HAPLOID, POLYPLOID AND ANEUPLOID PLANTS FROM CULTURED ANTHERS AND CALLUSES IN SPECIES OF *NICOTIANA* AND FORAGE CROPS

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I. INTRODUCTION

The knowledge of haploid plants dates back to 1922 when BLAKESLEE and his colleagues first reported the occurrence of a haploid plant in *Datura stramonium.* Since that time, haploid plants have been generally recognized for their usefulness for various approaches in cytogenetics, genetics, and plant breeding research.

In cytogenetics and evolutionary studies haploids may provide a useful clue in discerning the nature of the basic chromosome complement from an analysis of meiotic chromosome pairing relationships. In a cytogenetical study of haploid *Sorghum* plants ($n=10$), BROWN (1943) observed ten univalents at late prophase, whereas at metaphase I, approximately ten percent of the nuclei contained bivalents. Thus, the presence of these bivalents at metaphase I suggested that there may be some duplications present in the genome of *Sorghum* and that ten may not be the true basic chromosome number. Prior to this observation of BROWN, LONGLEY (1932) had reported the discovery of a five chromosome species in *Sorghum.* Therefore, it may be assumed that five is the basic chromosome number of *Sorghum* rather than ten. CHRISTENSEN and BAMFORD (1943) found a situation similar to that in *Sorghum* from a meiotic study of haploid pepper plants (*Capsicum annuum*) ($n=12$). At diakinesis twelve univalents were observed. However, all degrees of bivalent-univalent associations were occasionally observed, and in some cases complete pairing, or at least

associations, were present. Therefore, this finding also lead to the conclusion that diploid pepper plants are actually polyploids in which many of the genes may be duplicated. Recently, a number of meiotic studies on haploids with similar conclusions have been reported on haploids of rice plant (*Oryza sativa*) (CHU 1967), caster oil plant (*Racinus communis*) (NARAIN and SINGH 1968), *Triticum durum* (LACADENA and RAMOS 1968), tomato (*Lycopersicum esculentum*) (ECOCHARD *et al.* 1969), and pearl millet (*Pennisetum typhoides*) (PANTULU and MANGA 1969).

One of the outstanding usefulnesses of haploid plants is the combination with the recently advanced cell and tissue culture. Bacteria such as prokaryotes have played important roles for long time in sophisticated genetic analysis for several reasons as follows: (1) They are unicellular organism, (2) They generally have a quite short life cycle, (3) They are haploid, and (4) They have specific attributes such as transformation or transduction. Because cell and tissue cultures of eukaryotes have many advantageous traits as well as prokaryotes, many investigators in various fields are now using the culture technique in their studies. For instance, CARLSON (1970) reported methods for isolation of auxotrophic mutants in somatic cell cultures of haploid *Nicotiana tabacum* which was induced by anther culture. When large populations of haploid cells were treated with the mutagen ethyl methanesulfonate and grown on minimal medium for several cell generations, the non-mutant cells continued to grow and divide whereas mutant cells decreased or halted their growth. When the medium was supplemented with 5-bromodeoxyuridine (BUdR), the BUdR is incorporated into the DNA of the dividing cells and the cells were subsequently killed by exposure to visible light. The auxotrophic mutants were selected and were recovered on a nutritionally supplemented medium. By this method, 6 auxotrophic calluses were isolated from 119 calluses.

It is well established that purified DNA can be taken up by bacteria and that the recipient cells are capable of incorporating DNA into their chromosomes and expressing the genes thus acquired. An attempt to perform similar experiments with eukaryotes was carried out using haploid tomato and *Arabidopsis* calluses and by inoculating them with phages carrying wild type *Escherichia coli* genes prolonged its survival through several cell generations on normally fatal media (DOY *et al.* 1973). Inoculation with phage $\lambda Pgal^+$ enabled callus to grow slowly on 2% galactose medium, whereas untreated callus and those inoculated with $\lambda Pgal^-$ died within 3 weeks. The term "transgenosis" has been proposed for such phenomena even though it is not known whether gene transfer, mainte-

nance, transcription and function are similar to the transformation of bacteria.

Practical values of haploids in plant breeding have been illustrated by several investigators. For instance, HOUGAS *et al.* (1958a, b) successfully carried out matings between haploid *Solanum tuberosum* and several diploid *Solanum* species. They pointed out that the success of these matings was of particular interest from a breeding standpoint since the haploids presented a new approach for the transfer of desirable genes from diploid *Solanum* species to the common potato. In a study by SEARS (1954), haploid plants were used in the development of the monosomic series in common wheat. The complete establishment of the monosomic series was accomplished through the hybridization of haploid and diploid plants of *Triticum aestivum.* There is no doubt of the value of this monosomic series which has contributed not only to genetic studies but also to intervarietal and to alien chromosomal substitution programs.

Another desirable aspect of haploids in their practical use in plant breeding is notably the production and the exploitation of totally homozygous forms. Once the haploids are obtained, the chromosome number can be doubled by appropriate means. The resulting individuals would be completely homozygous for all genes, and would achieve complete homozygosity in only a single step that normally would require many generations to achieve through inbreeding. The efficiency of this haploid method of plant breeding has been discussed in detail by NEI (1963) who presented data on the frequencies of haploid occurrence and on the number of diploidizations that are necessary for the haploid method to be more efficient than the diploid method. NEI's study indicated that the haploid method is advantageous when the number of genes desired for breeding purposes is large and the frequencies of favourable alleles in the populations are small, even if the frequency of haploids is small. CHASE (1952) obtained about 700 homozygous diploid lines of *Zea mays* by selecting occasional haploids which had spontaneously doubled their chromosome complement. These haploid-derived homozygous lines were used practically in hybridization experiments in the production of hybrid corn in an equivalent manner to inbred lines.

Not only haploid but also aneuhaploid plants are sometimes very useful. For one instance, by using aneuhaploids and nullisomics of *Triticum aestivum*, RILEY and CHAPMAN (1958) and RILEY *et al.* (1959) have demonstrated the specific genetic mechanism controlling the diploid-like chromosome behavior of this species. They found that the 20-chromosome

wheat aneuhaploids, which subsequently proved to be deficient of chromosome 5B, have a pattern of meiotic pairing conspicuously different from that of the 21-chromosome euhaploids. The aneuhaploids, deficient of chromosome 5B, have bivalents and trivalents, whereas, there is little pairing of the chromosomes in the euhaploids. Furthermore, the formation of multivalents is often observed in the 40-chromosome nullisomic plants. Therefore, these results lead to the conclusion that the activity of chromosome 5B is clearly responsible for the chromosome isolation mechanism which restricts the formation of non-homologous pairing.

Thus, as mentioned above the haploid, homozygous diploid and aneuhaploid plants or the haploid state of cell and tissue cultures have many advantages in classical and modern genetics and plant breeding. A problem presented here is how to obtain these plants and cells or tissue at will.

In addition to the spontaneous occurrence of haploids, various artificial methods have been attempted to induce haploids ; such as cross pollination either intervarietal or interspecific, delayed pollination, X-ray or colchicine treatments and temperature shock. However, no single way has been found to overcome the difficulties encountered in order to obtain haploids in a high frequency and also to obtain them freely when they are required. Recently, however, the induction of haploid plants by means of anther cultures, gives promise for removing some of these difficulties in haploid production. GUHA and MAHESHWARI (1964, 1966) were the first to report the successful production of haploid plants from anther cultures in *Datura innoxia*. Following the report of this success, BOURGIN and NITSCH (1967) and NAKATA and TANAKA (1968) induced haploid *Nicotiana* plants from anther cultures. Also, NIIZEKI and OONO (1968) succeeded in producing haploid plants of *Oryza sativa* by culturing anthers of this species. Since then conditions have been established for the induction of growth in other some twenty genera of angiosperm pollens.

Since 1969, the author has carried out anther and callus culture of *Nicotiana* species and many species of forage crops. The investigations are mainly grouped into three parts as follows: (1) Haploid plant induction by anther culture in which the feature and mechanism of the induction, and the related problems were investigated, (2) Completely homozygous diploid and the other polyploid induction by use of cytogenetically unstable callus culture, instead of colchicin treatment. Also the possibility of maintenance of stable haploid callus is discussed for the requirement of somatic cell genetics, especially mutation and transformation research. (3) Exploitation of a new method of production of aneuploid, especially

aneuhaploid production by use of anther culture of plants with various polyploidy levels and chromosome constitutions. They are very useful for studies in genetics and plant breeding, because the majority of such aneuploid plants have not been reported or have been only rarely reported in a small number of species.

This paper presented here describes an outline of these experimental results.

II. LITERATURE REVIEW

1. Haploid production from male gametes

Recent work with plant cells has generally shown that cell differentiation is not carried out through genetic change but through the selective and programmed suppression or activation of certain parts of the genome. If so, under certain appropriate chemical or physical stimuli, the genome is capable to activate adult tissue to grow again and to differentiate into other tissues or organs *in vitro*, even in cases where such a phenomenon is not observed in nature. This view has been convincingly supported by advancements of cell culture studies *in vitro* (STREET 1969, STEWARD *et al.* 1969). Now, many examples of the regeneration of perfectly normal plantlets from callus tissues, and even from single somatic cell are available.

Recently, it has been shown that cell differentiation and morphogenesis in tissue or cell culture are not only found in somatic cells, but also in gametic cells. The pioneer study on the initiation of callus tissue from pollen grain culture was carried out by TULECKE (1953, 1957) with the gymnosperm, *Ginkgo biloba*. He noted that when a mass of pollen grains were cultured for several months on agar-solidified medium supplemented with coconut milk, or aqueous extract of the pollen tissue, approximately 4 percent of the pollen grains developed abnormalities. One type consisted of a coenocytic condition with many free nuclei within the gametophyte. Another type of abnormal development from the pollen grains was a multicellular condition at the exine region; infrequently, a several-celled tube was found. Some of these abnormally developed pollen grains lead to the proliferation of callus tissues. Observations on early stages of the formation of the callus tissue indicated that most of the tissue originated from the separated tube, whereas, the coenocytes were unable to form cell walls and to give rise to multicellular tissue. The basic chromosome number of the calluses was 12 (haploid number for *Ginkgo*), but 24 and 48 as well as other aneuploid numbers were also observed. Callus tissue formation has been also reported by TULECKE and SEHGAL (1963) from cultured pollen

grains of *Torreya nucifera* (Gymnospermae). KONAR (1963) noted that the normal germination of the pollen grains of *Ephedra foliata* (Gymnospermae) could be altered to form callus tissue on agar-solidified medium which was supplemented with coconut milk and 2, 4-dichlorophenoxyacetic acid (2, 4-D). In this case, callus tissues could be obtained in as many as 57 percent of the pollen cultures. He did not observe any intercalary divisions nor any diploid or polyploid cells in the tissue as had been observed in *Ginkgo biloba.*

On the other hand, conditions for triggering angiosperm pollens into active growth by direct planting procedures had not yet been established. However, mature pollen of *Brassica oleracea* and of the hybrid *B. oleracea* $\times$ *B. alboglabra* could be induced to form cell clusters in hanging drop cultures (KAMEYA and HINATA 1970). Culture medium inoculated with pollen (50–80 grains) was placed in hole slide glasses and sealed by cover slips with paraffin. The pollen was inoculated at a low temperature (5°C) to prevent the burst of pollen grains and the culture was carried out in a growth chamber at 20°C under darkness. Aeration was conducted by shaking gently by hand once a day. Cell clusters were formed after four weeks in medium supplemented with coconut milk. Subsequently, BINDING (1972) succeeded to induce the cell clusters of *Petunia hybrida* by suspension culture. In this instance, pollens inoculated both in the uninucleate microspore condition and in a more or less mature condition did not form cell clusters. Pollen inoculated just after mitosis, however, responded to the media containing kinetin and auxin [indole-3-acetic acid (IAA) and 2, 4-D] singly or in combination. Pollen of *Lycopersicon esculentum* could be stimulated into colony formation by nurse-culture (SHARP *et al.* 1972). Anthers of this species are placed on the surface of an agar medium and covered with a small disc of filter-paper. A drop of pollen-suspension (0.5 ml) containing about 10 grains is pipetted onto each disc. With incubation at 25°C under light, colonies of green parenchymatous cells appear on the discs within about 14 days. Plating efficiencies of up to 60% was obtained. Colony formation did not occur in cultures lacking the nurse-anther. In this instance, also immature pollen was used; flower buds 1 cm in length were surface-sterilized, and then anthers were removed aseptically and the pollen were released by cutting open the anthers in a small volume of liquid culture medium.

GUHA and MAHESHWARI (1964) were the first to obtain a plantlet from pollen grains by means of anther culture of angiosperm, *Datura innoxia.* They excised anthers from flower buds and aseptically cultured them on

NITSCH's or WHITE's basic media supplemented with coconut milk or kinetin. After 6 to 7 weeks and when the cultures were about to be discarded, numerous embryo-like structures abruptly projected out of the anthers from all sides. These embryoids possessed root and shoot axes and cotyledons. Subsequent cytological investigation by GUHA and MAHESHWARI (1966) showed that all of the embryoids and plantlets possessed a haploid chromosome number ($n=12$). Therefore, these embryoids and plantlets may definitely be said to originate from the pollen grains in the anthers and not from other somatic tissue such as connective tissue. Further studies of GUHA and MAHESHWARI (1967) indicated that the anthers of *Datura stramonium* also give rise to embryoids by the culture *in vitro*.

Subsequently, BOURGIN and NITSCH (1967) cultured anthers of *Nicotiana tabacum* (var. Wisconsin 38 and var. Maryland Mammoth), *N. sylvestris* and three hybrids; *N. sylvestris* × *N. tabacum* (var. Maryland Mammoth), *N. langsdorffii* × *N. glauca* and amphidiploid of *N. sylvestris* × *N. tabacum* (var. Maryland Mammoth). After being cultured for 6 to 10 weeks with a photoperiod of 16 hours, and under alternating temperature (30°C day, 22°C night), plantlets proliferated from some anthers of *N. tabacum* and *N. sylvestris*, but not from any of the hybrids or the amphidiploid. According to chromosome number determinations, there were 4 haploids and 1 diploid from *N. tabacum* (var. Wisconsin 38), 1 haploid and 1 diploid from *N. tabacum* (var. Maryland Mammoth), and 3 haploids and 1 diploid from *N. sylvestris*. Following this, NAKATA and TANAKA (1968) also reported haploid production of *N. tabacum* (var. Bright Yellow and var. Hicks) by anther culture.

Haploid production by means of anther culture in addition to that reported for *Datura* and *Nicotiana* was produced in *Oryza sativa* by NIIZEKI and OONO (1968). Anthers with immature pollen grains, one or two days prior to heading, were used for culturing. About three weeks after being cultured, the anthers turned black. Then pale yellow calluses proliferated from the anthers after 4 to 8 weeks of culturing. The appropriate medium for the induction of calluses from anthers in *Oryza* was found to contain IAA, kinetin and 2, 4-D. Chromosome counts on a squash preparation of the callus tissue proved that all of the cells possessed the haploid chromosome number ($n=12$). After the callus formation, shoots and roots were induced by transplanting the callus to a medium containing IAA and kinetin. In this manner, seven plants were grown from calluses of two rice varieties.

Since the successes in the anther culture of *Datura*, *Nicotiana* and *Oryza* many investigators have been extensively attempted haploid production in many species by this culture technique. The species in which anther culture has been successful are listed in Table 1.

TABLE 1. Species successful in anther culture

Species	Process of induction of haploid plant	Reference
Aegilops caudata × *Ae. umbellulata*	C	KIMATA and SAKAMOTO 1971, 1972.
Arabidopsis thaliana	C	GRESSHOFF and DOY 1972a.
Asparagus officinalis	C	PELLETIER *et al.* 1972.
Atropa belladonna	E	ZENKTELER 1971, MISIURA and ZENKTELER 1973.
Brassica oleracea	C	KAMEYA and HINATA 1970.
Brassica oleracea × *B. alboglabra* F_1	C	KAMEYA and HINATA 1970.
Bromus inermis	C	SAITO *et al.* 1973.
Capsicum annuum	C, E	WANG *et al.* 1973b, HARN *et al.* 1975.
Datura innoxia	E	GUHA and MAHESHWARI 1964, 1966, 1967, NITSCH 1972, NITSCH and NORREEL 1973, ENGVILD *et al.* 1972, SOPORY and MAHESHWARI 1972, 1973, SUNDERLAND *et al.* 1974.
D. metel	C, E	NARAYANASWAMY and CHANDY 1971, IYER and RAINA 1972.
D. meteloides	E	NITSCH 1972, KOHLENBACH and GEIER 1972.
D. muricata	E	NITSCH 1972.
D. stramonium	E	GUHA and MAHESHWARI 1967.
D. wrightii	E	KOHLENBACH and GEIER 1972.
Hordeum vulgare	C	CLAPHAM 1971, 1973.
Lilium longiflorum	C	SHARP *et al.* 1971b.
Lolium multiflorum	C	CLAPHAM 1971.
Lolium multiflorum × *Festuca arundinacea*	C	NITZSCHE 1970.
Lycium halimifolium	E	ZENKTELER 1972.
Lycopersicon esculentum	C	GRESSHOFF and DOY 1972b.
Nicotiana alata	E	NITSCH 1970.
N. clevelandii	E	VYSKOT and NOVÁK 1974.
N. glutinosa	E	NITSCH 1970, NAKAMURA and ITAGAKI 1973.
N. otophora	E	NITSCH 1972, NAKAMURA and ITAGAKI 1973.

Species	Process of induction of haploid plant	Reference
Nicotiana rustica	E	NITSCH 1970, NAKAMURA and ITAGAKI 1973.
N. sanderae	E	VYSKOT and NOVÁK 1974.
N. sylvestris	E	BOURGIN and NITSCH 1967, NITSCH 1970.
N. tabacum	E	BOURGIN and NITSCH 1967, NAKATA and TANAKA 1968, NITSCH *et al.* 1968, NITSCH 1969, 1970, NITSCH and NITSCH 1969, 1970, SUNDERLAND and WICK 1969a, b, BURK 1970, DEVREUX 1970, KOCHHAR *et al.* 1971, MII 1973.
Oryza sativa	C, E	NIIZEKI and OONO 1968, 1971, NIIZEKI 1968, NISHI and MITSUOKA 1969, HARN 1969, 1970a, b, GUHA *et al.* 1970, IYER and RAINA 1972, GUHA-MUKHERJEE 1973, OONO 1975.
Paeonia hybrida	E	SUNDERLAND 1974.
Pelargonium hortorum	C	ABO EL-NIL and HILDEBRANDT 1973.
Petunia axillaris	E	ENGVILD 1973.
Populus nigra	C	WANG *et al.* 1975a.
Setaria italica	C	BAN *et al.* 1971.
Solanam bulbocastanum	C, E	IRIKURA 1975.
S. demissum	C, E	IRIKURA 1975.
S. dulcamara	C, E	ZENKTELER 1973.
S. fendleri	C, E	IRIKURA 1975.
S. hjertingii	E	IRIKURA 1975.
S. nigrum	C	HARN 1971, 1972a, b, IRIKURA 1975.
S. phureja	E	IRIKURA 1975.
S. polytrichon	C, E	IRIKURA 1975.
S. stenotomum	E	IRIKURA 1975.
S. stoloniferum	E	IRIKURA 1975.
S. tuberosum	E	DUNWELL and SUNDERLAND 1973, IRIKURA 1975.
S. verrucosum	C, E	IRIKURA and SAKAGUCHI 1972, IRIKURA 1975.
S. verrucosum × *S. chacoense*	E	IRIKURA 1975.
S. verrucosum × *S. tuberosum*	E	IRIKURA 1975.
Triticale	C	WANG *et al.* 1973b, SUN *et al.* 1974.
Triticum aestivum	C, E	OUYANG *et al.* 1973, WANG *et al.* 1973a, CRAIG 1974.
Triticum vulgare × *Agropyron glaucum*	C	WANG *et al.* 1975b.

E : Haploid plant produced through embryoid formation from pollen.

C : Haploid plant produced through callus formation from pollen.

Pollens of *Atropa*, *Datura*, *Lycium*, *Nicotiana*, *Petunia* and *Paeonia* give rise to plantlets which develop through stages not unlike those of true zygotic embryos to form plantlets. Pollens of *Arabidopsis*, *Asparagus*, *Brassica*, *Bromus*, *Hordeum*, *Lilium*, *Lolium*, *Lycopersicon*, *Pelargonium*, *Populus*, *Setaria*, and *Triticale* give rise to callus before the anther opens. *Capsicum*, *Oryza*, *Solanum* and *Triticum* also give rise to callus predominatingly but occasionally give rise to plantlets in certain media or culture procedures (WANG *et al.* 1973a, GUHA *et al.* 1970, ZENKTELLER 1973, IRIKURA 1975, DUNWELL and SUNDERLAND 1973, WANG *et al.* 1973b, HARN *et al.* 1975).

2. Diploid and the other polyploid production by callus culture

Conversion of the haploid plant to the corresponding homozygous diploid plant have been usually carried out by colchicine treatment (STOKS 1963, TANAKA and NAKATA 1969, OONO 1975). The treatment is usually repeated several times, however, the technique is not always reliable. It has been reported by a number of investigators using different species of plants that the callus tissues and the cultured cells sometimes may alter their chromosome numbers to produce polyploid and aneuploid cells (STREET 1969, SUNDERLAND 1973a). MURASHIGE and NAKANO (1966) obtained tetraploid plants from the callus with polyploidized cells which were originally raised from single cells isolated from the pith of diploid tobacco plants. This observation leads us to believe that the technique of tissue culture may be useful in the production of homozygous diploid plants from the callus tissue derived from haploid plants. Indeed, KOCHHAR *et al.* (1971) attempted induction of diploid plant from the cultured haploid callus which originated from the stem of 7 to and 8-week-old haploid plantlets by anther culture of the tobacco plant. After about two weeks many shoots differentiated from this callus cultured on the medium described by MURASHIGE and SKOOG (1962) supplemented by 2 mg/l of kinetin and 2 mg/l of IAA. Cytogenetical studies on the root tips of these plants revealed the diploid ($2n=48$) chromosome number. The diploid plants raised *in vitro* are morphologically and cytologically similar to diploid plants produced from seed. Thus, it was postulated that these diploid plants differentiate from cells that have undergone autopolyploidy during the growth of the callus. NITSCH (1971) showed that a high concentration of IAA such as 10^{-5} M enhance the endomitosis of the callus. When no cytokinin was added to the medium, the occurrence of endomitosis was reduced. 6-benzylaminopurine (BAP) seems to be the cytokinin which

most favors the diploidization of the cells. These results were also confirmed by the work of TORREY (1961). This rapid method of producing homozygotes by callus culture may be potentially of importance in plant breeding and in programmes of crop improvement.

3. Aneuploid production from male gametes

Usefulness of aneuploid plants such as trisomic, monosomic and nullisomic plants for genetic studies and plant breeding are extensively discussed by KHUSH (1973). Besides spontaneous occurrences of these aneuploids, many methods have been attempted for production. The methods mainly are as follows: (1) Treatment with physical and chemical agents such as X-ray, gamma ray and ethyl methanesulfonate (EMS). (2) Using asynaptic and desynaptic disomic plant where a variable number of univalents are present at metaphase I of meiosis due to disturbances in normal pairing. Those univalents segregating at random to the two poles produce spores with irregular chromosome numbers and produce aneuploids upon fertilization by the haploid gametes. (3) Using various polyploid plants where triploids are good sources of trisomics and haploids are good sources of monosomics. (4) In aneuploids, monosomics are especially the best sources of nullisomics. (5) Using translocation heterozygous and other chromosomal abnormalities. However, because of the lack of proper artificial means in some cases, available plants with such useful aneuploid are severely restricted to certain varieties and numbers in their production.

Furthermore, haploid plants with aneuploid chromosome constitution have been recorded in only a small number of species such as *Triticum aestivum*, *Matthiola incana*, *Datura stramonium* and *Poa pratensis* (KIMBER and RILEY 1963). This may be due to the fact that aneuhaploid seldoms as compared with the euhaploid and other aneuploid in both spontaneous and artificial occurrences.

It is well known that the irregular distribution of chromosomes in the course of meiosis of autopolyploids such as autotriploid or autotetraploid results in irregular chromosome numbers in the gametes. By means of the developed anther culture technique, it may be reasonably surmised that these gametes are capable of differentiation into complete aneuploid plants. Thus, it is feasible that not only well known aneuploids such as trisomic, monosomic and nullisomic aneuploids but also various other aneuploids are produced, especially plants having chromosome numbers falling between haploid (n) and diploid ($2n$).

III. EXPERIMENTS

1. Production of haploid plants and calluses by anther culture

(1) Materials and methods

A. Materials

a. *Nicotiana species.*

Nicotiana tabacum (var. Wisconsin 38, var. Hicks Broadleaf and var. Delhi 34), *N. sylvestris* and *N. glutinosa.*

b. *Forage crops.*

Gramineae: *Bromus inermis* (*var.* Elsberry, var. Southland), *Dactylis glomerata* (var. Aries, var. Germinal and cv. Tsukisamu-Zairai), *Festuca arundinacea* (var. Festival, var. Gazelle, var. Grombaria, var. Ludion and var. Manade), *Festuca pratensis* (var. Daphné and var. Naïade), *Lolium hybridum* (Hybrid rygrass Io), *Lolium multiflorum* (var. Itaque, var. Rina, var. Rita and var. Tedis), *Lolium perenne* (var. Bocage, var. Primevere, var. Raidor and var. Real), *Phleum pratense* (var. Norin-Ichigo, P.I. 58382 and P.I. 206909) and *Sorghum sudanense* (var. Carifornia 23),

Leguminosae: *Lotus corniculatus* (var. Empire, B-534, var. Crassifolius and var. Viking), *Lotus caucasicus*, *Medicago sativa* [var. DuPuit and var. Hunter River $\times$ *M. falcata* (F_1)], *Trifolium pratense* (var. Alpilles and var. Triel).

B. Basic culture media

a. *Medium LS. RM-1964 medium described by* Linsmaier *and* Skoog *(1965).*

Mineral Salts:

Major elements:

NH_4NO_3	1,650 mg/l
KNO_3	1,900 ,,
$CaCl_2 \cdot 2H_2O$	440 ,,
$MgSO_4 \cdot 7H_2O$	370 ,,
KH_2PO_4	170 ,,
Na_2EDTA	37.3* ,,
$FeSO_4 \cdot 7H_2O$	27.8* ,,

* 5 ml/l of a stock solution containing 5.57 g $FeSO_4 \cdot 7H_2O$ and 7.45 g Na_2EDTA per litre of H_2O.

Minor elements:

H_3BO_3	6.2 mg/l
$MnSO_4 \cdot 4H_2O$	16.5 "
$ZnSO_4 \cdot 4H_2O$	8.6 "
KI	0.83 "
$Na_2Mo_4 \cdot 2H_2O$	0.25 "
$CuSO_4 \cdot 5H_2O$	0.025 "
$CoCl_2 \cdot 6H_2O$	0.025 "

Organic constituents:

Glycine	2.4 mg/l
Thiamine·HCl	0.1 "
Nicotinic acid	0.5 "
Pyridoxine·HCl	0.1 "
Myo-Inositol	100.0 "
Sucrose	30.0 g/l
Agar	10.0 "

b. *Medium M. Miller's basic medium (1963).*

Mineral salts:

Major elements:

KH_2PO_4	300 mg/l
KNO_3	1,000 "
NH_4NO_3	1,000 "
$Ca(NO_3)_2 \cdot 4H_2O$	347 "
$MgSO_4 \cdot 7H_2O$	35 "
KCl	65 "
Sodium ferric EDTA	32 "

Minor elements:

KI	0.8 mg/l
$ZnSO_4 \cdot 4H_2O$	1.5 "
H_3BO_3	1.6 "
$MnSO_4 \cdot 4H_2O$	4.4 "

Organic constituents:

Glycine	2.0 mg/l
Thiamine·HCl	0.1 "
Nicotinic acid	0.5 "
Pyridoxine·HCl	0.1 "

Sucrose	30.0 g/l
Agar	10.0 "

c. *Medium H. Described by* BURGIN *and* NITSCH *(1967).*

Mineral salts:

Major elements:

KNO_3	950 mg/l
NH_4NO_3	720 "
$MgSO_4 \cdot 7H_2O$	185 "
$CaCl_2 \cdot 2H_2O$	166 "
KH_2PO_4	68 "

To the above 5 ml/l of a solution of 7.45 g of Na_2EDTA and 5.57 g $FeSO_4 \cdot 7H_2O$ in 1 liter of distilled water was added.

Minor elements:

$MnSO_4 \cdot 4H_2O$	25.0 mg/l
H_3BO_3	10.0 "
$ZnSO_4 \cdot 7H_2O$	10.0 "
$Na_2MoO_4 \cdot 2H_2O$	0.25 "
$CuSO_4 \cdot 5H_2O$	0.025 "

Organic constituents:

Myo-Inositol	100.0 mg/l
Glycine	2.0 "
Nicotinic acid	5.0 "
Pyridoxine·HCl	0.5 "
Thiamine·HCl	0.5 "
Sucrose	20.0 g/l
Agar	8.0 "

All of the foregoing media were solidified with 8 to 10 g/l of Difco Bacto-agar. The procedure for the preparation of the culture media is as follows. Cylindrical glass bottles (capacity, 30 ml) with a plastic screw cap or test tubes (capacity, 27 ml) capped with aluminumfoil were used as the container for all culture studies. In order to leach out any compounds in the culture bottles or test tubes which might be suspected of interfering with the culture of the anthers, all new glass bottles and test tube were soaked in 10% HCl for 24 hours and then were boiled in a strong detergent and rinsed thoroughly. Before the Difco Bacto-agar was added, the

nutrient culture medium containing the supplemented mineral salts and organic substances was adjusted to a pH 5.5 to 6.0 with 1N HCl and 1N NaOH. Then, the agar was added to the medium and dissolved slowly by heating. Immediately after the agar was thoroughly dissolved, 10 ml of the medium was poured into each culture bottle or test tube and then autoclaved at 120°C for 15 minutes. After autoclaving, the culture bottles or test tubes were capped tightly and kept at 4°C in a refrigerator until they were used for culturing the anthers.

C. Anther culture procedures

In preparation for anther culture, the flower buds or florets were immersed in 70% ethanol in a Petri dish for several seconds, and then washed several times with sterilized distilled water to completely remove the ethanol. Subsequently, the flower buds or florets were immersed in a 7% solution of sodium hypochlorite for about 3 minutes and then thoroughly washed several times with sterilized distilled water to remove any traces of the sodium hypochlorite. Then the sterilized flower buds or florets were placed on sterilized filter paper for several minutes to remove the excess water. The excision of the anthers from the flower buds or florets was carried out aseptically with a dissecting knife and needle with the aid of a dissecting microscope. For each experiment, 5 to 10 anthers of *Nicotiana* species and 15 to 30 anthers of forage crops were planted on the surface of agar-solidified medium. Culture of pollen grains which were removed directly from the anther sacs was also tried with some species of *Lotus*. The pollen grains were squeezed aseptically from the anther sacs with the tip of a dissecting needle under a dissecting microscope and then planted directly on the surface of agar-solidified medium.

The incubator used for this culture study was maintained at a temperature of 26 ± 0.5°C and illuminated by fluorescent tubes with a light intensity of 4800 $1\times/m^2$.

In the case of the *Lotus* anther cultures, when shoot formation occurred on the calluses of the primary culture, the shoots were transplanted onto a new medium in order to promote root initiation. After the plantlets had developed an adequate root-system for their maintenance, they were transplanted into pots with a mixture of clay, sand and peat moss. Then, the plantlets were grown in a growth chamber maintained at 28°C (day) and 22°C (night), 70% humidity, and a photoperiod of 16 hours.

In the case of the *Nicotiana* species, the anthers sometimes gave rise to complete plantlets with well developed root-systems on the primary

cultures and therefore, they were transplanted directly into pots and grown in a green house until they commenced to flower. The plantlets lacking well developed roots were, however, transplanted onto medium without any growth regulators. After the plantlets developed a sufficient root-system, they were transplanted into pots. Plantlets transplanted into the pots were usually covered by polyethylene bags for the first two weeks to maintain sufficient humidity for their growth.

D. Chromosome number determinations of callus tissue and root tips

The chromosome numbers of callus tissue were determined as follows: Pieces of callus tissue were pretreated in 0.002 M 8-hydroxyquinoline for 2 to 4 hours at room temperature. They were then fixed in alcohol-acetic acid solution (3:1) for about 12 hours. The fixed callus tissues were macerated in 4% pectinase for 6 to 12 hours at room temperature, and then stained with alcoholic hydrochloric acid-carmine (SNOW 1963) for about 12 hours. Slides were prepared by a routine squashing method.

The chromosome numbers of root tips of regenerated plants were determined in the following way: Roots were pretreated in 0.002 M 8-hydroxyquinoline for 2 to 4 hours at room temperature, fixed with alcohol-acetic acid (3:1) for about 12 hours. They were then hydrolyzed with 1N HCl at 60°C in a water bath for 6 minutes and stained with leuco-basic fuchsin. Alcoholic hydrochloric acid-carmine was also used for staining the roots tips after pectinase treatment. Squashes were prepared in the usual manner using 45% acetic acid.

(2) Results

A. Anther culture of *Nicotiana*

a. *Production of plantlets in three Nicotiana species*

Three *Nicotiana* species, *N. tabacum* (var. Wisconsin 38, $2n=48$), *N. sylvestris* ($2n=24$) and *N. glutinosa* ($2n=24$) were used for anther culture on medium H supplemented with 0.1–2.0 mg/l of IAA and 0.1–2.0 mg/l of kinetin singly or in combination. All anthers used for this culture were in an immature stage around uninucleate pollen grain. The results are shown in Table 2. The frequencies of plantlet production in *N. tabacum* and *N. sylvestris* were considerably high, especially *N. tabacum* indicated a very high frequency, nearly 60% on the medium supplemented with 0.1 mg/l of IAA and 0.1 mg/l of kinetin. On the other hand, *N. glutinosa* was found to be quite difficult in producing plantlets although 4 types of media supplemented with low to high concentration of IAA and kinetin

TABLE 2. Anther culture of three *Nicotiana* species

Species	Chromosome number ($2n$)	Medium type*	Number of anther used for culture	Number of anther forming plantlets	% of anther forming plantlets
N. tabacum (var. Wisconsin 38)	48	H-1	120	69	57.50
N. sylvestris	24	H-1	100	22	22.00
		H-3	100	18	18.00
N. glutinosa	24	H-1	275	2	0.73
		H-2	250	0	0
		H-3	250	1	0.40
		H-4	65	1	1.54

* H-1, 0.1 mg/l IAA and 0.1 mg/l kinetin; H-2, 1 mg/l IAA and 1 mg/l kinetin; H-3, 2 mg/l IAA; H-4, 2 mg/l kinetin.

in combinations or singly were tried on this species. Therefore, this result indicates that different degrees of success in the anther culture of Genus *Nicotiana* largely depend upon the different species.

b. *Production of plantlets in three varieties of N. tabacum*

The flower buds were classified into three groups which correspond to three developmental stages of the anthers. The identification of the stages was carried out by a microscopic examination of the meiocytes stained with aceto-carmine. The stages of development of the anthers were found to be related with the length of the flower bud even though some variation was observed among anthers in the same flower buds and among flower buds (Table 3). Therefore, the length of the flower buds were used as arbitrary criteria in the selection of anthers prior to accurate cytological examination of one of five anthers in flower buds. The anthers were

TABLE 3. Relationship between bud length and stage of anther development in var. Wisconsin 38

Bud length (mm)	Developmental stage
~3	Meiosis
4~6	Quartet stage
6~13	Uninucleate stage
13~19	Mitosis
18~27	Early binucleate stage
25~	Mature binucleate stage with starch grains

TABLE 4. Organ formation from anthers of *Nicotiana tabacum*

Variety	Medium type*	Developmental stage of anthers**	Organ formation from anthers***	
			Shoot	Root
Wisconsin 38	H-1	1	+	+
	H-1	2	+	+
	H-1	3	−	−
	H-2	1	+	+
	H-2	2	+****	−
	H-2	3	−	−
	H-3	1	−	−
	H-3	2	−	−
	H-3	3	−	−
	H-4	1	−	−
	H-4	2	−	−
	H-4	3	−	−
Hicks Broadleaf	H-1	1	−	−
	H-1	2	−	−
	H-1	3	−	−
	H-2	1	−	−
	H-2	2	−	−
	H-2	3	−	−
	H-3	1	−	−
	H-3	2	−	−
	H-3	3	−	−
	H-4	1	−	−
	H-4	2	+	+
	H-4	3	−	−
Delhi 34	H-1	1	−	−
	H-1	2	+	+
	H-1	3	−	−
	H-2	1	−	−
	H-2	2	+	+
	H-2	3	−	−
	H-3	1	−	−
	H-3	2	−	−
	H-3	3	−	−
	H-4	1	−	−
	H-4	2	−	−
	H-4	3	−	−

* H-1, 0.1 mg/l IAA; H-2, 0.1 mg/l IAA and 0.1 mg/l kinetin; H-3, 4.0 mg/l IAA and 2.0 mg/l kinetin; H-4, 0.1 mg/l IAA, 0.1 mg/l kinetin and 1.0 mg/l GA.

** 1, Early prophase I to quartet stage of meiosis.
2, Individualized immatured pollen grains.
3, Mature pollen grains with starch grains.

*** −, No formation; +, Formation.

**** Shoot which developed was albino.

classified into three developmental stages as follows.

Stage 1. Early prophase I to the quartet stage of meiosis
2. Individualized immature pollen grains
3. Matured pollen grains accumulating starch grains

Three varieties of *N. tabacum*, var. Wisconsin 38, var. Hicks Broadleaf and var. Delhi 34 were used in this study. Six anthers were planted in each culture bottle on 4 types (H–1∼H–4) of basal medium H supplemented with various concentrations of IAA, kinetin and gibberellic acid (GA) singly or in combinations. After 4 to 6 weeks of incubation, plantlets emerged from inside the anthers of all varieties (Table 4). Some of the regenerated plantlets from var. Wisconsin 38 showed chlorophyll deficiencies. The culture media which were found suitable for the production of plantlets from var. Wisconsin 38 and var. Delhi 34 were H–1 and H–2. The former was supplemented with 0.1 mg/l of IAA, and the latter with both 0.1 mg/l of IAA and kinetin. On the other hand, medium H–4 which was supplemented with 0.1 mg/l of IAA and kinetin, and 1.0 mg/l of GA was found to be suitable for the production of plantlets from anthers of var. Hicks Broadleaf.

It was found that high concentrations of the growth regulators were inhibitory. Medium H–3 supplemented with 4.0 mg/l of IAA and 2.0 mg/l of kinetin completely inhibited the production of plantlets for all of the varieties of *N. tabacum*.

The numbers of plantlets which emerged from an anther varied even in the same variety, and in some cases, as many as 10 to 20 plantlets sprouted per one anther, in others, only 1 to 4 plantlets developed (Plate I-a, b). The anthers from which the earliest plantlets emerged were at developmental stage 2 and contained individualized immature pollen grains. In the case of var. Wisconsin 38, the anthers of developmental stage 1 also produced a few plantlets. However, plantlets from the anthers of developmental stage 1 emerged more than a week later than those from developmental stage 2. None of the anthers of developmental stage 3, which contained complete matured pollen grains, produced plantlets.

Two to three weeks after the proliferation of the plantlets, they produced several leaves and also a well developed root-system. A few plantlets which had chlorophyll deficiencies, however, were very slow in development and did not set forth roots even after two months of continued incubation.

Chromosome number determinations made on preparations from root tips excised from regenerated adult plants showed the plants to be haploid

with 24 somatic chromosomes (Plate II-a). Phenotypic evidence for the haploid condition was the overall smaller statue (about two-thirds the size of the parental plants) and smaller flowers (Plate I-c). The haploid plants were completely sterile in pollen grains (Plate II-c) and did not produce even a single seed, whereas the parental plants had 97.5% pollen fertility in the case of var. Wisconsin 38 (Plate II-d) and normal seed production.

c. *Microscopic observations of the site of origin of embryoid formation*

After six weeks of incubation of the cultured anthers, microscopic observations were carried out to determine if embryoid formation had commenced from the germ cells. These observations were carried out for three varieties of *N. tabacum*. It was observed that multicellular masses had developed from the pollen grains in all three varieties. The most active formations occurred in the pollen grains from anthers of developmental stage 2. Indeed, some of the anther cultures of this stage had multicellular masses forming from as high as 12% of the pollen grains. However, there was considerable variation in the formation of multicellular masses between anthers. Also, in one anther, various stages in the development of embryoids were observed from the initial cell division of the pollen grains to the cotyledon stage (Plate III). The development of the embryoids was observed to be proceeding normally, and several stages of embryogenesis could be clearly discerned, such as globular masses which establish polarity, followed by the "heart shape" stage and the "torpedo" stage. Subsequently, the embryoids germinated and gave rise to complete plantlets with cotyledons. Also, anthers in developmental stage 1 of var. Wisconsin 38 formed multicellular masses from pollen grains and developed into embryoids. The frequency of the latter, however, was lower than anthers in developmental stage 2. On the other hand, anthers of var. Hicks Broadleaf and var. Delhi 34 at developmental stage 1 did not form any multicellular masses and meiocytes in the quartet stage and remained in this same stage even after six weeks of incubation.

Further observations on the anthers of developmental stage 1 of var. Wisconsin 38 revealed that some of them contained pollen grains in a more advanced stage than the quartet stage, up to the early stage of individualized pollen grains which may be caused by incomplete synchronized stage of pollen grains, especially in pollen grains among different anthers in the same flower bud. It was considered that these pollen grains gave rise to the multicellular masses and embryoids, and not the cells of the quartet stage, or earlier stages.

A morphological change which occurred in the pollen grains was an accumulation of numerous starch grains. This was frequently observed in pollen grains within anthers of developmental stage 2. These pollen grains increased several times in size and the cell walls were prominently thicker than those of normal pollen grains (Plate IV-a).

In anthers of developmental stage 3, pollen tubes were observed arising from almost 60% of the pollen grains in all three varieties (Plate IV-b). Some of the pollen grains in anthers of developmental stage 2 continued their development and also developed pollen tubes.

d. *Effect of growth regulators on the initiation of plantlets or calluses*

The medium used in this study was medium H–2 (containing 0.1 mg/l of IAA and 0.1 mg/l of kinetin) which was the most successful in the previous experiment for the induction of haploid plants of var. Wisconsin 38 and var. Delhi 34. Medium H–0 (without supplement of growth regulators) was used as a control. Only var. Wisconsin 38 was used in this study.

Anthers used in this culture were classified according to developmental stages as follows:

Stage 1. Quartets and early individualized uninucleate pollen grains.
2. More mature pollen grains but still uninucleate to early binucleate stage.

TABLE 5. Plantlet formation from anthers of var. Wisconsin 38

Medium type*	Developmental stage of anthers**	Number of anthers used	Number of anthers forming plantlets	Percentage of anthers forming plantlets
H-0	1	18	0	0
	2	13	2	15.4
	3	8	0	0
H-2	1	59	10	16.9
	2	24	8	33.3
	3	22	0	0

* H-0, no growth regulator; H-2, 0.1 mg/l IAA and 0.1 mg/l kinetin.

** 1, Quartets and early individualized uninucleate pollen grains.
2, More mature pollen grains but still uninucleate and early binucleate stage.
3, Complete mature pollen grains with both a vegetative and a generative nucleus and with starch grains.

3. Complete mature pollen grains with a vegetative and a generative nucleus and accumulated starch grains.

After 4 to 6 weeks of incubation, plantlets developed from the anthers. The results are summarized in Table 5. On medium H–2, about 30% of anthers of developmental stage 2 produced plantlets. On the medium H–0, about 15% of the anthers of this same stage also produced plantlets. These results indicated that the growth regulators, IAA and kinetin, apparently promoted the production of plantlets from anthers of developmental stage 2, containing semi-mature uninucleate and early binucleate pollen grains, but that they were not absolutely essential factors. On the other hand, IAA and kinetin appeared to be essential for the anthers of developmental stage 1 (containing quartets and immature individualized uninucleate pollen grains), because about 17% of anthers of this stage produced plantlets on medium H–2, but none of them produced plantlets on medium H–0.

Anthers of the same variety, Wisconsin 38, containing individualized uninucleate pollen grains were planted on the basic medium M supplemented with three growth regulators α-naphthylacetic acid (NAA), IAA and BAP. The results are shown in Table 6. On the medium containing a low level of concentration of growth regulators, 0.1 mg/l of IAA and 0.1 mg/l of BAP, only haploid plantlets were induced directly from pollen grains. On the other hand, both haploid plantlets and calluses were induced on a medium containing a high level of concentration of growth regulators, namely 4.0 mg/l of IAA and 2.0 mg/l of BAP and only calluses were induced on the medium containing 4.0 mg/l of NAA and 2.0 mg/l of BAP. Determination of chromosome number of these calluses all showed a haploid state.

TABLE 6. Shoot and callus formation from anthers containing individualized uninucleate pollen grains of var. Wisconsin 38

Medium type*	Number of anthers used	Number of anthers forming plantlets	Number of anthers forming calluses
M-1	12	2 (16.7)**	0 (0)
M-2	12	1 (8.3)	5 (41.7)
M-3	12	0 (0)	9 (75.0)

* M-1, 0.1 mg/l IAA and 0.1 mg/l BAP; M-2, 4.0 mg/l IAA and 2.0 mg/l BAP; M-3, 4.0 mg/l NAA and 2.0 mg/l BAP.

** Indicates percentage.

Therefore, growth regulators may not be essential factors but are rather promoters for pollen differentiation and embryoid development, and concentrations and types of growth regulator play a determinative role in plantlet formation or callus formation.

e. *Relationship between anther stages and frequency of plantlet production*

Anthers of var. Wisconsin 38 taken from flower buds of different length were cultured on the medium H supplemented with 0.1 mg/l of IAA and 0.1 mg/l of kinetin. In this study at least 10 buds (about 50 anthers) were used for each bud length which was classified by a division of 2 mm unit. Frequency of plant production was shown as percentage of anthers forming plantlets (Fig. 1). The result showed that plantlet formation occurred from some stage of uninucleate stage to early binucleate stage. The most favorable stage for plantlet formation was at the time of first pollen mitosis. The anthers in this stage formed plantlets in 80% of the cases. Late uninucleate stage just before mitosis and early binucleate stage were also considerably favorable for plantlets formation, while the stage from late mitosis to initiation of binucleate pollen grain decreased slightly in their plantlet formation.

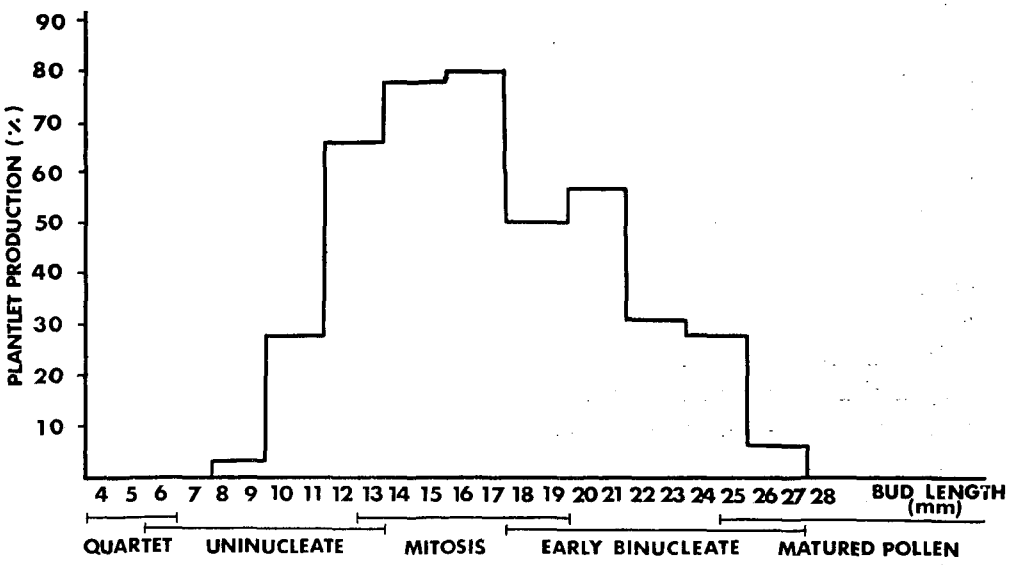

Fig. 1. Relationship between the stage of anther development and frequency in production of plantlets by anther culture.

f. *Critical stages of anthers for the initiation of embryoids*

The media used in this study and anthers classified into three groups were the same as those of the previous section d.

^{3}H-thymidine (sp. act. 3,000 mc/mM) was added to medium H-0 and H-2 at a final concentration of 0.5 μc/ml. Excised anthers of var. Wisconsin 38 were planted aseptically on the medium containing ^{3}H-thymidine and then incubated under illumination from fluorescent tubes and at a temperature of 26±0.5°C. Samples of anthers from each of the developmental stage for the autoradiographic preparations were removed at 48 hours, 1 week, 2 weeks, 3 weeks and 4 weeks after incubation. Anthers were then washed in cold thymidine (40 mg/l) and in several changes of distilled water. They were then fixed with alcohol-acetic acid solution (3:1) for 12 hours. Aceto-carmine was used for staining. A smear method was used for the preparation of slides. However, in order to reduce the risk of loosing specimens from the glass slide, a subbing solution containing gelatin (5.0 g/l) and chrom alum (0.5 g/l) was applied to the slides before they were used (GUDE 1968).

In order to prepare the slides for autoradiography, they were placed on a thermoelectric freezing attachment unit (Komatsu Electronics Inc.) for at least 5 minutes to lower the temperature to −40°C. Then the coverslips were popped off with a razor blade and the slides were left to dry in the air for about 1 hour. The application of liquid emulsion to the slides was carried out in a darkroom under a safe light using a 15 watt bulb and a Wratten Series 2 filter (Eastman Kodak Co.). A Coplin jar containing the liquid emulsion NTB_2 (Eastman Kodak Co.) was placed for approximately 1 hour in a water bath maintained at 42°C to melt the emulsion and to allow all the air bubbles to escape. Two slides placed back to back, or individual slides, were immersed vertically into the liquid emulsion three times within a period of 5 seconds. The dipped slides were then dried for 2 hours at room temperature in complete darkness before being placed into slide boxes. The slide boxes containing the dipped slides were then stored at 4°C in a refrigerator for 10 to 20 days to allow the ^{3}H-thymidine to expose the film. After the exposure, developing was carried out in a darkroom with a Wratten Series 2 filter in a safe light. The slides were immersed in the developer, D-19 (Eastman Kodak Co.) at 20°C for 1 to 3 minutes and then washed in an acid stopper bath (10% of acetic acid) for 5 seconds. They were then transferred to a fixing bath, F-5 (Eastman Kodak Co.) for fixation for about 10 minutes. Subsequently, the slides were washed in running water for about 1 hour

and then air dried. The slides were made permanent by mounting the coverslips on Euparal and then were examined microscopically. From 4 to 6 slides were examined at 10 to 20 days after exposure. The observations are as follows:

Germ cells in the anthers of developmental stage 1

During the first week of incubation, germ cells of this stage did not change morphologically and none of them were labelled with ^{3}H-thymidine (Plate V-a). By the second week of incubation, however, the individualized uninucleate pollen grains increased in size to several times the size of normal pollen grains (Plate V-b). At this time a single nucleus was visible in the pollen grain. Some of these pollen grains were labelled with ^{3}H-thymidine, indicating that DNA synthesis had taken place (Plate V-c). These labelled pollen grains were observed primarily on medium H–2 which contained the growth regulators, IAA and kinetin. On the other hand, morphological changes and DNA synthesis were not observed in germ cells of the quartet stage.

By the third week, the pollen grains, which had undergone DNA synthesis gave rise to globular masses with 5 to 12 cells in a frequency of 1.6% on medium H–2, and 0.6% on medium H–0, of the individualized pollen grains. The cell masses grew into globular embryoids displaying polarity by the fourth week of incubation on medium H–2, but this did not occur on medium H–0.

Germ cells on both media in the quartet stage, however, neither matured into normal pollen, nor differentiated into multicellular masses, even after four weeks of incubation.

Germ cells in the anthers of developmental stage 2

After 48 hours of incubation, using aceto-carmine to stain the nuclei, the generative nucleus, with its characteristic spindle shape, was observed clearly, and the vegetative nucleus was present but not as prominent (Plate V-d). The frequencies of binucleate pollen grains on medium H–0 and H–2 were 49.7% and 70.5%, respectively. Therefore, on the medium H–2 some of the uninucleate pollen grains in the anthers of this stage had underwent the first pollen mitosis and had become binucleate after 48 hours of incubation. This would suggest that 0.1 mg/l of IAA and 0.1 mg/l of kinetin were effective in the promotion of pollen grain mitosis in culture.

An autoradiographic study showed that pollens either uninucleate, or binucleate, were not labelled with ^{3}H-thymidine during the 48 hours of

incubation. This would indicate that the pollen in anthers of this stage had completed their DNA synthesis for the first pollen mitosis prior to incubation.

After 1 week of incubation, the pollen grains increased in size and became prominently vacuolated. Both nuclei were observed in these pollen grains (Plate V-e). At the same time, a few pollen grains which still remained in the uninucleate stage were observed and these were also vacuolated. These vacuolated binucleate and uninucleate pollen grains continued to undergo successive mitosis.

By the second week, globular masses of various numbers of cells were observed on both media H–0 and H–2 and their frequencies were 2.6% and 4.4%, respectively. Their nuclei were densely labelled with ^{3}H-thymidine (Plate V-f). By the fourth week, these cell masses had developed into embryoids, cotyledons, and plantlets.

After three weeks of incubation, a few pollen grains had formed pollen tubes. This suggested that culturing anthers on these media promoted the pollen grains not only to differentiate into embryoids but also in their normal development.

Germ cells in the anthers of developmental stage 3

By the end of the first week, pollen grains in this stage had germinated and pollen tubes into which the generative nucleus had migrated were frequently observed. However, not a single observation was made to indicate any further morphological development such as the formation of embryoids. There was no difference in pollen germination on medium H–0 and H–2.

These results indicate that the critical stage of pollen gained a totipotency for embryoid formation prior to DNA synthesis of the first pollen mitosis but not quartet and the totipotency continues until the early binucleate stage, probably before complete matured pollen grains with starch grains.

B. Anther culture of forage crops

The anthers used in this studies are all immature pollen grains in stages around individualized uninucleate pollen grains. The identification of the developmental stages of the anthers of each species was carried out by a microscopic examination of the meiocytes.

a. *Anther culture in Gramineae*

The mineral salts and organic constituents of the media used in this

TABLE 7. The composition of the media used for anther culture of Gramineae

Basal medium	Subtype	Growth regulator (mg/l)					Sucrose (g/l)
		IAA	NAA	2, 4-D	Kinetin	BAP	
L S	1		1				30
	2		1				60
	3		1				120
	4		1		1		30
	5		1		1		60
	6		1		1		120
	7		2				30
	8		2				60
	9		2				120
M	1	1.5			1.5		30
	2	1.5				1.5	30
	3	1.5	1.5		1.5		30
	4	1.5	1.5			1.5	30
	5	1.5		1.5		1.5	30
	6	2					30
	7	2			2		30
	8	3				1.5	30
	9		1.5		1.5		30
	10		1.5			1.5	30
	11		3				30
	12		3			1.5	30
	13		6				30
	14			2			30
	15			2			60
	16			2			120
	17			3			30
	18			6			30
	19				2		30
H	1	0.1				0.1	20
	2	4				2	20
	3	10			10		30
	4		4			2	20
	5		20				20
	6			20			20

TABLE 8. Anther culture in Gramineae

Materials	Medium used	Number of anthers used	Number of anthers forming calluses	Number of anthers forming plantlets
Bromus inermis				
var. Elsberry	M -5, 8, 12, 13, 18	135	0	0
	H -1, 2, 4	24	0	0
var. Southland	L S-7, 8, 9	90	0	0
Dactylis gromelata				
var. Aries	L S-1, 2, 3, 4, 5, 6	120	0	0
	M -6, 14, 15, 16, 19	80	0	0
var. Germinal	L S-1, 2, 3	75	0	0
cv. Tsukisam-Zairai	M -2, 4, 10, 11, 17	90	0	0
Festuca arundinacea				
var. Festival	L S-1, 2, 3	90	0	0
	M -6, 16, 19	60	0	0
var. Gazelle	L S-1, 2, 3, 7, 8, 9	102	0	0
var. Grombalia	M -2, 4, 10, 11, 17	150	0	0
	H -1, 2, 3*, 4, 5, 6	99	1	0
var. Ludion	M -6, 7, 19	150	0	0
var. Manade	L S-4, 5, 6	45	0	0
	M -6, 14, 15, 16, 19	110	0	0
Festuca pratensis				
var. Daphné	L S-7, 8, 9	90	0	0
var. Naïade	M -1, 3, 9, 11, 17	90	0	0
Lolium hybridum				
(Hybrid rygrass Io)	L S-1, 2, 3	270	0	0
	M -6, 16, 19	30	0	0
	H -3, 5, 6	81	0	0
Lolium multiflorum				
var. Itaque	L S-7, 8, 9	120	0	0
var. Rina	L S-2, 3, 7, 8, 9	170	0	0
	M -1, 3, 9, 11, 17	180	0	0
	H -1, 2, 4	108	0	0
var. Rita	L S-1, 2, 3	90	0	0
var. Tedis	L S-1, 2, 3*	520	7	1
Lolium perenne				
var. Bocage	M -1, 3, 9, 11, 17	90	0	0
var. Primevere	L S-1, 2, 3	165	0	0
	M -6, 16, 19	90	0	0
var. Raidor	L S-1, 2, 3	30	0	0
	M -14, 15	10	0	0
var. Real	L S-3, 7, 8, 9*	150	2	0
Phleum pratense				
var. Norin-Ichigo	L S-3	75	0	0
	M -1, 3, 9, 11, 17*	270	5	0
P. I. 58382	M -5, 8, 12, 13*, 18	267	3	0
P. I. 206909	M -1, 3, 9, 11, 17	135	0	0
Sorghum sudanense				
var. California 23	M -1, 3, 9, 11, 17	135	0	0
	H -1, 2, 4	135	0	0

* Successful medium for callus or plantlet formation.

study are medium LS, medium M and medium H. Various concentrations and combinations of growth regulators used, and sucrose contents are shown in Table 7.

The results of the anther culture are shown in Table 8. Among nine species in Gramineae, four species, *Festuca arundinacea* (var. Grombalia), *Lolium multiflorum* (var. Tedis, autotetraploid), *Lolium perenne* (var. Real) and *Phleum pratense* (var. Norin-Ichigo and P. I. 58382) formed calluses on the cultured anthers after three to four weeks. In *Festuca arundinacea* only 1 of 99 anthers formed a callus on the basic medium H which contained a rather high concentration of growth regulators such as 10 mg/l of IAA and 10 mg/l of kinetin. The growth of the callus, however, was very slow and finaly appeared to cease after two months of continuous incubation. In *Phleum pratense* 5 of 270 anthers of var. Norin-Ichigo and 3 of 267 anthers of P. I. 58382 formed calluses vigorously on the basic medium M which contained considerable high concentrations of growth regulators such as 3 mg/l of 2, 4-D or 6 mg/l of NAA. Microscopic observations of the cultured anthers of this species revealed various developmental stages from pollen grains to embryoids (Plate VI-a, b, c, d, e, f). The pollen grains at the uninucleate stage firstly differentiated into globular masses of multicells and then developed to embryoids. Later, the embryoids developed distinct cotyledon and roots. However, complete plantlets were not developed from the embryoids and all of them differentiated into calluses (Plate VI-g, h). Several root formations were observed on these calluses in continuous incubation. In *Lolium multiflorum* (autotetraploid var. Tedis) formed 7 calluses and 1 very small plantlet of 520 anthers on medium LS which contained an extraordinary high concentration (120 g/l) of sucrose. The small immatured plantlet proliferating from the dehisced anther was immediately transferred to the medium H supplemented with no growth regulator and after sufficient growth of roots the plant was transferred to a pot (Plate VII-a). The obtained plant showed 14 somatic chromosomes which are in a haploid state of autotetraploid in *Lolium multiflorum*. Pollen fertility of the obtained plant was very high 92.2%, while the parent autotetraploid plant showed 70.5% (Plate VII-b, c). This may depend upon the fact that the var. Tedis is an artificially induced autotetraploid and the plant produced by anther culture reverts to the original diploid state ($2n=14$). In *Lolium perenne* (var. Real) 2 calluses from 150 anthers were also obtained only on the medium LS supplemented with a high concentration of sucrose as high as 120 g/l.

TABLE 9. The composition of the media used for anther culture of Leguminosae

Basal medium	Subtype	Growth regulator (mg/l)						Remarks
		IAA	NAA	2, 4-D	Kinetin	BAP	GA	
L S	1	0.1			0.1			
	2	10.0			10.0			
	3	1.5			1.5			myo-inositol was eliminated
M-I	1	1.5			1.5			
	2	1.5			4.0			
	3	4.0			1.5			
	4	1.5		1.5	1.5			
	5	1.5				1.5		
	6	1.5				4.0		
	7	4.0				1.5		
	8	1.5		1.5		1.5		
	9			3.0				
	10		3.0					
	11		1.5			1.5		
	12	1.5	1.5			1.5		
	13			6.0				
	14		6.0					
	15	3.0				1.5		
	16		3.0			1.5		
M-II	1	1.5			1.5			all organic supplements were eliminated
	2	1.5			4.0			do.
	3	4.0			1.5			do.
	4	1.5		1.5	1.5			do.
H-I	1							
	2	0.1						
	3	1.5			1.5			
	4	1.5			4.0			
	5	4.0			1.5			
	6	1.5		1.5	1.5			
	7	0.1				0.1		
	8		4.0			2.0		
	9		20.0					
	10			20.0				
	11	10.0			10.0			
H-II	1							plus 0.5 mg/l each of folic acid and biotin
	2	0.1						do.
	3	1.0						do.
	4	4.0						do.
	5	0.1			0.1			do.
	6	2.0			0.05			do.
	7	0.05			2.0			do.
	8	4.0			2.0			do.
	9	0.1			0.1		1.0	do.
	10	0.1					1.0	do.
	11	0.1				0.1	1.0	do.

TABLE 10. Anther culture of Leguminosae

Species	Medium type	Number of anthers used	Number of anthers forming calluses	Percentage of anthers forming calluses	Organ formation from calluses*	
					Shoot	Root
Lotus corniculatus						
var. Viking	L S -1	84	0	0		
	2	67	38	56.7	−	+
	3	60	12	20.0	−	−
	M-I-1	73	27	37.0	+	−
	2	71	13	18.3	−	−
	3	65	5	7.7	−	−
	4	63	18	28.6	−	+
	5	88	36	40.9	+	−
	6	77	24	31.2	+	−
	7	71	36	50.7	+	−
	8	79	45	57.0	−	−
	M-II-1	78	3	3.8	−	−
	2	66	14	21.2	−	−
	3	63	16	25.4	−	−
	4	67	21	31.3	−	−
	H-II-1	42	0	0		
	2	39	0	0		
	3	42	0	0		
	4	56	0	0		
	5	49	0	0		
	6	49	0	0		
	7	49	0	0		
	10	47	0	0		
	11	47	0	0		
var. Empire	L S -3	45	8	17.8	−	−
	M-I-1	72	11	15.3	−	−
	2	67	25	37.3	+	+
	3	65	10	15.4	−	−
	4	63	21	33.3	−	−
	5	85	30	35.3	+	−
	6	69	16	23.2	+	−
	7	86	15	17.4	+	−
	8	88	33	37.5	−	−
	M-II-1	68	28	41.2	−	−
	2	77	31	40.3	−	−
	3	62	22	35.5	−	−
	4	78	41	52.6	−	−

Species	Medium type	Number of anthers used	Number of anthers forming calluses	Percentage of anthers forming calluses	Organ formation from calluses*	
					Shoot	Root
Lotus corniculatus						
var. Empire	H-II-1	47	0	0		
	2	43	0	0		
	3	56	0	0		
	4	52	0	0		
	5	46	0	0		
	6	47	3	6.4	−	−
	7	59	19	32.2	−	−
	10	43	0	0		
	11	40	6	15.0	−	−
var. crassifolius	M-II-1	67	33	49.3	−	−
	2	73	28	38.4	−	−
	3	71	39	54.9	−	−
	4	81	40	49.4	−	−
B-534	H-I-2	22	0	0		
	3	30	4	13.3	−	−
	4	32	7	21.9	−	−
	5	28	8	28.6	−	−
	6	27	13	48.1	−	−
Lotus caucasicus						
	LS-1	98	2	2.0	−	−
	2	82	12	14.6	−	−
	3	45	6	13.3	−	−
	M-I-1	75	16	21.3	−	−
	2	70	12	17.1	−	−
	3	59	13	22.0	−	−
	4	63	23	36.5	−	−
	5	70	23	32.9	−	−
	6	69	29	42.0	+	−
	7	68	30	44.1	+	−
	8	69	31	44.9	−	−
	M-II-1	69	15	21.7	−	−
	2	85	16	18.8	−	−
	3	74	22	29.7	−	−
	4	60	13	21.7	−	−

Species	Medium type	Number of anthers used	Number of anthers forming calluses	Percentage of anthers forming calluses	Organ formation from calluses*	
					Shoot	Root
Lotus caucasicus						
	H-II-1	42	0	0		
	2	40	0	0		
	3	45	0	0		
	4	46	0	0		
	5	53	0	0		
	6	44	0	0		
	7	53	0	0		
	10	58	0	0		
	11	39	0	0		
Medicago sativa						
var. DuPuit	M-I-5	18	2	11.1	−	−
	8	18	3	16.7	−	−
	9	18	1	5.6	−	−
	10	18	2	11.1	−	−
	11	18	6	33.3	−	−
Medicago sativa						
var. Hunter River	M-I-5	81	1	1.2	−	−
× *M. falcata* (F_1)	6	81	2	2.5	−	−
	7	81	12	14.8	−	−
	H-I-1	27	0	0		
	7	27	0	0		
	8	27	5	18.5	−	−
	9	27	0	0		
	10	27	4	14.8	−	−
	11	27	1	3.7	−	−
Trifolium pratense						
var. Alpilles	M-I-8	9	0	0		
	13	9	0	0		
	14	9	0	0		
	15	9	1	11.1	−	−
	16	9	0	0		
	H-II-9	9	0	0		
	10	9	0	0		
	11	9	0	0		
var. Triel	H-II-9	9	0	0		
	10	9	0	0		
	11	9	0	0		

* +, Formation, −, No formation.

b. *Anther culture in Leguminosae*

The mineral salts of the media used in this study are the same as those used in the anther culture of Gramineae. Various concentrations and combinations of growth regulators, and organic substances used are shown in Table 9.

The results of the anther culture are shown in Table 10.

Anther culture of Lotus species

On medium LS-1 containing a low concentration (0.1 mg/l) of IAA and kinetin, callus formation occurred from none of the *L. corniculatus* (var. Viking) anthers, and from 2% of the *L. caucasicus* anthers. However, on medium LS-2, containing a high concentration (10 mg/l) of IAA and kinetin, the anthers of both *L. corniculatus* and *L. caucasicus* proliferated into brownish calluses during the first 4 weeks of incubation (Plate VIII-a, b). After 4 to 5 months of continuous culture, two to four roots developed from the calluses of *L. corniculatus*, but no shoots were initiated. During the 4 weeks of incubation on medium LS-3, calluses proliferated from the anthers of each species but continued growth was less vigorous. After 2 to 3 weeks of incubation on medium M-I, brownish calluses proliferated from the anthers in each species. The medium subtypes supplemented with BAP were more effective in callus formation and provided increasingly vigorous growth than those supplemented with kinetin. After 3 months of culturing, roots initiated from calluses of *L. corniculatus* (var. Empire) on medium M-I-2 which contained kinetin. Subsequently shoots were formed from these calluses (Plate VIII-c). On medium M-I-1, the calluses of *L. corniculatus* (var. Viking) formed several shoots. Also on media subtypes M-I-5, M-I-6, and M-I-7, all supplemented with BAP, the calluses of *L. corniculatus* (var. Viking and var. Empire) formed a number of shoots after 3 months of incubation, but no root formation occurred (Plate VIII-d). Also, after the calluses of *L. caucasicus* had been cultured on subtypes M-I-6 and M-I-7 for 9 to 10 months a number of shoots were produced. The ability to initiate shoots was greater on media supplemented with BAP than those supplemented with kinetin. On the other hand, BAP did not promote root formation. Medium subtypes containing 2, 4-D suppressed root and shoot formation, although callus growth was extremely vigorous.

When callus formation was first observed on these media, the anthers were stained with aceto-carmine and examined under a microscope to determine if the calluses arose from the germ cells in the cultured anthers.

However, none of the multicellular masses were found to originate from the pollen but rather from the somatic tissue of the anthers. Pollen grains merely hypertrophied and developed thick cell walls.

Thus, with the intention of promoting growth from the germ cells and suppressing growth from the somatic cells of the anthers, the vitamin components were eliminated, which resulted in medium M–II. The frequency of callus formation on this medium was almost the same as on medium M–I. However, callus growth on medium M–II slowed down and finally became necrotic. No roots or shoots developed from any calluses.

Furthermore callus formation occurred from the anthers of *L. corniculatus* (B–534) in medium H–I with the exception of medium H–I–2 supplemented with 0.1 mg/l of IAA only. In continued incubation for 6 months, no root or shoot development occurred from any of the calluses in any of the medium subtypes.

In medium H–II, folic acid and biotin were added to the vitamin components of medium H–I, but no callus formation occurred on subtypes containing 0 to 4.0 mg/l of IAA as the only growth regulator. Subtypes containing IAA plus kinetin or BAP and GA induced calluses from anthers of *L. corniculatus* (var. Empire) but not from those of var. Viking or of *L. caucasicus.*

Microscopic examinations of the anthers growing on the different media indicated that there was still no growth or initiation of cell masses from the germ cells.

Anther culture of Medicago and Trifolium species

In *Medicago sativa* (var. DuPuit) and F_1 of *Medicago sativa* (var. Hunter River) × *M. falcata,* high frequencies of callus formation was generally observed on medium M–I or some subtypes of medium H–I, while in *Trifolium pratense* the induction of the calluses was rare on any media. No shoot and root formation were observed in both species in any calluses. Microscopic observations of the cultured anthers of these species indicated that some of the pollen grains swelled up enormously and occasionally hypertrophied examples were observed bursting from the cell walls. Also, pollen grains were frequently observed with numerous starch grains and thickened cell walls. However, there was no evidence that cell divisions or multicellular formations were initiated from the pollen grains.

Therefore, in the case of Leguminosae, all of the calluses formed by the anther culture were considered to have originated from somatic tissues of the anthers such as connective tissues or filament residues rather than the germ cells.

c. *Relationship between anther stages and feature of pollen in Lotus species*

The media used in this study, classification of the developmental stages of anther and the method of the applications of ^{3}H-thymidine were completely the same as those used in culturing the anthers of *Nicotiana tabacum* in the previous section A-f. Anthers from *Lotus corniculatus* (var. Empire) were used in this experiment. By the fourth week of incubation, the anthers produced neither calluses nor plantlets on either media, namely medium H–0 without supplemental growth regulators and medium H–2 containing 0.1 mg/l of IAA and 0.1 mg/l of kinetin. The detailed results are as follows.

Germ cells in the anthers of developmental stage 1

The quartet cells and the early immature uninucleate pollen grains did not undergo any further development even after four weeks of incubation. Autoradiography did not show any DNA synthesis of the nuclei (Plate IX-a). The pollen, likewise ceased to develop further on both media H–0 and H–2. Some metabolic disturbance of unknown origin caused by removing the anthers from the flower buds at this stage might be responsible for the failure of the pollen to further mature.

Germ cells in the anthers of developmental stage 2

The pollen grains in the anthers of this stage were labelled with ^{3}H-thymidine, indicating that DNA synthesis had occurred (Plate IX-b). The frequency of the labelled pollen grains are given in Fig. 2. Within 48 hours of incubation, 1.5 to 3% of the pollen grains were labelled and the level of this frequency was maintained, or slightly increased, during the four weeks of incubation. The frequency of DNA synthesis on medium H–2 was slightly higher than that of the control. These results indicated that some of the pollen grains in the anthers of developmental stage 2 had undergone DNA synthesis and that DNA synthesis was not prevented from taking place on this culture medium. Furthermore, some pollen grains in which DNA synthesis had occurred underwent mitosis indicating that nuclear division was possible and that they could develop into mature pollen grains (Plate IX-c, d). However, these mature pollen grains neither germinated nor initiated multicellular masses.

Germ cells in the anthers of developmental stage 3

Neither pollen tube development nor the initiation of multicellular masses were observed during the four weeks of incubation of the germ

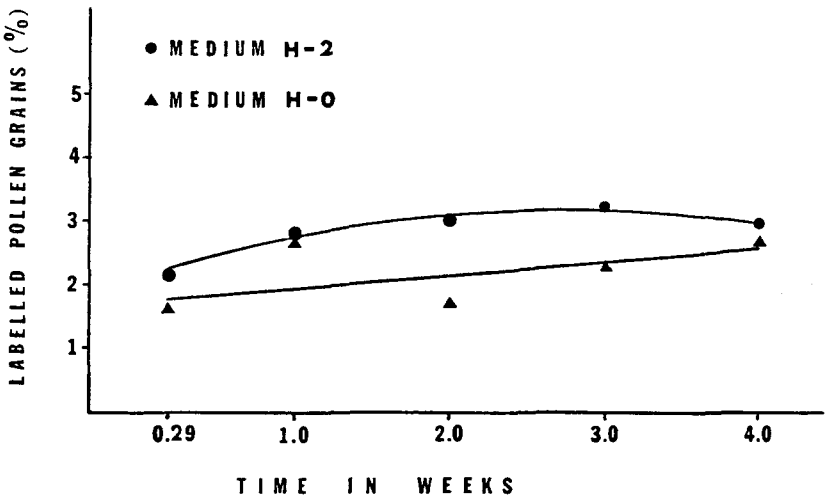

Fig. 2. Percentage of ^{3}H-thymidine labelled pollen grains in anthers of developmental stage 2 of *Lotus corniculatus* (var. Empire).

cells in the anthers of this developmental stage. Only a few pollen grains were observed which were hypertrophied and contained starch grains.

Hence, in conclusion, it may be said that the germ cells in the anthers from *L. corniculatus* in all developmental stages were unable to differentiate into multicellular masses and embryoid forms. In the anthers of developmental stage 2, normal pollen development was clearly observed insofar as DNA synthesis and subsequently nuclear division are concerned. However, there was no sign of any cellular differentiation from the pollen observed during the four weeks of incubation, as was seen in *N. tabacum*.

d. *Pollen grain culture of Lotus species*

From the large number of anthers of *Lotus* species cultured on many different media, as was described in the previous sections, the objective of the study to induce calluses and embryoids from germ cells was not achieved. One reason for the lack of success may be that the somatic tissues of the anthers which readily initiated growth and continued to grow vigorously may have either quickly depleted the nutritional requirements or had some antagonistic action towards the induction of calluses by the germ cells.

In order to rule out any such effects by the somatic cells of the anthers, pollen grains were removed from the anther sacs and directly cultured on the surface of agar-solidified medium. Two *Lotus* species, *L.*

corniculatus (var. Viking and var. Empire) and *L. caucasicus*, were used in this trial. Pollen grains at two stages were used: (1) immature pollen in which the pollen grains were individualized but uninucleate, and (2) mature pollen with a vegetative and a generative nucleus. The pollen was squeezed from the anthers and planted on media M-I-1 to M-I-4. The culture bottles were kept in an incubator without illumination and at a temperature of 26±0.5°C.

After four weeks of incubation, two morphological changes were observed affecting the pollen grains. One change was a hypertrophy of the pollen grain in which an increase from 2 to 4 times its normal size was seen. This condition occurred frequently, especially, in mature pollen grains and occurred on all of the subtype media. A second observation was that the protoplasm of some of the pollen grains which had hypertrophied burst through the cell walls.

Another change which was observed, but only for *L. corniculatus* (var. Viking), was nuclear divisions which occurred mainly in pollen grains which were uninucleate. The nucleus divided into a vegetative and a generative nucleus as in normal pollen development, then, the movement of these 2 nuclei to opposite poles was followed by cytokinesis (Plate X-a). A further division of one of the nuclei occurred which resulted in a 3-celled cluster in which the cells were in tandem within the original pollen cell wall (Plate X-b). The frequency of this type of cell division within the pollen grains, however, was extremely low, being less than 0.5 percent. Advanced stages with more than 3 cells were not observed even after prolonged incubation. These results, however, would indicate that pollen grains of *L. corniculatus* appear to have a totipotency which gives rise to the multicellular condition. These pollen grains were cultured on medium M-I-3 supplemented with 4.0 mg/l of IAA and 1.5 mg/l of kinetin.

At present, it is not clear whether the expression of totipotency to form a multicellular mass from the cultured pollen grains is due to some release from the suppression of growth by somatic cells of the anthers or whether some other factor is responsible. Nevertheless, it may be concluded that the cultured pollen grains had aquired some unknown factor which appears to be lacking when the anthers possessing the pollen grains were cultured. This factor is effective at least in the initiation of the multicellular condition, although it is not sufficiently effective for the continued development into a callus or an embryoid.

e. *Regeneration of Lotus plants from shoots*

Transplantation of shoots onto new media

When shoot formation occurred from the primary calluses on medium M–I, the shoots were transplanted onto a series of new media (Table 11). The shoots were cut off from the primary calluses with the aid of a dissecting knife and then transplanted to the culture media by inserting 2 to 3 mm of the basal part of shoot into the medium. Generally, after 2 to 4 weeks of subculture, tap roots appeared from around the basal part of the shoots.

Media with no additional growth regulators or which contained low concentrations of growth regulators such as 0.1 mg/l of IAA and 0.1 mg/l of kinetin were considerably more suitable for root regeneration and developed good root-systems (Table 12). At concentrations of 2.0 mg/l of IAA, or 2.0 mg/l of kinetin, root initiation appeared to be suppressed and even if a few roots were formed, the later development of the root-system was very poor. The addition of 1.0 mg/l of 2, 4–D in combination with 1.0 mg/l of IAA and 1.0 mg/l of kinetin completely inhibited root development, but regrowth of the calluses occurred around the basal part of the shoots.

Young seedlings with well developed root-systems were transplanted to pots and grown in a growth chamber. In this manner, 4 mature plants of *L. corniculatus* (var. Viking), 36 of *L. corniculatus* (var. Empire), 2 of *L. caucasicus* were obtained successfully.

Chromosome numbers of regenerated plants

About 5 root tips were collected from each regenerated plant for each species and the chromosome numbers were determined from at least 5 good metaphase figures. The results are given in Table 12.

TABLE 11. The composition of the media used for the root differentiation

Basal medium	Subtype	Growth regulator (mg/l)		
		IAA	Kinetin	2,4-D
M-III	1	–	–	–
	2	0.1	0.1	–
	3	2.0	0.05	–
	4	0.05	2.0	–
	5	1.0	1.0	1.0

TABLE 12. Regeneration of *Lotus* plants from shoots on subculture

Species	Medium type of primary culture	Medium type of subculture	Number of regenerated plants with 24 chromosomes	Number of regenerated plants with 48 chromosomes	Chromosome numbers of original callus
L. corniculatus					
var. Viking	M-I-1	M-III-1	1	0	not examined
		M-III-2	2	0	
		M-III-3	0	0	
		M-III-4	0	0	
		M-III-5	0	0	
	M-I-5	M-III-1	1	0	not examined
		M-III-2	0	0	
		M-III-3	0	0	
		M-III-4	0	0	
		M-III-5	0	0	
	M-I-6	M-III-1	0	0	not examined
		M-III-2	0	0	
		M-III-3	0	0	
		M-III-4	0	0	
		M-III-5	0	0	
var. Empire	M-I-2	M-III-1	5	0	15-88
		M-III-2	1	0	
		M-III-3	1	0	
		M-III-4	2	2	
		M-III-5	0	0	
	M-I-5	M-III-1	10	0	24
		M-III-2	7	0	
		M-III-3	1	0	
		M-III-4	0	0	
		M-III-5	0	0	
	M-I-7	M-III-1	5	0	24 and 48
		M-III-2	2	0	
		M-III-3	0	0	
		M-III-4	0	0	
		M-III-5	0	0	
L. caucasicus	M-I-7	M-III-1	2	0	not examined
		M-III-2	0	0	
		M-III-3	0	0	
		M-III-4	0	0	
		M-III-5	0	0	

All four regenerated plants of *L. corniculatus* (var. Viking) had 24 somatic chromosomes, the normal diploid chromosome number. The average pollen fertility of these plants was 98.0%. These results indicated that there was no change in the chromosome constitution of the regenerated plants. In the case of *L. corniculatus* (var. Empire), two of the regenerated plants from the total of 36 had 48 somatic chromosomes, the tetraploid chromosome number, whereas, all the others had 24 (Plate XI-a, b, c). The average pollen fertility of the plants with 24 chromosomes was 97.6%, whereas, the pollen fertility of the plants with 48 chromosomes was 63.1% (Plate XI-d, e). Two regenerated plants of *L. caucasicus* also had 24 chromosomes and their average pollen fertility of 98.0% was likewise high.

f. *Chromosome numbers of callus cells*

Six month old callus tissues of *L. corniculatus* (var. Empire) which had given rise to shoots on primary cultures on medium M–I, were investigated for their chromosome numbers. Since the frequency of mitotic metaphases of these callus cells was extremely low, callus tissue was transferred onto medium M–I–4 containing 1.5 mg/l of IAA, 1.5 mg/l of kinetin and 1.5 mg/l of 2, 4–D to promote cell division of the callus tissue. Mitotic activity of the callus cells increased during the first week of subculture and a sufficient number of cells with metaphase figures were obtained by which to determine the chromosome numbers. In total, figures in 20–60 cells were examined for var. Empire. The results are given in Table 12. Three types of chromosome behaviour in the callus tissues were observed as follows: (1) The callus tissue did not vary in chromosome number and consisted only of cells with 24 somatic chromosomes. The callus tissues derived from anthers cultivated on medium M–I–5 had 24 somatic chromosomes in all the cells examined. In addition, there was no variation from the 24 chromosomes observed in any of the regenerated plants which arose from these calluses. (2) The callus tissue consisted of two kinds of cells, with 24 and 48 somatic chromosomes, respectively. The callus tissue derived from anthers cultured on medium M–I–7 was of this type. In this callus, 43.9% of cells had tetraploid chromosome number of 48, the remainder had 24 somatic chromosomes, the diploid chromosome number. However, unexpectedly, all seven of the regenerated plants grown from this callus tissue had only 24 somatic chromosomes. (3) In this third type, the callus tissue consisted of cells with different numbers of chromosomes including both euploid cells and those with different de-

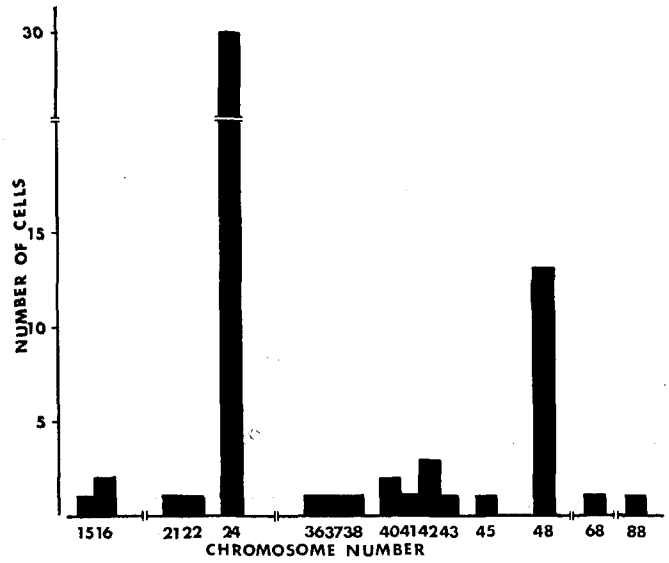

Fig. 3. Chromosome numbers found in callus tissues of *Lotus corniculatus* (var. Empire) derived from cultured anthers on medium M-I-2.

grees of aneuploidy. The callus derived from anthers cultured on medium M-I-2 belonged to this type and the chromosome numbers ranged from 15 to 88 (Plate XII). There were two peaks in the distribution of the chromosome numbers, at 24 and 48, with a frequency of 48.5% and 21.0%, respectively (Fig. 3). All other chromosome numbers were considered aneuploids. Of the 36 plants which developed from the calluses, two had 48 somatic chromosomes and 34 had a somatic chromosome number of 24. None of the regenerated plants were aneuploids.

It is possible that the variation in chromosome numbers arose in some manner, during the subculturing of the callus tissue while inducing nuclear divisions for chromosome number determinations. However, it is considered that the tetraploid chromosome numbers observed existed at least in the cells of the callus prior to subculturing since plants with 48 chromosomes were regenerated from the primary callus.

There was a considerable range in chromosome numbers found in the callus lines from different primary cultures, even though they had originated from the same genotype. The factors responsible for causing the variation in chromosome numbers will be disscussed in the next chapter.

(3) Discussion

A. Anther culture of *Nicotiana* species

a. *Different intensity of plantlet formation in Nicotiana species and varieties*

The frequencies of plantlet formation in *N. tabacum* and *N. sylvestris* were considerably high, especially *N. tabacum* indicated nearly 60% of plantlet formation. On the other hand, *N. glutinosa* showed a considerable difficulty in the production of haploid plantlets even on the four types of media tested for the anther culture. NITSCH (1970, 1972) succeeded in the induction of haploid plants of these three *Nicotiana* species and NAKAMURA and ITAGAKI (1973) also succeeded in *N. tabacum* and *N. glutinosa.* Their results also indicated species difference in haploid plantlet production, namely *N. glutinosa* showed lower success than the other species investigated. One may wonder why the results were different in different species. NITSCH (1970) gave the speculation of the causativeness in which *N. tabacum* is presumably an amphidiploid between *N. sylvestris* and *N. otophora* (GOODSPEED 1954). From his results obtained with *N. glutinosa* in which plantlets were obtained in higher numbers from tetraploid anthers than diploid anthers, he suggested that polyploid pollen may yield viable embryos more readily than diploid pollen. Furthermore from his observation in *N. alata,* the self-incompatible species, which presumably contains many lethal genes, gives rise to a limited number of poorly-growing haploid plantlets. In any case, true haploids have been obtained with *N. sylvestris,* self-compatible species, in considerable high frequencies, but not so high as *N. tabacum.* This species which possibly contains less lethal genes. Furthermore, the anther culture of the varieties of *N. tabacum* (vars. Wisconsin 38, Delhi 34 and Hicks Broadleaf) in this study indicated that the three varieties required different growth regulator conditions for the suitable haploid plantlet production. Therefore, it can be assumed that the capacity to form haploid embryoids probably depends upon the genetic conditions such as ploidy level or autogamous and allogamous of the species, and small differences of so-called genotype in relation to its nutritional requirement.

b. *Culture media for anther culture*

Basal culture media usually consists of the same ingredients as used in the culture of somatic tissues. They are mainly several media set forth

by BOURGIN and NITSCH (1967), MURASHIGE and SKOOG (1962), MILLER (1963) and LINSMAIER and SKOOG (1965).

The induction of haploid tobacco plants [*Nicotiana tabacum* (vars. Wisconsin 38, Delhi 34 and Hicks Broadleaf)], from cultured anthers was successful on the basic medium described by BOURGIN and NITSCH (1967). The growth regulators, 0.1 mg/l of IAA alone, 0.1 mg/l of IAA and 0.1 mg/l of kinetin in combination, and 0.1 mg/l of IAA, 0.1 mg/l of kinetin and 1.0 mg/l of GA in combination, were suitable for the induction of plantlets from the cultured anthers. High concentrations of growth regulators, 4.0 mg/l of IAA and 2.0 mg/l of kinetin, were inhibitory. NITSCH and NITSCH (1969) reported that they found the optimal concentration of IAA was 0.1 mg/l, and that 0.2 mg/l of kinetin was generally inhibitory. However, it was found in the present study that high concentrations of growth regulators which were supplied to basic media other than that of BOURGIN and NITSCH (1967) were not inhibitory. When 4.0 mg/l of IAA and 2.0 mg/l of BAP were supplied in the basic medium described by MILLER (1963), the cultured anthers of *N. tabacum* (var. Wisconsin 38) produced the plantlets. In these cases, besides haploid plantlets some anthers produced whitish calluses. When 4.0 mg/l of IAA was substituted by 4.0 mg/l of NAA all anthers produced calluses which were determined as haploid calluses by chromosome counting. NAKATA and TANAKA (1968) also succeeded in producing plantlets from anthers of *N. tabacum* (var. Bright Yellow) on the modified RM–1964 medium of LINSMAIER and SKOOG (1965) containing a rather high concentration of growth regulators such as 2.0 mg/l of IAA and 4.0 mg/l of kinetin. SUNDERLAND (1974) also reported that embryoid formation of anthers of *Paeonia hybrida* showed greatly different frequencies on eight media differing only in the basic media, sucrose and vitamins. Therefore, these results suggest that the concentration of exogenous growth regulators supplied to the medium is not the only decisive factor for the production of plantlets and calluses from cultured anthers of *N. tabacum*. The interaction between growth regulators and different types of basic medium also appeared to play an important role for the production of plantlets.

Although the growth regulators, 0.1 mg/l of IAA and 0.1 mg/l of kinetin when present in the medium are considered to have promoted the initial increase in pollen grain size and the subsequent multicellular condition and embryoid formation, they were not absolutely essetial since individualized pollen grains at the late uninucleate to early binucleate stage could give rise to complete plantlets on media lacking growth regulators

while pollen grains at the early uninucleate stage could not form entire embryoids. Other investigators also obtained the same results in which haploid plantlets growth occurs in the absence of exogenous growth regulalors (NITSCH and NITSCH 1969, NITSCH 1970). Furthermore, an exogenous supply of a growth regulator was not always essential in *Datura innoxia* to trigger the development of pollen in the direction of embryoid formation (SOPORY and MAHESHWARI 1972). All these results seem to indicate that the exogenous growth regulators themselves do not function as a trigger for differentiation from the pollen but act as a promotor of the multicellular condition and of embryoid development. In anther cultures of *Nicotiana* and *Datura*, therefore, some complexity can be assumed to exist in the triggering mechanism for differentiation which may be regulated by the exogenous supply of some unknown elements in the anther culture.

c. *Anther stages for plantlet formation*

In addition to the component of the medium, the stage in microsporogenesis at which the pollen is inoculated may be critical for the anther culture.

The results of this study showed that plantlet formation can be induced in tobacco anthers of bud length between 9 mm to 27 mm in which the pollen grains underwent from midle uninucleate to early binucleate stage. The pollen stage at which plantlets were most readily induced was at the time of the first pollen mitosis. This result agreed with study on tobacco by SUNDERLAND and WICKS (1971) and SUNDERLAND (1971, 1973b).

A more detailed investigation carried out with the aid of autoradiography using ^{3}H-thymidine showed that the earliest totipotency gained to differentiate from normal pollen to the embryoid was after the quartet stage and before DNA synthesis of the first pollen mitosis. Differentiation was initially accompanied by an increase in pollen grain size and vacuolization. SUNDERLAND and WICKS (1969a, b) also reported the occurrence of vacuolization of the pollen grains after the first microspore mitosis and before multicellular formation.

Plantlet formation also arose from binucleate pollen grains. If this is so the question inevitably arises as to how long binucleate pollen grains can remain susceptible to the induction properties of the culture treatment. NITSCH *et al.* (1968) and SUNDERLAND and WICKS (1971) stated that the latest stage for ensuring plantlet formation occurs just before starch is deposited in the grains of tobacco. This experiment showed, however,

that plantlet formation occasionally appeared to occur just after the initiation of starch accumulation. Generally in all instances where starch was formed, either before or after the first mitosis in the culture, further development was consistently prevented. Therefore, the results of this experiment might have arisen by the variation of anther stage in the same size of flower bud. SUNDERLAND and WICKS (1971) also reported a slight difference of anther stage in the same length of petal while some anthers mainly showed quartet, and others, mainly free microspores.

There are, however, some discrepancies in the anther stages for embryoid formation. NAKATA and TANAKA (1968), by using different varieties of *N. tabacum* and different culture media, successfully raised plants from anthers cultured when the pollen were mainly at the quartet stage. GRESSHOFF and DOY (1972a, b) reported that meiotic stages are essential for the induction of haploid callus in *Arabidopsis thaliana* and *Lycopersicon esculentum*. In *Brassica oleracea*, anthers taken from mature buds give rise to pollen callus (KAMEYA and HINATA 1970). The work on gymnosperm pollens also indicates that the pollen can be triggered into callus growth when the grains are as far advanced in microsporogenesis as the 4- or 5-celled stage (TULECK 1953, 1957). Therefore, these results obtained so far suggest that the critical stage may vary from species to species, and possibly from variety to variety; it may also be influenced by the culture conditions and the environment under which the donor plants are grown.

d. *Mechanism of plantlet induction*

Initial pattern of the pollen into embryoids in *Nicotiana tabacum* was represented by SUNDERLAND (1973b) and SUNDERLAND and DUNWELL (1974). There were mainly two types. The first was that the embryoid is formed from the vegetative cell. The cell divides repeatedly within the framwork of the exine to form a multicellular mass. During the early stages, divisions follow each other rapidly and as many as twenty to thirty cells come to occupy the volume of the mother cell. With continued division, the exine ruptures and the liberated embryoid continues its development (SUNDERLAND and WICKS 1971). *Hordeum* follows a similar pattern except that, after rupture of the exine, organized growth breaks down and a callus is formed (CLAPHAM 1971). The generative cell may also divide, but the number of divisions is limited. The few cells formed do not contribute to the future embryoid or callus; they are probably lost when the exine ruptures. On *Hordeum* multicellular grains are formed with up to six generative daughter cells (CLAPHAM 1971), and in *Nicotiana tabacum*,

with up to four (SUNDERLAND and WICKS 1971). Similar degeneration of the generative cell was also reported in *Datura metel* (IYER and RAINA 1972), *Triticum aestivum* (WANG *et al.* 1973a) and *Triticale* (WANG *et al.* 1973b). The second type of embryoid initiation was that in which certain microspores may be diverted into an irregular mitosis which results in the formation of two equal diffuse nuclei instead of the usual condensed generative and diffuse vegetative types. This type of grain was most frequently observed in the anthers cultured at the beginning of individualized uninucleate stage in *Datura innoxia* (NITSCH and NORREEL 1973, SUNDERLAND *et al.* 1974). *Triticum aestivum* (WANG *et al.* 1973a) and *Tritical* (WANG *et al.* 1973b).

The initiation of pollen differentiation into embryoid does not start immediately after inoculation on the culture medium. In *Nicotiana tabacum* of this experiments there was a lag of two to three weeks in the early uninucleate pollen grains (before DNA synthesis of the first pollen mitosis) and of one to two weeks in the late uninucleate (after DNA synthesis of the first pollen mitosis) to early binucleate pollen grains. The uninucleate to early binucleate pollen grains differentiated into embryoid, increased in size and became prominently vacuolated during the lag period. This lag period may be very important as a inductive period during which the triggering mechanism comes into operation. By the culture of anthers at the stage of the first pollen mitosis of *Nicotiana tabacum* SUNDERLAND and WICKS (1969a, b, 1971) observed that after a lag period mitosis commences in the normally quiescent vegetative cell and during the lag period the cytoplasm of the vegetative cell gradually loses its affinity to stains such as aceto-carmine, and as the cell enters mitosis the staining of the cytoplasm becomes decidedly weak. In contrast, non-embryogenic pollen in the same anther shows a dense staining. Subsequently, BHOJWANI *et al.* (1973) observed that by removal of the anther from the plant at the stage of the first pollen mitosis and by placing it in contact with a simple nutrient medium the pollen responds in one of two ways; it accumulates either relatively large amounts of RNA and protein or apparently none at all. In the former situation the grains are non-embryogenic, and in the latter, embryogenic. The amount of RNA and protein in non-embryogenic grains are in fact similar to those attained *in vivo* at anthesis but in the giant class of non-embryogenic grains, which appeared occasionally, the amount is considerably in excess of those produced *in vivo*, sometimes as much as two- to three-fold. In contrast, the embryogenic class is one in which the gametophytic program is suppressed and replaced by a new

program concerned with sporophytic differentiation, and this is a change which must also be related in some way to the changed environment of the pollen. The switch of program followed by the degradation of RNA and protein occur at a certain period prior to mitosis of the vegetative cell in embryogenic grains.

BENNETT and HUGHES (1972) found that a chemical, ethrel, known to be a substance which induces male sterility, gives rise frequently to additional nuclear divisions in pollen grains of *Triticum aestivum* (var. Chinese Spring). Anthers from heads estimated to be at, or after meiosis at the time of ethrel treatment completed normal pollen development and the anthers dehisced at the expected time. Anthers from heads estimated to be from one to five days in the premeiosis at the time of ethrel treatment exhibited normal meiosis and early pollen development up to and including the first pollen grain mitosis. Abnormal development first appeared just before second pollen grain mitosis. The vegetative and generative nuclear products of the first pollen grain mitosis differentiated normally. About 24 hours before the second pollen grain mitosis would normally occur, however, later both nuclei divided in many cases synchronously. Examination of pollen grains with more than three nuclei showed that none subsequently elongated to the shape of sperm nuclei. At times pollen grains containing up to 8 nuclei were observed in anthers which had undergone meiosis 10–12 days previously. Ethrel is known to break down in plant cells at pH below 4.0 releasing ethylene, a molecule widely acknowledged to have hormonal properties in plants. Hormones are thought to act sometimes by directly affecting gene transcription. A possible explanation of the effect of ethrel is therefore that it alters the production of long-living messenger RNA transcribed before meiosis but it is necessary for normal pollen development several days later.

A common feature of the anther culture and ethrel treatment is that the pollen grains are switched from the normal microsporogenesis to mitotic nuclear division, while the time of the occurrence of nuclear division is somewhat different between them. In the case of anther culture the physical stimulation to the anthers disected from the plant or some change of endogenous hormones may disturb the normal microsporogenesis through RNA and protein degradation. Indeed, in *Nicotiana tabacum* and *Datura innoxia* in the anther culture exogenous growth regulators are not essential for the triggering mechanism but probably act as promoters of embryoid development. Therefore, it can be postulated that some RNA such as long-living messenger RNA transcribed at the stage of mitosis of

pollen mother cells and possessing a roll of normal microspore development may be inactivated or lost by removing the anthers from plant and planting in proper culture medium. The pollens which lost such RNA required for operation of microsporogenesis, lack the ability of protein synthesis required for pollen development and thus return to the mitotic cell cycle again which results in pollen differentiation into embryoids or calluses. If it could be actually considered that the most important triggering key is the loss of some RNA required for normal microspore development in the anther culture, the sporophytic course may switch on the mitosis and give rise to embryoids or calluses during various stages of anther culture, sometimes at meiosis or matured pollen stages observed in several species by the proper culture conditions.

B. Anther culture of forage crops

a. *Anther culture in Gramineae*

With species of Gramineae, anther culture has been less successful than with in Solanaceae. NIIZEKI and OONO (1968), and GUHA, *et al.* (1970) obtained haploid plants from *Oryza sativa*, BAN *et al.* (1971) from *Setaria italica*, OUYANG *et al.* (1973) and WANG *et al.* (1973a) from *Triticum aestivam*, WANG *et al.* (1973b) from *Triticale* and CLAPHAM (1971) from *Hordeum vulgare*. All of these species produced haploid calluses predominatingly and thereafter haploid plants were obtained from the calluses.

In forage crops NITZSCHE (1970) reported the production of polyhaploids from *Festuca-Lolium* hybrids, CLAPHAM (1971) obtained albino plants from the pollen of *Lolium multiflorum* and SAITO *et al.* (1973) obtained haploid plants from *Bromus inermis.* In this study, four species, namely *Festuca arundinacea*, *Lolium multiflorum*, *Lolium perenne* and *Phleum pratense*, formed a few calluses which were considered to have originated from germ cells. The process of embryoid formation in the anther culture of *Nicotiana* species was similar to that of normal embryogenesis. The embryoids formed in the anthers germinated and gave rise to complete plantlets. In contrast to *Nicotiana* species, in the present study on *Phleum pratense* the embryoids from the pollen grains differentiated into the calluses instead of germinating into complete plantlets. My previous study on *Nicotiana tabacum* indicated that the difference between the induction of plantlets or calluses from the pollen grains depends upon the difference of growth regulators in the medium. Indeed, in *Lolium multiflorum* when the anther in which the incomplete germinated embryoid

formed was transferred onto the medium without any growth regulators, the callus initiation from the embryoid was inhibited and germinated a complete plantlet. GUHA *et al.* (1970) and GUHA-MUKHERJEE (1973) also succeeded in the production of haploid rice plants directly from the embryoid by using a transplantation technique in which the embryoids were dissected from the anthers onto a fresh medium without auxin. WANG *et al.* (1973a) also reported that one of the anthers of *Triticum aestivum*, which predominantly produces calluses in general, inoculated onto a medium containing 20% of coconut milk or 2.0 mg/l of IAA and 2.0 mg/l of kinetin produced complete haploid plantlets. Therefore, it can be assumed that in *Oryza*, *Triticum* and *Lolium* the initiation of differentiation of pollen grains from microsporogenesis does not give rise to callus formation but at first produces embryoid formation as observed in *Phleum pratense* in this study. Thus, it is likely in the future that improved techniques of anther culture such as early transplantation of anther involving the embryoid or introduction of a suitable media which would provide the basis for direct plantlet formation will enable workers to produce haploid plants in sufficient amounts for practical use in these species.

Sucrose concentration in the media is generally 2 to 3% for successful anther culture. *Lolium multiflorum* and *Lolium perenne*, however, required a very high concentration of sucrose such as 12% for induction of haploid calluses. CLAPHAM (1971 and 1973) used also 12 to 15% of sucrose in their culture of *Lolium multiflorum* and *Hordeum vulgare*. OUYANG *et al.* (1973) recommended 6% of sucrose for *Triticum aestivum*, at which concentration there is an enhancement of pollen callus formation and an inhibition of somatic callus formation. However, still higher concentration of sucrose such as 10 to 15%, while promoting pollen callus formation in tomato, have a deleterious morphogenetic effects in tobacco (SHARP *et al.* 1971a).

Suitable concentrations of sucrose are different among different species. CLAPHAM (1973) made an attempt to determine whether the favourable effect of high sucrose concentration in *Hordeum* was osmotic; if so, a neutral substance, such as polyethylene glycol together with 3% sucrose as a carbon source, would give a high rate of callus formation. However, this medium was unsuccessful. This result suggests that the pollen generally requires a high concentration of sucrose itself rather than a medium with a high osmotic pressure, while the precise reason is still unknown.

b. *Anther culture in Leguminosae*

In contrast to Gramineae *Lotus* species and *Medicago sativa* belonging

to the Leguminosae formed calluses at high frequencies. However, it appeared that all calluses of the species in the Leguminosae probably originate from the somatic tissues rather than the germ cells.

The regenerated *Lotus* plants which developed from the callus tissues in this study were plants with the same diploid chromosome number as the parents with the exception of a few plants which werc tetraploids. Haploid plants, however, were not obtained. In the case of the chromosome number determinations carried out on the callus tissues of *Lotus corniculatus*, no cells were observed with a haploid chromosome number, whereas cells with diploid, tetraploid and various aneuploid chromosome numbers were observed. Two factors related to the formation of the callus tissues may be postulated as reasons for the failure to find haploid cells: (1) The calluses originated from somatic tissues of the anthers rather than from germ cells, or (2) The calluses originated from germ cells but polyploidization followed, and chromosome irregularities developed during the period of incubation. However, the possibility of calluses originating from the germ cells of the *Lotus* species may be ruled out from the observations which were carried out directly on the germ cells in the cultured anthers. No growth initiated from the pollen and the only morphological changes observed were hypertrophied pollen grains with thicker cells walls and some pollens filled with starch grains. Sucn changes were primarily observed in immature uninucleate pollen and mature pollen grains. Thus, there was no evidence to indicate that the germ cells had undergone division to produce multicellular masses as was observed in the cultured anthers of the *Nicotiana* species. Therefore, it may be stated with considerable certainty that the proliferation of callus tissues from anthers of the *Lotus* species was from somatic tissues rather than from germ cells. In one other study, KONOR and NATARAJA (1965a) reported the proliferation of callus tissues from somatic tissue in cultured anthers of *Ranunculus sceleratus*. They observed that the calluses originated from connective tissue cells of the anthers and not from the pollen grains. Furthermore, SAUNDERS and BINGHAM (1972) and WATANABE *et al.* (1972) also obtained calluses from connective tissue cells of the anthers and not from the pollen grains in *Medicago sativa* and several *Chrysanthemum* species, respectively. Similarly, it may be assumed that the callus tissues of the *Lotus* species originated from such cells, however, it is not certain what somatic tissue gave rise to the calluses since histological analysis on callus development were not performed.

The autoradiographic study of the cultured anthers of *L. corniculatus*

(var. Empire) using ^{3}H-thymidine provided precise information on the time of development of the germ cells within the anthers. Quartets and the early immature individualized uninucleate pollen grains were not labelled with ^{3}H-thymidine even after four weeks of incubation. Their further development beyound this stage appeared to be entirely blocked after the anthers were excised from the flower buds. On the other hand, a number of the individualized pollen grains at the late uninucleate stage, probably just prior to the nuclear division of the first pollen mitosis, were labelled with ^{3}H-thymidine indicating that DNA synthesis had occurred in nucleus. Subsequently, the nuclei underwent division and formed pollen grains with a vegetative and generative nucleus. The autoradiographic studies with *Lilium longiflorum* by TAYLOR and MCMASTER (1954) and with *Tradescantia paludosa* by MOSES and TAYLOR (1955) indicated that DNA synthesis in the course of normal pollen development takes place at three specific stages: (1) At a preleptotene stage before meiotic prophase, (2) at late interphase preceding mitosis in the microspore and (3) during early to mid-interphase in the generative nucleus of the pollen grain. In the cultured anthers of the *Lotus* species, the individualized pollen grains at the late uninucleate stage were able to synthesize DNA and develop to advanced stages. Presumably, they underwent the same course of normal pollen development as was observed in *Lilium* and *Tradescantia* (TAYLOR and MCMASTER 1954, MOSES and TAYLOR 1955). However, the factor which is required for differentiation beyond this normal pollen development, and which induces cell division and the formation of multicellular masses, was apparently lacking from the media in the case of the *Lotus* anthers.

In several studies, successful inductions of calluses from pollen grain cultures have been reported for several species, namely, *Ginkgo biloba* (TULECKE 1953, 1957), *Torreya nucifera* (TULECKE and SEHGAL 1963), *Ephedra foliata* (KONAR 1963), *Brassica oleracea* (KAMEYA and HINATA 1970), *Petunia hybrida* (BINDING 1972) and *Lycopersicon esculentum* (SHARP *et al.* 1972). In this study on the pollen grain culture of *Lotus* species, cell division was initiated and subsequently 2 to 3 celled pollen grains were formed when the pollen was cultured on agar-solidified medium which contained Miller's basic medium, and IAA and kinetin. This would suggest that the totipotency for the continuation of cell division of the pollen could be provided by supplying the missing component, or components, by pollen culture. Some of the factors involved might be simply the suppression of some nutritional requirement caused by the initiation

and vigorous growth of the somatic callus or the disappearance of some inhibitor which exists in the anthers. However, it can not be concluded as to which factor or factors are responsible for the continuation of cell division in the pollen grain cultures. At the present time at least, the initiation of cell division in the pollen grains of the *Lotus* species would indicate that it would be possible for the pollen to develop further into more complex tissues, namely, calluses and embryoids, and finally into complete haploid plants, if the proper medium and cultural conditions were supplied.

2. Production of polyploid cells and plants by callus culture of *Nicotiana* species

(1) Materials and Methods

Haploid plants of three species, *N. tabacum*, var. Wisconsin 38 ($2n=48$), *N. sylvestris* ($2n=24$) and *N. glutinosa* ($2n=24$) were induced from anther culture in the previous chapter of this paper. Calluses of these three species of haploid plants and their parental diploid plants were induced from young stem cultures. About 3–4 mm of young stems were disected from the 3–4 cm of plantlets derived by anther culture and of seedlings, which germinated aseptically on medium without any growth regulators, which were cultured on the basic medium of MILLER (1963) supplemented with 2.0 mg/l of IAA and 2.0 mg/l of kinetin. About 3 to 4 weeks after culture, whitish calluses proliferated from the stems. These obtained calluses were thereafter subjected to subcultures at intervals of about one month on the same medium which was used for the induction of these calluses. Furthermore, haploid calluses of *N. tabacum* (var. Wisconsin 38) were directly induced on a medium containing high concentration of NAA and BAP, which was reported in the previous chapter, this was transplanted to a series of Miller's basic media containing or not containing the growth regulators as follows ; no growth regulator (medium M–1), 4.0 mg/l of kinetin (medium M–2), 4.0 mg/l of IAA (medium M–3) and 2.0 mg/l of kinetin and 2.0 mg/l of IAA (medium M–4). At intervals of one month of subculture, the calluses cultured on medium M–4 were successively transplanted to the same series of media as above. 100 ml of flask was used as the container for all of the callus cultures. All cultures were kept under dark condition at 26 ± 0.5°C.

Cytological observations of the callus tissues performed are the same as described in chapter 1.

After one month of the 8th subculture of *Nicotiana tabacum* on the

four types of medium, all callus cultures were transferred into light condition at 26±0.5°C. After 10 to 20 days of sustained culture, shoots or shoots primordia developed from the calluses. The regenerated shoots on each medium were severed from the calluses and then transplanted onto MILLER's basic medium without any growth regulators. After 10 to 20 days of culturing, the shoots generally proliferated a number of roots, and thereafter the obtained plantlets were transplanted into pots. The determination of chromosome numbers of these regenerated plants was carried out by the same way as described in chapter 1.

(2) Results

A. Cytogenetical instability of callus tissue in culture

a. *Karyotypic changes in calluses of haploid and diploid plants of three Nicotiana species*

The following changes in chromosome number and structure in the cultured callus tissues of three *Nicotiana* species were observed after 10

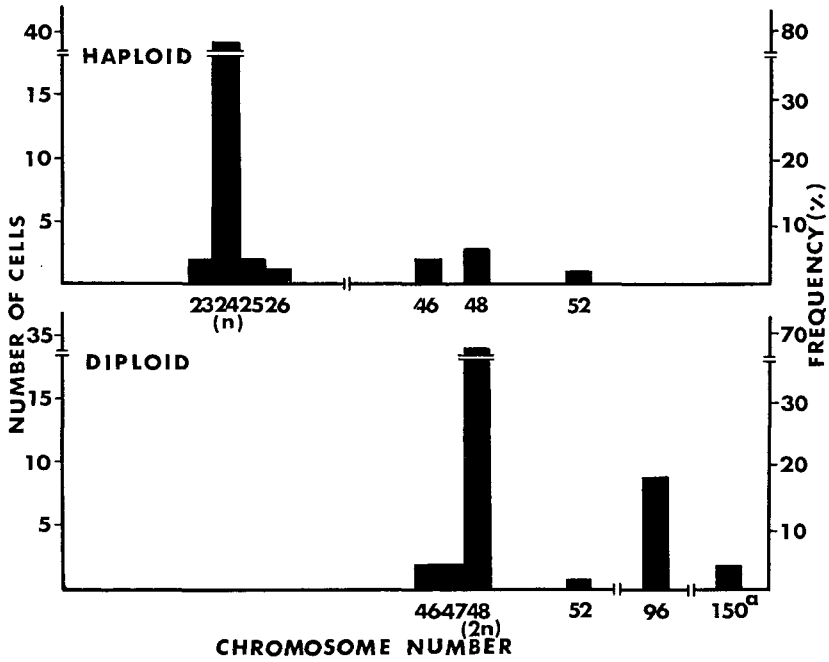

Fig. 4. Frequencies of the cells with various chromosome numbers in haploid and diploid calluses of *Nicotiana tabacum* ($2n=48$). a, More than 150 chromosomes.

days of the second subculturing passages. 50 metaphase cells were examined in each haploid and diploid calluses of three species.

N. tabacum ($2n=48$)

Haploid callus tissues maintained the original haploid cell ($n=24$) line in more than 70%, 10 days after transplantation onto the subculture (Fig. 4). About 6% of the cells in the callus tissues showed diploid chromosome numbers ($2n=48$) which might have arisen from the multiplication of the haploid chromosomes by the process of endomitosis. The diploid callus line also maintained the original diploid cells at a high frequency of about 70%, but a considerable number of cells showed tetraploid chromosome numbers ($4n=96$). In both haploid and diploid callus tissue lines a few aneuploid cells were observed and sometimes cells with more than 150 chromosomes were found in the diploid callus line.

N. sylvestris ($2n=24$)

In the line of haploid callus tissues 50% and 18% of the cells examined for their chromosome number showed diploid ($2n=24$) and

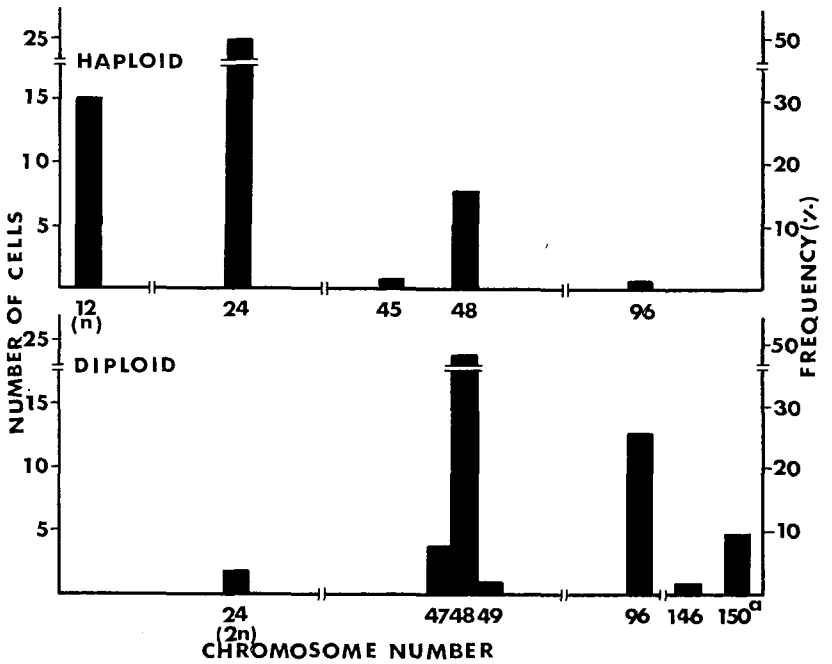

Fig. 5. Frequencies of the cells with various chromosome numbers in haploid and diploid calluses of *Nicotiana sylvestris* ($2n=24$). a, More than 150 chromosomes.

tetraploid ($4n=48$) chromosome numbers, respectively. The cells with the original haploid chromosome number ($n=12$), on the other hand, remained at only less than 30% (Fig. 5). In the line of diploid callus tissues 48% and 26% of the cells exhibited tetraploid ($4n=48$) and octoploid ($8n=96$) chromosome numbers, respectively, and about 10% of the cells showed more than 150 chromosomes, while only 4% of the cells preserved the original chromosome numbers of diploid state ($2n=24$). Therefore, a high frequency of the multiplication of chromosome numbers was considered as a conspicuous characteristic in both ploidy levels of the callus cultures of this species.

N. glutinosa ($2n=24$)

The line of haploid calluses contained 22%, 22% and 6% of the haploid ($n=12$), diploid ($2n=24$) and tetraploid ($4n=48$) cells, respectively (Fig. 6). The most remarkable characteristic of the calluses of this species was the highly frequent appearence of aneuploid cells such as 13 ($n+1$), 25 ($2n+1$) and 26 ($2n+2$) chromosomes. Especially, cells with 13 chromo-

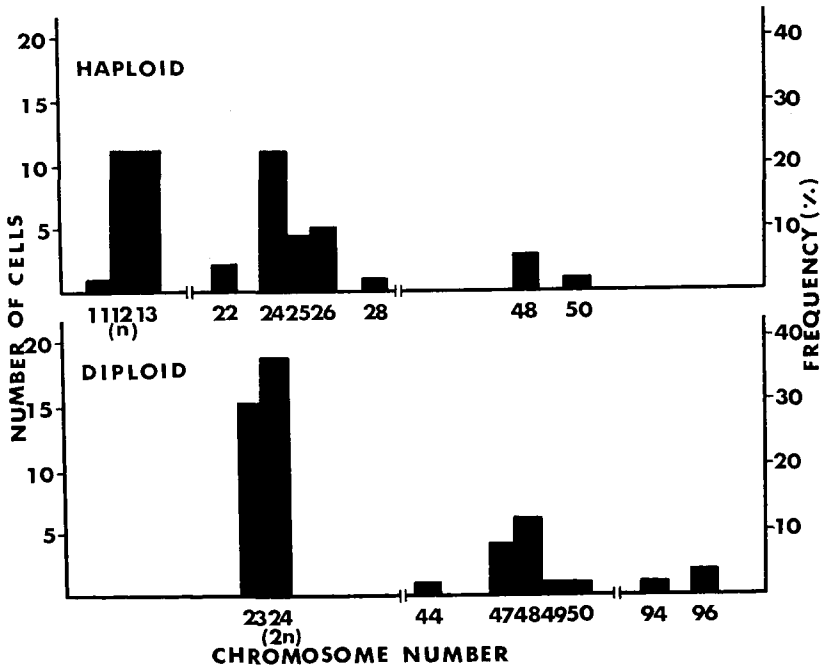

Fig. 6. Frequencies of the cells with various chromosome numbers in haploid and diploid calluses of *Nicotiana glutinosa* ($2n=24$).

TABLE 13. Changes in chromosome number and structures in the cells of *Nicotiana glutinosa* (2*n*) in callus culture

Chromosome number of cell	Number of cells	Remarks	Chromosome number of cell	Number of cells	Remarks
21	1	All monocentric	47	9	One dicentric
22	3	All monocentric	48	24	All monocentric
22	1	Two dicentric	48	1	Two dicentric
23	19	All monocentric	48+F*	1	
23	14	One dicentric	49	1	All monocentric
24	55	All monocentric	50	1	Two dicentric
			54	1	All monocentric
44	1	One dicentric			
46	1	One dicentric	94	1	All monocentric
46	1	Two dicentric	96	2	All monocentric
47	4	All monocentric			

* Chromosome fragment.

somes showed 22% of the same frequency as the original euhaploid cell with 12 (*n*) chromosomes (Plate XIII-a). In the diploid callus line 38%, 12% and 4% were diploid ($2n=24$), tetraploid ($4n=48$) and octoploid ($8n=96$) cells, respectively. The occurrences of aneuploid cells were also significantly high in this diploid callus line, especially 23 ($2n-1$) and 47 ($4n-1$) which were 30% and 8%, respectively. Chromosome structual changes which showed dicentric chromosomes were observed as a special feature in this diploid callus line (Table 13 and Plate XIII-c, d). Cells with one dicentric chromosome were most frequently observed, but at times a few of the cells showed two dicentric chromosomes. The formation of such chromosomes in tissue and cell cultures is not a rare event and has been reported in several species (KAO *et al.* 1970 and SUNDERLAND 1973a). SUNDERLAND (1973a) interpreted the origin of this dicentric chromosome to be the result of a breakage-fusion-bridge cycle similar to that originally described by MCCLINTOCK (1951) in maize.

As far as this experiment is concerned, no single line having a stable nuclear state was found in these cultured calluses in the three species, although the degree and pattern of chromosomal variation was quite different among them.

b. *Effect of different kinds of media on the variation of chromosome numbers in haploid callus of N. tabacum*
Chromosome numbers of callus tissues on 1st and 2nd subculture

Primary calluses derived directly from pollen grains by anther culture consisted of haploid cells of 24 chromosomes alone. Even at three weeks after the induction of the calluses, they did not show any variation in chromosome numbers in all cells examined.

The callus growth after the first transplantation was mainly affected by the addition or lack of growth regulators. The greatest growth was observed on the calluses cultured on medium M–4 containing both IAA and kinetin. Medium M–2 and M–3 containing IAA and kinetin, respectively, promoted the callus growth to a lesser extent than medium M–4. The calluses on medium M–1 containing no growth regulator grew very slowly and appeared to become gradually quiescent.

One month after the first transplantation, the callus tissues cultured on medium M–1 containing no growth regulator did not show any variation in chromosome numbers and consisted of cells with only 24 somatic

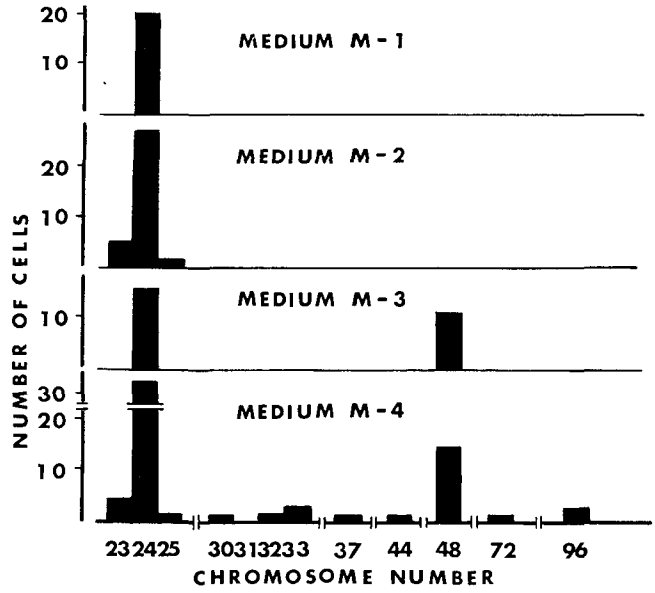

Fig. 7. Chromosome numbers in the first subculture of callus tissues induced by anther culture of *Nicotiana tabacum* (var. Wisconsin 38). Medium M–1, no growth regulator; Medium M–2, 4 mg/l of kinetin; Medium M–3, 4 mg/l of IAA; Medium M–4, 2 mg/l of kinetin and 2 mg/l of IAA.

chromosomes. On the other hand, a considerable range of variation in chromosome numbers was observed in the calluses cultured on medium M–2, M–3 and M–4 (Fig. 7, Plate XIV). The widest variation in chromosome numbers occurred in the callus tissues on medium M–4. There were two peaks in the distribution of the chromosome numbers at 24 and 48, with a frequency of 53.2% and 24.2%, respectively. Tetraploid cells were also rarely observed. All others were cells of aneuploid chromosome number. The calluses cultured on medium M–2 mainly consisted of cells with a haploid chromosome number of 24, but several cells were aneuploids with numbers such as 23 and 25. In the calluses cultured on medium M–3, 40.7% of cells had a diploid chromosome number of 48, while the remainder had a haploid chromosome number of 24. In these calluses, no aneuploid cells were seen.

Thus, the variation in chromosome numbers was found in the callus lines cultured on media containing IAA alone or both IAA and kinetin, even though they were derived from the same line which originally had uniform cells with 24 chromosomes. Therefore, the polyploidy and aneuploidy may possibly be interpreted to be the result of response to the growth regulator of IAA. The supplement of kinetin to IAA promotes further variation of the chromosome numbers as compared against the addition of IAA alone to a medium.

The calluses, which were cultured for one month on medium M–4 and which had the widest variation of chromosome numbers, were again transferred to the same series of media as those of the first subculture. One month after the second transplantation, the determination of chromosome numbers of callus tissues on each medium revealed the same tendency of chromosome variation as those of the first subculture (Fig. 8). In the calluses cultured on medium M–1 and M–2, only haploid and diploid chromosome numbers of 24 and 48, were observed, even though the calluses on the first subculture contained cells of a higher level of polyploids and various aneuploids. This means that only the cells of 24 and 48 chromosomes are active in cell division on medium M–1 and M–2. On the other hand, medium M–3 and M–4 promote the division of the cells of various chromosome numbers including polyploids and aneuploids. Furthermore, on these media, the variation of chromosome numbers increased beyond those of the first subculture, and the highest number of 192 ($8n$) was counted on medium M–4.

Many types of alterations of cell morphology in the cultured calluses were observed. A prominent one was the so-called giant cell which some-

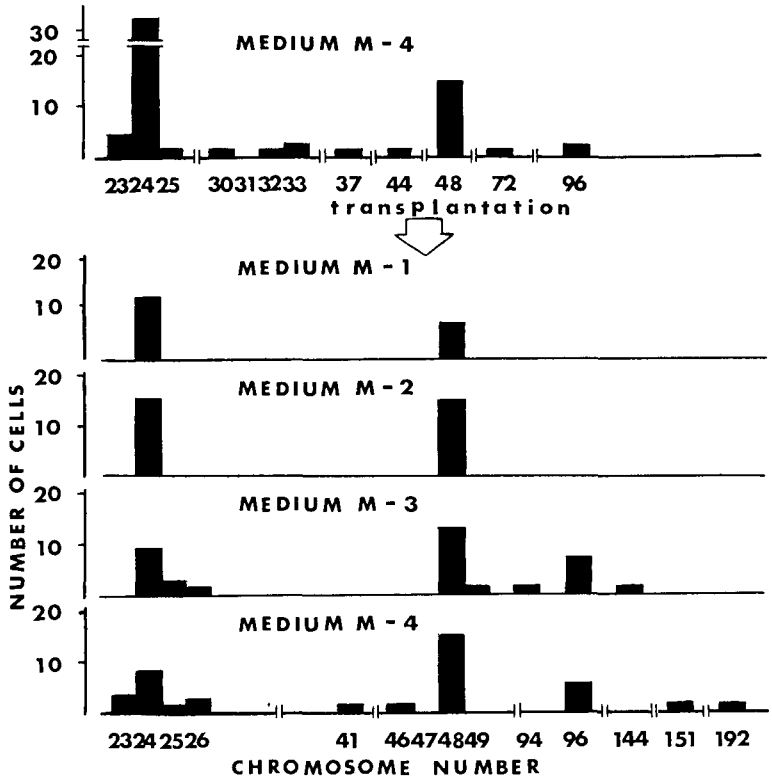

Fig. 8. Chromosome numbers in the second subculture of callus tissues of *Nicotiana tabacum* (var. Wisconsin 38) derived from the first subculture on medium M-4. Medium M-1, no growth regulator; Medium M-2, 4 mg/l of kinetin; Medium M-3, 4 mg/l of IAA; Medium M-4, 2 mg/l of kinetin and 2 mg/l of IAA.

times increased from 5 to 20 times in cell size of ordinary cells (Plate XV). Other prominent cell morphology was the change of nuclear behaviours. Multinuclei were frequently observed in either the giant cells or the ordinary cells (Plate XVI). They might have originated from the failure of cytokinesis. However, it was not clarified as to how they were precisely originated. Some cells consist of up to four nuclei and in some cases they were not equal in size and some of them had an appearance of micronuclei. Frequently, nuclear fusion was observed in the multinucleate cells.

Chromosome numbers of callus tissues on 8th subculturing passage

Determination of chromosome numbers of the calluses was performed after 10, 20, and 30 days of the 8th passage of subcultures. The results are shown in Fig. 9. The callus tissue cultured on medium M–1 without growth regulators showed the most stable chromosomal state maintaining the constituent of cells with only 24 (n) and 48 ($2n$) chromosomes throughout the entire period of the culture. Only changes such as a slight increase of the cells with 48 chromosomes and decrease of the cells with 24 chromosomes were noted. No aneuploid cell was found in this callus tissue. On the other hand, considerable variations in the chromosome numbers were observed in the callus tissues cultured on medium M–2, M–3, or M–4, which contained IAA and kinetin singly or in combination.

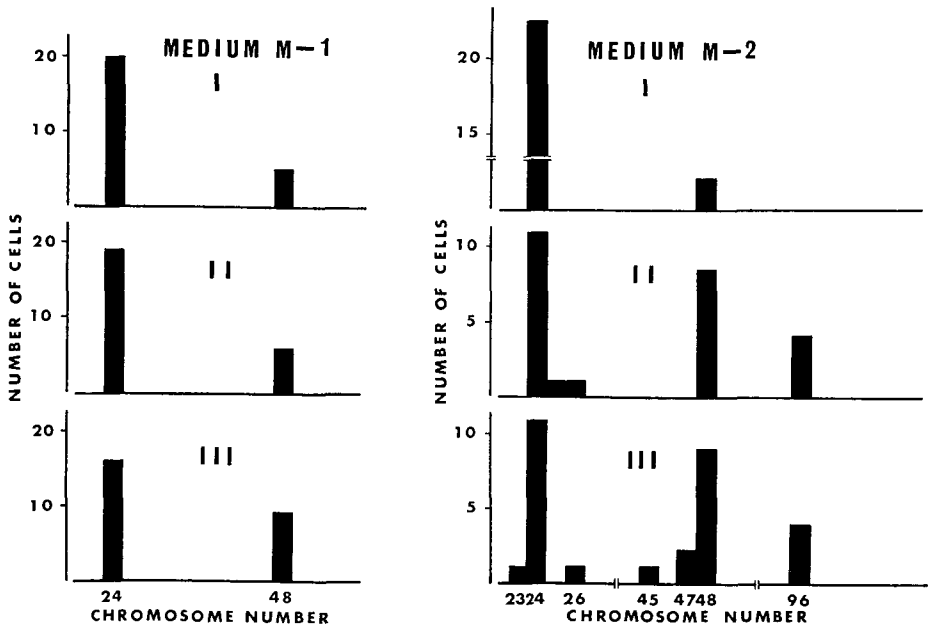

Fig. 9. Chromosome numbers in the 8th subculture of haploid callus tissues of *Nicotiana tabacum* (var. Wisconsin 38). The callus tissues cultured on the medium M–4 successively transplanted into four types of the next subculturing media at interval of 30 days. Medium M–1, no growth regulator; Medium M–2, 4 mg/l of kinetin; Medium M–3, 4 mg/l of IAA; Medium M–4, 2 mg/l of kinetin and 2 mg/l of IAA. I, 10 days after transplantation; II, 20 days after transplantation; III, 30 days after transplantation.
a, More than 300 chromosomes.

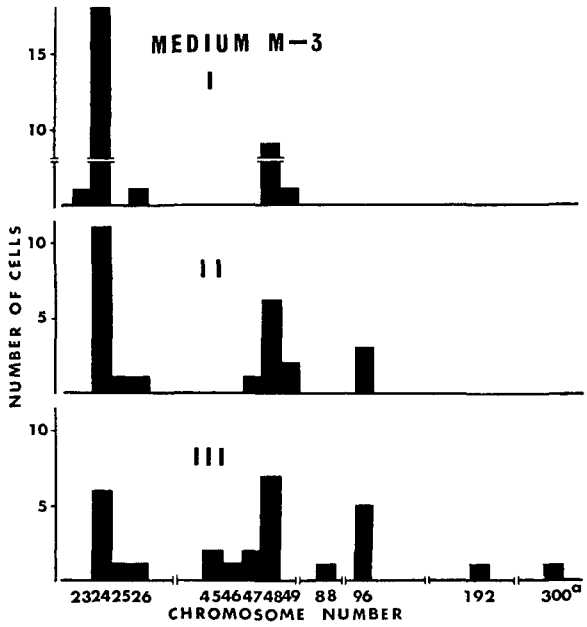

Fig. 9. continued.

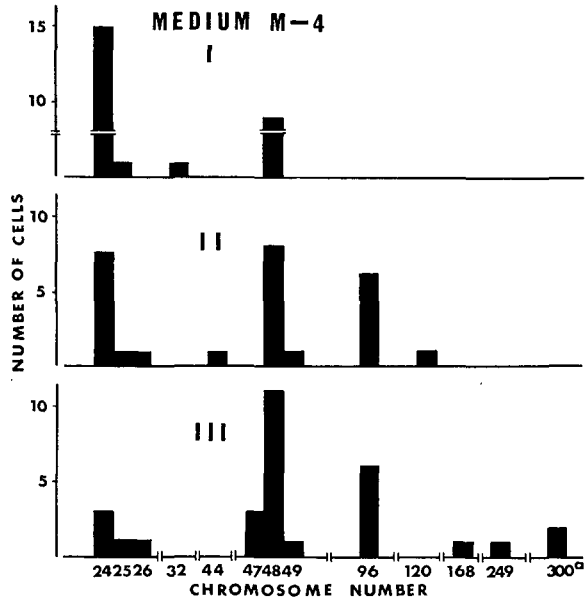

Fig. 9. continued.

As a common feature of the calluses on these three types of media, the variations of chromosome numbers were generally small in the early stages after transplantation and became wider in advanced stages of the subcultures. In general, final variations of chromosome number after one month of culturing were wider than those of the first and second subculture and some cells show numerous chromosome numbers (Plate XVII). Most of the cells of the callus tissues on these media showed 24 and 48 chromosomes and a few cells were aneuploids after 10 days of incubation. However, in the late period of the cultures, namely after 20 to 30 days of incubation, the variations of chromosome numbers drastically increased, involving 96 ($4n$), 192 ($8n$) chromosomes, and various aneuploids. The widest variation was observed in the callus tissues cultured on medium M-4 containing both IAA and kinetin and chromosome numbers exceeding 300 were sometimes counted in several of the cells. The callus tissues cultured on medium M-3 containing IAA alone also showed a considerable variation in the chromosome numbers. The calluses on medium M-2 containing kinetin alone showed a somewhat smaller variation than those cultured on medium M-3 and M-4.

Frequencies of cell division on 8th subculturing passage

About 1 to 2×10^3 cells of meristematic parts of callus tissues were examined for mitotic frequencies immediately prior to transplantation and at two intervals at 10 and 20 days of the 8th subculture. The number of cells at the metaphase and anaphase stage in the cell cycle was shown as the relative activity of the mitotic cell division in each callus tissue cultured on four different types of media. The results are shown in Table 14.

TABLE 14. Frequencies of mitotic cells at meristematic parts of callus tissues cultured on four types of media

Medium type*	Days in culture		
	0**	10	20
M-1	0.235%	0.539%	0.315%
M-2	0.235	1.201	0.587
M-3	0.235	1.284	0.874
M-4	0.235	1.390	0.890

* M-1, no growth regulator; M-2, 4 mg/l kinetin; M-3, 4 mg/l IAA; M-4, 2 mg/l kinetin and 2 mg/l IAA.

** At the time just before transplantation of callus cultured on medium M-4 to subculture.

The frequencies of metaphase and anaphase cells at both periods after 10 and 20 days of incubation were higher in the order of calluses cultured on medium M–4, M–3, M–2, and M–1. In other words, the calluses cultured on medium M–4 containing both IAA and kinetin showed the highest frequency of cell division at both periods of culture. The cell division on medium M–2 or M–3 containing IAA or kinetin alone was somewhat lower as compared to those on the medium M–4. The lowest cell division was observed on the callus tissues cultured on medium to which no growth regulator was added. As a common characteristic of the calluses cultured on four different types of media the frequencies of mitotic cell division showed a tendency to decrease with the advanced periods in all cultures.

B. Morphogenesis in callus culture

30 plants regenerated from each callus tissue cultured on four types of media were determined for their chromosome numbers by using root tips (Table 15). Totipotency of the callus tissues seems to be affected by different medium types. A high potentiality of regeneration of plants from calluses appeared to occur on medium M–1 and M–2, while medium M–3 and M–4 containing IAA led to a considerable decrease in the ability to produce shoot initiation of the calluses (Table 16). On medium M–1 containing no growth regulator, 28 individuals of regenerated plants had 24 (n) chromosomes and the remaining 2 had 48 ($2n$) chromosomes. On medium M–2 containing kinetin alone, 18, 9 and 3 of the regenerated

TABLE 15. Number of regenerated plants with different chromosome numbers from callus tissues cultured on four different types of media

Medium type*	Number of regenerated plants			
	Chromosome number			
	24 (n)	48 ($2n$)	96 ($4n$)	Total
M–1	28 (93.3)**	2 (6.7)	0 (0)	30 (100)
M–2	18 (60.0)	9 (30.0)	3 (10.0)	30 (100)
M–3	27 (90.0)	3 (10.0)	0 (0)	30 (100)
M–4	23 (76.7)	7 (23.3)	0 (0)	30 (100)

* M–1, no growth regulator; M–2, 4 mg/l kinetin; M–3, 4 mg/l IAA; M–4, 2 mg/l kinetin and 2 mg/l IAA.

** Indicates percentage.

TABLE 16. Frequency of regenerated plants from each callus tissue cultured on four types of media

Medium type	Regenerated plant
Medium M-1	++
Medium M-2	+++
Medium M-3	+
Medium M-4	+

+++ High frequency.
++ Medium frequency.
+ Low frequency.

plants had 24, 48 and 96 ($4n$) chromosomes, respectively (Plate XVIII). These various ploidy levels of regenerated plants derived from the callus on medium M-2 could be naturally expected from callus tissues containing various polyploid cells. On the other hand, 27 and 3 of the regenerated plants had 24 and 48 chromosomes, respectively, on medium M-3 containing IAA, 23 and 7 of the regenerated plants had 24 and 48 chromosomes, respectively, on medium M-4 containing IAA and kinetin. Tetraploid plants and much higher levels of polyploidy did not regenerate from the callus tissues on medium M-3 and M-4 even though these calluses had a higher frequency of the higher levels of polyploid cells than those on medium M-2. No single aneuploid plant was derived from all calluses cultured on the above described four types of media.

(3) Discussion

A. Cytogenetical instability of callus tissues in culture

a. *Effect of different kinds of media*

The occurrence of polyploidy, aneuploidy and chromosome aberrations in plant and animal cells cultured *in vitro* are widespread phenomena.

The majority of mammalian cell cultures can be grouped into two classes, depending upon the variation in chromosome complements. First homonuclear lines are characterized by the fact that there is very little variation among cells in their chromosome complement; next heteronuclear lines, on the other hand, show a marked variation. Homonuclear lines can be started from benign (i.e., noncancerous) solid tissue of any individual living body, while heteronuclear lines can be induced quite frequently from malignant tissues. The growth of homonuclear cell lines

have in general a finite life time of about 50 generations after the separation of the cells from the explant. On the other hand, the growth of heteronuclear cell lines are capable of indefinite life time *in vitro*, mostly with malignant properties, exhibiting chromosome variation even between cells of a single clone (see rev.: KROOTH *et al.* 1968).

In contrast to mammalian tissue culture, plant cell cultures are capable of indefinite growth *in vitro* even without tumorous transformation such as crown-gall tumor or habituation. Furthermore, these normal cultures of plant cells frequently exhibit an extensive chromosome variation, such as polyploidy, aneuploidy and various chromosome aberrations.

The question arises as to how the polyploidy, aneuploidy and various other chromosomal aberrations originate in the cultured calluses and cells of plant. There is evidence that single-celled clones which were initially diploid, or tetraploid, may increase in ploidy during the course of culture. COOPER *et al.* (1964) examined cytologically the clones of tissues of single-cell origin, isolated from the crown gall callus of *Nicotiana tabacum* which was previously grown on a medium supplemented with NAA, coconut milk, 2, 4-D and calcium panthothenate for eight years. Chromosome count revealed numbers of 48, 96, and 192 in the clones. On the other hand, TORREY (1961) observed that when seedling roots of *Pisum sativum* were cultivated on a medium containing yeast extract and auxin (2, 4-D), or kinetin, callus tissues were predominantly composed of tetraploid chromosome numbers even though the initial tissues were from roots known to consist of cells also with diploid chromosome numbers. On a medium containing a mixture of vitamins, amino acids, amides and urea, the entire population of cells remained diploid. Therefore, he concluded that certain growth regulators used in the culture medium, such as yeast extract, 2, 4-D or kinetin, induce mitosis in dormant polyploid cells that are present in a tissue when it is introduced into the culture.

These findings suggest that the causativeness in the widespread occurrence of polyploidy in the cultured calluses and cells may be preferential promotion of division in polyploid cells either arising during normal cell expansion or being already present in the original explant and that this promotion may be a function of growth regulators in the culture medium.

It has also been well established by the other experiments that growth regulators such as auxin and cytokinin play an important role in the polyploidization of cultured tissues or cells (BLAKELY and STEWARD 1964, COOPER *et al.* 1962, DE TOROK and RODERICK 1961, 1962, DE TOROK and WHITE 1960, MITRA *et al.* 1960, TORREY 1958). One explanation of the

occurrence of polyploidy in the cultured tissues or cells is the failure of cytokinesis in mitosis, which leads to cells of multinuclei which sometimes fuses into uninucleus at a later stage (MITRA and STEWARD 1961). Another possible explanation is endoreduplication, which involves chromosomal reproduction during the interphase and is manifested by the presence of diplochromosomes (4-chromatids), quadruplochromosomes (8-chromatids), or polychromosomes (polyteny) (D'AMATO 1952). This endoreduplication is observed commonly in differentiated tissues of higher plants and is regarded as the most widespread mechanism of somatic polyploidization. The observation of the multinuclei in the present study on the haploid callus tissues of tobacco, however, agrees with the former explanation. The precise mechanism by which growth regulators induce such a change, however, are not well understood. STERN (1960) gave an interpretation involving the physiological events which surround chromosomal behaviour in the cultured tissues or cells. Factors which determine respiration, oxygen and energy utilization and which have been invoked to explain different phases of mitotic and meiotic activity, may lead to abnormal chromosomal behaviors. However, the extent to which such ideas may be applicable to the observation made on the cultured haploid callus tissues of tabacco remains to be seen.

The cultured calluses in this study were found not only to contain polyploid cells, but also to show, more or less infrequently, chromosomal aberrations such as dicentric chromosomes and aneuploids. MITRA *et al.* (1960) observed dicentric and tricentric bridges in mitosis of cultured carrot cells. Similarly, nuclei showing pseudochiasmata, chromosome breaks, reunions, and bridges were observed in suspension cultures of *Haplopappus gracilis* by MITRA and STEWARD (1961). TORREY (1958, 1959, 1961, 1965) in his studies on polyploidy in pea root callus found that, in older cultures maintained on media containing yeast extract or kinetin, there could be detected not only various degrees of polyploidy up to $12n$ but also aneuploids (especially around $4n+1$) and cells showing anaphase bridges, chromosome loops and rings. Whether the mechanism for the occurrence of such chromosomal aberrations and aneuploids are the same as those which have undergone structural alterations as a result of treatment with irradiations or other agents which promote chromosomal breakage is uncertain. It does seem, however, probable that division, and subsequent multiplication of such aberrant nuclei, is promoted in culture medium containing growth regulators such as auxin and cytokinin.

It is clear now that the growth regulators in the culture medium

have a determinative role of chromosomal behaviour in callus tissues, although the precise mechanism(s) largely are unknown at present. In this study on haploid callus of *N. tabacum*, the occurrence of polyploid cells such as diploid, tetraploid and octoploid were frequent in the calluses cultured on the media containing IAA alone or IAA and kinetin in combination. Also, aneuploid cells on the media containing both IAA and kinetin were more frequent than any other media. In contrast, almost all cells cultured on the media containing no growth regulator resulted in haploid and diploid cells. Furthermore, when the callus tissues containing many polyploid and aneuploid cells were transferred onto media containing no growth regulator or kinetin alone, only haploid and diploid cells were active in mitosis. It is, therefore, concluded that IAA, a kind of auxin, has a function related to the formation and division of polyploid and aneuploid cells and that kinetin, a kind of cytokinin, intensifies the function of auxin. SUNDERLAND (1973a) reported that a suspension culture of *Haplopappus* cotyledon cells grown in a medium containing 2, 4-D changed entirely from diploid to entirely tetraploid over a period of less than 6 months of subculture. Therefore, it may be concluded that 2, 4-D as an auxin component contributes by accelerating the rate of polyploidization. Thus, it may be stated that chromosomal constituents of callus tissues and cultured cells are possibly controlled by such media containing appropriate growth regulators and particular cells of desired chromosomal constituents, for instance, complete homozygous diploid cells originated from haploid calluses or cells can be obtained in the cultured calluses and cells.

The frequencies of the metaphase and anaphase cells which may indicate the relative activity of the mitosis and may be closely related to the rate of callus growth were significantly high on media containing IAA as compared to those on medium containing no growth regulator or kinetin alone. The highest cell division was observed in the callus tissues on medium containing IAA and kinetin in combination. Therefore, it seems to be an incontrovertible fact that a condition such as the medium containing auxin such as IAA enhances the growth of calluses and simultaneously affects the variations of chromosome numbers. However, it still remains unclarified as to whether the association of the rapid callus growth and the high chromosomal instability depends upon a mere coincidence of two independent phenomena in a common environment or has a certain cause and effect relationship in such a way that the higher callus growth strikingly gives rise to wider variations in the chromosome numbers.

b. *Cytogenetically stabilized callus culture*

The chromosome instability found in the callus tissue culture provides a useful source of desired polyploid plants which are required for crop improvement. On the contrary, this chromosome instability may be considered sometimes as a serious barrier, when the cultured cells applied to several approaches such as somatic cell genetics and mutation research which require the preservation of a stable chromosome state. The difficulty to obtain a callus culture which is cytogenetically stable may partly depend upon the plant species. In other words, the chromosome stability and instability of plant callus cultures are subjected in part to plant species from which these cultures originated. The present study on the comparison in nuclear behaviour and karyotypic changes did not show a sufficiently stable culture line in the haploid and diploid plants in three *Nicotiana* species, *N. tabacum, N. sylvestris* and *N. glutinosa*. However, it may still be considered important to pay attention to the fact that some cultures in certain species actually remain stable without any visible chromosomal changes. For instance, REINERT and KÜSTER (1966) observed no variation in chromosome number after one year in cells derived from *Crepis capillaris*. SACRISTÁN (1971) also reported that in the same species 34.1% of the subcultures derived from the haploid plants and 78.5% from the diploid plants maintained the original ploidy levels for more than a year. Therefore, there is still hope of achieving chromosome stability in certain species for considerable long periods of subculturing.

The question inevitably arises as to whether or not perpetual maintenance of the stable nuclear state in any species can be achieved by the available culture techniques alone at present time. In this study, some culture media supplemented with defined growth regulators render the callus tissues to reduce the increased variations of chromosome numbers. It is, however, still unknown as to how to select and maintain only desired ploid cells from a mixed population of various chromosomal constituents. Insofar as the past experiments by many investigators are concerned the answer appears to be very pessimistic. Therefore, we must seek alternative methods, for instance, preserving clones in non-growing condition or the use of some specific chemicals which have a selective effect on a particular nuclear state. CAPLIN (1959) succeeded in maintaining cultures for long periods without loss of viability by covering them with a layer of mineral oil. More recently some successes have been reported in freezing of cultures in the presence of protecting agents such as glycerol or liquid nitrogen (LATTA 1971, NAG and STREET 1973). Such a controlled freezing and

low temperature storage of the cultured plant cells might enable the initial characters of newly established cultured to be preserved.

An amino acid analogue, para-fluorophenylalanine (PFP), has been reported as an inducer of mitotic haploidization in diploid lines of some genera of fungi (LHOAS 1961). GUPTA and CARLSON (1972) found that this PFP specifically inhibits the growth of the diploid callus tissues and has no effect on the haploid callus tissues plants, *Nicotiana tabacum, in vitro.* The auther's experiment (1974) substantially supported their results as to the function of PFP. However, the results were somewhat in controversary with them, namely the PFP not only hinders the growth of diploid but also haploid callus tissues (Table 17). In other words, even haploid calluses are not free from a considerable suppression of their growth at increasing concentrations of PFP, although the haploid calluses are less damaged than the diploid calluses. Cytogenetical observations of the haploid callus tissues revealed that the haploid cells effectively increase in frequencies at some range of concentration of the PFP such as 40 to 100 mg/l and the haploid cells attain more than 70%, while the haploid cells were less than 50% of the control medium containing on PFP (Table 18). This may be caused by the prevention of polyploidization of the haploids and simultaneously the preferential growth of haploid cells. Therefore, it may be suggested that the possibility of using this amino acid analogue may result not only in the promotion of preferential growth of the haploid

TABLE 17. Growth of haploid and diploid calluses of *Nicotiana tabacum* (var. Wisconsin 38) on media containing various concentrations of PFP after one month

	Concentrations of PFP (mg/l)	Weight of initial callus (mg)	Weight of callus after one month (mg)	Fold increase	Rate of growth inhibition (%)
Haploid	0	400	5,483	13.708	0
	50	400	4,800	12.000	12.5
	100	400	3,417	8.543	37.7
	200	400	1,033	2.583	81.2
Diploid	0	400	7,300	18.250	0
	50	400	3,733	9.333	48.9
	100	400	2,900	7.250	60.3
	200	400	633	1.583	91.3

TABLE 18. Frequencies (%) of different chromosome numbers in calluses cultured on media containing various concentrations of PFP

		Concentrations of PFP (mg/l)						
		0	5	10	20	40	80	200
10 days in culture	Haploid (24)	50	62	62	50	60	60	96
	Diploid (48)	38	32	26	39	34	34	4
	Others*	12	6	12	11	6	6	0
20 days in culture	Haploid (24)	42	50	58	55	82	74	—
	Diploid (48)	44	44	36	45	16	24	—
	Others*	14	6	6	0	2	2	—

* Including various polyploids and aneuploids.
— No more callus growth occurred.

calluses but also to select and maintain the haploid cells from the mixed populations of varying polyploids. To date, the effect of PFP has been examined in only tobacco plant cells, but if the effect of PFP is not specific to this species, they may well be a generally useful method of maintenance of haploid cells of higher plants in culture passages. Further work should be performed to determine the extent of applicability of PFP for the preferential selection of haploid cells of other species *in vitro*.

B. Morphogenesis in callus culture

a. *Mechanism of morphogenesis*

The regeneration of roots in callus cultures was first reported by NOBÉCOURT (1939) in work with the carrot. In the same year, WHITE (1939) succeeded in inducing leafy buds in callus tissues derived from the interspecific hybrid of tobacco (*Nicotiana glauca* × *N. langsdorffii*).

This study showed a tendency in which a high potentiality of regeneration of plants from calluses appeared to occur on medium with no growth regulator or kinetin alone and that an addition of IAA to the medium led to a decrease in ability to produce shoot initiation from the calluses. The initiation of organs from callus tissues was studied precisely by SKOOG and MILLER (1957). They also showed that growth responses were greatly affected by plant growth regulators such as auxin and kinetin and that changes in the auxin-kinetin balance markedly altered the growth expression of an auxin-kinetin synergism in tobacco pith callus. At low

kinetin to auxin ratios roots developed in callus tissue, but bud and shoot formation were suppressed, whereas at higher ratios development of buds and shoots was induced. At intermediate ratios, essentially undifferentiated callus tissues resulted. Thus, the formation of buds and shoots is one of the several possible modes of expression of growth induced by the auxin-kinetin interaction and it has been found that it is not simply induced solely by kinetin and suppressed by auxin.

However, the observation of SASTRI (1961) with callus derived from *Armoracea rusticana* is rather different from that of SKOOG and MILLER (1957). He found that an addition of auxin stimulated bud induction, whereas, an addition of kinetin suppressed bud formation. Thus, SASTRI's results implied that the callus has a critically low endogenous auxin level relative to its endogenous level of cytokinin. On the other hand, the tobacco calluses in this study and that of SKOOG and MILLER may be markedly deficient in some endogenous cytokinin.

In addition to the above studies, a number of recent examples describing the development of plants (morphogenesis) from single cells, or callus tissues, have been demonstrated in various other plants. For instance, we have examples from callus tissue of *Nicotiana tabacum* and *N. alata* (NISHIYAMA and TAIRA 1966, TABATA 1967), endive (*Cichorium endivia*) and parsley (*Petroselinum hortense*) (VASIL and HILDEBRANDT 1966a, b), geranium (*Pelargonium*) (CHEN and GALSTON 1967, PILLAI and HILDEBRANDT 1969), *Convolvulus ervensis* (EARLE and TORREY 1965), *Chrysanthemum* (HILL 1968), aspen (*Populus tremuloides*) (MATHES 1964, WINTON 1968, 1970, WOLTER 1968) and *Petunia* (RAO *et al.* 1973). Plants have been obtained from single carrot (*Daucus carota*) cell suspensions (STEWARD *et al.* 1958a, b, KATO and TAKEUCHI 1963, HALPERIN 1964), from cell suspension culture of *Ranunculus sceleratus* (KONAR and NATARAJA 1965a) and from single isolated *Nicotiana* plant cells (CHANDRA and HILDEBRANDT 1967, VASIL and HILDEBRANDT 1967). Morphogenesis in monocotyledonous plants has also been demonstrated, in rice (*Oryza sativa*) (KAWATA and ISHIHARA 1968, NISHI *et al.* 1968), oats (*Avena sativa*) (CARTER *et al.* 1967), asparagus (*Asparagus officinalis*) (WILMAR and HELLENDOORN 1968), *Saccharum* (HEINZ and MEE 1969, BARBA and NICKELL 1969), *Haworthia* (KAUL and SABHARWAL 1972) and BUCKWHEAT (*Fagopyrum esculentum*) (YAMANE 1974).

A common feature of the totipotency of plant cells is exemplified by the ability of the cells to behave in a similar manner to that of a zygote and to undergo morphogenesis. STREET (1969) suggested that two steps

are required for the expression of cell totipotency. These are the release of the controlling factors from the cells which operate in the original plant organs and secondly, the induction of rapid cell division within the meristematic nodules. Both of these may be necessary preliminaries to the expression of totipotency of plant cells. Furthermore, certain hormonal stimuli, may be also required. LETHAM (1969) pointed out in his review that by using plant hormones, or synthetic compounds which have a similar activity as natural occurring hormones, that it is possible to promote, suppress, or modify, almost any phase of plant development. The functional aspect of growth regulators in cells have been described recently in detail. Auxin (ROYCHOUDHURY *et al.* 1965, CHERRY 1967, MAHESHWARI *et al.* 1966, MATTHYSSE and PHILLIPS 1969), gibberellin (ROYCHOUDHURY *et al.* 1965, JOHRI and VARNER 1968) and cytokinin (ROYCHOUDHURY *et al.* 1965) have all been reported to stimulate DNA dependent RNA synthesis in isolated nuclei or chromatin. Thus, it may be reasonably assumed that growth regulator function at, or very near the level of the genes may act as gene derepressors. Furthermore, the occurrence of cytokinin in certain species of tRNA (ZACHAU *et al.* 1966, MADISON and KUNG 1967, HALL *et al.* 1967, ROBINS *et al.* 1967) raises the intriguing possibility that cytokinin action may be intimately connected with the translational regulation of protein synthesis. Therefore, growth regulators seem to be capable of playing an important role in the controlling mechanisms in cell differentiation and morphogenesis by acting at the site of transcription or translation, although their precise mechanisms are still largely obscure.

The totipotency of cell cultures to initiate roots and shoots is sometimes decreased or lost by cytological and genetic factors. In many cases, a decrease in growth and morphogenetic capacity is associated with a callus which is continuously cultured. Such a progressive depletion in the capacity of root initiation of pea callus has been described by TORREY (1959). He noted that failure of root initiation was accompanied by a shift in the chromosome number of the tissue in which there had been a change-over from a diploid cell type to that of a tetraploid one. MURASHIGE and NAKANO (1965) reported marked declines in growth of tobacco callus and differentiation of roots and shoots with advancing age in tissue culture. Chromosome number determinations of callus tissue showed that the callus lines in the morphogenetically reduced state were associated with a highly variable aneuploidy. The totipotent lines, on the other hand, were tetraploids or diploids, with no apparent difference in capacity between the two euploids. One scheme to account for the aging process of plant cells was

proposed by MURASHIGE and NAKANO (1965) as follows: there is a progressive accumulation in the plant cells with anomalous chromosome numbers which increases with development. These cells, which have reduced the morphogenetic potential, adversely influence the overall physiology of the plant cells. However, this study is somewhat contrary to the situation as mentioned above. The callus tissues after eight subcultures at intervals of one month showed sufficient totipotency for plant regeneration even though they had considerable wide variation of chromosome numbers. YAMABE and YAMADA (1973) reported that the callus of *Haworthia* subcultured over 3 years showed remarkable bud formation. Therefore, these results indicate that the control of morphogenesis in plants is not likely to be so simple as to be interpreted by the general scheme mentioned by MURASHIGE and NAKANO (1965).

b. *Chromosome number of regenerated plants*

Studies by TORREY (1959), and MITRA *et al.* (1960) have shown that root meristems induced from highly polyploid cultured tissues are normally diploid. In this study of *Lotus corniculatus*, most of the regenerated plants from the calluses containing a number of the various polyploid and aneuploid cells were diploid and only a few of them were tetraploid. In the case of *Nicotiana tabacum*, media containing IAA, which was employed for accelerating the polyploidization of chromosome numbers, appeared to have a considerably high inhibiting effect on the regeneration of plants from the calluses as compared with the medium containing kinetin alone or no growth regulator. Furthermore, the regenerated plants from the calluses cultured on media containing IAA were mainly haploids and a few of them were diploids, even though the callus tissues showed a high degree of polyploidization of the chromosome numbers. On the other hand, the callus cultured on medium containing kinetin alone produced a considerable number of diploids and even tetraploids, although the calluses did not indicate such a high polyploidization as those on media containing IAA. These facts suggest that the conditions which are favorable for polyploidization of the calluses probably are unfavorable to the morphogenesis of higher levels of polyploids. In contrast, it seems that suppressible conditions of polyploidization of the callus tissues are rather preferential to morphogenesis of polyploid levels of plants.

Another feature of the morphogenesis of the calluses found in this study is that no single plant showing aneuploidy was obtained even from the calluses showing high aneuploidy. Cells such as aneuploids in the

calluses might be naturally screened for survival under *in vitro* cultures, while genetic systems necessary for totipotency had no selective advantage under these conditions and could not be regenerated from the cultured cells. Furthermore, it can be possibly assumed that the loss of chromosomes or the loss of a part of chromosomes might be a partial, but not total explanation for the loss of totipotency. On the other hand, SACRISTÁN and MELCHERS (1969) succeeded in the regeneration of plants with grossly aneuploid chromosome complements from a number of long established aneuploid tobacco calluses. This provides striking evidence that even though cell populations may reach a highly aneuploid state, they are still able to retain their morphogenetic potentiality. In this case, it could be concluded that they still have a whole set of genes required for the totipotency in spite of high chromosomal irregularities of the callus tissues.

3. Production of aneuploid plants by anther culture in *N. tabacum*

(1) Materials and methods

Four levels of ploidy, haploid ($n=24$), diploid ($2n=48$), triploid ($3n=72$), tetraploid ($4n=96$) and various aneuploid plants of *Nicotiana tabacum* (var. Wisconsin 38) were used for the study of anther culture. Haploid plants were derived from the anther culture of diploid plants. Tetraploid plants were obtained by the regeneration from a callus which originated from haploid tissue but showed atainment of various polyploid and aneuploid cells in the course of subcultures on medium M containing 4 mg/l of kinetin (previous chapter 2). Triploid plants were obtained by tetraploid plants crossed with diploid plants. Among 23 plants of F_1 examined for their chromosome numbers, 5 plants were triploids and the others showed various chromosome numbers ranging from 67 to 73. Furthermore, various aneuploid plants with 54 to 63 chromosomes were obtained by triploid plants crossed with diploid plants.

In this study the ability of pollens with various chromosome numbers in fertilization to diploid plants and androgenetic plant formation by anther culture were also compaired by using several kinds of polyploid and plants with various chromosome constitutions. Disomic haploid ($n+1$) plants produced by these anther cultures were collected and classified into 12 types depending upon their flower morphology. All media used for the anther culture are the basic medium of BOURGIN and NITSCH (1967) (medium H) supplemented with 0.1–2.0 mg/l of IAA and 0.1–2.0 mg/l of kinetin singly or in combination. All anther cultures were kept at 26 ± 0.5°C under light condition. The method of chromosome determination of

obtained plants are the same as described in chapter 1.

(2) Results

A. Anther culture of plants of various ploidies and chromosome constitutions

a. *Anther culture of four ploidy levels of plants*

Pollen fertility, androgenetic plant production and length of flower buds used for the anther culture of four ploidy levels of plants are shown in Table 19.

At least 500 pollen grains of four flowers of each plant used for the anther culture were examined for their fertilities by staining with aceto-carmine. The diploid plants as expected showed the highest pollen fertility in excess of 96 percent. On the other hand, all haploid plants examined exhibited complete sterility. The pollen fertilities of 5 triploid plants were generally less than 40 percent. In these triploids, however, a large fluctuation in the pollen fertility was observed among flowers of the same plant and also among 5 plants. Three tetraploid plants used for the anther culture also showed a considerably low pollen fertility of less than 35 percent and a large deviation was again observed among flowers and among the 3 plants used. The causativeness of the large fluctuation in the pollen fertility of triploid and tetraploid plants was not clear in this study.

The frequency of plant production by anther culture was usually higher in the order of diploid, triploid and tetraploid plants. Whereas, no single androgenetic plant was successfully produced in the anther culture of haploid plants. In the diploid plants more than 50 percent of the anthers produced androgenetic plantlets on medium H supplemented 0.1 mg/l of IAA and 0.1 mg/l of kinetin. In this case while some of the anthers produced numerous numbers of plantlets, some of them produced only one or two. The triploid plants also showed a relatively high production of androgenetic plantlets although large differences in productibility were observed among the 5 plants used. Although 4 types of medium containing various concentrations and combinations of IAA and kinetin were tested, anther culture of the tetraploid plants was generally less successful than the diploid and triploid plants while an exception was observed in $4n-3$ which showed about 11 percent of the anthers forming plantlets on medium supplemented 2 mg/l of kinetin. In general, however, only several plants from each anther were produced from the successful

TABLE 19. anther culture of four ploidy levels of plants in *Nicotiana tabacum* (var. Wisconsin 38)

Ploidy level	Pollen fertility (%)	Medium type*	Number of anther used for culture	Number of anther forming plantlets	% of anther forming plantlets	Length of flower bud used for anther culture (mm)	Length of flower bud forming plantlets (mm)
Haploid	0	H-1	190	0	0	5-36	
		H-2	85	0	0	5-35	
		H-3	250	0	0	5-38	
Diploid	97.6 (96.8-98.0)**	H-1	120	69	57.50	5-25	9-23
Triploid							
3*n*-1	0.8 (0.3-1.1)	H-1	200	11	5.50	9-23	11-17
3*n*-2	3.3 (0.4-7.4)	H-1	245	10	4.08	10-26	12-18
3*n*-3	3.3 (0.7-8.6)	H-1	310	68	21.94	9-25	10-17
3*n*-4	4.4 (0.6-8.7)	H-1	380	72	18.95	9-30	12-22
3*n*-5	16.9 (2.7-36.5)	H-1	400	53	13.25	9-32	11-19
Tetraploid							
4*n*-1	9.3 (2.2-29.9)	H-1	25	1	4.00	9-26	12
		H-2	165	1	0.61	5-25	13
		H-3	30	1	3.33	9-30	13
		H-4	485	8	1.65	6-33	12-15
4*n*-2	10.9 (3.0-18.8)	H-1	110	3	2.73	11-22	14-15
4*n*-3	14.8 (7.2-33.6)	H-4	205	23	11.22	10-28	10-23

* H-1, 0.1 mg/l IAA and 0.1 mg/l kinetin; H-2, 1 mg/l IAA and 1 mg/l kinetin; H-3, 2 mg/l IAA; H-4, 2 mg/l kinetin.

** Range of variation.

anthers of the tetraploid plants.

The length of flower buds was measured on all flower buds used for the anther culture. Diploid plant flower buds with lengths ranging from 9 mm to 23 mm produced androgenetic plantlets on anther culture. Androgenetic plantlets from the triploid and tetraploid plants were also produced from flower buds of the same length range as the diploids. Microscopic determination of the flower buds of this length range which fromed plantlets showed an uninucleate stage to early binucleate stage prior to an accumulation of starch grains.

b. *Chromosome numbers of androgenetic progenies*

Chromosome numbers of the androgenetic progenies produced by the anther culture of three ploidy levels of plants, namely diploids, triploids and tetraploids, are shown in Fig. 10.

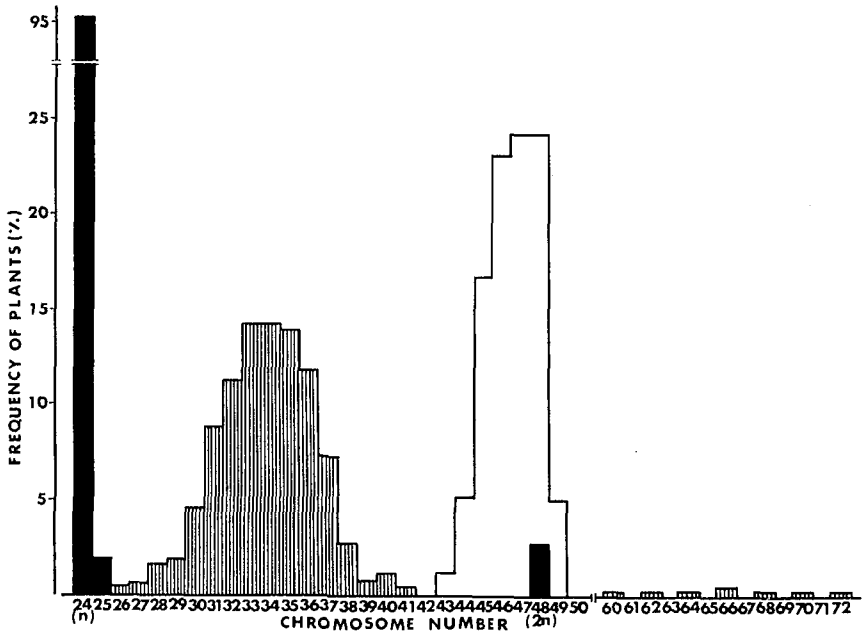

Fig. 10. Chromosome numbers of androgenetic plants produced by anther culture of $2n$, $3n$ and $4n$ tobacco plants. ■ androgenetic plants from $2n$, ▤ androgenetic plants from $3n$ and □ androgenetic plants from $4n$ plants. 107, 415 and 78 plants were examined for their chromosome numbers in $2n$, $3n$ and $4n$, respectively. 7 chromosomal chimeric plants obtained in the $3n$ plants are excluded from this figure.

107 androgenetic progenies from the diploid plants were examined for their chromosome numbers. Two of them were disomic haploid, 25 ($n+1$), and three of them were in a diploid state, having 48 ($2n$) chromosomes. All other plants showed 24 (n) chromosomes of haploid state. Whether the production of the unexpected 25 chromosome plants was due to a non-disjunction of chromosomes during the gamete formation or an unexpected irregularity during the differentiation of plantlets in the anther culture was not ascertained. On the other hand, the induction of polyploid plants in addition to the expected haploids has been reported in the anther culture of several species by many investigators. Thus, it may be said that the androgenetic diploid plants are likely to have originated in the multiplication of gametic chromosomes or fusion of vegetative and generative nucleus during the anther culture although the precise mechanism remains obscure.

415 androgenetic progenies from the triploid plants showed a variation in their chromosome numbers from 26 to 41. The progenies in the most frequent appearance showed 33 and 34 chromosome numbers. The theoretical distribution of chromosomes in the gametes of the triploids was probably spread out between 24 (n) and 48 ($2n$) with a mode of 36 chromosomes. Therefore, the characteristics of the distribution in the actual differentiated androgenetic plants was the lack of plants having higher levels of chromosome numbers. In fact, only about 24 percent of all regenerated plants exhibited more than 36 chromosomes. Some of the plants showed more than 60 chromosomes which might be derived from the multiplication of original chromosome numbers of the gametes *in vitro*. Occasional chimeric plants with regard to chromosome numbers such as (30, 60) or (32, 64) which are different among root tips of the same individuals and infrequently in the same root tips were also obtained and it seems evident that endomitosis occurred at some stage in the development of the androgenetic plants.

Among 78 androgenetic progenies from the tetraploid plants examined for their chromosome numbers, 94.8 percent of the total showed less than 48 ($2n$) chromosomes. The minimum chromosome number was 43 and the maximum was 49 which were the only plants showing over 48 chromosomes.

c. *Anther culture of androgenetic progenies induced by tetraploid plants*

Plants with various chromosome numbers which were obtained by

TABLE 20. Anther culture of androgenetic plants induced from tetraploid plants

Plant no.	Number of chromosome	Pollen fertility (%)	Number of anther used for culture	Number of anther forming plantlets	% of anther forming plantlets	Length of flower bud used for anther culture (mm)	Length of flower bud forming plantlets (mm)
I	45	0.1 (0 - 0.2)*	100	0	0	10-26	
II	45	0.6 (0 - 2.2)	270	0	0	9-25	
III	45	12.5 (8.3-17.5)	180	47	26.11	11-27	12-18
IV	45	21.0 (17.4-25.0)	90	6	6.67	11-26	15-21
V	46	2.9 (1.4- 4.0)	175	1	0.57	10-37	15
VI	46	2.9 (1.8- 4.0)	165	2	1.21	12-31	17-18
VII	46	11.3 (4.5-16.6)	185	0	0	12-30	
VIII	46	12.3 (7.9-18.0)	155	7	4.52	12-28	13-17
IX	46	19.4 (12.5-27.2)	150	65	43.33	11-23	11-23
X	46	25.4 (21.1-31.7)	165	44	26.67	10-25	10-18
XI	46	34.3 (31.0-39.5)	205	1	0.49	11-42	15
XII	47	1.9 (0.4- 4.2)	190	68	35.79	11-25	11-18
XIII	47	6.1 (3.8- 8.1)	205	0	0	9-24	
XIV	47	8.0 (5.6-10.0)	155	22	14.19	11-28	12-19
XV	47	21.8 (19.0-28.1)	170	3	1.76	12-26	15-24
XVI	47	27.1 (19.2-32.4)	50	9	18.00	11-32	13-22
XVII	48	16.7 (7.4-28.3)	185	47	25.41	13-35	13-30
XVIII	48	18.1 (5.0-26.6)	205	29	14.15	10-30	10-20
XIX	48	23.0 (20.3-25.5)	95	32	33.68	12-25	13-23
XX	48	34.0 (24.5-52.1)	180	87	48.33	11-27	12-27
XXI	48	34.1 (27.8-39.7)	160	74	46.25	14-30	14-28
XXII	48	73.5 (56.6-81.0)	125	32	25.60	10-26	12-26
XXIII	49	40.1 (12.8-57.2)	190	6	3.16	12-31	14-21
XXIV	49	62.5 (60.4-66.3)	150	55	36.67	11-25	11-25

All media used for the anther culture were supplemented with 0.1 mg/l of IAA and 0.1 mg/l of kinetin.

* Range of variation.

means of anther culture of tetraploid plants were again subjected to anther culture on medium H supplemented with 0.1 mg/l of IAA and 0.1 mg/l of kinetin (Table 20). Some of the plants with aneuploid chromosome numbers generally displayed low pollen fertilities and rarely showed success in the production of plants by anther culture. Some aneuploids, however, showed relatively high pollen fertilities and at times produced a number of the androgenetic progenies. On the other hand, the plants with diploid chromosome numbers as a whole showed considerably high pollen fertilities and plant production but were not so high with respect to both traits as compared to the original diploid parents which are shown in Table 19. There was, however, no strict relationship found between the pollen fertility and androgenetic plant production by anther culture.

d. *Chromosome numbers of progenies reinduced by anther culture of androgenetic plants of tetraploid plants*

Of the reinduced androgenetic progenies, even plants having diploid chromosome numbers, became segregated into several kinds of chromosome numbers, namely 24 (n), 25 ($n+1$) and 26 ($n+2$) (Table 21). The flower morphology between reinduced diploid plants by the anther culture of tetraploid plants and original parent diploid plants was clearly different (Plate XIX). Therefore, the majority of these aneuploid and diploid plants used for the anther culture are likely to be chromosome substituted types in which some chromosomes might be substituted by other homologous chromosomes, although irrevocable evidence remains unavailable. Besides these hyperhaploids some of the androgenetic plants occasionally displayed multiple chromosome levels such as 48, 50 and chromosomal chimera among root tips such as (24, 48), (25, 50) or (26, 52) chromosomes. Occasionally, some aneuploid plants produced androgenetic plants with 45, 46 and 47 chromosomes, although how they originated is unknown at the present time. Furthermore, two triploid plants ($3n=72$) were obtained by the anther culture of aneuploid plants with 47 chromosomes.

B. Effect of different chromosome numbers of pollen on plant formation by anther culture and fertilization

a. *Cross compatibility between diploid plants and several polyploid, aneuploid, and chromosome substituted diploid plants*

The results of reciprocal crosses between diploids and tetraploids, triploids, aneuploids, and chromosome substituted diploids are shown in Table 22. The plants possessed extremely high chromosome numbers such

TABLE 21. Chromosome numbers of progenies reinduced by anther culture of androgenetic progenies of tetraploid plants

Plant no.	Chromosome number of parental plant	Number of androgenetic progenies												
		Chromosome number												
		24	25	26	45	46	47	48	50	52	72	(24, 48)	(25, 50)	(26, 52)	Total
III	45	66 (94.3)						2 (2.9)				2 (2.9)			70 (100)
IV	45	1 (11.1)	3 (33.3)	3 (33.3)						1 (11.1)		1 (11.1)			9 (100)
V	46	1 (100)													1 (100)
VI	46	3 (42.9)	1 (14.3)	1 (14.3)		1 (14.3)			1 (14.3)						7 (100)
VIII	46	2 (11.1)	9 (50.0)			2 (11.1)		4 (22.2)				1 (5.6)			18 (100)
IX	46	60 (64.5)	32 (34.4)									1 (1.1)			93 (100)
X	46	14 (38.9)	17 (47.2)		1 (2.8)	2 (5.6)		1 (2.8)	1 (2.8)						36 (100)
XI	46		1 (50.0)	1 (50.0)											2 (100)
XII	47	27 (40.3)	28 (41.8)	1 (1.5)				4 (6.0)	6 (9.0)		1 (1.5)				67 (100)

XIV	47	24 (40.7)	26 (44.1)	2 (3.4)	2 (3.4)	1 (1.7)	2 (3.4)		1 (1.7)	1 (1.7)			59 (100)
XV	47	3 (100)											3 (100)
XVI	47	5 (38.5)	8 (61.5)										13 (100)
XVII	48	42 (47.7)	41 (46.6)			1 (1.1)	1 (1.1)			2 (2.3)	1 (1.1)		88 (100)
XVIII	48	3 (8.6)	29 (82.9)	2 (5.7)				1 (2.9)					35 (100)
XIX	48	18 (25.7)	32 (45.7)	18 (25.7)		1 (1.4)						1 (1.4)	70 (100)
XX	48	28 (35.9)	43 (55.1)	4 (5.1)		1 (1.3)	1 (1.3)			1 (1.3)			78 (100)
XXI	48	18 (28.6)	36 (57.1)	4 (6.3)		4 (6.3)	1 (1.6)						63 (100)
XXII	48	2 (3.4)	50 (84.7)	2 (3.4)			2 (3.4)				3 (5.1)		59 (100)
XXIII	49	3 (27.3)	5 (45.5)	3 (27.3)									11 (100)
XXIV	49	47 (52.8)	26 (29.2)	11 (12.4)		3 (3.4)	1 (1.1)			1 (1.1)			89 (100)

(24, 48), (25, 50) and (26, 52) indicate chromosomal chimera.

( ) Indicates percentage.

TABLE 22. Compatibility of reciprocal crosses between plants with various chromosome constituents and diploid plant, and chromosome number of plants produced by the cross to $2n$ plants

Plant number	Chromosome number	Pollen fertility (%)	Compatibility		Chromosome number of plants produced by the cross to $2n$ plants	Number of plants examined for their chromosome number
			cross to $2n$	cross with $2n$		
I	96 ($4n$)	11.7	−	+		
II	73	0.2	−	+		
III	73	2.4	−	+		
IV	73	30.5	−	+		
V	72 ($3n$)	5.7	−	+		
VI	67	15.4	−	+		
VII	67	23.2	−	+		
VIII	59	45.1	+	+	48-54 (24-30)**	110
IX	54	78.9	+	+	48-52 (24-28)	161
X	54	87.4	+	+	48-52 (24-28)	85
XI	48 ($2n$)	97.6	+	+		
XII	48*	23.0	+	+	48-50 (24-26)	84
XIII	48*	34.1	+	+	48-50 (24-26)	67
XIV	48*	73.5	+	+	48-50 (24-26)	74
XV	48*	16.7	+	+	48-49 (24-25)	87

\+ Compatible; − Incompatible.
* Chromosome substituted type.
** Chromosome number of pollens contributed to the fertilization.

as tetraploids ($4n=96$), triploids ($3n=72$) and aneuploids with 73 and 67 chromosomes were incompatible in the crosses to the maternal diploid plants. In these crosses, all artificially pollinated flowers usually dropped off after about one week. On the other hand, all these reciprocal crosses produced fruitful capsules with fertile seeds. This result, however, hardly means that all pollen grains of the plants with such high chromosome numbers lack the potentiality of fertilization, because a considerable percentage of pollens appeared to be fertile when they were stained by aceto-carmine (Table 22) and the selfing of some of these plants produced fertile seeds although they were not so many seeds in general. On the other hand, the plants with considerably low chromosome numbers such as 59 or 54 chromosomes (plant no. VIII, IX and X in Table 22), were capable of crossing to the maternal diploid plants ($2n=48$). The progenies derived

from the crosses of plants with 59 and 54 chromosome numbers to the normal diploids indicated a range of 48 to 54 and 48 to 52 chromosome numbers, respectively. Therefore, this result indicates that the pollens which may possibly cross with normal diploid plants have less than 30 chromosome in numbers. The chromosome substituted diploid plants (plant no. from XII to XV in Table 22) were also capable of crossing with the normal maternal diploid plants and their progenies showed a range of 48 to 50 chromosome in numbers (Plate XX, XXI).

b. *Anther culture of plants with various chromosome constitutions*

Frequencies of androgenetic plant formation and chromosome numbers of the plants obtained by anther culture of all plants used for the

TABLE 23. Chromosome number of androgenetic plants produced by anther culture of plants with various chromosome constituents

Plant number	Chromosome number	% of anther forming plants	Chromosome number of plants produced by anther culture*	Number of plants examined for their chromosome number
I	96 ($4n$)	3.67	43-49	78
II	73	8.81	31-40	72
III	73	7.03	29-40	25
IV	73	29.09	27-40	42
V	72 ($3n$)	13.25	26-41	399
VI	67	12.77	28-39	75
VII	67	5.88	28-39	32
VIII	59	41.67	24-32	140
IX	54	29.71	24-29	103
X	54	42.07	24-29	138
XI	48 ($2n$)	57.50	24-25***	102
XII	48**	33.68	24-26	68
XIII	48**	46.25	24-26	58
XIV	48**	25.60	24-26	54
XV	48**	25.41	24-25	83

* Chromosomal chimeric plants such as (24, 48) or (25, 50) chromosomes and multiple chromosome plants such as 48 or 50 chromosomes obtained occasionally are excluded from this table.

** Chromosome substituted type.

*** Normal diploid plant occasionally produced androgenetic plants with 25 ($n+1$) chromosomes.

experiment of cross compatibility are shown in Table 23. The plants with high chromosome numbers, which showed crossing inability to the maternal diploid plants, were not incapable of differentiating into androgenetic plants from pollen grains by the anther culture. The androgenetic plant production showed, however, a considerably low success rate with one exception of the plant with 73 chromosomes (plant no. IV). The chromosome numbers of these plants obtained by the anther cultures of plants with 96, 73, 72 and 67 chromosome numbers varied from 43 to 49, 27 to 40, 26 to 41 and 28 to 39, respectively. Other plants with considerably low chromosome numbers such as 59 and 54 chromosomes usually showed a high degree of success in the anther culture as compared with plants of high chromosome numbers and they produced the androgenetic plants with chromosome numbers from 24 to 32. The chromosome substituted diploid plants also produced many androgenetic plants with 24 to 26 chromosomes (Plate XX, XXI). Therefore, it seems that the totipotency of pollens differentiating into plants was affected to a lesser degree by their chromosome numbers than their fertilization, while the ability of fertilization of pollens with various chromosome numbers was restricted by the relative chromosome numbers of the maternal plants.

c. *Ability of plant formation by anther culture and fertilization of pollens*

Comparisons between the ability of plant formation by anther culture and fertilization of pollens of plants with 59, 54 chromosomes and auto-substituted chromosomes, all of which can be crossed with maternal diploid plants, are shown in Table 24. More than 50 plants produced by anther culture and by crossing seeds were carried out for the determination of their chromosome numbers. The results indicated that the pollens fertilized to the diploid plants in general have an advantage in the lower chromosome numbers as compared with the pollens differentiated into the plants by anther culture. This is possibly due to the fact that the pollens with the lower chromosome numbers have an additional advantage of fertilization in the competition among pollens with various chromosome numbers, in other words, the pollens with the lower chromosome numbers have a higher ability of fertilization than those of the higher chromosome numbers. On the other hand, no competition or at least not so high competition as found at the time of the fertilization, exists in the differentiation of plants from pollens with various chromosome numbers, although it could not clearly proved because of the lack of direct determination of pollen chromosome numbers used for the anther culture.

TABLE 24. Frequency (%) of different chromosome numbers of pollens differentiated into plants by anther culture and fertilized to diploid plant

Plant number	Chromosome number	A and B	Chromosome number of pollens contributed to plant formation and fertilization*									Number of plant examined for their chromosome number
			24	25	26	27	28	29	30	31	32	
VIII	59	A	4.3	10.0	20.7	20.7	17.1	14.3	10.0	2.1	0.7	140
		B	9.3	15.6	26.5	24.6	11.0	9.2	3.7	0	0	110
IX	54	A	12.6	27.2	23.3	27.2	6.8	1.9	1.0			103
		B	19.9	28.6	29.8	18.0	3.7	0	0			161
X	54	A	18.1	21.0	28.3	23.2	8.7	0.7				138
		B	22.4	37.6	27.1	11.8	1.2	0				85
XII	48**	A	26.5	47.1	26.5							68
		B	58.3	29.8	11.9							84
XIII	48**	A	31.0	62.1	6.9							58
		B	62.7	19.4	17.9							67
XIV	48**	A	3.7	92.6	3.7							54
		B	23.0	70.3	6.8							74
XV	48**	A	50.6	49.4								83
		B	74.7	25.3								87

A Frequency (%) of androgenetic plants by anther culture.
B Frequency (%) of pollens fertilized to diploid plant.
* Chromosomal chimeric plants such as (24, 48) or (25, 50) chromosomes and multiple chromosome plants such as 48 or 50 chromosomes obtained occasionally are excluded from this table.
** Chromosome substituted type.

C. Production of disomic haploid ($n+1$) series by anther culture

a. *Comparison of various parts of flowers in different seasons*

Depending upon the general observation of morphology in tobacco plants growing in a green house over a year, vegetative characters such as plant height, leaf number, leaf size and leaf shape appeared to be highly variable from season to season even in the same individual plant. Furthermore, characters such as leaf size and shape growing from different positions of the stem appeared to be significantly different even in the same seasons. In general, the leaves at a high position of the stem, which are immature, were smaller in size and narrow in shape and those at a low position of the stem, which are mature, were large in size and wide in shape. In addition to these, it was difficult to obtain data conserning such characters in a number of the same growing stage of plants, because the time of plant transplantation from test tube to pot in a green house differed among the obtained plants by the anther culture. Therefore, characters of various parts of flowers such as length of corolla and calyx, width of corolla and calyx and length of style and filament (Fig. 11), which are considered to be more stable characters compared with the vegetative characters in different seasons and conditions, were used for morphological analysis. However, preliminary tests were carried out to determine to what extent these characters are stable in two

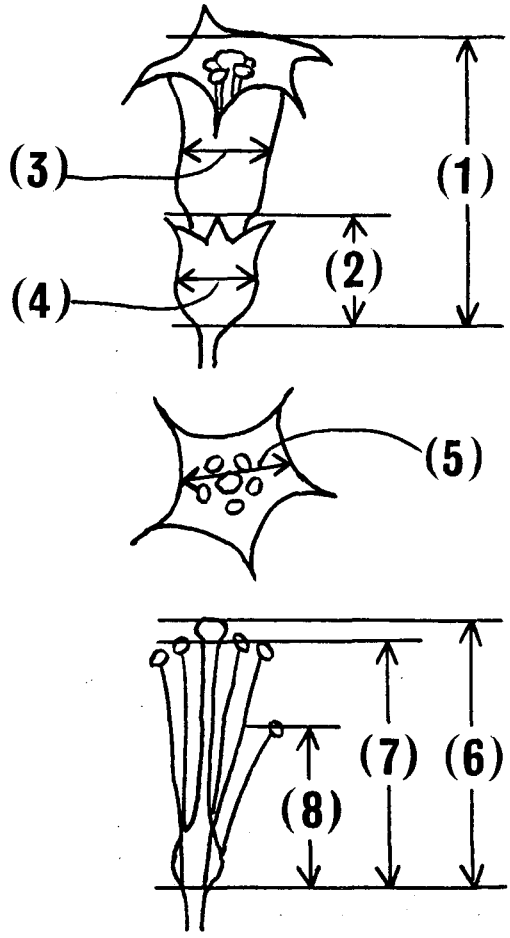

Fig. 11. Various parts of flower measured for morphological analysis.
(1) to (8) are corresponding to those in Table 25 and 26.

TABLE 25. Comparison of size in various parts of flower in different seasons

Material	Season	Character					
		(1)	(2)	(3)	(6)	(7)	(8)
		mm	mm	mm	mm	mm	mm
$2n$	Dec.-Jan.	42.6	16.3	8.5*	39.9***	38.9	33.6
	Apr.-May	43.0	15.8	7.9	38.0	38.6	33.2
A ($n+1$)	Dec.-Jan.	46.9	19.6***	8.0***	45.5	38.4**	29.8**
	Apr.-May	48.2	17.0	7.2	46.3	41.3	32.6
E ($n+1$)	Dec.-Jan.	34.8	13.8***	7.1	31.7	24.0	19.9*
	Apr.-May	35.1	10.7	7.0	32.7	25.4	21.5
n	Dec.-Jan.	37.7	15.6*	7.4*	37.5	29.2	24.5
	Apr.-May	37.2	13.9	6.9	36.9	29.1	25.4

*, ** and ***; exceeding significantly at 5%, 1% and 0.1% levels, respectively.
(1), (2), (3), (6), (7) and (8) are corresponding to Fig. 11.

different seasons, winter (December to January) and Spring (April to May) by using the same individuals of haploid, diploid and disomic haploid ($n+1$) plants. The results are shown in Table 25. The results indicated that no significant difference was observed in the length of corolla between two seasons, but some other parts of flower such as length of calyx, width of corolla, length of style, and length of the longest and shortest filament were significantly different in haploid, diploid and disomic haploid plants tested, although a regular tendency of difference among plants with different chromosome numbers could not be found. Thus, this result suggests that even in flower morphological comparisons should be performed with each other in a confined season and condition.

b. *Disomic haploid plants classified by flower morphology*

71 disomic haploid plants which flowered from April to May were collected among various aneuploid progenies derived by anther culture of aneuploids produced by triploid plants crossed with diploid plants, and chromosome substituted diploids and aneuploids produced by anther culture of tetraploid plants. These could be classified into 12 types by observation of flower morphology. Five flowers of all of these plants were sampled and measured for the size of eight parts of flowers (Table 26, Plate XXII). All of these plants showed some parts of flower which were not significantly different but some parts were significantly different from each other, for instance, corolla lengths were not significantly different between 2 types

TABLE 26. Disomic haploid ($n+1$) plants grouped into 12 types by comparison of size in various parts of flower

Type	(1) mm	(2) mm	(3) mm	(4) mm	(5) mm	(6) mm	(7) mm	(8) mm	Number of plant
n	36.8 a	13.2 e f	7.0 b	7.0 b c	12.4 c d	37.0 b c	28.4 c	24.8 c	
A	48.2 b	16.6 a b	7.0 b	7.4 a b	13.0 b c	45.4 a	41.2 a	32.4 a	8
B	42.6 c	16.0 a b c	6.8 b	7.0 b c	15.8 a	38.2 b	31.4 b	27.0 b	4
C	37.6 c d	15.4 b c d	7.0 b	7.0 b c	13.0 b c	35.8 c d	26.2 d	22.2 d e	3
D	36.4 c d	17.2 a	6.6 b c	7.8 a	12.4 c d	36.0 c	27.0 c d	24.0 c d	1
E	36.0 d	11.2 g h	7.0 b	7.0 b c	12.8 c	33.2 e f	26.0 d	22.4 d e	46
F	34.2 e	14.2 d e	6.8 b	6.6 c d	12.0 c d	35.8 c d	24.0 e	20.4 e f	1
G	34.0 e	11.8 f g h	8.0 a	7.2 a b c	14.4 a b	32.0 f	26.8 c d	22.2 d e	1
H	34.0 e	10.8 h	7.0 b	6.0 d e	12.4 c d	29.8 g	23.8 e	21.2 e	1
I	34.0 e	11.2 g h	5.6 d e	5.6 e f	8.4 e	30.2 g	22.0 f	18.0 g h	1
J	33.0 e f	14.8 c d	6.0 c d	6.0 d e	9.4 e	34.2 d e	19.2 h	16.6 h	1
K	33.2 f	12.4 f g	6.0 c d	5.2 f	9.4 e	32.2 f	21.0 f g	18.8 f g	1
L	31.8 f	16.2 a b c	5.0 e	7.0 b c	11.0 d	32.0 f	20.0 g h	17.4 g h	3
Total									71

Two values with different letters in the same column differ at 5% level after Duncan's multiple range test.

(1), (2), (3), (4), (5), (6), (7) and (8) are corresponding to Fig. 11.

but the lengths of calyx were significantly different. Some types of disomic haploids, namely type A and B were significantly larger in fiower size than the haploid plants and especially type A was larger even than normal diploid plants. The other types of disomic haploids were almost the same size of haploid or smaller than haploid plants. The occurrence of type E was very high, namely 46 of 71 plants were grouped into type E. The origination of 71 plants was indicated in Table 27. This shows that most of these disomic haploid plants (57 of 71 plants) were derived from the aneuploids and diploids which were derived by anther culture of tetraploids and which were considered to be chromosome autosubstituted type. These chromosome substituted type of plants extensively produced plants of type E. Therefore, in the plants of chromosome autosubstituted plants it can be assumed that the chromosomes concerned with the substitution by the course of irregular meiosis of tetraploid plants are restricted to a small number of certain chromosomes. However, the possibility that type E plants including various types of $n+1$ plants which were indistinguishable by the morphology of flowers can not be ruled out.

TABLE 27. Origin of disomic haploid plants

Parent Plant	Chromosome number	Number of disomic haploid												Total
		A	B	C	D	E	F	G	H	I	J	K	L	
IV*	45					1								1
VIII*	46					5								5
IX*	46			3		2								5
X*	46				1	4								5
XII*	47					5								5
XIV*	47					3								3
XVI*	47					5								5
XVII*	48					4								4
XVIII*	48					5								5
XIX*	48	4												4
XX*	48					3					1			4
XXI*	48	4												4
XXII*	48					4								4
XXIV*	49												3	3
IX**	54		3			4	1	1				1		10
X**	54		1			1			1	1				4
Total		8	4	3	1	46	1	1	1	1	1	1	3	71

* The same plant number used in Table 21 and all of them are chromosome substituted type.

** The same plant number used in Table 22.

(3) Discussion

A. Anther culture of plants of various ploidies and chromosome constitutions

a. *Anther culture of four ploidy levels of plants*

Spontaneous or artificial occurrences of aneuploid plants are common and widespread phenomena in the plant kingdom. Some of them are useful for studies in genetics and plant breeding. Because of the lack of proper artificial means in some cases, however, available plants with such useful aneuploid chromosomes sometimes are severely restricted to certain varieties and numbers in their productions.

Tissue culture technique was applied to the production of various aneuploid plants by regeneration from tumorous callus cultures (SACRISTÁN and MELCHERS 1969). Furthermore, the recent development of anther

culture technique shows great promise in the production of a number of haploid plants in many genera. Therefore, it may be possible to consider that this anther culture technique may be applied to the production of aneuploid plants including the aneuhaploids by using proper materials such as various polyploids producing gametes with irregular chromosome numbers. Indeed, this new method of anther culture of triploids, tetraploids and androgenetic progenies from tetraploids of *Nicotiana tabacum* has clarified beyond doubt that the gametes of not exact multiples of the basic numbers are capable of differentiation into complete plants as well as the euhaploid state of gametes. MATTINGLY and COLLINS (1974) also reported that the gametes with nullihaploid chromosome numbers can possibly differentiate into complete plants by the anther culture of monosomic tobacco plants.

It may be expected in the gamete formation of autotriploid that two groups of genome separate evenly at each pole and one more additional genome segregates randomly at each pole in the course of meiosis. Therefore, in autotriploid tobacco plants ($3n=72$) various aneuploid gametes containing from 24 to 48 chromosomes are predicted to be formed by random segregation of the additional 24 chromosomes. The most frequent theoretical appearance of gametes may be 36 chromosomes. The distribution of the actual differentiated plants from the anther of triploid plants, however, was between 26 and 41 with a mode of around 33 and 34 chromosomes which falls into the lower portion of the theoretical distribution of gametes. This incline towards the lower chromosomes numbers was at first assumed to be due to the differences of totipotency of the gametes possessing numerous varieties of chromosome numbers or suspected to be result of competition among the gametes during the time of regeneration into plants, namely it was surmised that the gametes with the lower chromosome numbers showed higher totipotency or competition. No evidence confirming this explanation, however, was obtained from this study. The other possibility is illustrated by the facts that DARLINGTON and MATHER (1944) in *Hyacinthus* and ISHIMURA (1971) in *Beta vulgaris* observed the appearance of lagging chromosomes in the meiosis of triploid plants, which frequently disappeared at the late stage of meiosis, thus some gametes show a reduced state of chromosome numbers. If the same phenomenon occurred in the meiosis of the triploid tobacco plants, the distribution of chromosome numbers in the gametes itself might incline towards the lower state of chromosome numbers. The results which show that most of the androgenetic progenies from tetraploid plants also showed lower parts of

chromosome nnmbers, less than 48 chromosomes, while only a few of them showed over 48 chromosomes, may be possibly again considered to depend upon the disappearance of some of the lagging chromosomes during the meiosis.

b. *Anther culture of androgenetic progenies induced by tetraploid plants*

Androgenetic progenies from both aneuploid and diploid plants derived from the anther culture of tetraploid plants produced a large number of plants possessing hyperhaploid chromosome numbers besides euhaploid. They were mainly disomic $(n+1)$ and trisomic $(n+2)$ haploids. As indicated in Fig. 10, most of the androgenetic progenies from eudiploid parent plants showed haploid chromosome numbers even though a few of them showed spontaneous disomic haploid chromosome numbers which might have originated in the unreduced chromosomes during meiosis or an unexpected irregularity of chromosomes during the differentiation from pollen grains. On the other hand, the occurrences of aneuploid from the anther culture of the plants having diploid chromosome numbers derived from the tetraploid was significantly high as compared with those from eudiploid plants. This may be caused, therefore, by these androgenetic diploid plants derived from the tetraploid which are probably not eudiploid but are rather of the chromosome autosubstitution type. In other words, these plants presumably have one or several sets of three or four homologous chromosomes which might have originated in the irregular segregation of chromosomes in the meiosis of tetraploid plants. A set of homologus chromosomes related to the substitution may be segregated irregularly in the meiosis. Thus, these irregular segregations may result in the gametes itself with disomic or trisomic haploid chromosome numbers which regenerate into plants with aneuhaploid chromosomes by means of the anther culture. Hyperhaploid plant segregation from parents with aneuploid chromosomes such as 45, 46, 47 and 49 chromosomes may also have arisen by further complicated substitution in the parent chromosomes.

c. *Mechanism of polyploid plant induction in anther culture*

There have been many reported instances in which the anther culture produced polyploids besides haploid plants. For instance, diploid plants reported in the anther culture of *Nicotiana tabacum* (BOURGIN and NITSCH 1967, TANAKA and NAKATA 1969, COLLINS *et al.* 1972, ENGVILD 1974) and *Nicotiana otophora* (COLLINS *et al.* 1972), diploid and triploid plants in

Atropa belladonna (ZENKTELER 1971, NARAYANASWAMY and GEORGE 1972) and *Datura metel* (NARAYANASWAMY and CHANDY 1971), diploid, triploid and tetraploid plants in *Datura innoxia* (ENGVILD *et al.* 1972). SUNDERLAND *et al.* (1974) reported from haploid to hexaploid plants in *Datura innoxia.* The plants obtained through the calluses derived by the anther culture also showed various ploidies, for instance, diploid plants in *Oryza sativa* (IYER and RAINA 1972), *Setaria italica* (BAN *et al.* 1971) and *Solanum melongena* (RAINA and IYER 1973), diploid and triploid plants in *Datura metel* (IYER and RAINA 1972) and *Solanum nigrum* (HARN 1971), diploid and tetraploid in *Hordeum vulgare* (CLAPHAM 1973), triploid in *Petunia hybrida* (RAQUIN and PILET 1972), triploid and tetraploid plants in *Petunia axillaris* (ENGVILD 1973), from diploid to tetraploid plants in *Oryza sativa* (NIIZEKI and OONO 1971) and from diploid to pentaploid plants in *Oryza sativa* (NISHI and MITSUOKA 1969). IRIKURA (1975) reported from haploid to tetraploid plants directly or through calluses derived by anther culture of 6 species of genus *Solanum* and of an interspecific hybrid. Insofar as the anther culture of *Nicotiana* species studied by many investigators, there has not been a single report of induction of odd numbered polyploid plants. However, this study indicated that the induction of triploid plants in this species besides haploid and diploid plants could have occurred from the anther culture of plants with aneuploid chromosome numbers which were derived from the anther culture of tetraploid plants.

NARAYANASWAMY and CHANDY (1971) based on their observations suggested from their microscopic observations that the origin of diploid plants in *Datura metel* is the multiplication of chromosomes in germ cells in the early period of anther culture or the fusion of vegetative and generative nucleus. Further, SUNDERLAND *et al.* (1974) made a precise analysis on the process of polyploid formation in the anther culture of *Datura innoxia.* In the microspores of the cultured anthers at 28°C for 24 hours, two forms of haploid pollen grains were produced, namely one with typical unequal generative and vegetative nuclei (A), and the other with equal vegetative type nuclei (B). Haploid plants usually originated by division of the vegetative cell in A grains accompanied by rapid degeneration of the generative cell or simultaneous mitosis of the nuclei in B grains. At times endoreduplicated generative nucleus gave rise to diplochromosomes which are postulated to divide on a common spindle together with the vegetative chromosomes to yield triploid proembryoid. Simultaneous mitosis of an endoreduplicated generative nucleus and two vegetative daughter

nuclei occurred at times. Nuclear fusion in these grains was envisaged to lead to a tetraploid proembryoid. Diploid proembryoids probably arose from nuclear fusion in A and B grains.

In contrast to the postulation in which the polyploid formation occurs by nuclear fusion, it is considered that the endomitosis in the early stage of embryoid differentiation can not be ruled out as a possible mechanism of polyploid production. This study indicated that many types of chromosomal chimeric plants such as (24, 48) or (25, 50) chromosomes frequently occurred in the anther culture of triploids and plants with various aneuploid and chromosome constitutions. These chimeric plants may have originated in the endomitosis of considerable later stages of embryoids or plantlets. If this endomitosis can occur at the time of initiation of embryoid formation, it would give rise to complete polyploid plants instead of haploid plants.

B. Effect of different chromosome numbers of pollen on plant formation by anther culture and fertilization

There are many instances in plants which show a cross incompatibility caused by the different chromosome numbers of parent plants. They are mainly grouped into two types of the cross incompatibility arising from the conditions in which first, paternal plants have more chromosomes than the maternal plants and second, paternal plants with less chromosomes than the maternal plants. *Nicotiana tabacum* may be considered to belong to the former type because of the condition in which the paternal plants with more chromosomes than the maternal plants are in general difficult to produce the hybrid plants.

In this study the plants showing a crossing ability to maternal diploids were considerably low in chromosome numbers such as 59 and 54 and chromosome substituted diploids. In these plants the pollens which are capable of substantial crosses to diploid plants had as a prerequisite less than 30 chromosome numbers. If so, it would give rise to a question as to why the plants with high chromosome numbers such as 73, 72 and 67 are incompatible with the maternal diploid plants, because they may be also able to produce pollens with less than 30 chromosomes. Indeed, the fact that the plants with high chromosome numbers (plant no. from III to VII in Table 23) formed androgenetic plants with less than 30 chromosomes by anther culture, means beyond doubt that they also produce pollens with less than 30 chromosomes and thus have cross competence. The frequencies of androgenetic plants with less than 30 chromosomes by

TABLE 28. Frequency (%) of androgenetic plants with less than 30 chromosome numbers by anther culture

Plant number	Chromosome number	Plants with less than 30 chromosomes (%)
I	96 (4*n*)	0
II	73	0
III	73	4.0
IV	73	11.9
V	72 (3*n*)	9.8
VI	67	26.7
VII	67	28.1
VIII	59	93.9
IX	54	100
X	54	100
XI	48 (2*n*)	100
XII	48*	100
XIII	48*	100
XIV	48*	100
XV	48*	100

* Chromosome substituted type.

anther culture of each plant are shown in Table 28. This indicates that the frequencies of the androgenetic plants with less than 30 chromosomes are extremely different between the plants which are compatible or incompatible with diploid plants. All of the plants which showed crossing ability to the diploid plants produced more than 90 percent of androgenetic plants with less than 30 chromosomes, while all plants showing crossing inability produced less than 30 percent. If the amount of androgenetic plants with less than 30 chromosomes are almost in pararell with the amount of pollens with less than 30 chromosomes in the anthers, it must be considered a fact that a certain amount of pollens with a crossing ability, for instance in this study more than 90 percent, are neccessary for successful crosses to diploid plants. Thus, in plants which can not produce a certain amount of pollen with crossing ability probably fail in their crosses to diploid plants and the production of fruitful capsules, although the precise mechanism for the failure of the crosses was not clarified in this study.

TABLE 29. Frequency (%) of different chromosome numbers of pollens differentiated into plants by anther culture and fertilization

Plant number	Chromosome number	A B and C	Chromosome number of pollens contributed to plant formation and fertilization*									Number of plant examined for their chromosome number
			24	25	26	27	28	29	30	31	32	
VIII	59	A	4.3	10.0	20.7	20.7	17.1	14.3	10.0	2.1	0.7	140
		B	4.1	11.0	17.8	20.6	26.0	6.9	6.9	4.1	2.7	73
		C	9.3	15.6	26.5	24.6	11.0	9.2	3.7	0	0	110

A Frequency (%) of androgenetic plants by anther culture.

B Frequency (%) of megaspores fertilized with diploid plant pollens.

C Frequency (%) of pollens fertilized to diploid plant.

* Chromosomal chimeric plants such as (24, 48) or (25, 50) chromosomes and multiple chromosome plants such as 48 or 50 chromosomes obtained occasionally are excluded from this table.

NAKATA (1971) reported in the anther culture of tobacco plants that no competition could be observed among pollen grains for haploid plant formation as to leaf color, green and albino, which are the major genic characters. Subsequently, NAKATA and KURIHARA (1972) obtained the same results dealing with the other characters such as tobacco mosaic virus (TMV) resistance, wildfire disease resistance and leaf base shape character. In this study also the pollens possessing various chromosome numbers appeared to differentiate into plants at least without so severe competition, although it was difficult to conclude that absolutely no competition existed among pollens, because of the lack of the direct determination of chromosome numbers of pollens used for the anther culture. Both progenies of reciprocal crosses between one plant (plant no. VIII in Table 24) with 59 chromosomes and diploid plant were examined for their chromosome numbers (Table 29). The distributions of chromosomes in female gamete formed fertile seeds and those of male gamete formed androgenetic progenies were quite similar, while the distribution of the chromosomes in male gamete contributed to the fertilization to diploid plants showed an extreme inclination toward low chromosome numbers. In general, it is well known that there is less or no competition among female gametes with no extra and with extra chromosomes. If the distributions of chromosomes in female and male gametes are almost the same, the above result could be circumstantial evidence for the production of androgenetic progenies with various chromosome numbers without obvious competition.

Some of the aneuploid plants obtained by the anther culture of various polyploid and chromosome substituted plants were, however, occasionally observed to terminate their growth and subsequently to die at a young stage in the test tubes or soon after the transplantation to pots. This fact seems to show a possibility that at the early stages of the anther culture such as at the time of initiation of embryoid differentiation or during the embryoid formation stage the pollens or embryoids of certain aneuploid chromosome constitutions may sometimes lead to a lethality which possibly may not be caused by the competition among pollen grains but rather by the genic imbalance due to certain chromosome combinations. Therefore, it seems to be somewhat difficult to conclude that all pollens with any chromosome constitutions are equally capable of differentiation into plants although there is undoubtedly not so high a competition as found at the time of fertilization to the diploid plants.

C. Production of disomic haploid plants by anther culture

In the plant kingdom aneuploid plants in general are more likely to

differ from the diploids than eupolyploids. In an attempt to explain this phenemenon, BRIDGES (1922) presented the theory of genic balance. This theory assumes that the genetic complement of a normal diploid permits the individual to develop and function as an integrated organism. Additions of whole genomes to the basic complement do not affect this genic balance or little if at all. However, when individual chromosomes are added to the normal complement of some species, the genic balance is greatly disturbed. Imbalance is reflected in physiological disturbance and morphological and developmental deviations.

Trisomic morphology, however, is not seen in any species well defined and distinct from each other. The extra chromosomal types of some species are as vigorous and fertile as the normals and are morphologically indistinct from them as well as from each other. KHUSH (1973) grouped the above into three categories on the basic of trisomic morphology.

(1) Species in the first group have trisomics that are morphologically distinct and can be distinguished from each other phenotypically.
(2) The trisomics of the second category can not be distinguished from each other or from the disomic sibs by phenotype.
(3) The species in the third category are somewhere in between the extremes of the other two groups.

The species in the first group are basic diploids with few duplications in their genomes. The imbalance caused by the presence of an extra chromosome is expressed generally in phenotypic deviations, lowered vigor, and reduced fertility.

In the second group, usually the species are polyploids which carry many gene duplications in their genomes, which is evident by their tolerance to monosomy, and in the case of *Triticum aestivum* and *Avena sativa*, for nullisomy. Further duplication by an addition of a single chromosome alters the genic balance but little and there is no effect on the phenotype, vigor, or fertility of the trisomic. *Nicotiana tabacum* belongs to the second category. Indeed, this species is allopolyploid regarding which CLAUSEN and GOODSPEED (1924) described that all trisomics are indistinct from each other, and from normal diploids except for only three trisomics which differ slightly in flower size.

If the theory of genic balance is true, they should be distinct from each other in disomic haploids corresponding to the trisomics in *Nicotiana tabacum*. In fact, this study showed that 12 disomic haploids were classified and all of them differ in the morphology of flowers from that of haploid plants. This result supports convincingly the theory of genic

balance in polyploid species.

The usefulness of these disomic haploids is that it is a source of tetrasomic plants by duplication of the disomic haploids by appropriate treatment such as with colchicin or regeneration of callus culture. The selfing of the tetrasomic plants will give rise to trisomic plants. The trisomic plants will be the maintainer for disomic haploid plants, because the anther culture of the trisomic plants may produce disomic haploids. In the future, tetrasomic, trisomic and disomic haploids with the same extra chromosomes will be compared morphologically or physiologically and may well give rise to fruitful results for genetical studies.

IV. GENERAL DISCUSSION

The pollen grain is programmed for gamete and tube formation, but these processes are clearly not irrevocable and can be altered by anther and pollen grain culture and the pollen grain can be given an entirely new role for haploid plants or haploid callus formation. It is evident that the switch for the alteration comes into being as a result of the changed physical and nutritional environment of the pollen grain, namely the anther or pollen grain are removed from the flower bud or anther and transferred onto a proper medium. Precise factor or factors concerned in triggering the switch mechanism, however, are not fully understood. Nevertheless, in some economically important crops, especially rice, wheat and tobacco plant, a way to produce sufficient amounts of haploid plants by anther culture for practical uses and at will when required has been established. At present, some twenty genera in plants are available in the production of haploid plant and callus by the pollen and anther culture, however, in a large number of species the results remain unsuccessful. Some species such as *Lotus*, *Medicago* and *Trifolium* belonging to Leguminosae in this study are difficult to handle, because the diploid parts of somatic tissue readily formed callus, while pollen differentiation into the plantlet or callus could not be initiated in the media tested. The same conditions have been also found for triggering callus formation from somatic tissues in anthers of several species (KONOR and NATARAJA 1965b, FOWLER, *et al.* 1971, WATANABE *et al.* 1972, SUNDERLAND 1971). The study on *Lotus* species indicated that in the cultured anthers the individualized pollen grains at the late uninucleate stage were able to synthesize DNA for the first pollen mitosis and developed into mature pollen grain. However, the factor required for further differentiation beyond the formation of normal pollen grain was lacking in this case. SUNDERLAND (1971)

suggested, however, that simultaneous development of haploid and diploid callus is not impossible, and that although the diploid callus will probably outgrow the haploid there seems to be a possibility that haploid plants might be regenerated from such a mixed tissue. In fact, he observed mixed development in *Nicotiana* where the entire anther wall switched to callus growth and produced many diploid roots, while at the same time the pollen formed embryoids within the callus mass; these embryoids developed into plantlets and eventually grew out of the callus. Also, the initiation of cell division in the pollen grain culture of *Lotus* species in this study suggested that totipotency for differentiation is not completely lacking and that it may be possible for the pollen to develop further into more complex tissues. Therefore, there is an expectation to exploit the haploid plant or callus by anther or pollen grain culture in unsuccessful species if suitable culture conditions or appropriate medium is discovered. Further extensive investigations may be required to lead to success without abandoning the idea.

Doubling of the chromosome complement of an androgenetic haploid plant leads directly to the formation of a fertile and completely homozygous plant. As indicated in chapter 3 spontaneous chromosome doubling occurred in *Nicotiana tabacum*. The frequency of them was, however, low namely in less than 3 percent. Also, about 1 percent of the plants examined by SUNDERLAND (1970) gave rise to haploid-diploid mixed inflorescences and about 2% of those were examined by KASPERBAUER and COLLINS (1972). On the other hand, the method of doubling by regeneration from pollen callus which attained various polyploids and aneuploids in the process of culture promises good results. In this study, the most suitable media for the plant with doubling chromosome was medium H (described by BOURGIN and NITSCH 1967) supplemented with 4.0 mg/l of kinetin. Although the characteristics of plant obtained by doubling of haploids have not been extensively investigated, some data show that genetic changes may occur in the process of haploid plant formation and chromosome doubling. Furthermore, this genetic alteration appears to occur in both plants through treatment of the haploid with a spindle inhibitor such as colchicine and through regeneration from callus. BURK *et al.* (1972) observed the difference of agronomic and chemical characters among colchicine induced diploid lines of tobacco. This result suggested the occurrence of mutation. NAKAMURA *et al.* (1974) also reported variation among diploid lines obtained by colchicine treatment in some tobacco varieties for agronomic and chemical characters such as number of leaves

and alkaloid content. NITSCH (1969, 1972) obtained a spontaneous mutant of haploid tobacco with malformed leaves as well as mutants for leaf shape and flower color induced by chemical treatment or gamma-ray irradiation. Furthermore, he pointed out that anomalies could be generated during the tissue culture for doubling of chromosome number. OINUMA and YOSHIDA (1974) also obtained the result that three of nine diploid line of tobacco by doubling through the callus culture showed genetically different characters from the normal diploid lines in such points as plant height, leaf number and final yield. Because the changed characters were not segregated in the following generation, they suggested that mutations may have occurred in the haploid stage. OONO (1975) reported in *Oryza sativa* that some androgenetic diploid plants induced by both colchicine and callus culture were carrying mutant genes for dwarf or liguleless character in homozygous or heterozygous condition, which were respectively pressumed to be induced at haploid or diploid stage before or after redifferentiation. Thus, these results indicate that homozygotes produced by the haploid callus derived by anther culture should be subjected to rigorous testing for gene and at times chromosome defects, while this rapid method of producing homozygotes has a high potential in plant breeding research and in programs of crop improvement.

This study indicated that the anther culture technique can be undoubtedly applied to produce various varieties of aneuploid plants in tobacco by using plants with various polyploid chromosomes and chromosome constitutions. This method has a great advantage as compared with the past routine methods of aneuploid production because there is no strong competition found in fertilization among pollens with various chromosome numbers and chromosome constitutions. Besides collection of aneuploid occurrences spontaneously in natural populations, the usual methods of aneuploid production have been carried out by selfing or outbreeding of proper materials such as asynaptic or desynaptic disomics, various polyploids and plants with chromosomal abnormalities. Aneuploid production by these methods may be restricted by a lesser viability or competitive ability of aneuploid gametes than normal gametes. This tendency generally is more conspicuous in the male gametes than the femal gametes. On the contrary, the anther culture method in this study of tobacco proved that various varieties of aneuploid plants which have already well known aneuploids such as trisomics, monosomics and nullisomics or various aneuhaploids which have not been reported or rarely reported so far were produced without the restriction found in the past routine

methods. Some species, which are economically important, are restricted to certain varieties of aneuploid. In *Oryza sativa*, for instance, the reported aneuploids are mainly trisomics (NAKAMORI 1932, RAMANUJAM 1937, KARIBASAPPA 1961, KATAYAMA 1963, SEN 1965, WATANABE *et al.* 1969 and IWATA *et al.* 1970). Fortunately, *Oryza sativa* is one of the crops in which the anther culture technique has rapidly progressed over the past several years. Therefore, if the anther culture method for aneuploid production can be applied to such a crop it has a possibility to give various types of aneuploids which have not been reported hitherto and may contribute to advances in the genetic studies and plant breeding research. From the view point mentioned above, further investigation of aneuploid production by the anther culture of crops other than tobacco must be required extensively.

V. SUMMARY AND CONCLUSION

Anther and callus tissue culture could be recognized by this study as a highly useful and reliable means for production of haploids, complete homozygous diploids and polyploids and aneuploids, all of which have very important roles in genetics and plant breeding. The summary and conclusion of this study are as follows:

1. Production of haploid plants and calluses by anther culture

(1) Anther culture of three *Nicotiana* species, *N. tabacum* ($2n=48$), *N. sylvestris* ($2n=24$) and *N. glutinosa* ($2n=24$) was carried out on medium H (described by BOURGIN and NITSCH in 1967) supplemented with 0.1–2.0 mg/l of IAA and 0.1–2.0 mg/l of kinetin singly or in combination. Differences in the frequency of plantlet production were observed among species, namely *N. tabacum* and *N. sylvestris* were considerably high, while *N. glutinosa* was very low in plantlet production.

Anthers of three varieties of *N. tabacum*, Wisconsin 38, Hicks Broadleaf and Delhi 34 were cultured on medium H supplemented with various concentrations of IAA, kinetin and GA singly or in combinations. Different varieties required different media for suitable plantlet production. These studies reveal that the capacity to form plantlets probably depends upon genetic differences of the species or varieties in relation to its requirement of nutrition and growth regulator.

However, it was found that the growth regulators are not absolutely essential factors for the androgenetic plantlet initiation of *N. tabacum*. Considerable late uninucleate to early binucleate stages of anther produced

haploid plantlets on medium without any growth regulators. Exogenous growth regulators such as IAA and kinetin, therefore, appeared to be promoters but not initiators for multicellular development and subsequent embryoid and plantlet formation.

(2) After six weeks of incubation, microscopic observations on the cultured anthers of *N. tabacum* showed that in one anther different stages in the development of embryoids from the pollen grains were observed from a few initial cell divisions up to the cotyledon stage. Globular masses formed in which polarity was established, and the "heart shape" and the "torpedo" stages followed. The development of the embryoids, therefore, occurred in the same way as in normal embryogenesis. Matured plantlets produced from the anther and which developed good root-systems were then transplanted to pots and grown in a green house. Chromosome number determinations on regenerated adult plants showed them to be haploid with 24 somatic chromosomes which showed complete pollen sterility.

The androgenetic plantlet formation sometimes changed into callus formation depending on certain concentrations and types of growth regulators. On medium M (described by MILLER in 1963) containing 0.1 mg/l of IAA and 0.1 mg/l of BAP, only haploid plantlets were induced directly from pollen grains. On the other hand, both haploid plantlets and haploid calluses were induced on a medium containing a high level of concentration of growth regulators, namely 4.0 mg/l of IAA and 2.0 mg/l of BAP and only haploid calluses were induced on the medium containing 4.0 mg/l of NAA and 2.0 mg/l of BAP. Thus, whether plantlets or calluses predominantly proliferate from the anther depends upon the type and concentration of growth regulators in the medium.

(3) A study on the relationship between the development stage of anthers of *N. tabacum* and frequencies of androgenetic plantlet production indicated that the plantlet formation occurred from the anthers from the uninucleate pollen grain stage to binucleate stage prior to the accumulation of starch grains and that the most favorable stage for plantlet formation was at the time of the first pollen mitosis in which almost 80% of anthers used formed plantlets.

The autoradiographic study with ^{3}H-thymidine on cultured anthers clarified the critical stage at which the germ cells within the anthers gained totipotency for embryoid formation and also the effect that growth regulators (0.1 mg/l of IAA and 0.1 mg/l of kinetin) have on DNA synthesis and embryoid formation. The earliest pollen differentiation into

multicellular masses occurred before DNA synthesis in the first mitosis of the early individualized uninucleate pollen grains, but not in the quartet stage. The initial morphological changes observed in differentiation were an increase in pollen grain size and vacuolization. In pollen grains in which the nucleus was in the post-DNA synthesis stage, differentiation occurred one week earlier than for those pollen grains in which the nucleus was in the pre-DNA synthesis stage. The exogenous growth regulators, IAA and kinetin, appeared again to be responsible for multicellular development and subsequent embryoid formation, although they were not essential factors for the initiation.

(4) Six genera in Gramineae; *Bromus*, *Festuca*, *Lolium*, *Dactylis*, *Phleum* and *Sorghum* were used in the present study on the anther culture.

Four species, *Festuca arundinacea*, *Lolium multiflorum*, *Lolium perenne* and *Phleum pratense* formed several calluses from individualized uninucleate pollen grains in the cultured anthers. Microscopic observations indicated that the development of embryoids from the pollen grains occurred as in normal embryogenesis. However, all of the embryoids failed to germinate to complete plantlets but differentiated into calluses.

One very small immature plantlet proliferated from the anther of autotetraploid variety of *Lolium multiflorum*. The plantlet was immediately transferred to medium H supplemented with no growth regulator and thereafter it grew to a complete plantlet with a sufficient root-system. It was then transferred to a pot. Chromosome determination revealed that the obtained plant showed a haploid state of the autotetraploid plant. This early transplantation to another proper medium suggests the possibility of direct production of plantlets from pollen grains instead of calluses in other species in which matured embryoid differentiates into calluses in the course of anther culture.

(5) Three genera in Leguminosae; *Lotus*, *Medicago* and *Trifolium* were used for the anther culture.

Genus *Lotus* and *Medicago* formed calluses on the cultured anthers at high frequencies, while *Trifolium* was very low.

In genus *Lotus* the callus formation was studied in detail. In general, during the first four weeks of incubation, callus tissues proliferated readily from the anthers on media containing either two or three growth regulators. In an examination of the chromosome numbers of the callus tissues of *L. corniculatus*, cells with a haploid chromosome number were not found, but cells with diploid, tetraploid and various aneuploid chromosome numbers were observed. A microscopic examination of the germ cells in

the cultured anthers, indicated some morphorogical changes such as hypertrophy of the pollen grains and pollen with numerous starch grains. However, there was no evidence that cell division nor multicellular tissue had initiated from the germ cells. As a result, it was concluded that the calluses growing on the cultured media originated from somatic tissues of the anthers rather than from the germ cells.

The autoradiographic study with ^{3}H-thymidine on cultured anthers of *L. corniculatus* indicated that in the anthers containing individualized pollen grains at the late uninucleate stage, normal pollen grain mitosis (confirmed by *in vivo* observations insofar as DNA synthesis occurred) and maturation of the pollen occurred. However, there was no sign of any further cellular differentiation from the pollen as had occurred in the germ cells of *N. tabacum*.

(6) Shoot and root formation frequently occurred after 3 to 10 months of incubation from primary calluses of *Lotus* species which were cultured on medium M supplemented with IAA and kinetin. The shoots were transplanted onto a series of medium H with IAA, kinetin and 2, 4-D, in various combinations. In general, media which either lacked growth regulators or which contained low concentrations, such as 0.1 mg/l of IAA and 0.1 mg/l of kinetin, were found suitable for root regeneration. Young plantlets with well developed root-systems were transplanted to pots and grown in a growth chamber. In this manner, a number of mature plants of *L. corniculatus* and *L. caucasicus* were obtained successfully. These regenerated plants were mainly diploid, but in the case of *L. corniculatus*, some tetraploid plants were obtained.

Six month old callus tissues of *L. corniculatus* which had given rise to shoots on primary cultures showed a wide variation in chromosome numbers including diploid, tetraploid and various aneuploid chromosome numbers. Also, there was a considerable variation in chromosome numbers in cells of callus lines from a different primary cultures, even though each callus line had originated from one genotype.

(7) On pollen grain culture of *L. corniculatus* and *L. caucasicus*, many pollen grains, especially mature grains, hypertrophied and in some cases, the protoplasm burst through the cell walls. Nuclear and cell divisions occurred in some pollen grains of *L. corniculatus* which were in the uninucleate stage to form 3-celled tiers or clusters. Pollen with more advanced stages were not observed even after prolonged incubation. From these observations it was suggested that the pollen grains of *L. corniculatus* at least have the totipotency to give rise to the multicellular condition.

2. Production of polyploid cells and plants by callus culture of *Nicotiana* species

(1) Chromosome behaviours and karyotypic changes were investigated in the calluses of haploid and diploid plants in three *Nicotiana* species, *N. tabacum* ($2n=48$), *N. sylvestris* ($2n=24$) and *N. glutinosa* ($2n=24$). The medium used in this study was medium M supplemented with 2.0 mg/l of IAA and 2.0 mg/l of kinetin. Both haploid and diploid cultured calluses in *N. tabacum* showed a considerably high stability in the nuclear state as compared with the other two species, although a considerable amount of the cells with polyploid and aneuploid chromosomes were also observed in both ploidy levels of this species. On the other hand, in *N. sylvestris* the degree of polyploidization of the haploid or diploid cultures was significantly higher than those of the other species. The original haploid and diploid cells in each line were maintained at rate of only 30% and 4%, respectively. In *N. glutinosa*, the appearance of aneuploid cells was very remarkable, especially the cells with $n+1$ or $2n+2$ chromosomes in haploid and $2n-1$ or $4n-1$ chromosomes in diploid culture line were observed at high frequencies. Furthermore, dicentric chromosomes which might have originated from chromosome rearrangement were frequently observed in the diploid cultured callus of this species. As far as this experiment is concerned, in conclusion, no single line of cultured calluses having a stable nuclear state was found in these cultured calluses in the three species, although the degree and pattern of chromosomal variation was quite different among them.

(2) Haploid calluses of *N. tabacum* were cultured on four types of medium M, namely containing no growth regulator, 4.0 mg/l of kinetin, 4.0 mg/l of IAA, and 2.0 mg/l of IAA and kinetin. The calluses cultured on the medium containing 2.0 mg/l of IAA and kinetin were repeatedly subjected to transplantations to fresh media in the same manner as the above described series of media at intervals of 30 days. The occurrence of polyploid cells in the callus tissues was promoted by the media containing IAA alone or IAA and kinetin in combination. In addition, various kinds of aneuploid cells were observed, especially in the callus tissues cultured on the medium containing IAA. Several types of aberrations of nucleus such as multinuclei (multikaryon), micronucleus and nuclear fusion were accompanied by the occurrence of the polyploid and aneuploid cells. On the contrary, the medium without any growth regulators maintained a considerable stability of the original nuclear state.

The relative callus growth measured by the frequencies of the metaphase and anaphase cells was significantly high on media containing IAA as compared to those on media containing no growth regulator or kinetin alone. Thus, it may be concluded that a condition such as the medium containing IAA as auxin promotes the callus growth and simultaneously causes variations of the chromosome numbers.

(3) Media containing IAA, which was employed for accelerating the polyploidization of chromosome numbers, in calluses of *N. tabacum*, however, appeared to have a considerably high inhibiting effect on the regeneration of plants from the calluses as compared with the medium containing kinetin alone or no growth regulator. Most of the regenerated plants from the calluses cultured on the media to which IAA was added were haploids and the remaining were diploids, even though the callus tissues showed high polyploidization. On the other hand, the callus cultured on the medium containing kinetin alone produced higher numbers of diploids and even tetraploids, although the calluses did not show such high polyploidization as those on media containing IAA. These facts suggest that the conditions which are favorable for polyploidization of the calluses probably are unfavorable on the morphogenesis of higher levels of polyploids. In contrast, it seems that suppressible conditions of polyploidization of the callus tissues are rather preferential to morphogenesis of polyploid levels of plants.

Even though we are in a quandary between chromosome number of calluses and regenerated plants as mentioned above, it is still a useful way to obtain a number of diploid plants by duplication of the haploid chromosome number.

3. Production of aneuploid plants by anther culture in *N. tabacum*

(1) The anthers of four ploidy levels of *N. tabacum*, haploid ($n=24$), diploid ($2n=48$), triploid ($3n=72$) and tetraploid ($4n=96$) were cultured on medium H containing IAA and kinetin. Frequencies of plant production by the anther culture were higher in the order of $2n$, $3n$ and $4n$ plants. No single androgenetic plant was, however, obtained by anther culture of the haploid plants.

Androgenetic progenies from the diploid plants mostly had 24 (n) chromosomes while a few of them occassionally showed 25 ($n+1$) or 48 ($2n$) chromosomes. On the other hand, androgenetic plants from the triploids showed a variation in their chromosome numbers between 26 and 41 and the most frequent appearances were 33 and 34 chromosomes. In

the tetraploid plants the androgenetic plants also varied in their chromosome numbers from 43 to 49. A common characteristic of these androgenetic plants from both triploids and tetraploids was the decline to lower chromosome levels of plants and the lack of the higher chromosome levels of plants.

Androgenetic plants from aneuploid and diploid plants, which were induced from the anther culture of tetraploids, segregated into several kinds of chromosome numbers such as 24 (n), 25 ($n+1$) and 26 ($n+2$). Therefore, from this fact most cases of these plants were assumed to be chromosome autosubstitution types although irrevocable evidence is yet to be found.

Thus, it is now possible to produce various varieties of aneuploid plants some of which have hitherto not been or rarely reported in both spontaneous and artificial occurrences.

(2) There are many instances of cross incompatibility due to the different chromosome numbers of parents in the plant kingdom. In reciprocal crosses between diploid tobacco plants and polyploid, aneuploid, and chromosome substituted diploid plants, the plants with high chromosome numbers such as 96 ($4n$), 72 ($3n$), 73 and 67 were incompatible with the maternal diploid ($2n=48$) plants but the reversal crosses were all compatible. The plants with considerably low chromosome numbers such as 59 and 54, which have, however, still more chromosome numbers than diploid plants, were capable of crossing to the maternal diploid plants. In this case the chromosome numbers of pollens fertilized to the diploid plants were less than 30. The chromosome substituted diploid plants which were obtained by anther cultures of tetraploid plants were also capable of crossing to the maternal diploid plants. The progenies from the crosses indicated a range of 48 to 50 chromosomes. In anther culture, on the other hand, even the pollens with high chromosome numbers formed from 96 ($4n$), 72 ($3n$), 73 and 67 chromosome plants were all capable of differentiation into the plants, although the frequencies of plantlet formation are considerable lower than in the other plants with low chromosome numbers or substituted chromosomes. These results indicate, therefore, that the crossing ability of the pollens with various chromosome numbers may be restricted by the chromosome numbers of maternal plants, while the totipotency of the pollens into plants by the anther culture is likely to be less affected by the various chromosome numbers themselves. Furthermore, a significant difference was observed between the ability of plant formation and fertilization of pollens in plants with 59, 54 and substituted chromo-

somes, in other words, the pollens fertilized to the maternal diploid plants have the advantage of lower chromosome numbers as compared against the pollens differentiated into plants by anther culture.

However, from the observation of the cessation of growth during the young stages of some aneuploid plants obtained by anther culture of various polyploid or chromosome substituted plants, it is difficult to assume that all pollens with any chromosome constitutions have the same extent of totipotency into plant formation.

(3) There are many instances of trisomic plant series $(2n+1)$ reported in plant species. They are classified into three types, namely, the first, trisomic plants are distinguishable from each other and from normal diploids, the second, trisomic plants are not distinguishable from each other and from normal diploids and the third category are somewhere in between the extremes of the first and second category. *N. tabacum* comes under the second category. The interpretation of the second category in which each trisomic plant is not distinguishable is that the species are polyploids which carry many gene duplications in their genome, thus further duplication by an addition of a single chromosome alters the genic balance but little and there is no effect on the phenotype. This interpretation by genic balance was proved by the production of 12 types of disomic haploid plants where gene duplications may be reduced and magnifying the effect of addition of a single chromosome on genic balance and the phenotype. It is an interesting fact that the flowers of some disomic haploids are larger than haploids and even larger than normal diploid plants. However, most of them were less vigorous in flower size than the normal haploid plants.

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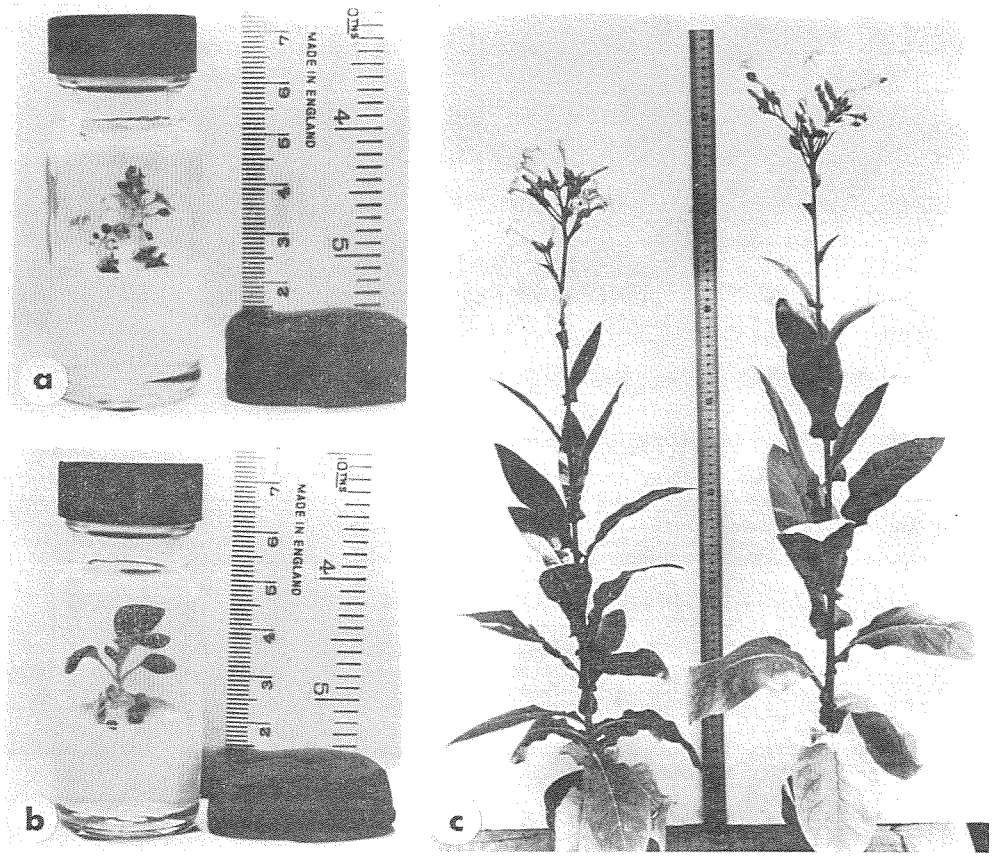

Plate I. Production of haploid plants from cultured anthers of *N. tabacum* (var. Wisconsin 38).

a. Proliferation of more than 10 plantlets from a single anther cultured on medium H-2 after 6 weeks of incubation.

b. A single plantlet growing from an anther cultured on medium H-1 after 6 weeks of incubation.

c. An adult haploid plant (left) produced by anther culture and its parent diploid plant (right).

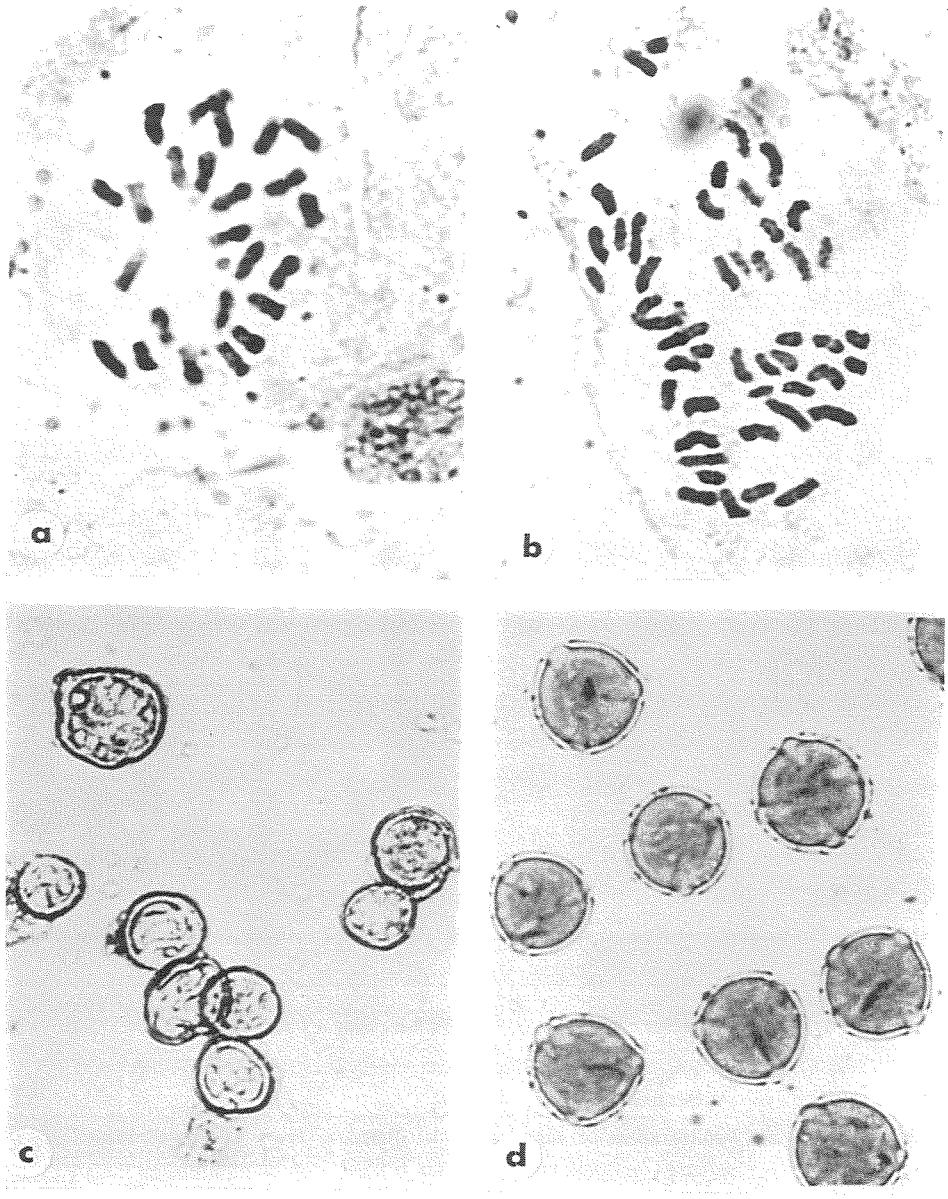

Plate II. Chromosome numbers in root tips and pollen fertility of haploid and parental diploid plant of *N. tabacum* (var. Wisconsin 38).

a. 24 somatic chromosomes. × ca. 1600.
b. 48 somatic chromosomes. × ca. 1600.
c. Pollen grains of haploid plant. × ca. 480.
d. Pollen grains of diploid plant. × ca. 480.

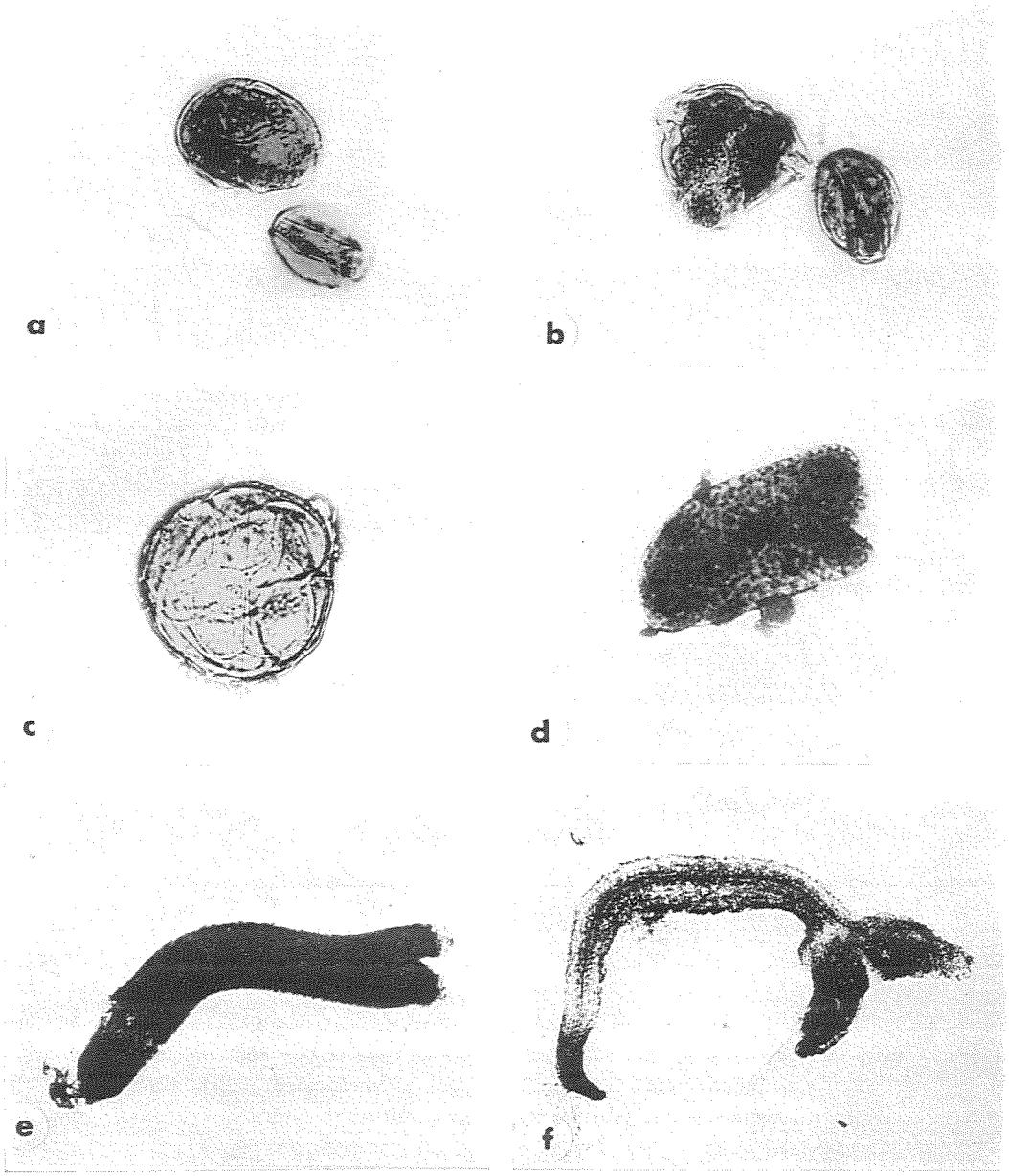

Plate III. Various stages in the formation of multicellular masses and embryoids in organ development from pollen grains in cultured anthers of *N. tabacum* (var. Wisconsin 38).

a. Initiation of cell division within a pollen grain. × ca. 520.
b. A cluster of several cells. × ca. 520.
c. A spherical mass with more than 10 cells. × ca. 520.
d. Heart shape stage. × ca. 95.
e. Torpedo stage. × ca. 60.
f. Cotyledonary stage. × ca. 30.

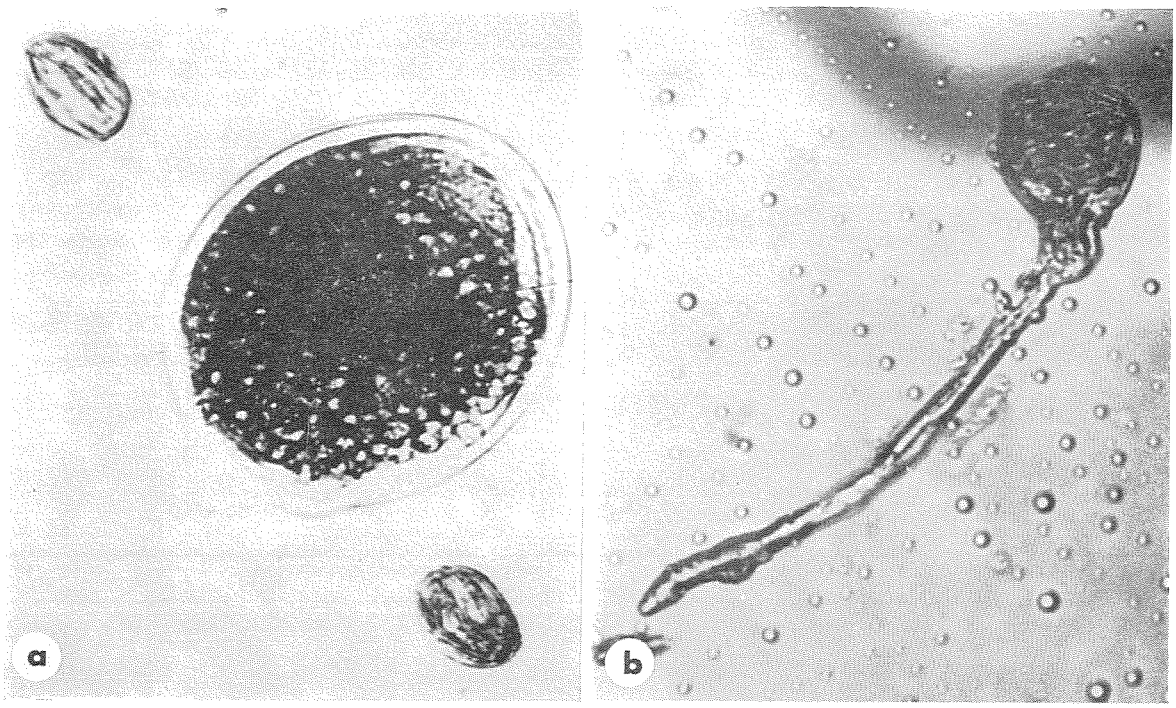

Plate IV. A pollen grain which had undergone hypertrophy and contained numerous starch grains (left), and a germinated pollen grain showing pollen tube (right). × ca. 550.

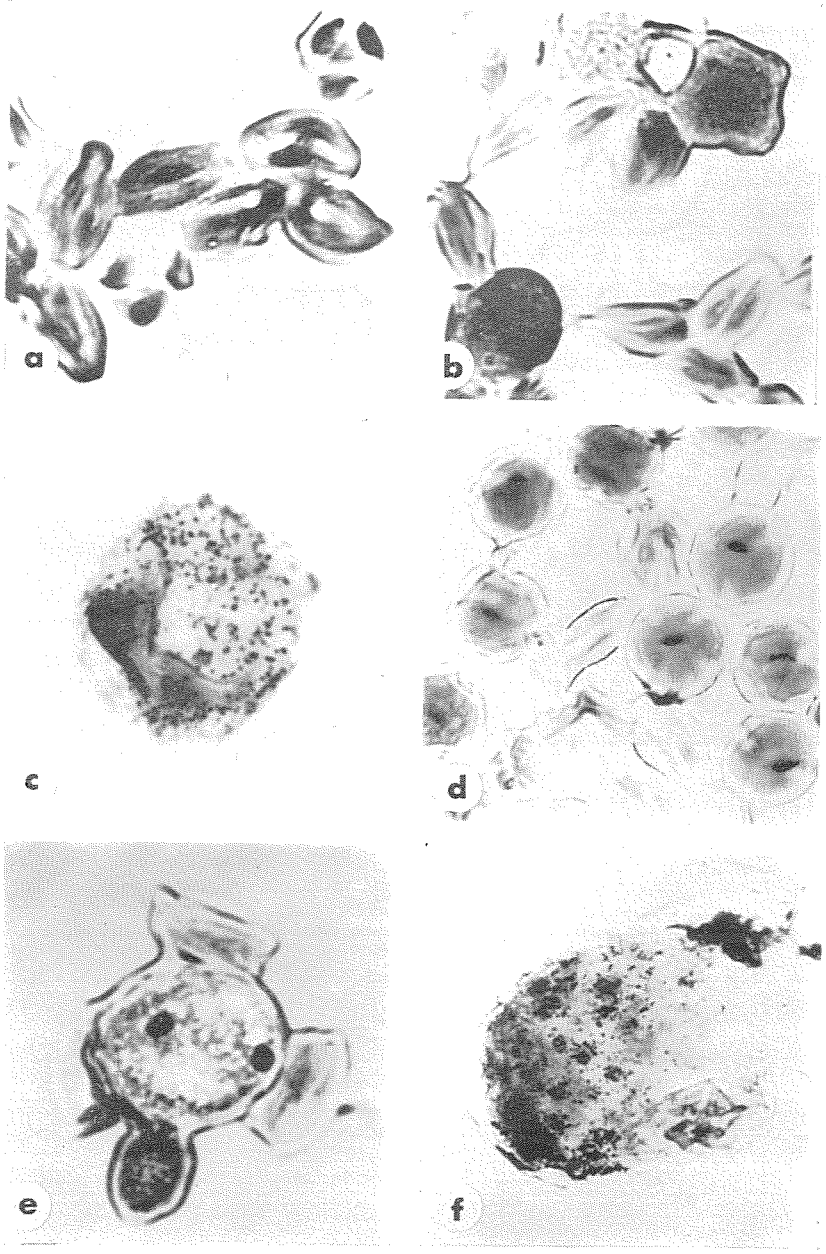

Plate V. Autoradiographs of cultured anthers of *N. tabacum* (var. Wisconsin 38) after ^{3}H-thymidine application.

a. No labelling of quartets and individualized pollen grains in the anthers of developmental stage 1 after 48 hours of incubation. × ca. 540.

b. Increased size of pollen grains in anthers of developmental stage 1 after 2 weeks of incubation. × ca. 540.

c. Pollen grain with a labelled nucleus in an anther of developmental stage 1 after 2 weeks of incubation. × ca. 610.

d. Binucleate pollen grains from an anther of developmental stage 2 after 48 hours of incubation. × ca. 540.

e. Increased size of a pollen grain with an unlabelled binucleus in an anther of developmental stage 2 after 1 week of incubation. × ca. 610.

f. A globular mass with labelled nuclei in the anther of developmental stage 2 after 2 weeks of incubation. × ca. 480.

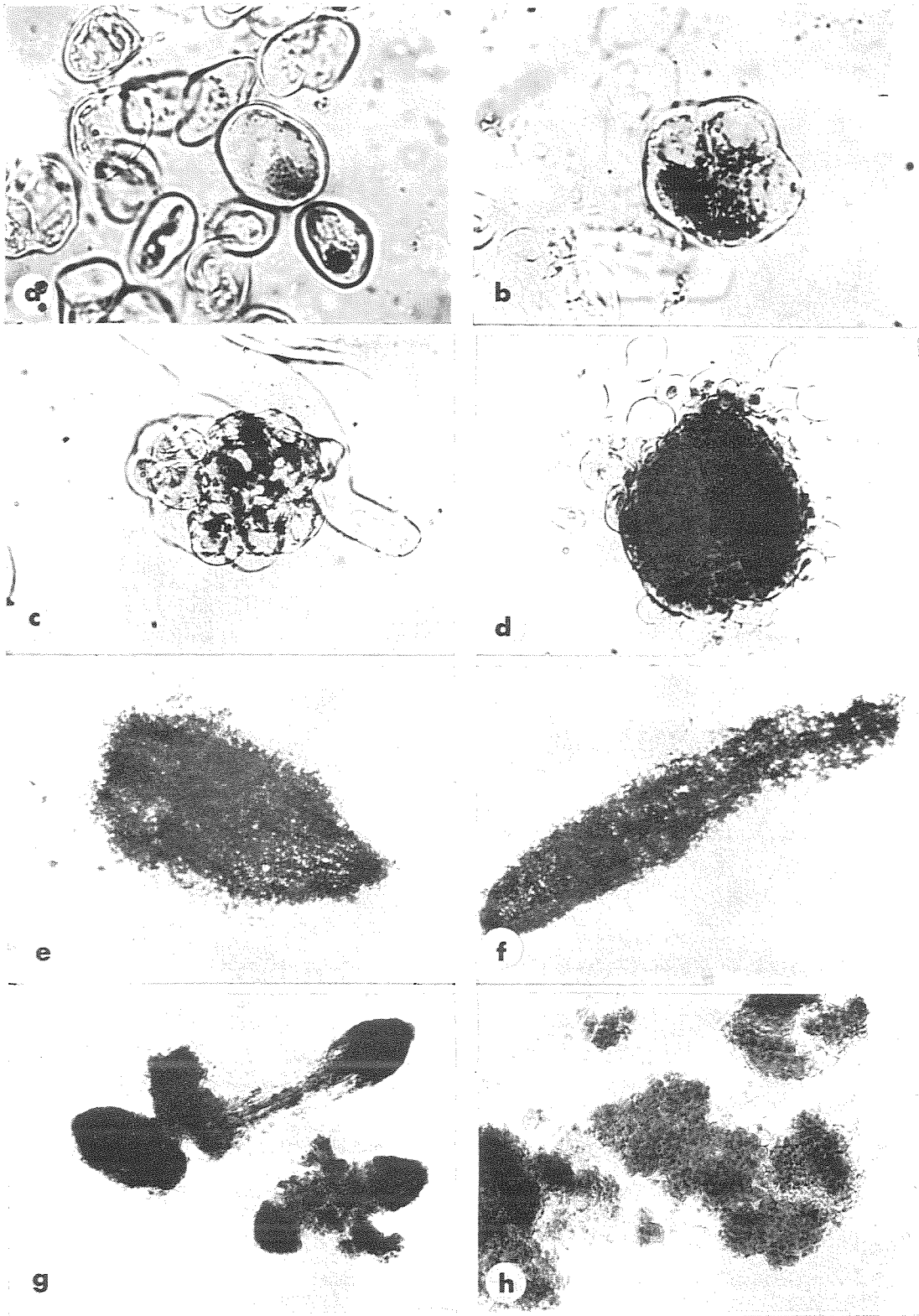

Plate VI. Various stages in the formation of embryoids and calluses from pollen grains in cultured anthers of *Phleum pratense* (var. Norin-Ichigo). a. Individualized uninucleate pollen grains. × ca. 650. b. A spherical mass with several cells. × ca. 650. c. A cluster with about 10 cells. × ca. 650. d. Initiation of polarity. × ca. 400. e. Establishment of polarity. × ca. 150. f. Cotyledonary stage. × ca. 75. g. Initiation of calluses from embryoids. × ca. 60. h. Establishment of calluses from embryoids. × ca. 60.

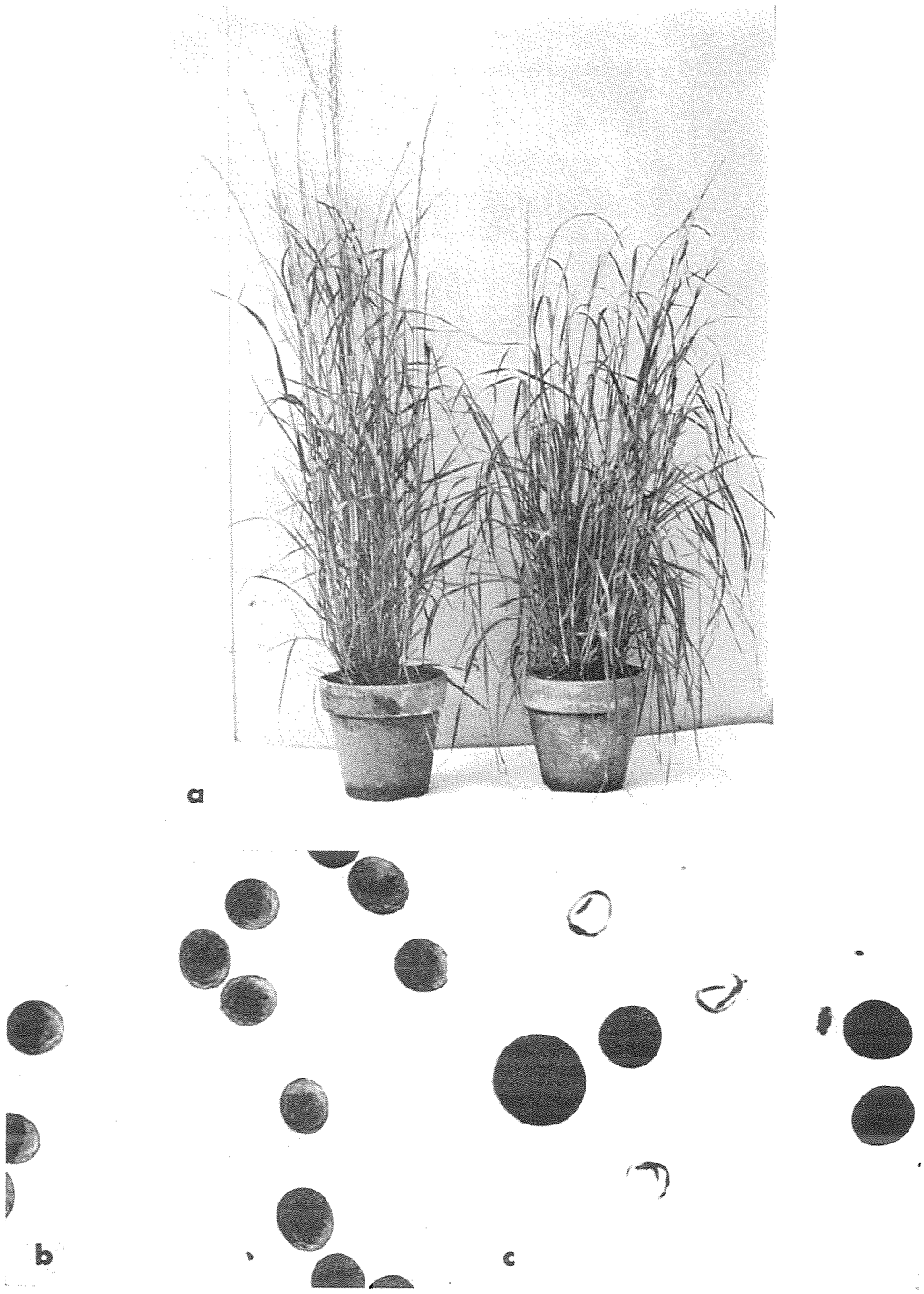

Plate VII. Production of diploid (haploid state) plants from cultured anthers of *Lolium multiflorum* (autotetraploid var. Tedis).

a. An adult diploid plant (left) produced by anther culture and its parent tetraploid plant (right).
b. Pollen grains of diploid plant. × ca. 200.
c. Pollen grains of tetraploid plant. × ca. 200.

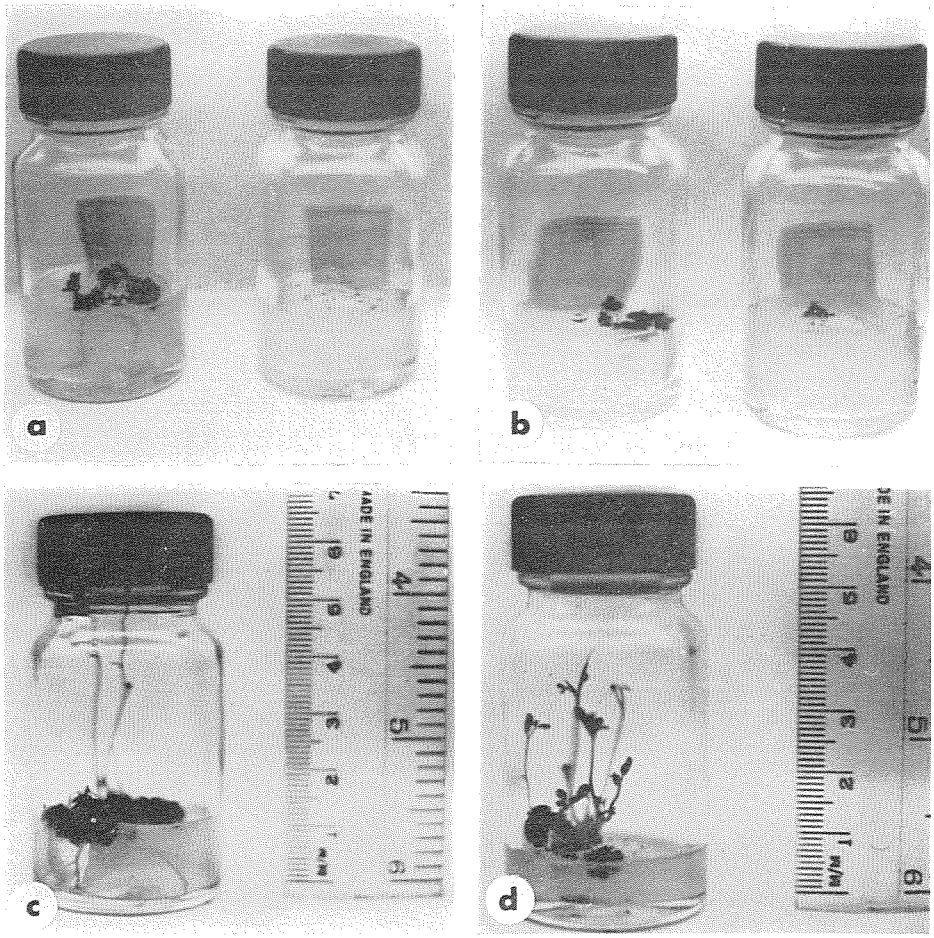

Plate VIII. Anther culture of *Lotus* species.

a. Anther culture of *L. corniculatus* (var. Viking). Left culture bottle, callus formation from anthers and root regeneration from calluses of medium LS-2; right culture bottle, no callus formation from the anthers on mdeium LS-1.

b. Anther culture of *L. caucasicus*. Left culture bottle, callus formation from anthers on medium LS-2; right culture bottle, callus formation from anthers on medium LS-1.

c. Root and shoot regeneration from calluses of *L. corniculatus* (var. Empire) in medium M-I-2 after 3 months of incubation.

d. Shoot regeneration from calluses of *L. corniculatus* (var. Empire) on medium M-I-7 after 3 months of incubation.

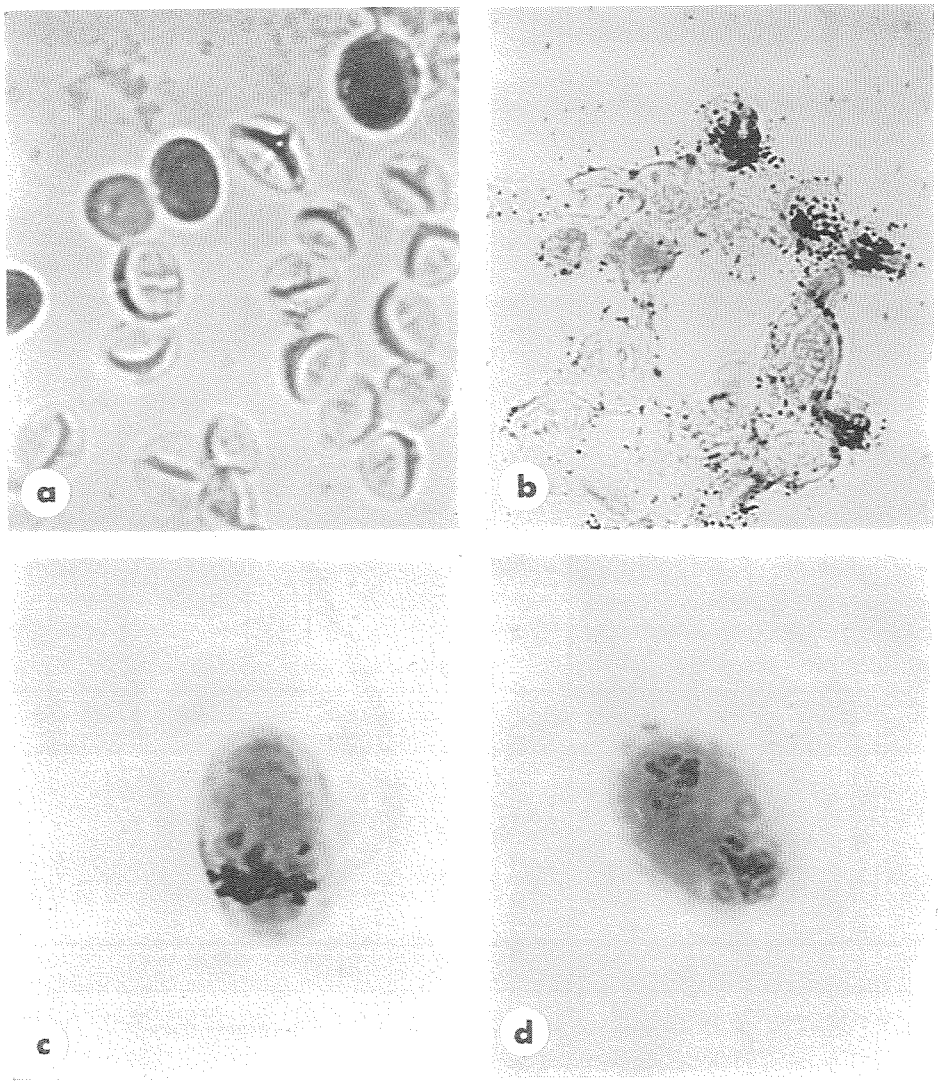

Plate IX. Autoradiographs of cultured anthers of *L. corniculatus* (var. Empire) from ^{3}H-thymidine application and after 48 hours of incubation.

a. No labelling of pollen grains in an anther of developmental stage 1. × ca. 720.

b. Labelled pollen grains in an anther of developmental stage 2. × ca. 720.

c. A labelled pollen grain in an anther of developmental stage 2. × ca. 2380.

d. Occurrence of nuclear division after DNA synthesis in an anther of developmental stage 2. × ca. 2380.

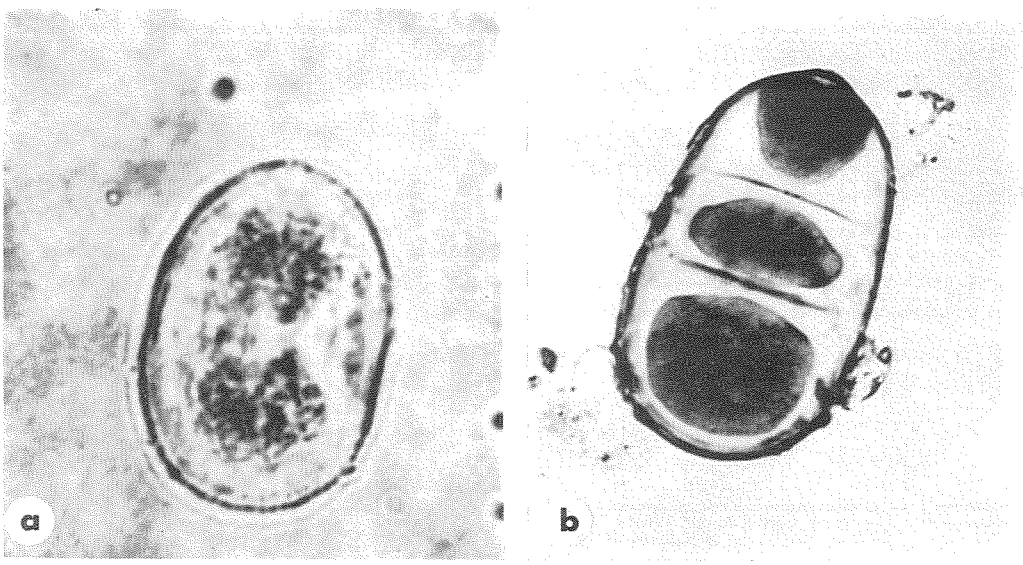

Plate X. Cell division in a pollen grain of *L. corniculatus* (var. Viking) on medium M-1-3.

a. Pollen grain, 2 nucleate stage. × ca. 1516.

b. 3 cell stage.

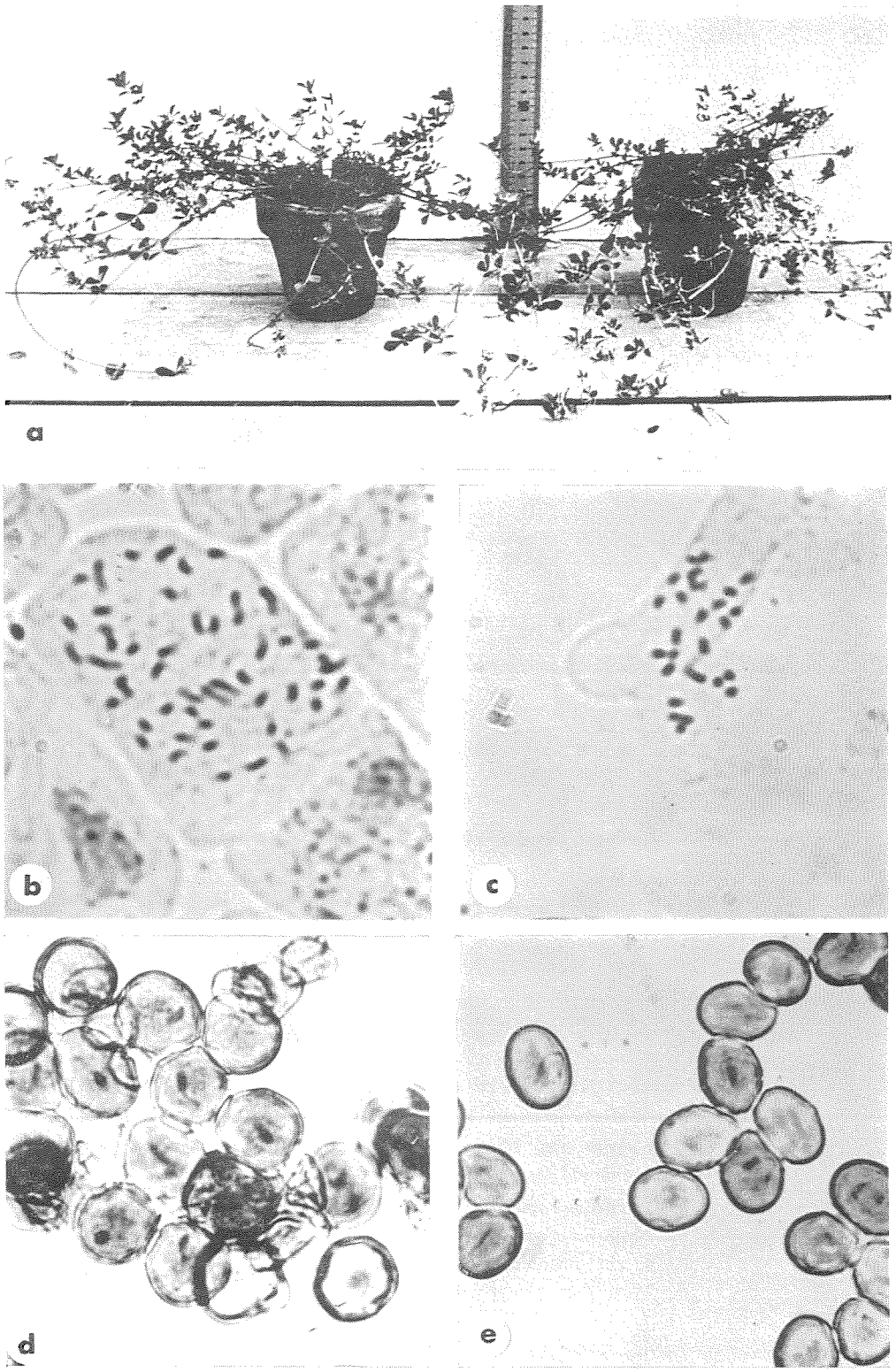

Plate XI. Regenerated plants of *L. corniculatus* (var. Empire) from calluses after anther culture. a. Left, a plant with 48 chromosomes; right, a plant with 24 chromosomes. b. Chromosome numbers in root tips of a plant with 48 somatic chromosomes. × ca. 1230. c. Chromosome numbers in root tips of a plant with 24 somatic chromosomes. × ca. 1230. d. Pollen grains from a plant with 48 somatic chromosomes. × ca. 640. e. Pollen grains from a plant with 24 somatic chromosomes. × ca. 640.

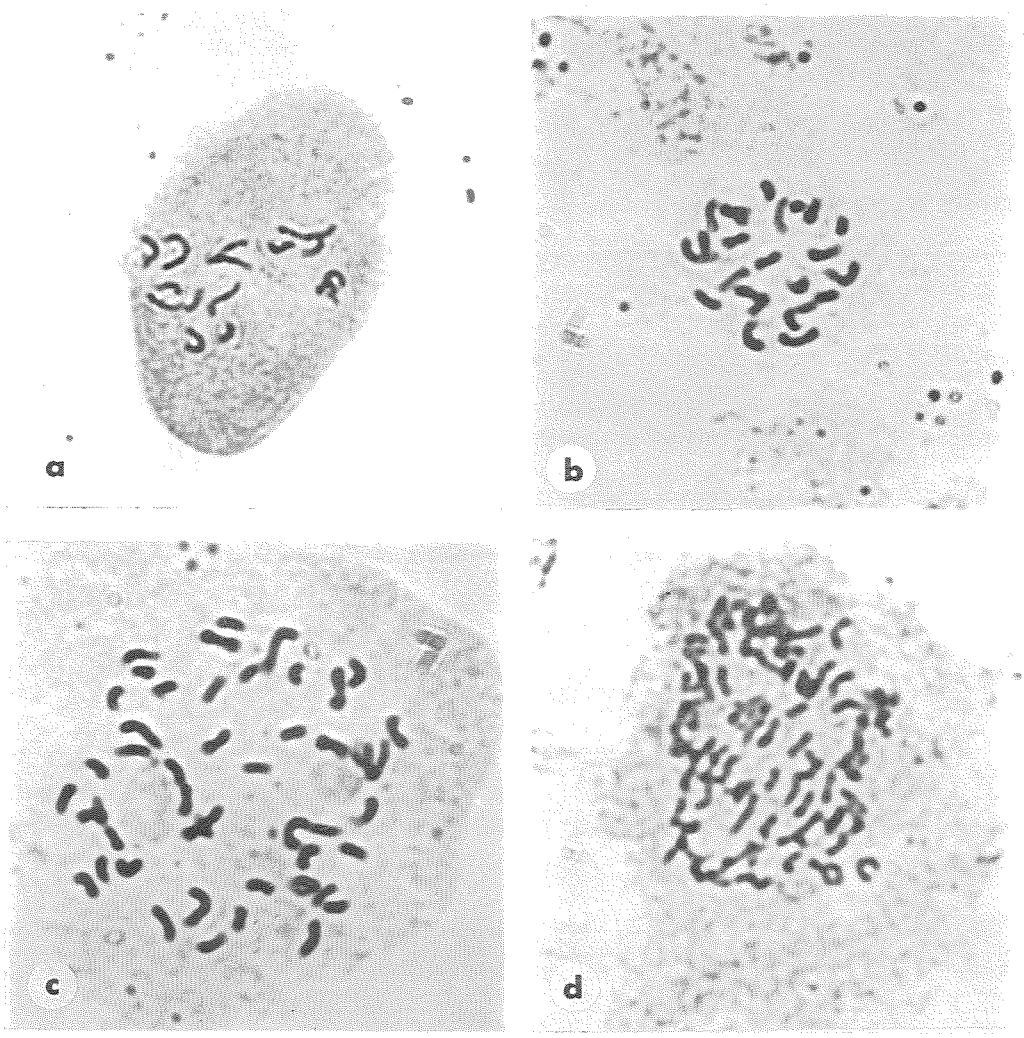

Plate XII. Different chromosome numbers found in callus tissues of *L. corniculatus* (var. Empire) which originated from anthers of developmental stage 2 on medium M-I-2.

a. 15 chromosomes. × ca. 1340.
b. 24 chromosomes. × ca. 1340.
c. 48 chromosomes. × ca. 1340.
d. More than 80 chromosomes. × ca. 1340.

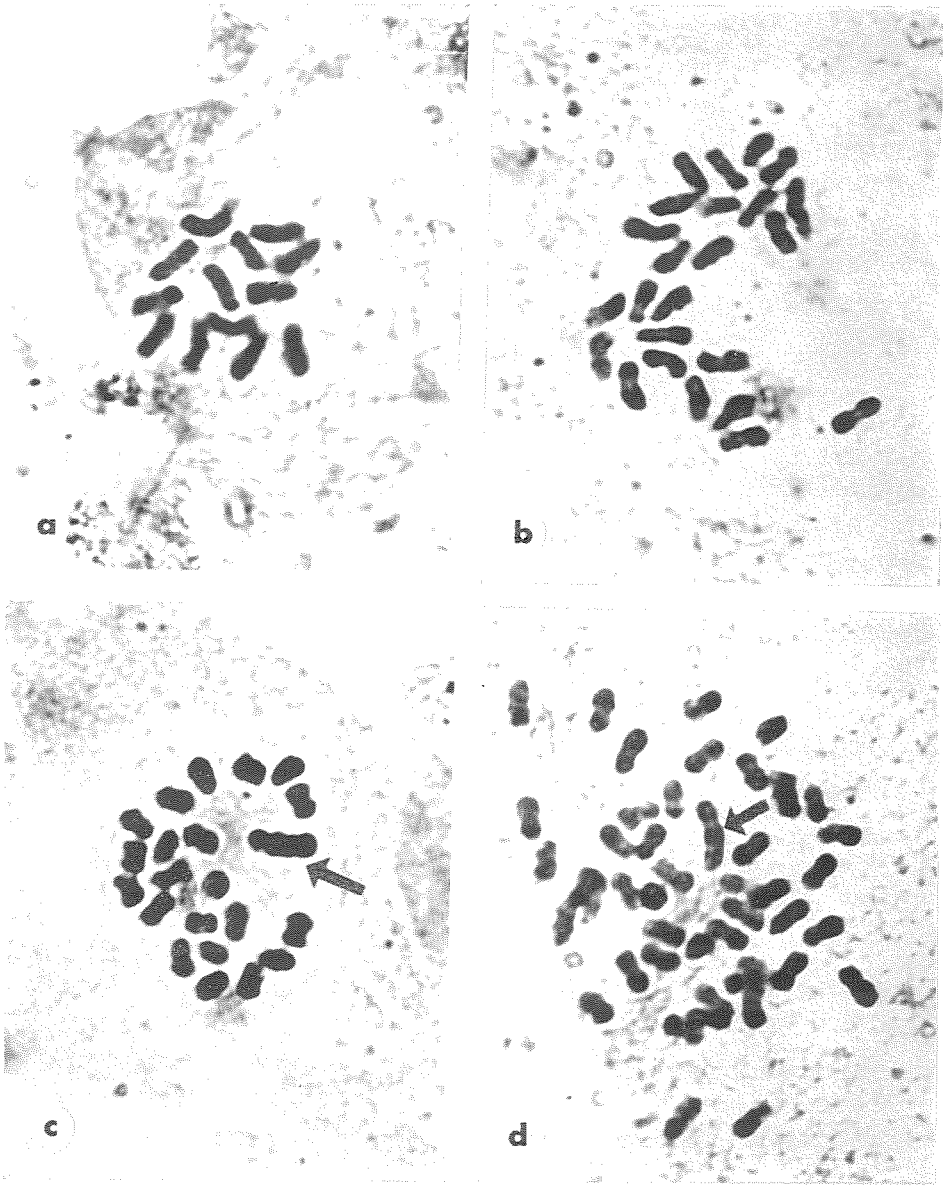

Plate XIII. Changes in chromosome number and structure found in haploid and diploid callus lines of *N. glutinosa*.

a. 13 chromosomes in the haploid callus line. × ca. 2000.
b. Normal chromosome complement in the diploid callus line. × ca. 2000.
c. One dicentric chromosome (indicated by arrow) and 22 monocentric chromosomes in the diploid callus line. × ca. 2000.
d. One dicentric chromosome (indicated by arrow) and 46 monocentric chromosomes in the diploid callus line. × ca. 2000.

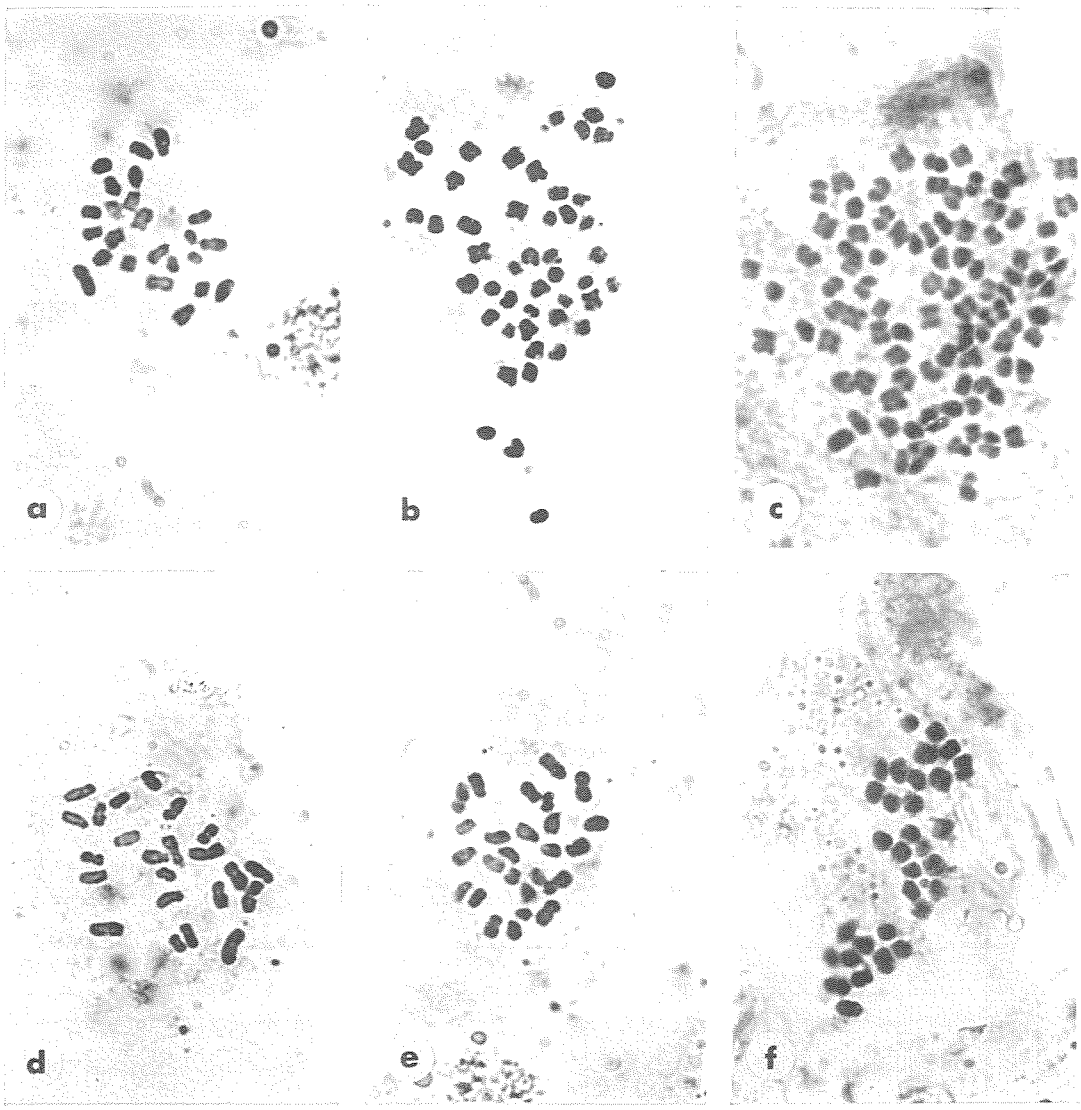

Plate XIV. Different chromosome numbers in the first and the second subculture of callus tissues induced by anther culture of *N. tabacum* (var. Wisconsin 38).

a. 24 chromosomes. × ca. 1300.
b. 48 chromosomes. × ca. 1300.
c. 96 chromosomes. × ca. 1300.
d. 23 chromosomes. × ca. 1300.
e. 26 chromosomes. × ca. 1300.
f. 32 chromosomes. × ca. 1300.

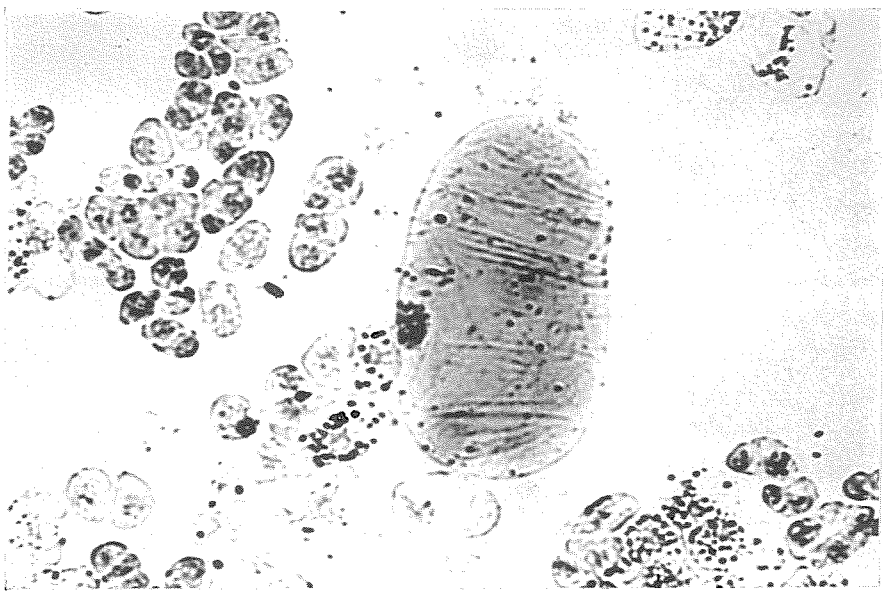

Plate XV. Giant cell found in the first subculture of callus tissues induced by anther culture of *N. tabacum* (var. Wisconsin 38). × ca. 200.

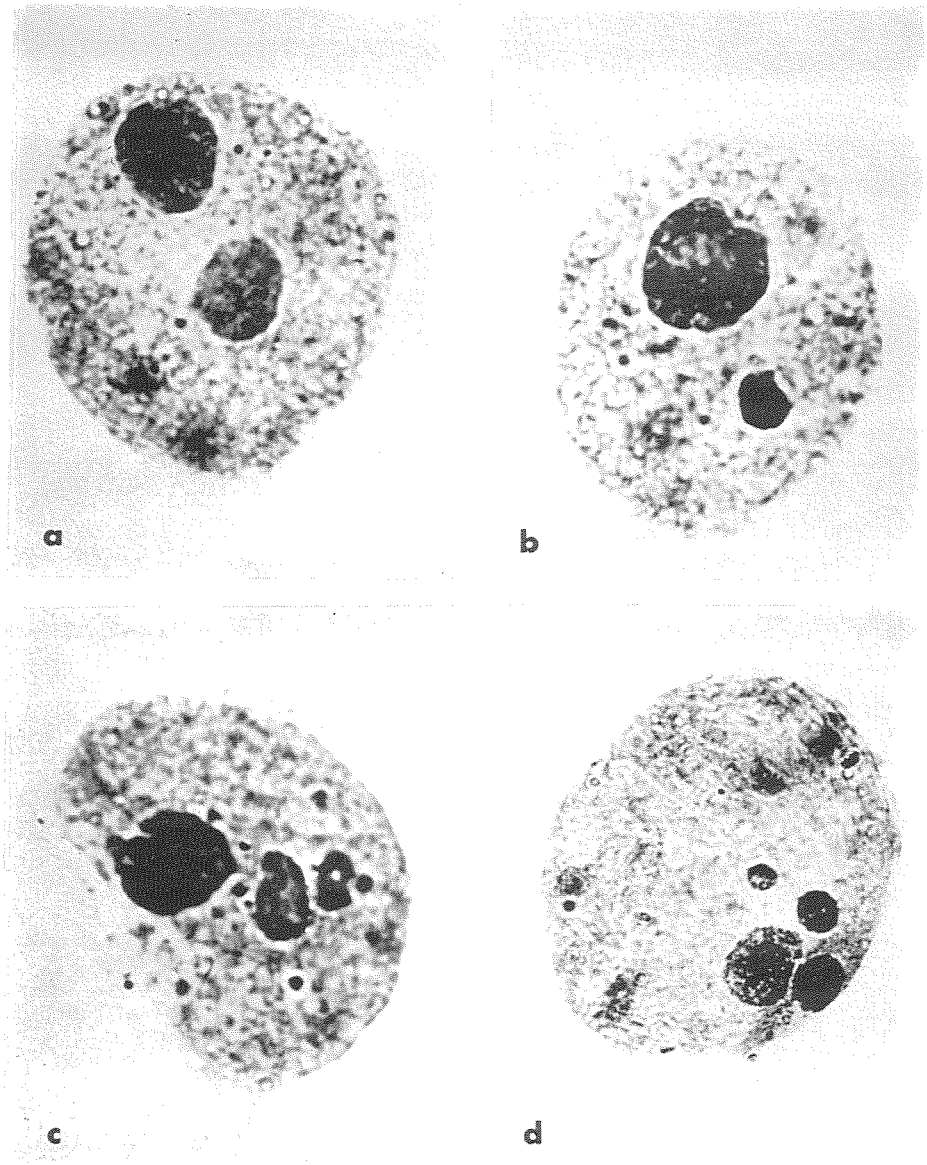

Plate XVI. Cells with multinuclei in the first and the second subculture of callus tissues of *N. tabacum* (var. Wisconsin 38) derived from cultured anthers.

a. Two nuclei in equal size. × ca. 1600.
b. Two nuclei in different size. × ca. 1600.
c. Three nuclei in different size. × ca. 1600.
d. Four nuclei in different size. × ca. 800.

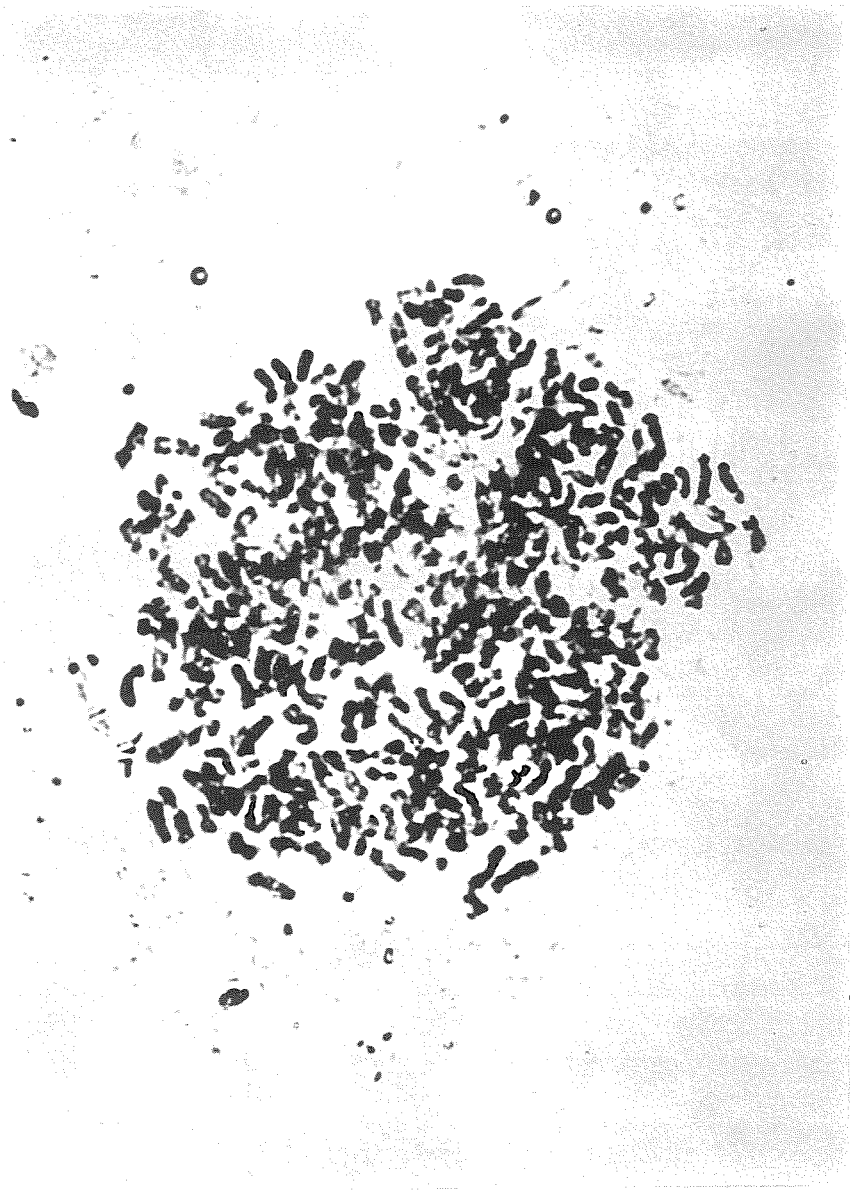

Plate XVII. Numerous chromosome numbers found in the cell cultured on the second subculture of callus tissues of *N. tabacum* (var. Wisconsin 38). × ca. 1400.

Plate XVIII. Plants regenerated from cultured callus of *N. tabacum* (var. Wisconsin 38) on medium M-2.

a. Haploid plant.
b. Diploid plant.
c. Tetraploid plant.

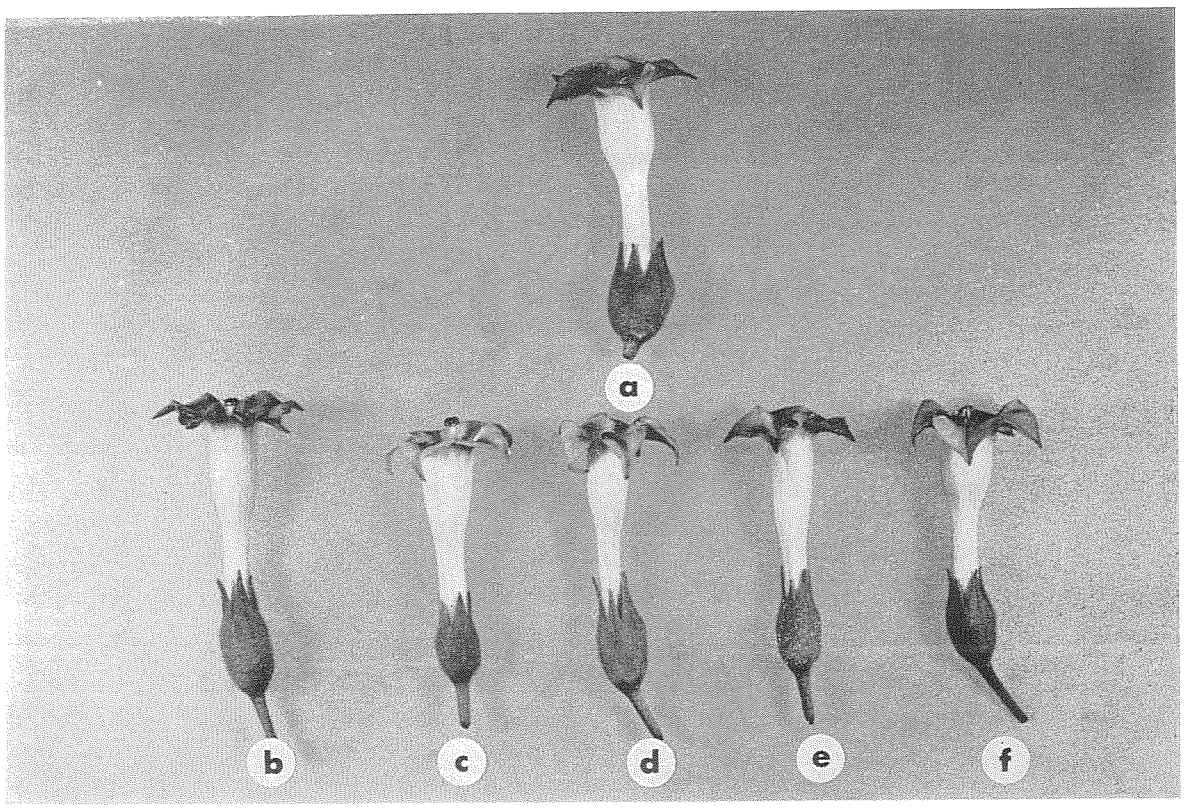

Plate XIX. Flower of a normal diploid plant and autosubstituted diploid plants derived from anther culture of tetraploid plants of *N. tabacum* (var. Wisconsin 38). × ca. 0.8.

a. Normal diploid.
b. Plant no. IX in Table 21.
c. Plant no. XVIII in Table 21.
d. Plant no. XVII in Table 21.
e. Plant no. XX in Table 21.
f. Plant no. XXII in Table 21.

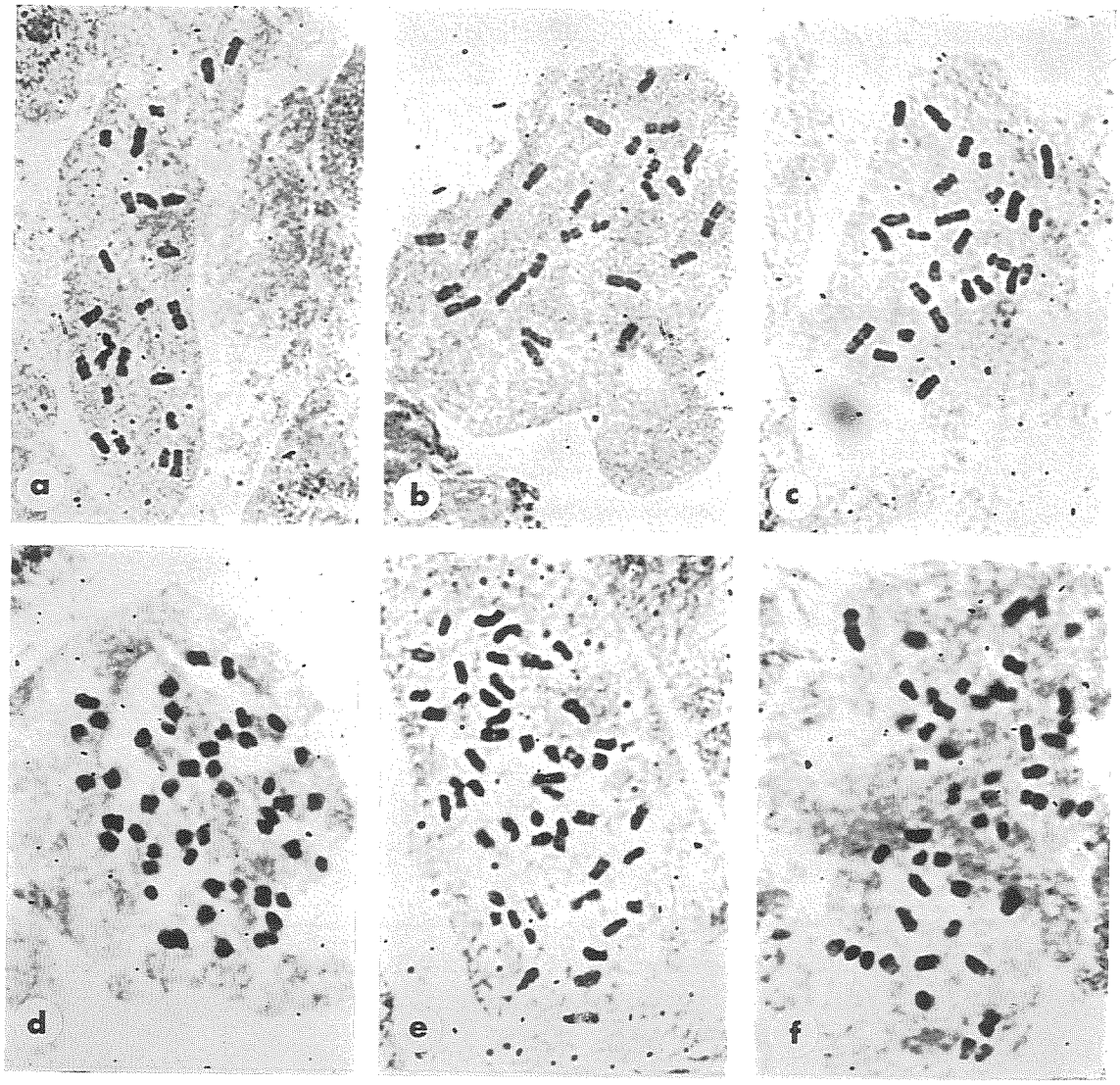

Plate XX. Chromosome number of plants produced by anther culture of plant no. XII in Table 24 and by crossing seeds to diploid plant of *N. tabacum* (var. Wisconsin 38). × ca. 1300.

a, b and c. 24, 25 and 26 chromosomes of androgenetic plants by the anther culture, respectively.

d, e and f. 48, 49 and 50 chromosomes of plants produced by the crossing seeds, respectively.

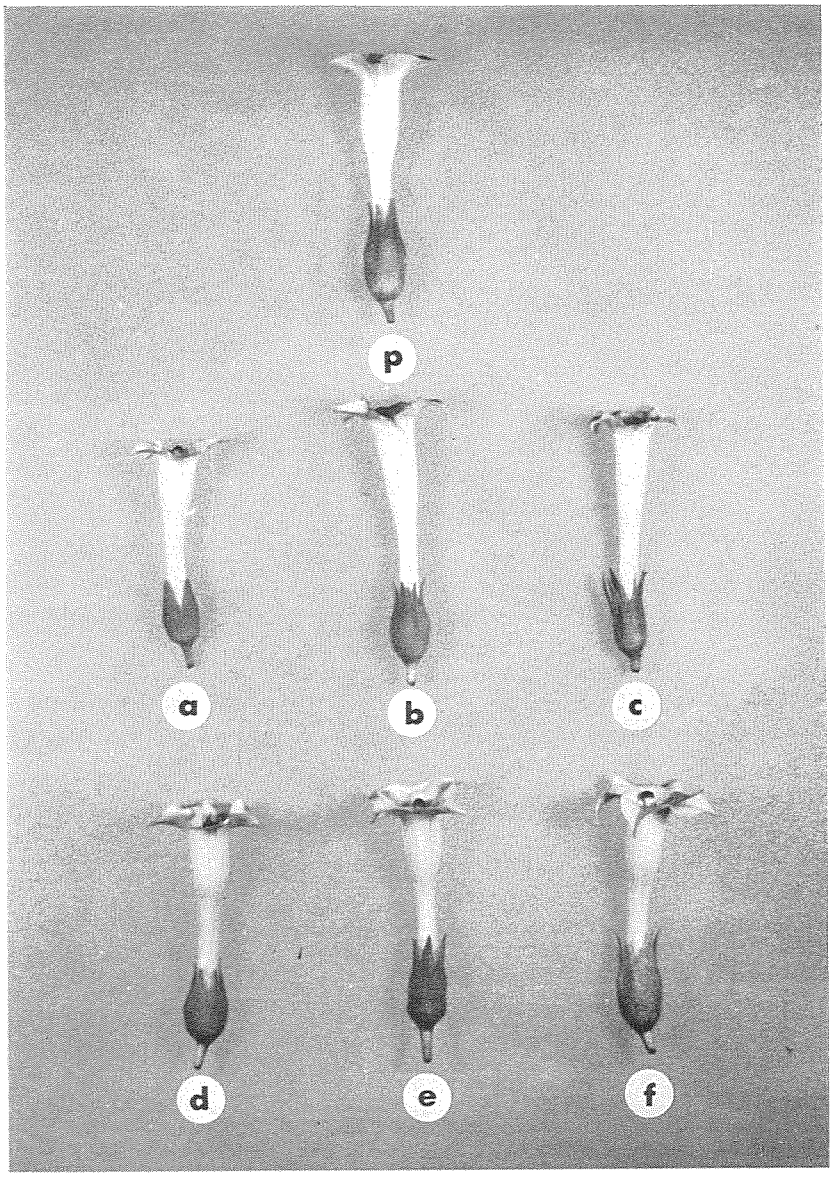

Plate XXI. Flowers of plants produced by anther culture of plant no. 12 in Table 24 and by crossing seeds to diploid plant of *N. tabacum* (var. Wisconsin 38). × ca. 0.7.

p. Parent plant (plant no. XII in Table 24).

a, b and c. Flowers of androgenetic plants with 24, 25 and 26 chromosomes, respectively.

d, e and f. Flowers of plant with 48, 49 and 50 chromosomes which were produced by the crossing seeds.

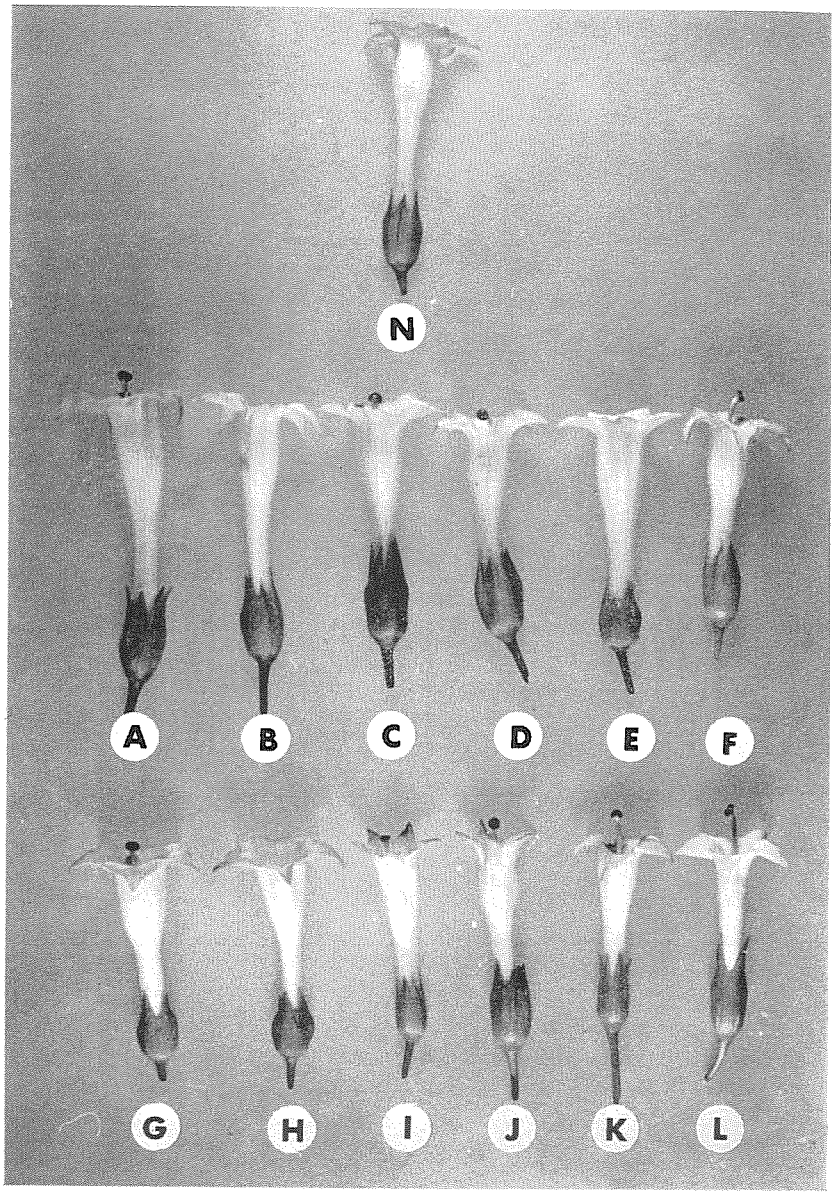

Plate XXII. Flowers of normal haploid and disomic haploid plants of *N. tabacum* (var. Wisconsin 38). × ca. 0.8.

N. A normal haploid plant.

A~L. Disomic haploid plants.