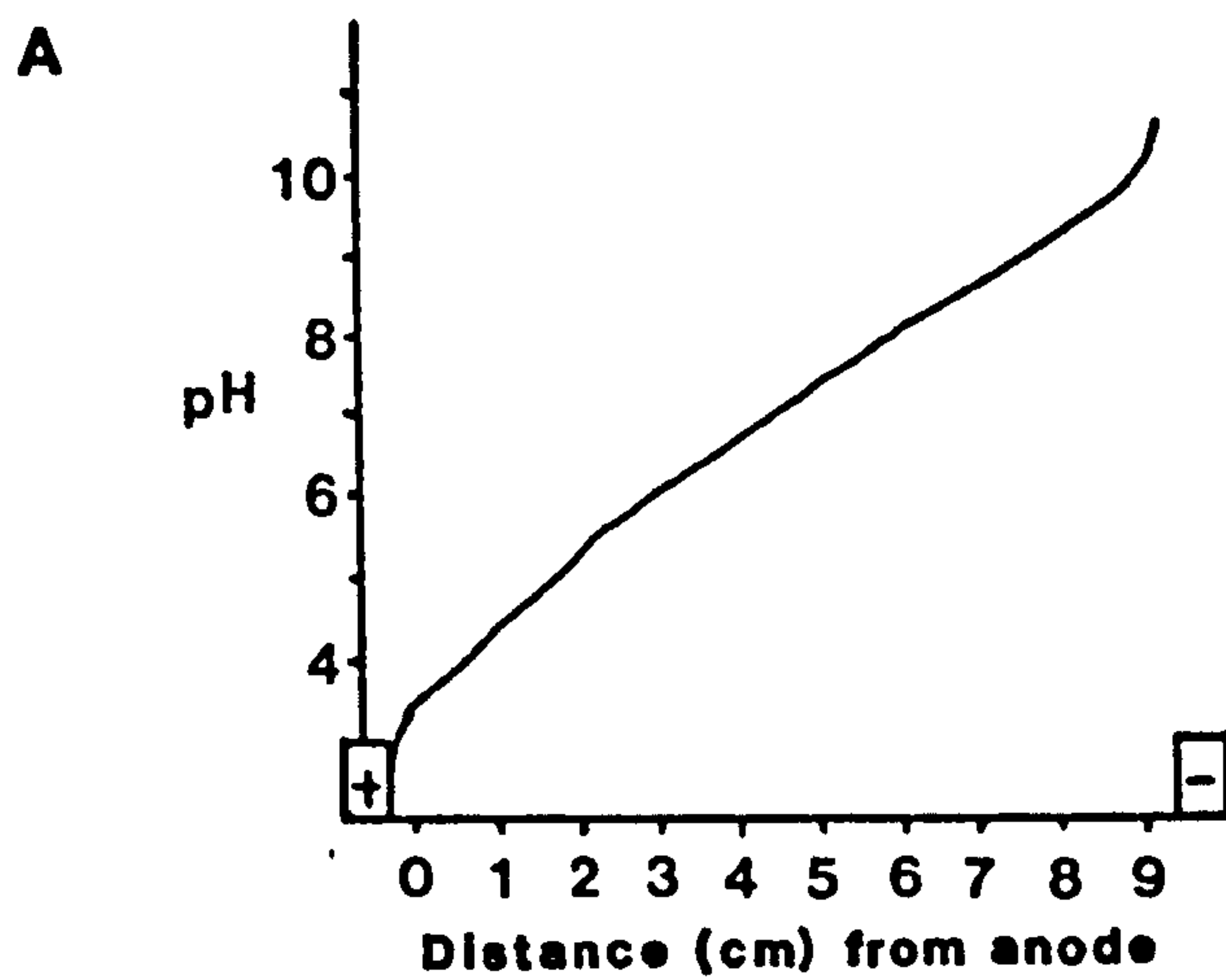
CALIBRATION CURVES

2.1 A Calibration curve for protein determination by the method of Lowry et al. (1951)

Transmission of light at 500 nM and 700 nM was recorded.

2.1 B Calibration curve for the nitrate reductase activity assay of Jaworski (1971)

Absorption of light at 540 nM was recorded.



2.2 Typical pH gradients formed in LKB PAG-Plates used for
isoelectric focusing of protein extracts in isoenzyme
analysis

The pI of prominent isoenzyme bands was determined from these calibration curves. Reproduced from information provided by the manufacturer (LKB).

2.2 A pH 3.5 - 9.5

2.2 B pH 4.0 - 6.5

2.2 C pH 5.5 - 8.5