STUDIES OF *IN VITRO* FLOWERING AND *DE NOVO* FLOWERS OF *NICOTIANA TABACUM*

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(ABSTRACT)

The objectives of this research were to examine factors influencing *de novo* flowering of *Nicotiana* on 2-3 X 10mm explants consisting of epidermal and 3-6 layers of subjacent cells (thin cell layers, TCLs) and to compare *de novo* to *in vivo* flowers.

TCLs from short-day and long-day tobacco plants were compared with TCLs from day-neutral species to examine *in vitro* floral photoinduction and graft transmissibility of floral promoters and inhibitors. TCLs from photoperiodic species of tobacco did not form flowers *de novo* , whereas TCLs from day-neutral plants did flower. When TCLs were removed from photoperiodic plants and grafted *in vitro* to TCLs from day-neutral plants, there was no indication that a floral-promoter or inhibitor was transported through the

non-vascular graft union. In vitro photoinduction of TCLs removed from photoperiodic plants was not possible under conditions conducive to in vitro flowering of TCLs from day-neutral species.

TCLs taken from intraspecific F_1 and F_2 hybrids between short-day and day-neutral cultivars of N. tabacum were examined to assess the importance of genotype and photoperiod to de novo flowering. Flowering of the F_2 population occurred over a 9 week period under naturally decreasing photoperiod. Photoperiodic response and in vitro flowering were correlated in the F_2 population with fewer flowers produced per TCL with increasing short-day reaction. F_2 segregates whose TCLs did not yield de novo flowers were found among both day-neutral and short-day phenotypes.

When de novo flowers were compared to in vivo flowers of diploid (2n=4x=48) N. tabacum 'Samsun' and haploid (2n=2x=24) plants derived from 'Samsun' anther culture, major morphological differences were found. Flower and anther sizes were reduced in de novo flowers and the numbers of anthers and pistils produced per flower were variable. TCLs from haploid plants produced more flowers in a shorter period of time than TCLs from diploid plants. Anthers cultured from de novo

haploid plants were embryogenetic resulting in mixoploid plants; anthers from *in vivo* haploid flowers were not embryogenetic. Anthers from *in vivo* diploid plants were five times more embryogenetic than anthers from either *de novo* haploid or diploid flowers. Meiotic analysis revealed similar abnormalities from both *in vivo* and *de novo* microsporogenesis of haploids.

DEDICATION

To my wife,

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Since I first began graduate school, has acted as my mentor and I sincerely appreciate all that he has done on my behalf. He was never pretentious and was always willing to help; for this, I will always be grateful.

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CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Thin cell layers (TCLs) are sections of epidermal and subepidermal tissue which are cultured on a nutrient medium *in vitro* for the production of *de novo* roots, vegetative shoots, flowers, and callus. There are several plant species which respond to the TCL system (Table 1). Vegetative buds and callus can be differentiated from most of the listed species (Tran Thanh Van, 1980). Flowers have been observed to differentiate from TCLs of *Cichorium*, *Nicotiana*, and *Torenia* and a new form of morphological expression, unicellular hairs, has been obtained from *Begonia* (Chlyah and Tran Thanh Van, 1975) and *Torenia* (Tran Thanh Van et al., 1974).

First attempts to culture monolayers of epidermal cells from leaf petioles of *Begonia rex* (Chlyah and Tran Thanh Van, 1975) yielded unicellular hairs; when the same tissue was excised and cultured with subjacent cells, buds and roots formed. In later work with *Nautilocalyx*, Tran Thanh Van and Drira (1970)

Table 1. Plant species which are responsive to the thin cell layer system.

Genus	Response				
	Unicellular Hairs	Roots	Callus	Vegetative Shoots	Flowers
Begonia	X	X	X	X	
Catharanthus		X	X	X	
Chrysanthemum				X	
Chicorium		X	X	X	X
Nautilocalyx		X	X	X	
Nicotiana		X	X	X	X
Psophocarpus			X	X	
Saintpaulia		X	X	X	
Solanum		X	X	X	
Torenia	X	X	X	X	X
Vicia		X	X		

obtained direct root, bud, or callus formation from epidermal cells, depending on which growth regulator was included in the culture medium. A slightly thicker explant including 3 to 6 layers of epidermal and subjacent collenchyma tissue devoid of vascular bundles from the main leaf of Begonia was found to yield more consistent bud formation in addition to unicellular hairs and roots (Chylah and Tran Thanh Van, 1975). The most obvious difference between the epidermis of the petiole and that of the leaf vein was the presence of underlying chlorophyllous parenchyma adjacent to the collenchyma layers in the leaf vein. Presence of this photosynthetic layer was hypothesized as the reason for differences in TCL cultures. Later, histological (Chlyah, 1974b; Tran Thanh Van and Thi Dien, 1975), histoautoradiographic (Thi Dien and Tran Thanh Van, 1974), and electron microscopic (Tran Thanh Van and Chlyah, 1976) studies demonstrated conclusively that de novo organs and callus arise from the subepidermal layer.

Organogenetic expression in the TCL system has been attributed to the absence of interorgan and intertissue correlations (Chlyah, 1974a; Tran Thanh Van, 1980; Tran Thanh Van, 1981). Explants larger than TCLs retain more of the residual growth and nutritional substances which were present in the tissues of the whole plant. These substances may suppress or

enhance expression of certain morphogenetic forms (Tran Thanh Van, 1980). The smaller size of TCLs would be expected to carry a lower level of endogenous substances, and hence, TCLs should be more responsive to exogenous growth substances in the medium.

There appears to be a delicate balance of unidentified residual substances within TCLs. Explants smaller than TCLs, eg. protoplasts or isolated cells, have not demonstrated morphogenetic capability in vitro. Only callus, with possibly subsequent embryos or vegetative shoots, has been observed from culture of single cells. Therefore, it appears that some intercellular and intertissue correlations are important for the control of morphogenesis (Chlyah, 1974a,1978; Tran Thanh Van, 1980,1981).

Attempts to demonstrate the source of meristematic activity in TCLs of Nicotiana have been made by culture of various combinations of the component tissues: epidermis and subepidermis, cortex, vascular tissue, cambium and medullary tissue (Fig. 1) (Tran Thanh Van and Trinh, 1978a). De novo bud formation has been observed only in the epidermal or subepidermal tissues and in the inner cambial tissues of stems or roots (Tran Thanh Van, 1980). Cultured alone or together, cambial

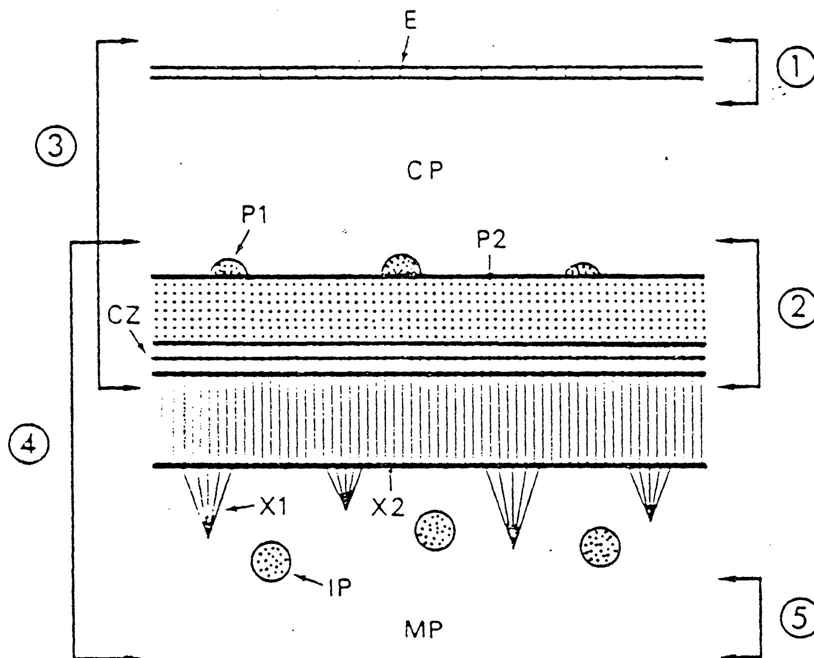

Figure 1. Diagram showing different types of thin cell layers and their composition. (From Tran Thanh Van and Trinh, 1978a).

1. Explant composed of epidermis (E).
2. Explant composed of cambial zone (CZ), phloem 1 (P1) and phloem 2 (P2).
3. Explant composed of 1, 2 and intermediate parenchyma cells (CP).
4. Explant composed of 2 and medullary parenchyma (MP).
5. Explant composed of only medullary parenchyma (MP).

cells and medullary cells will not flower. However, if cambial cells are joined with epidermal and subepidermal cells, flowering can be observed. If medullary cells are joined to cambial cells, neither will flower. Apparently, cambial cells possess the capacity for _de novo_ flower formation but will only express it when correlated with the epidermal tissue (Tran Thanh Van, 1980). Similar results have been obtained with _Torenia_ (Chlyah, 1974a; Chlyah and Tran Thanh Van, 1975).

TCL procedures have been extensively studied in _Nicotiana_ (Tran Thanh Van, 1977,1981; Tran Thanh Van and Cousson, 1982; Tran Thanh Van et al., 1974; Trinh and Tran Thanh Van, 1981) due to its flexibility for differentiation of vegetative shoots (Tran Thanh Van and Trinh, 1978b), roots (Trinh, 1978), callus (Ha Ngoc et al., 1979), and flower buds (Tran Thanh Van, 1973a). The present studies have centered on _de novo_ flowering of tobacco. In addition to the aforementioned features of the TCL system, other advantages of the system for the study of flowering and organogenesis include:

1. several treatments and replications per treatment can be examined at one time

2. homogeneous explants are utilized

3. strict environmental control exists _in vitro_

4. uniform response occurs under inductive conditions

5. genetic homogeneity exists

6. variable morphogenetic responses can occur depending on experimental treatment

A brief review of TCL procedures follows, with emphasis on Nicotiana.

THIN CELL LAYER PROCEDURES

Explants generally consist of 3 to 6 layers of epidermal and subepidermal cells (Tran Thanh Van, 1980; Tran Thanh Van and Trinh, 1978b; Tran Thanh Van, 1977). Their size has varied from 1 X 1mm (Tran Thanh Van, 1977; Tran Thanh Van, 1980) to 2 X 10mm (Tran Thanh Van, 1980; Tran Thanh Van, 1977), with 2-3 X 10mm most commonly used.

Nicotiana plumbaginofolia (Trinh and Tran Thanh Van, 1981; Tran Thanh Van, 1980) and Nicotiana tabacum cv. Wisconsin 38 (Tran Thanh Van, 1977; Tran Thanh Van et al., 1974; Tran Thanh Van and Thi Dien, 1975), cv. Samsun (Tran Thanh Van and Trinh, 1980; Cousson and Tran Thanh Van, 1983), cv. Xanthi (Kamate et al., 1981) and cv. Lacerata (Kamate et al., 1981) have responded to the TCL system by flowering in vitro. Floral

organs are macroscopically visible on TCLs within 8 to 14 days (Tran Thanh Van et al., 1974; Tran Thanh Van, 1980), vegetative buds can be observed macroscopically after 11 days, and roots after 15 days (Tran Thanh Van et al., 1974). Photoperiodic species of Nicotiana however, have responded only with vegetative buds or not at all. Interspecific F_1 hybrids between photoperiodic and day-neutral genotypes can flower but have a much reduced in vitro flowering capacity (Tran Thanh Van and Cousson, 1982; Kamate et al., 1981).

In general, the physiological state of the mother plant from which an explant is removed is important to consider with TCLs (Thorpe, 1978; Tran Thanh Van, 1981; Trinh et al., 1981). For de novo floral bud formation, TCLs should be removed from an inflorescence at the green fruit stage (Tran Thanh Van et al., 1974). Explants taken from inflorescences at the brown fruit stage result largely in de novo root formation (Tran Thanh Van et al., 1974). Formation of vegetative buds on callus can be observed on TCLs regardless of flowering or fruiting of the source plant.

The genotype of the donor plant has also been demonstrated to be important for de novo organogenesis (Tran Thanh Van and Trinh, 1980). Morphogenetic capability of N. tabacum

'Mammoth', except for root formation, was not expressed when backcrossed with Nicotiana rustica. TCLs from 3 interspecific F_1 hybrids between day-neutral and photoperiodic tobacco species flowered irregularly with approximately 30% flowering (Kamate et al., 1981; Tran Thanh Van and Trinh, 1978).

For in vitro flowering, TCLs should be removed from the distal positions of inflorescences (Tran Thanh Van and Trinh, 1978b; Chouard and Aghion, 1961; Tran Thanh Van et al., 1974). The proximal positions of the inflorescences and stem sections yield vegetative buds but do not flower under the same environmental conditions (Chouard and Aghion,1961). It has been suggested that DNA from flowering plant parts may differ from that of vegetative organs (Wardell, 1976,1977; Wardell and Skoog, 1973; Skoog, 1970) and that an acropetal floral gradient might be due to a basipetally increasing level of endogenous IAA (Wardell and Skoog, 1969a,b). Isoperoxidases have also been examined because of their involvement in the metabolism of auxin. Qualitative and quantitative differences were observed with a decrease in peroxidase content observed acropetally (Gaspar et al., 1977; Thorpe et al., 1978).

THIN CELL LAYER MEDIA

Thin cell layers have generally been cultured on a medium consisting of Murashige and Skoog (1962) macro- and micronutrients plus 100 mg/l myoinositol (Tran Thanh Van, 1973b). Thiamine-HCl has been used at 0.4 mg/l (Cousson and Tran Thanh Van, 1983), 1.0 mg/l (Tran Thanh Van, 1980), or 0.1 mg/l (Tran Thanh Van, 1973b) with 0.4 mg/l used most often for flowering. The pH of the medium has most frequently been adjusted to 5.6 (Tran Thanh Van, 1980).

The carbohydrate source included in TCL media has varied depending upon the type of organogenesis desired. For floral buds, callus, or vegetative shoots, 30 g/l glucose or sucrose can be used. For root differentiation, 10 g/l glucose or sucrose have been used (Table 2). Comparison of glucose, saccharose, fructose, and mannitol for organ formation revealed glucose as most conducive to formation of flower buds and mannitol as most conducive to vegetative bud formation. Saccharose + glucose at 1/12 M and saccharose + fructose treatments at 1/12 M produced more flowers than glucose, saccharose, fructose, or a combination of mannitol and saccharose (Tran Thanh Van, 1977).

Table 2. Reported growth regulators and carbohydrate sources for *Nicotiana* thin cell layer organogenesis.

Form of Organogenesis	Carbohydrate (g/l)	Growth Regulator: Auxin (M)	Growth Regulator: Cytokinin (M)	References[z]
Vegetative Shoots	Glucose (30)	IBA (10^{-6})	KIN (10^{-5})	3,7
	Glucose (30)	IAA (10^{-6})	BA (10^{-5})	5
	Sucrose (10)	IAA (10^{-6})	BA (10^{-5})	4
	Sucrose (30)	IAA (10^{-6})	BA (10^{-5})	2,4
Flowers	Glucose (30)	IBA (10^{-6})	KIN (10^{-6})	3,7
	Glucose (30)	IAA (10^{-6})	KIN (10^{-6})	5
	Sucrose (30)	IAA (10^{-6})	KIN (10^{-6})	1,2,4
Callus	Glucose (30)	IBA (10^{-6})	KIN (10^{-7})	7
	Glucose (30)	IBA ($3x10^{-6}$)	KIN (10^{-7})	3
	Glucose (30)	2,4D($5x10^{-6}$)	KIN (10^{-7})	5
	Sucrose (30)	2,4D($5x10^{-6}$)	KIN (10^{-7})	6
Roots	Sucrose (10)	IBA (10^{-5})	KIN (10^{-7})	2,3,7
	Sucrose (10)	IAA (10^{-5})	KIN (10^{-7})	4

[z]1= Tran Thanh Van, 1973

2= Tran Thanh Van, 1977

3= Tran Thanh Van, 1980

4= Tran Thanh Van, Chlyah, and Chlyah, 1974

5= Tran Thanh Van and Cousson, 1982

6= Tran Thanh Van, Thi Dien and Chlyah, 1974

7= Tran Thanh Van and Trinh, 1978

In a medium containing 1 uM IBA (indole 3-butyric acid) and kinetin, an optimum balance for flower bud formation, organogenesis was delayed until day 18 if glucose was reduced below 30 g/l; even after 3 weeks, only a small percentage of buds was obtained on low glucose medium (Tran Thanh Van, 1980). To determine if glucose was essential to morphogenesis at some critical time, TCls were transferred onto media with or without glucose (30 g/l) at day 0, 2, 4, 8, and 10. Absence of glucose for the first 4 days had no effect on flower formation. However, the lack of glucose from day 6 to day 10 resulted in abnormal meristems; this suggests an absolute requirement for carbohydrate at later stages for normal organ differentiation. Electron microscope studies showed an accumulation of starch in plastids starting from the first hour of culture (Tran Thanh Van and Chlyah, 1976).

The altered function of floral meristems when deprived of an exogenous supply of glucose indicates that glucose has a more dramatic impact on flower formation and organogenesis than does light (Tran Thanh Van, 1980). Tobacco callus which has been induced to form vegetative buds shows an increase in glucose oxidation, indicating that bud initiation is a high energy-requiring process. It is expected that flower formation would need an even higher level of energy as floral meris-

tems show enhanced levels of respiration (Tran Thanh Van, 1980).

Although specific organ-forming substances have not yet been isolated, the auxin:cytokinin ratio has been shown to regulate bud or root formation, shoot development, and leaf formation in tissue cultures (Skoog and Miller, 1957; Thorpe, 1978). Organogenesis on TCLs can be precisely controlled by manipulation of the auxin:cytokinin ratio in the medium (Tran Thanh Van and Trinh, 1978a; Tran Thanh Van, 1980). IBA has been the auxin and kinetin the cytokinin most often used (Table 2). The auxin:cytokinin ratio for tobacco TCL development is 1.0 (10^{-6} M each) for floral buds, 0.1 (10^{-6} M auxin, 10^{-5}M cytokinin) for vegetative shoots, 100.0 (10^{-5} M auxin, 10^{-7} M cytokinin) for root development, and 30-50 (3.1×10^{-6} M auxin, 10^{-7} M cytokinin) for callus (Tran Thanh Van, 1977,1980).

TCL medium is usually semi-solid by addition of 10 g/l agar (Tran Thanh Van and Trinh, 1978). TCLs have been observed to produce only callus if grown on a liquid medium without support; organ formation on liquid medium by TCLs supported on glass beads, however, has been equivalent to that of TCLs on agar-solidified medium (Cousson and Tran Thanh Van, 1981). The percentage of flower bud formation of TCLs has been shown to

decrease from 100% to 85% to 0% as the percentage of agar in the medium is decreased from 1% to 0.5% to 0.25%, respectively (Tran Thanh Van and Trinh, 1978). A role for osmotic potential of the culture medium as a determinant of de novo organogenesis is suggested.

THIN CELL LAYER ENVIRONMENT

Light is required for all forms of organogenesis on TCLs with the exception of root formation (Tran Thanh Van et al., 1974). Organogenesis is initiated in the chlorophyllous cells of the subepidermal layers, in which both photosynthesis and respiration occur (Tran Thanh Van ,1980; Cousson and Tran Thanh Van, 1983). In the presence of 30 g/l glucose, continuous light of 50 watts m^{-2} is required for optimal flower differentiation in vitro (Cousson and Tran Thanh Van, 1983). However, the same light intensity supplied continuously to TCLs from day 6 to day 11 of culture has been sufficient to trigger flower formation in 80% of the explants. This is the same critical period of culture during which an absolute requirement for carbohydrate as energy source has been identified (Cousson and Tran Thanh Van, 1983; Tran Thanh Van, 1980). Light without carbohydrate has resulted either in no organogenesis or formation of abnormal structures.

To determine which pigments are critical for <u>de novo</u> organogenesis, fluorescent and incandescent light was applied to TCLs on flower medium at high (150,000 erg cm^{-2} sec^{-1}), medium (80,000 erg cm^{-2} sec^{-1}), and low (10,000-20,000 erg cm^{-2} sec^{-1}) energy; for the latter, monochromatic light from 350 to 740 nm was tested. The high energy light treatment inhibited all morphogenesis, even callus. The minimum and maximum levels for flower formation were 15,000 and 90,000 erg cm^{-2} sec^{-1}, respectively. Monochromatic light at 625 nm (11,000 erg cm^{-2} sec^{-1}) stimulated flower bud formation compared with broad spectrum control at 20,000 erg cm^{-2} sec^{-1}. Only callus and/or vegetative buds were observed at 475-500 nm (10,000 erg cm^{-2} sec^{-1}) (Tran Thanh Van, 1980).

To determine critical time for stimulation of organogenesis by light, as was the case for carbohydrates, a mixture of incandescent and fluorescent lights of medium energy was applied or removed sequentially at days 0, 2, 4, 6, 8, and 10. The critical phase during which light was absolutely needed for flower formation occurs from day 7 to day 10 (Tran Thanh Van, 1980). Darkness applied at day 7 resulted in vegetative rather than flower bud formation. This critical time for light coincides with the glucose dependent phase (Cousson and Tran Thanh Van, 1983).

For flower bud formation, TCLs have been cultured most often under 16 hour photoperiod (Tran Thanh Van, 1977). However, 100% flower bud formation on tobacco TCLs has been reported under photoperiods varying from 12 to 24 hours (Trinh and Tran Thanh Van, 1981).

The temperature under which donor plants are grown or TCLs cultured may also affect TCL development in vitro. This has been demonstrated with TCLs of Begonia rex, Saintpaulia ionantha (Tran Thanh Van et al., 1978), and Nicotiana (Trinh and Tran Thanh Van, 1981). TCLs taken from Saintpaulia grown at 22°C form vegetative buds over a larger range of in vitro temperatures than the other 2 species (Tran Thanh Van, 1980). Best and fastest floral development on TCLs of Nicotiana tabacum has been reported to occur at 22-24°C (Trinh, 1978).

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CHAPTER 2

STUDIES OF *DE NOVO* FLOWER INITIATION FROM THIN CELL LAYERS OF *NICOTIANA* SPP.

ABSTRACT

The potential for *in vitro* floral photoinduction of epidermal and subepidermal thin cell layers (TCLs) taken from the short-day plant *Nicotiana tabacum* L. 'Maryland Mammoth', the long-day plant *Nicotiana sylvestris* L. and the day-neutral plant *Nicotiana tabacum* L. 'Samsun', in both the floral and vegetative states, was examined. Whether cultured under long days (16 H) or short (8 H), only TCLs from the flowering day-neutral species flowered *in vitro*; these were termed responsive TCLs. TCLs from photoperiodic plants yielded only vegetative buds; these were termed non-responsive. Vegetative bud formation in non-flowering TCLs was generally greater than in flowering TCLs but did not approach the number of flower buds on flowering TCLs. *In vitro* grafts of responsive TCLs to non-responsive TCLs resulted in flowering only in the responsive portion regardless of the position of the graft. Just as the non-responsive TCLs were not induced to flower by some

graft-transmissible substance, responsive TCLS were not inhibited from flowering when grafted to non-responsive TCLs.

INTRODUCTION

The culture of 3 to 6 layers of cytologically differentiated epidermal and subepidermal cells (Thin Cell Layers, hereafter TCLs) on a nutrient medium *in vitro* has been described as an ideal system for the study of the control of flowering in plants (Tran Thanh Van, 1981). A range of organogenetic phenomena can be induced from TCLs of *Nicotiana tabacum* by manipulation of the culture medium. Although roots, callus, and vegetative shoots may differentiate *in vitro* from TCLs of both day-neutral and photoperiodic cultivars of tobacco, flower buds have been restricted to the former (Tran Thanh Van, 1981; Kamate et al.,1981).

De novo flowers develop on TCLs of day-neutral species of tobacco if the cell layers are taken from the inflorescence and cultured on a Murashige and Skoog (1962) basal medium containing 0.166M glucose, 1uM IBA, and 1uM kinetin. *In vitro* flowering is maximized if TCLs are taken from distal portions of the inflorescence; a decreasing capacity for *in vitro* flower formation occurs with increasing distance from the inflorescence

(Tran Thanh Van, 1973). An endogenous gradient of floral promoters or inhibitors or both along the stem and inflorescence has been hypothesized (Chouard and Aghion, 1961).

A substance or group of substances that are involved with flower initiation has been demonstrated to be graft transmissible between tobacco plants _in vivo_, but its identity is still unknown (Lang, 1965). By grafting whole plants together, flowering day-neutral plants can induce non-flowering short-day plants, flowering short-day plants can induce non-flowering long-day plants and vice versa, and flowering short-day plants can induce long-short-day plants (Lang, 1965).

Several daylength-sensitive species have been induced to flower through _in vitro_ photoperiodic induction. However, this has not been possible with any photoperiodic species of _Nicotiana_ (Scorza, 1982). Hillman (1959) found that whole plants of _Lemna_ could be induced to flower _in vitro_ when subjected to short days. Harada (1967) induced vegetative shoot apices of _Pharbitis_ and _Chrysanthemum_ to flower through _in vitro_ photoinduction, and Rossini and Nitsch (1966) accomplished the same using leaf discs of _Streptocarpus nobilis_. Stem internodes, hypocotyl sections, and root segments have also been reported on several species to flower through _in_

vitro photoinduction (Scorza, 1982). In these experiments, the explants taken from vegetative plants responded to photoperiodic induction; hence, reception of the photoperiodic stimulus does not depend upon the presence of leaves, meristems, or intact plants.

The relationship between photoperiodism in the source plant and organogenetic capacity of its TCLs cultured under two photoperiods has been examined in the following report. The possibility of a graft-transmissible substance from TCLs of day-neutral tobacco capable of *in vitro* flowering to TCLs of non-responsive tobacco has also been studied with pairwise cultures.

MATERIALS AND METHODS

Seeds of *Nicotiana tabacum* 'Samsun' (day-neutral plant), *N. tabacum* 'Maryland Mammoth' (short-day plant), and *N. sylvestris* (long-day plant) were obtained from the USDA-ARS Tobacco Research Laboratory in Oxford, N.C. Two plants of each cultivar were grown in the greenhouse (24°C) under both long days (16 hours) and short days (8 hours) from March to July, 1983. Long days were provided by incandescent lighting and short days by shading with black cloth. Every 10 days, a new set of plants

was started under each photoperiod to ensure that plants of each species would be simultaneously in the appropriate stage of development when needed.

TCLs in all experiments were cut to approximately 2 X 10mm and cultured in Bellco glass tubes (150 X 25mm) containing 30 ml of a medium (pH 5.6) consisting of the Murashige and Skoog (1962) macro- and microelements, 100 mg/l myo-inositol, 30 g/l glucose, 0.4 mg/l thiamine-HCl, 9 g/l agar, 1.0 uM IBA, and 1.0 uM kinetin. Cultures were incubated at 24-26°C under 60-75 uE s^{-1} m^{-2} light produced by a combination of cool white fluorescent and incandescent bulbs. A photoperiod of 16 hours per day was used unless otherwise stated.

De novo flower buds and vegetative shoots visible under 10X magnification were counted on TCLs after 28 days in culture. Data were analyzed by SAS GLM procedure with mean separation by the Student Newman-Keuls' test (Council and Helwig, 1982).

EXPERIMENT 1. Influence of the physiological state of the mother plant on *de novo* organogenesis *in vitro*. TCLs were removed from all 3 plant types in both the vegetative and floral states. TCLs were taken from flowering plants in the green fruit stage of development. For disinfestation, flowers and

fruits were removed from the inflorescence and the peduncles were cut into 6 cm lengths. Peduncle sections were disinfested by a wash in distilled water followed by a 5 second dip in 70% ethanol and 5 minutes in 2.1% sodium hypochlorite. Plant sections remained in a sterile distilled water rinse until cultured. On vegetative plants, TCLs were taken from internodal sections along the top 20 cm of the plant. They were disinfested as described above.

Half of the TCLs in each treatment were cultured under short days (8 hours) and half under long days (16 hours). The entire experiment was conducted twice with 12 replications in each of the 12 treatments using a randomized complete block design.

EXPERIMENT 2. In vitro grafting experiments. TCLs from the distal portion of an inflorescence (floral-responsive) of 'Samsun' were grafted to TCLs taken from the stem of the same plant, 3 nodes below the inflorescence (non-floral responsive). De novo flowers have not been observed in vitro on cultures of the latter (Tran Thanh Van, 1977). Stem-stem and inflorescence-inflorescence grafts as well as ungrafted stem and inflorescence TCLs (controls) were compared. The 6 treatments were replicated 12 times in a randomized complete block design.

Floral-responsive TCLs of 'Samsun' were grafted to non-responsive TCLs from inflorescences of 'Maryland Mammoth' or N. sylvestris. Neither of the latter species has exhibited de novo flower formation from TCLs (Kamate et al., 1981). Each pair of TCLs was cultured reciprocally, i.e., with the floral-responsive member in either the acropetal or basipetal position (Fig. 1). Ungrafted TCLs of all 3 species, as well as 'Samsun'-'Samsun' grafts, were used as controls. Careful positioning of freshly cut TCLs in the culture tube so that parenchyma cells of both TCLs were closely aligned was sufficient association for graft union formation. The 8 graft treatments were replicated 18 times in a randomized complete block design.

RESULTS

EXPERIMENT 1. In vitro flowering was observed only on TCLs taken from flowering plants of N.tabacum 'Samsun' regardless of the photoperiod under which they were cultured (Table 1). TCLs taken from N. tabacum 'Maryland Mammoth' and N. sylvestris did not flower regardless of the physiological state of the mother plants or the photoperiod under which they were cultured.

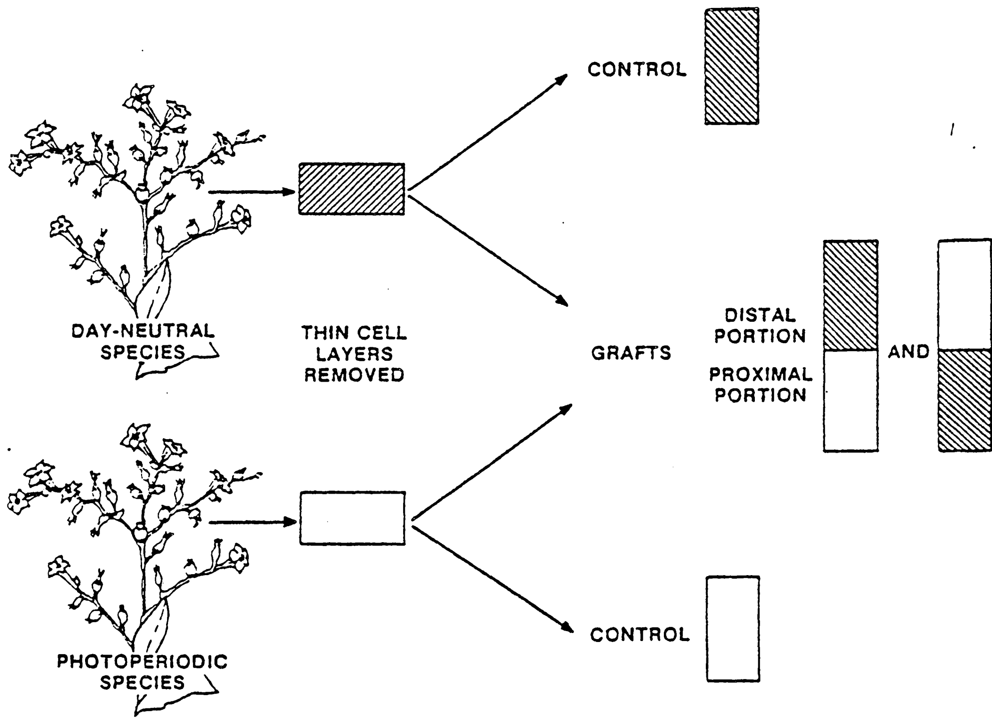

Figure 1. Positioning of thin cell layers in grafting experiments.

Table 1. Influence of photoperiod on flower and vegetative shoot production from tobacco thin cell layers.

Explant source		Daylength of source plant (hr)	Average number of flower buds per TCL[zy]		Average number of vegetative shoots per TCL[zy]	
			Short days[x]	Long days[w]	Short days[x]	Long days[w]
Nicotiana tabacum 'Samsun' (day-neutral)						
	Flowering	8 or 16	10.7 a	10.3 a	0.4 b	0.9 a
	Vegetative	8 or 16	0.0 b	0.0 b	1.1 a	1.3 a
Nicotiana tabacum 'Maryland Mammoth' (short-day)						
	Flowering	8	0.0 b	0.0 b	1.3 a	0.9 a
	Vegetative	16	0.0 b	0.0 b	1.4 a	1.4 a
Nicotiana sylvestris (long-day)						
	Flowering	16	0.0 b	0.0 b	1.5 a	1.5 a
	Vegetative	8	0.0 b	0.0 b	1.6 a	1.4 a

[z] TCL = thin cell layer.

[y] Mean separation within columns by the Student Newman-Keuls' test, 1% level.

[x] Short days = 8 hours.

[w] Long days = 16 hours.

De novo vegetative shoot formation was observed on all TCLs. However, fewer vegetative shoots were formed on 'Samsun' TCLs taken from flowering plants and cultured under short days than on any other TCL. The number of vegetative shoots produced by TCLs of the 2 photoperiodic species did not differ with the explant source or photoperiod during culture.

Experiment 2 When TCLs taken from the inflorescence of 'Samsun' were grafted to TCLs taken from stem tissue of the same plant, flowering was observed only on the explant taken from the inflorescence (Table 2). TCLs taken from stem tissue did not flower in any treatment whereas flowering of inflorescence-derived TCLs was equivalent in all treatments. The number of *de novo* vegetative shoots formed on TCLs taken from the inflorescence was less than the number formed on TCLs taken from stem tissue.

When reciprocal grafts were made between TCLs from inflorescences of *N. tabacum* 'Samsun' to TCLs of *N. tabacum* 'Maryland Mammoth' or *N. sylvestris*, only the explant from 'Samsun' flowered *in vitro* (Table 3). TCLs taken from inflorescences of *N. sylvestris* and 'Maryland Mammoth' did not form *de novo* flowers when cultured singly or in graft combination. The average number of vegetative shoots per TCL was less for TCLs taken

Table 2. Flower and vegetative shoot production from grafted thin cell layers taken from the inflorescence and stem tissues of *Nicotiana tabacum* 'Samsun'.

Graft		Average number of flower buds per TCL[zy]	Average number of vegetative shoots per TCL[zy]
Inflorescence TCL (control)		10.0 a	0.6 c
Stem TCL (control)		0.0 b	1.1 ab
Inflorescence TCL grafted acropetally to Stem TCL			
	Inflorescence portion	9.8 a	0.7 bc
	Stem portion	0.0 b	0.8 ab
Stem TCL grafted acropetally to inflorescence TCL			
	Stem portion	0.0 b	1.2 ab
	Inflorescence portion	9.9 a	0.6 c
Inflorescence TCL grafted to inflorescence TCL			
	$Inflorescence_1$ portion	9.8 a	0.6 c
	$Inflorescence_2$ portion	9.8 a	0.6 c
Stem TCL grafted to stem TCL			
	$Stem_1$ portion	0.0 b	1.2 ab
	$Stem_2$ portion	0.0 b	1.3 a

[z]TCL = thin cell layer

[y]Mean separation within columns by Student Newman-Keuls, 1% level.

Table 3. Flower bud and vegetative shoot production on individual thin cell layers (TCL) of *Nicotiana tabacum* 'Samsun', *Nicotiana sylvestris*, and *Nicotiana tabacum* 'Maryland Mammoth'.

Graft	Average number of flower buds per TCL	Average number of vegetative shoots per TCL[z]
Nicotiana sylvestris (long-day plant)	0.0 b	1.5 ab
Nicotiana tabacum 'Samsun' (day-neutral plant)	9.7 a	0.5 cd
Nicotiana tabacum 'Maryland Mammoth' (short-day plant)	0.0 b	1.6 a
'Samsun' TCL grafted acropetally to 'Mammoth' TCL		
'Samsun' portion	9.4 a	0.4 cd
'Mammoth' portion	0.0 b	1.4 ab
'Mammoth' TCL grafted acropetally to 'Samsun' TCL		
'Mammoth' portion	0.0 b	1.2 abc
'Samsun' portion	8.7 a	0.5 cd
'Samsun' TCL grafted to 'Samsun' TCL		
'Samsun'$_1$ portion	9.1 a	0.4 cd
'Samsun'$_2$ portion	9.0 a	0.3 d
'Samsun' TCL grafted acropetally to *N. sylvestris* TCL		
'Samsun' portion	8.6 a	0.5 cd
N. sylvestris portion	0.0 b	1.0 abcd
N. sylvestris TCL grafted acropetally to 'Samsun' TCL		
N. sylvestris portion	0.0 b	0.8 bcd
'Samsun' portion	9.4 a	0.3 cd

[z] Mean separation within columns by the Student Newman-Keuls' test, 1% level.

from 'Samsun'.

DISCUSSION

Although genotypic control of flowering is well established, specific environmental conditions such as temperature and photoperiod are often prerequisites to floral evocation (Lang, 1965). In vivo flowering of N. tabacum 'Maryland Mammoth' and N. sylvestris requires short and long days, respectively. Because leaves are not the sole plant organs responsive to photoinduction (Lang, 1965) and because in vitro photoinduction of daylength-sensitive species other than Nicotiana has been reported (Harada, 1967; Rossini and Nitsch, 1966; Hillman, 1959; Scorza, 1982), TCLs of 'Maryland Mammoth' and N. sylvestris tobacco were examined for their response to photoinductive conditions in vitro. However, under the experimental conditions reported, TCLs taken from short-day 'Maryland Mammoth' and long-day N. sylvestris in either the floral or vegetative state, could not be induced to flower through in vitro photoperiodic manipulations. Only TCLs from inflorescences of 'Samsun' responded by in vitro flowering.

These results indicate that there is a substance responsible for flowering present in TCLs of 'Samsun' and not in the

others. If, as hypothesized, flowering is evoked *in vivo* by reaching some critical balance of hormones (Zeevaart,1976), the differential response of TCLs from day-neutral and photoperiod-sensitive species of tobacco may reflect a difference in the period of time during which that critical balance is maintained. A sequential derepression of flowering genes has also been hypothesized to explain the ontogeny of flowering (Heslop-Harrison, 1964). The combination of culture conditions and physiological state of the explant results in evocation of flowering of 'Samsun' TCLs; however the physiological state of TCLs from daylength-sensitive tobacco does not permit floral evocation under the same conditions.

Recent work has demonstrated that the concentration of gibberellins increases and the amount of abscisins decreases in *N. sylvestris* during long-day induction; on the other hand, the amount of gibberellins decreases and abscisin increases in Mammoth tobacco during short-day induction (Loznikova et al., 1982). The critical balance of hormones required to evoke flowering *in vitro* may well differ for different photoperiodic species.

Grafts between whole plants of induced and non-induced *Nicotiana* have successfully transferred floral promoters from

the induced plants to the non-induced (Melchers and Lang, 1941; Zeevaart, 1958). We attempted to do the same using responsive and non-responsive TCLs on the hypothesis that the substance(s) responsible for floral-induction in plants of different response types is(are) identical physiologically and chemically (Lang, 1965). The hypothesis also contends that these response types do not differ with respect to the presence or absence, and the nature of, the photoperiodic control of flowering substance(s). However, our work suggests that neither promoters nor inhibitors were transferred between TCLs. Stimulation of flowering was not observed in non-responsive TCLs grafted to responsive ones, nor was inhibition of flowering observed in the responsive TCLs. These results appear to support Zeevaart's (1976) critical balance hypothesis.

It may be possible that a floral inhibitor or promoter was present in the TCLs and was transported through the graft, but not in sufficient quantity to exert an effect on morphogenesis in the receiving TCL; it may also be possible that this substance was present but was not transported through the grafts into the epidermal and subepidermal cells. The majority of evidence indicates that transmission through grafts occurs only after tissue union, and specifically, the union of phloem (Zeevaart, 1958); phloem is not present in TCLs. However, the

substance that is transported in the phloem also moves in continuous, living tissue with no preferential movement acropetally or basipetally (Lang, 1965). On removal of pairs of co-cultured TCLs, the original explant along with de novo organs formed in vitro were always united into a single unit; this was taken as evidence of graft union formation sufficient for transmission. The substance or group of substances responsible for flowering in day-neutral TCLs apparently is not transportable through epidermal and subjacent cells.

The number of vegetative shoots on non-flowering TCLs, although generally greater than on flowering TCLs, did not approach the number of flower buds on flowering TCLs. Therefore, there was no pre-existing limit to the number of meristematic centers that could develop on a given TCL. Vegetative and floral meristems appear to have arisen independently. Floral meristems appeared to exert limited suppression on the development of vegetative meristems.

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CHAPTER 3

GENOTYPIC AND PHOTOPERIODIC REGULATION OF *DE NOVO* FLOWER BUD PRODUCTION ON THIN CELL LAYERS OF *NICOTIANA TABACUM* L.

ABSTRACT

Thin cell layers taken from intraspecific F_1 and F_2 hybrids of short-day and day-neutral cultivars of *Nicotiana tabacum* were examined to assess the importance of genotype and photoperiod to *de novo* flowering. All thin cell layers taken from the day-neutral parents and F_1 hybrids produced *de novo* flowers; the thin cell layers taken from the short-day parent did not. The reciprocal F_1 hybrids flowered *in vitro* and produced equivalent numbers of *de novo* buds. The F_2 genotypes flowered over a range of photoperiods (11.4-13.6 hours of light/day). Thin cell layers taken from F_2 genotypes, which flowered at the same time or prior to the day-neutral parent plants, exhibited an *in vitro* flowering response equivalent to the parents and F_1 hybrids. Photoperiodic response and *in vitro* flowering were correlated in the F_2 population with fewer flowers produced per TCL with increasing short-day reaction.

However, F_2 segregates whose TCLs did not yield *de novo* flowers were found among both day-neutral and short-day phenotypes.

INTRODUCTION

Flower initiation is ultimately determined by genotype (Lang, 1965), with environmental factors such as temperature and photoperiod sometimes exerting a significant effect on the flowering process (Zeevaart, 1976). Short-day and long-day plants (SDP and LDP, respectively) are so defined because of their strong flowering reaction to photoperiod. However, within each category, considerable genotypic variability exists for control of flowering. Therefore, the SDP and LDP definitions may become blurred under marginally inductive conditions (Lang, 1965).

The thin cell layer (TCL) system of plant tissue culture has been used to induce flower buds on epidermal and subepidermal strips of *Nicotiana*, *Torenia*, and *Begonia* (Tran Thanh Van, 1980). This system has been extensively outlined for *Nicotiana*. Three to 6 layers of epidermal and subepidermal cells, when removed from peduncles of a day-neutral tobacco plant (DNP) and placed *in vitro* on a nutrient medium consisting of the Murashige and Skoog (1962) macro- and microelements,

0.166 M glucose, 1 uM IBA, and 1 uM kinetin, form macroscopic flower buds in approximately 15 to 21 days (Tran Thanh Van and Trinh, 1978). This has been successful with several tobacco species and cultivars (Table 1).

Compared with day-neutral species or cultivars, TCLs of long-day or short-day tobacco have not been found to flower *in vitro*. The only exceptions are the F_1 hybrids, *N. sylvestris* X *N. tomentosiformis* (LDP x SDP), *N. plumbaginifolia* X *N. sylvestris* (DNP x LDP), and *N. tabacum* 'Samsun' X F_1 (*N. sylvestris* X *N. tomentosiformis*)(DNP X LDP) (Kamate et al.,1981; Tran Thanh Van and Trinh, 1978; Tran Thanh Van and Cousson, 1982). All of these hybrids are LDPs and have responded to the TCL procedure with only minimal flowering (Table 1). An intergenomic interaction has been suggested as an explanation.

Short-day tobacco has been called "mammoth" because of its massive vegetative growth compared with day-neutral plants under typical long-day temperate field conditions (Garner, 1912). The short-day flowering response of mammoth tobacco has been shown to be under control of a single gene with mammoth recessive to day-neutral (Allard, 1919). The following study used the mammoth character to evaluate the potential for *in vitro* flowering in day-neutral and short-day cultivars of *N.*

Table 1. *Nicotiana* genotypes which have been tested for *de novo* flowering from thin cell layers[z].

Species or cultivar	Photoperiod	Chromosome Number	Per cent TCLs with flowers
Floral-responsive			
N. tabacum 'Wisconsin 38'	Day neutral	48	100
'Samsun'	Day neutral	48	100
'Lacerata'	Day neutral	48	82
'Xanthi'	Day neutral	48	100
F_1 [*N. tabacum* 'Samsun' X *N. sylvestris*]	Day neutral	36	60
F_1 [*N. tabacum* 'Samsun' X *N. tomentosiformis*]	Day neutral	36	75
F_1 [*N. sylvestris* X *N. tomentosiformis*]	Long day	24	30
N. rustica 'Brasilia'	Day neutral	48	25-35
F_1 [*N. plumbaginifolia* X *N. sylvestris*]	Long day	22	10
F_1 [*N. suaveolens* X *N. tabacum*] X *N. tabacum*	Day neutral	48	89
F_1 [*N. debneyi* X *N. tabacum*] X *N. tabacum*	Day neutral	48	100
Non floral-responsive			
N. tabacum 'Maryland Mammoth'	Short day	48	0
N. alata	Day neutral	18	0
N. debneyi	Day neutral	48	0
N. plumbaginifolia	Day neutral	20	0
N. sylvestris	Long day	24	0
N. tomentosiformis	Short day	24	0
F_1 [*N. plumbaginifolia* X *N. tomentosiformis*]	Long day	22	0
N. otophora	Short day	24	0

[z] References: Kamate et al., 1981
Tran Thanh Van and Cousson, 1982
Tran Thanh Van and Trinh, 1978

tabacum, in their reciprocal F_1 hybrids, and in their F_2 populations which were expected to segregate for photoperiodic reaction. The objectives of this study were:

1. to determine the influence of the simply inherited mammoth character on *de novo* flowering within a species,

2. to determine if there is a maternal influence on *de novo* flower bud production on TCLs, and

3. to assess the relative importance of genotype and photoperiod to *de novo* flower formation.

MATERIALS AND METHODS

The populations of *Nicotiana tabacum* L. used in this experiment were assigned the following codes:

DNC = 'Samsun' (day-neutral control)

DNP_1 = 'McNair 944' (day-neutral parent)

DNP_2 = 'North Carolina 82' (day-neutral parent)

SDP = 'NC 22 NF' (short-day mammoth parent)

$F_1 1$ = DNP_2 x SDP

$F_1 2$ = SDP x DNP_2

$F_1 3$ = DNP_1 x SDP

$F_1 4$ = SDP x DNP_1

$F_2 1$ = selfed progeny of a single $F_1 1$ plant

$F_2 2$ = selfed progeny of a single $F_1 3$ plant

Seed of all cultivars except DNC was obtained from Dr. R. Terrill, Associate Professor of Agronomy, at the Southern Piedmont Research and Education Center in Blackstone, Virginia. Selfed seed of DNC were harvested from inbred stock plants at VPI&SU horticulture greenhouses.

To determine the photoperiodic requirement of each genotype, 2 plants of each parent cultivar and F_1 hybrid were grown under both long days (16 hours) and short days (8 hours) in the greenhouse in 2 separate experiments from August, 1982, to April, 1983. Long days were provided by incandescent lighting and short days by pulling of black cloth.

In May, 1983, seeds of all 10 lines were sown for field planting. In mid-June, 30 plants of each F_2 and 4 plants of each parent cultivar and F_1 were transplanted to the field at the VPI horticulture research farm. Each F_2 population was planted as a block with 2 plants of each parent and F_1 randomized within the block. These plants remained in the field until October 5; at that time they were transplanted into 60 cm plastic pots and moved to the VPI greenhouses where they remained under natural photoperiod. The anthesis date of the

first flower on each plant was recorded.

When each plant reached the green fruit stage, 10 pedicels were removed from the third and fourth peduncle from the base of the inflorescence. These were disinfested by a wash in distilled water followed by 5 seconds in 70% ethanol and 5 minutes in 2.1% sodium hypochlorite. Pedicels remained in a sterile distilled water rinse until TCLs were taken. TCLs, 2 X 10mm, were excised with a scalpel, removed and cultured in Bellco glass tubes (150 x 25mm) containing 30 ml of medium (pH 5.6) consisting of the Murashige and Skoog (1962) macro- and microelements, 30 g/l glucose, 1uM IBA, 1uM kinetin, 0.4 mg/l thiamine-HCL, 100 mg/l myo-inositol, and 8.0 g/l agar. Cultures were incubated at 24-26°C under 16 h light (60-75 uE s^{-1} m^{-2}) produced by a combination of cool white fluorescent and 60 watt incandescent bulbs. After 21 days in culture, the numbers of flower buds and vegetative structures per TCL were recorded. Data were analyzed by SAS GLM and regression procedures (Council and Helwig, 1982); segregation in the F_2 populations was tested by Chi-square.

RESULTS

The cultivars DNC, DNP_1, DNP_2, and all 4 F_1 hybrids flowered

in the greenhouse under both long and short days confirming their day-neutral status. SDP plants flowered only under short days and hence are qualitative short-day plants.

Plants in both F_2 populations flowered over a wide range of natural photoperiods (Fig. 1). If plants which flowered between photoperids ranging from 13.7 HOL (hours of civil twilight per day) to 12.1 HOL are considered DNP and those which flowered only when the photoperiod was less than 12.1 HOL are considered SDP, a segregation pattern of 18 DNP:12 SDP and 17 DNP:13 SDP was observed for $F_2$1 and $F_2$2, respectively. A 3 DNP:1 SDP segregation ratio for monofactorial inheritance was accepted for $F_2$1 (x^2=3.6, .10>p>.05), but rejected for $F_2$2 (x^2=5.38, .05>p>.01). There was an excess of SDPs in both families. All parents bloomed in the field under their expected photoperiods. TCLs taken from DNC, DNP_1, DNP_2, and all 4 F_1 hybrid plants flowered 100% of the time (Table 2). Reciprocal F_1 hybrids formed an equivalent number of de novo flowers (Table 2).

The average number of flowers formed per TCL of F_1 hybrids derived from DNP_2 was equal to DNP_2 and less than DNC; however, the average number formed with the F_1 hybrids derived from DNP_1 was greater than either parent and equal to DNC plants (Table

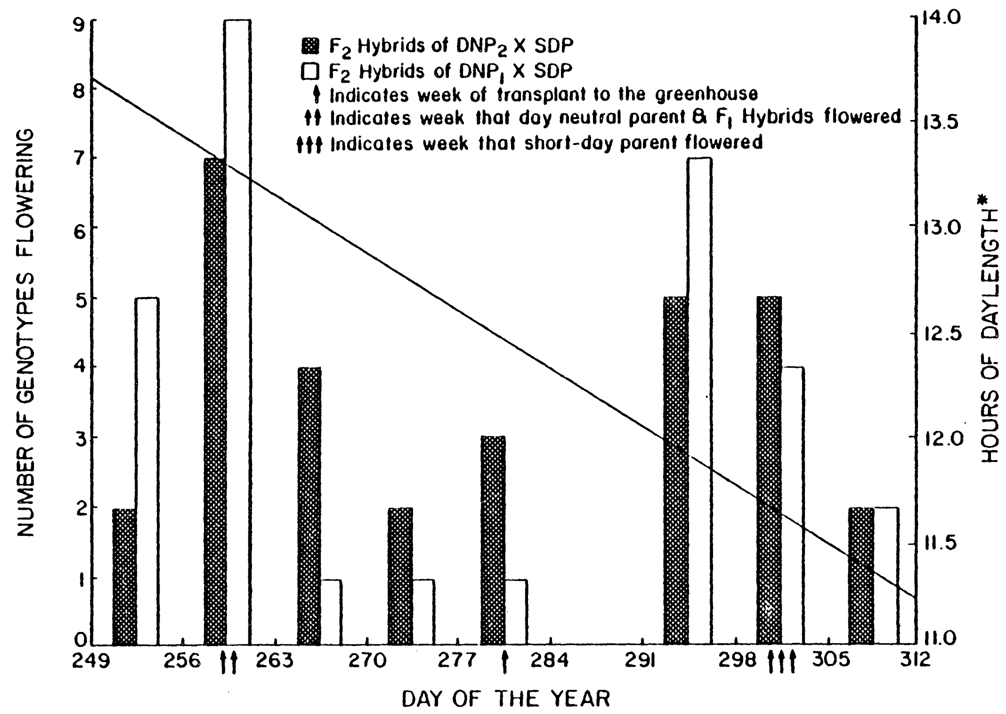

Figure 1. Flowering responses of F_2 hybrids.

Table 2. Photoperiodic and morphogenetic responses from *Nicotiana tabacum* thin cell layers.

Cultivar	Symbol	Photoperiod	% TCLs with *de novo* flowers	Flower number per TCL mean (± SD)
'Samsun'	DNC	day neutral	100.0	3.5 (1.2) a[z]
'McNair 944'	DNP_1	day neutral	100.0	2.1 (0.8) b
'NC 82'	DNP_2	day neutral	100.0	2.0 (0.7) bc
'NC 22 NF'	SDP	short day	0.0	0.0 (0.0) d
DNP_2 X SDP	$F_1 1$	day neutral	100.0	1.8 (1.2) bc
SDP X DNP_2	$F_1 2$	day neutral	100.0	1.9 (0.8) bc
DNP_1 X SDP	$F_1 3$	day neutral	100.0	3.5 (0.9) a
SDP X DNP_1	$F_1 4$	day neutral	100.0	3.5 (0.7) a
F_2 (DNP_2 X SDP)	$F_2 1$	variable	44.2	0.8 (1.2) cd
F_2 (DNP_1 X SDP)	$F_2 2$	variable	66.8	1.8 (1.8) bc

[z] Mean separation within column by Student Newman-Keuls, 5% level.

2). This observation along with the fact that all TCLs from the 4 F_1 hybrids flowered, demonstrates that there was no maternal influence.

The only F_2 plants which exhibited an *in vitro* flowering response equivalent to DNP (3-4 buds/TCL) bloomed at the same time or prior to DNP (Figs. 2 & 3). TCLs taken from F_2 plants which bloomed after the date of DNP flowering exhibited less than 1.5 flowers per TCL. Most F_2 genotypes which flowered at the same time as the SDP produced floral-responsive TCLs. The 6 F_2 genotypes which did not flower *in vitro* responded to a wide range of natural photoperiods (Figs. 2 & 3). Regression analysis revealed that the mean number of flowers per TCL decreased as the days shortened (Fig. 2 & 3).

DISCUSSION

The importance of genetic determinants on *de novo* morphogenetic differentiation has been demonstrated mostly among rather than within species (Kamate et al., 1981). Certain genotypes of *Nicotiana* have responded by flowering under the TCL system whereas others have yielded only vegetative buds (Table 1). In all cases, except for 3 interspecific F_1 hybrids, the TCLs which demonstrated a *de novo* flowering capacity have come

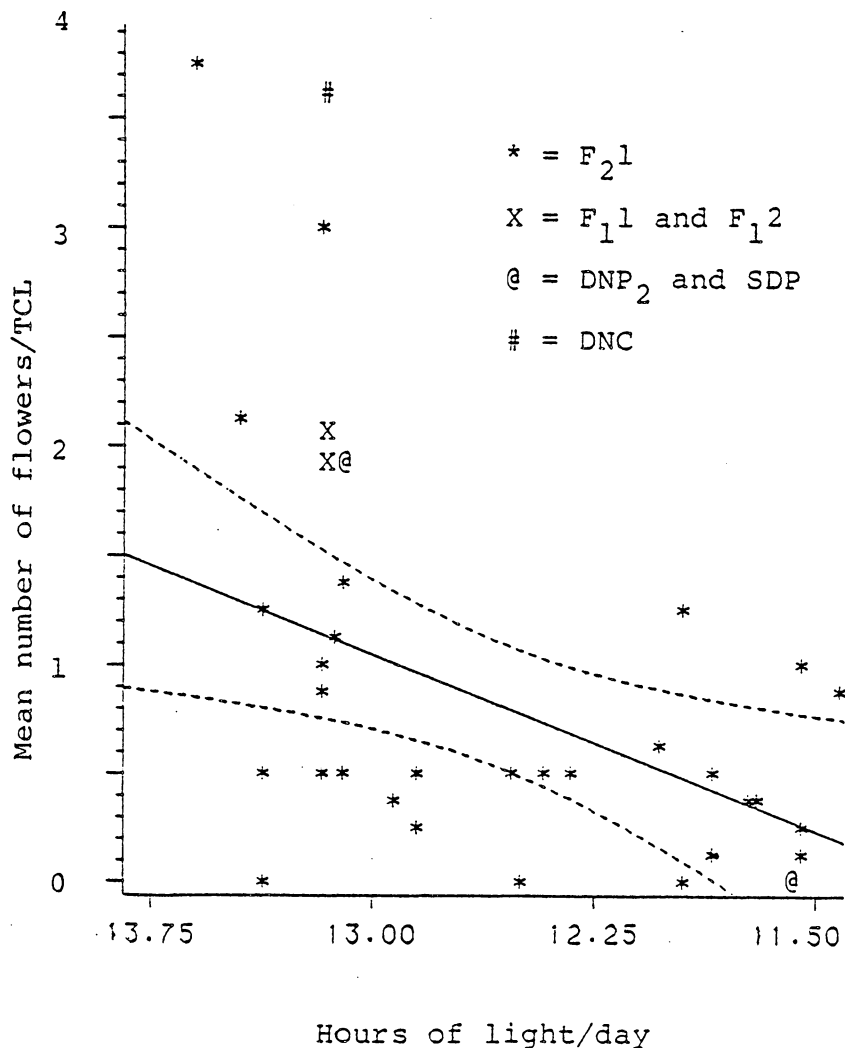

Figure 2. Number of flowers formed per thin cell layer of intraspecific hybrids from Nicotiana tabacum 'North Carolina 82' (DNP_2) and 'NC 22 NF' (SDP). ($y = (0.009 \pm 0.003)X - (5.99 \pm 2.5)$, $p < .05$, $r^2=0.2$).

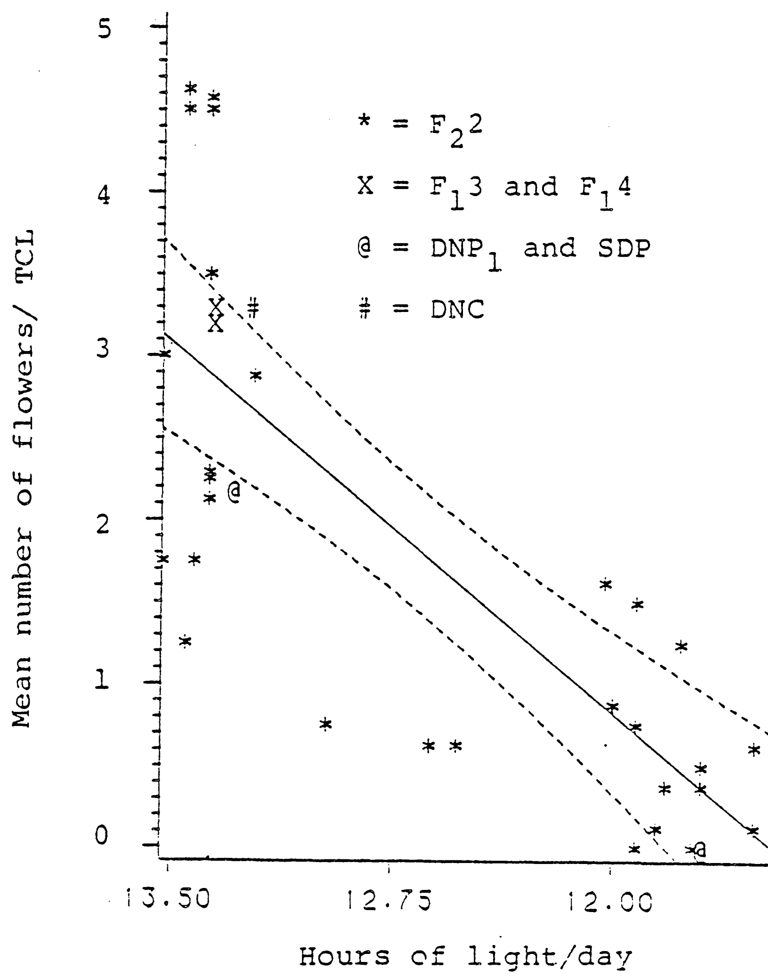

Figure 3. Number of flowers formed per thin cell layer of intraspecific hybrids from <u>Nicotiana tabacum</u> 'McNair 944' (DNP_1) and 'NC 22 NF' (SDP).
($y= (0.025 \pm 0.004)X - (17.25 \pm 3.0)$, $p < .01$, $r^2=0.5$)

from DNPs. The exceptions, although flowering only minimally in vitro (less than 30% of cultured TCLs developed de novo flower buds), indicate that interactions between genomes can be essential determinants of in vitro flower formation; a recent study which compared the morphology of flowers formed in vivo to those formed in vitro supports this statement (Trinh and Tran Thanh Van, 1983). The fact that short-day types of N. tabacum have not flowered in vitro and day-neutral types have, has led to a presumed relationship between photoperiodism, or rather lack of it, and the capacity for in vitro flowering. This has been supported by the observation that the theorized photoperiodic parents of the DNP N. tabacum, i.e. N. sylvestris (LDP) and N. tomentosiformis (SDP), do not form flowers from TCLs (Kamate et al., 1981; Tran Thanh Van and Cousson, 1982).

A dosage effect of genomes has been suggested by the observation that day-neutral hybrids (2n=36) of N. tabacum 'Samsun' (2n=48) will only express 60% de novo flowering when crossed with N. sylvestris (LDP, 2n=24) (Tran Thanh Van and Trinh, 1978); 'Samsun' normally responds 100% of the time. This indicates that de novo flower formation is not inherent in all day-neutral germplasm. The hypothesis that decreasing ploidy level is associated with decreased de novo flowering capacity

is also inadequate. Even TCLs of DNPs with a chromosome number of 48 (N. tabacum 'Lacerata', N. rustica, N. debneyi, and (N. suaveolens X N. tabacum) X N. tabacum have demonstrated a reduced flowering capacity (Table 1). On the other hand, anther-derived haploids (2n=2x=24) of 'Samsun' have demonstrated a de novo flowering capacity equal to or greater than 'Samsun' (refer to Chapter 4).

Our F_2 hybrid populations demonstrated some independence of the photoperiodic and in vitro flowering characteristics. The apparent natural break betwen LDPs and SDPs in Fig. 1 occurred at the time of transplanting field plants to the greenhouse. Hence, recovery from transplant shock may have falsely given the appearance of discontinuous variation for photoperiodic reaction (Fig. 1). Nevertheless, the fact remains that only F_2 plants that flowered under long days exhibited the same intensity of in vitro flowering as the DNP plants.

TCLs taken from short-day F_2 segregates had the ability to flower in vitro although they exhibited fewer flowers per TCL than DNPs or F_1 hybrids. However, reduced de novo flowering on TCLs from F_2 was not restricted to SDPs but was observed on several day-neutral F_2 hybrids as well. Under the experimental conditions, only 6 F_2 genotypes demonstrated no capac-

ity for _in vitro_ flowering. One of these flowered under the same daylength as the DNP plants. Four others were in the range of the SDP and the last was intermediate. An interaction between genomes rather than a quantitative effect of genome number is again indicated.

Apparently, there is no maternal factor involved in the ability to form flowers _de novo_ as reciprocal F_1 hybrids reacted similary _in vitro_ (Table 2). Not only did reciprocal crosses in both cases produce hybrids which formed _de novo_ flowers 100% of the time, but they also produced equivalent numbers of flower buds per TCL for each of the respective crosses.

In summary, this study of intraspecific hybridization between day-neutral tobacco plants, which produce _de novo_ flowers, and short-day mammoth tobacco, which do not, has demonstrated that there is no maternal influence on _de novo_ flowering and that the _de novo_ flowering capacity is qualitatively independent of photoperiod. However, the number of flower buds per TCL is generally greater for DNPs.

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CHAPTER 4

A COMPARISON OF THE EMBRYOGENETIC CAPACITY OF POLLEN AND SUBSEQUENT EMBRYOS FROM ANTHERS OF *DE NOVO* AND *IN VIVO* FLOWERS FROM DIPLOID AND HAPLOID PLANTS OF *NICOTIANA TABACUM* L. 'SAMSUN'

ABSTRACT

Thin cell layers (TCLs) were cultured from diploid (2n=4X=48) and haploid (2n=2X=24) *Nicotiana tabacum* 'Samsun' and the subsequent *de novo* flowers compared. The *de novo* flowers were also compared to *in vivo* flowers of both diploid and haploid plants. The TCLs of haploid plants produced more flower buds than diploid TCLs, and did so in a shorter period of time. *De novo* flowers and anthers of both genotypes were considerably smaller than the *in vivo* flowers with a variable number of anthers and pistils. The embryogenetic capacity of anthers taken from *in vivo* diploid flowers was five times greater than *de novo* diploid and haploid anthers. *In vivo* haploid anthers produced no embryos. Mitotic cells in root tips of plants derived from anther cultures of *de novo* haploid flowers revealed a mixoploid nature. Diploid meiosis was regu-

lar and haploid meiosis was found to express a similar array of irregularities regardless of the origin (*de novo* or *in vivo*) of the flowers.

INTRODUCTION

Aseptic techniques have been used to study various aspects of flowering including flower initiation, floral development, the flowering gradient, photoinduction, and vernalization of plants (Scorza, 1982). *In vitro* techniques have allowed researchers to isolate flowering sites and to study the process of flowering without the influences of other plant parts. Flowers produced *in vitro*, however, are typically abnormally small and malformed (Ganapathy, 1969; Scorza, 1982).

Variation in chromosome numbers has often been reported among plants derived from tissue culture. Phenovariants, ie. plants with changes in their phenotype but no change in their chromosome number, can also occur (Tran Thanh Van and Trinh, 1978). Such features of *in vitro* experimentation erode the reliability of findings based on *de novo* flowers and limit the applicability of such results to whole plant processes.

To help assess the validity of experimentation on flowering

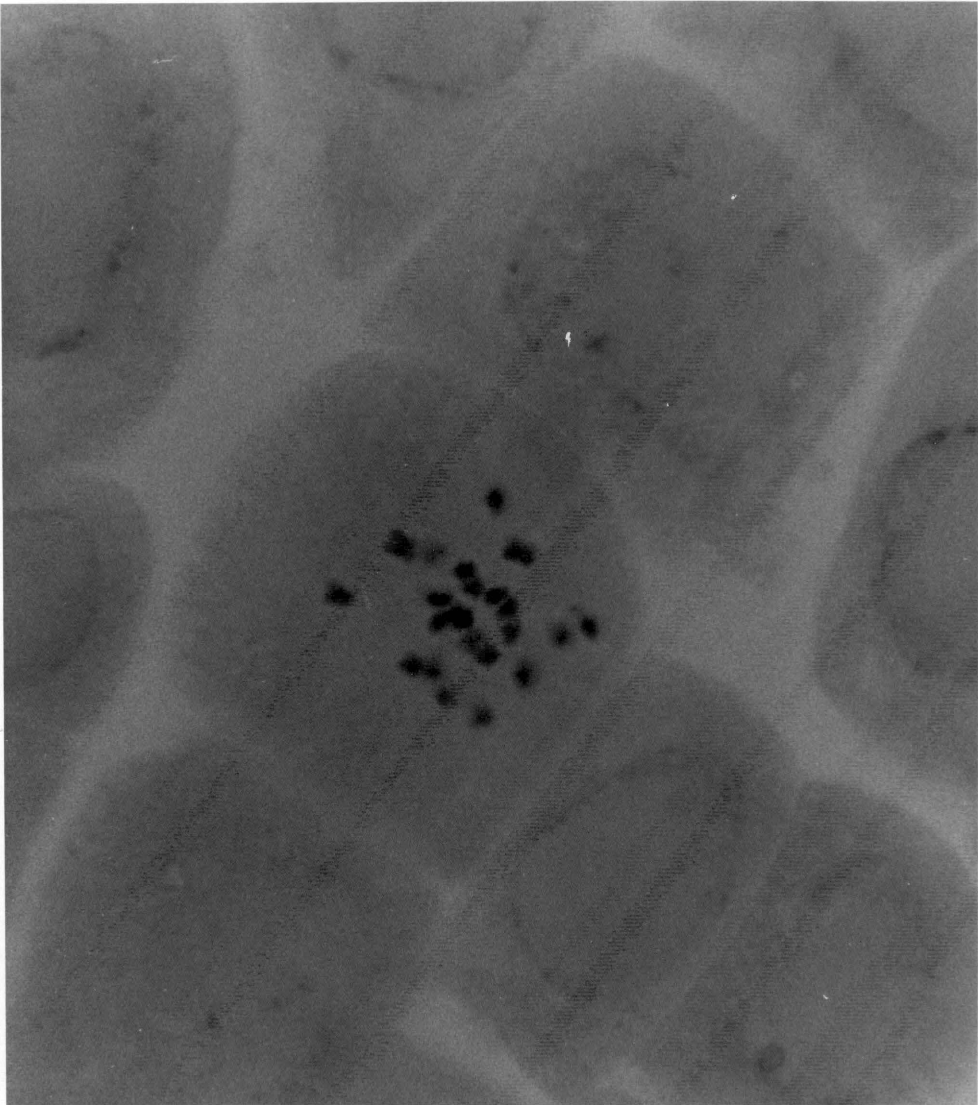
in vitro, the following study describes comparisons of de novo with in vivo flowers of both haploid and diploid Nicotiana tabacum L. 'Samsun'. The embryogenetic capacity of immature microspores in cultured anthers taken from these 4 types of flowers is also estimated. Cytogenetic analysis of microsporogenesis in the 4 types of flower buds, and the resulting anther-derived plantlets, has been undertaken to attempt to explain differences in responsiveness to anther culture.

MATERIALS AND METHODS

Diploid plants (2n=4x=48) of Nicotiana tabacum L. 'Samsun' were grown from seed under natural prevailing photoperiod (August-October, 1983) in the VPI&SU greenhouses. Haploid plants (2n=2x=24) were obtained by culturing anthers of 'Samsun' at the uninucleate stage of microspore development (Nitsch, 1969) on a nutrient bilayer (Johansson et al., 1982) and subsequently confirmed as haploid by chromosome counts in root tip preparations (Fig. 1).

When haploid and diploid plants reached the green fruit stage, peduncles were removed, rinsed in distilled water for 5 minutes, dipped in 70% ethanol, and placed in 2.1% sodium hypochlorite for 5 minutes. After a single rinse in sterile dis-

Figure 1. Root squash of haploid plant derived from the culture of anthers from diploid *Nicotiana tabacum* 'Samsun'. (24 chromosomes).

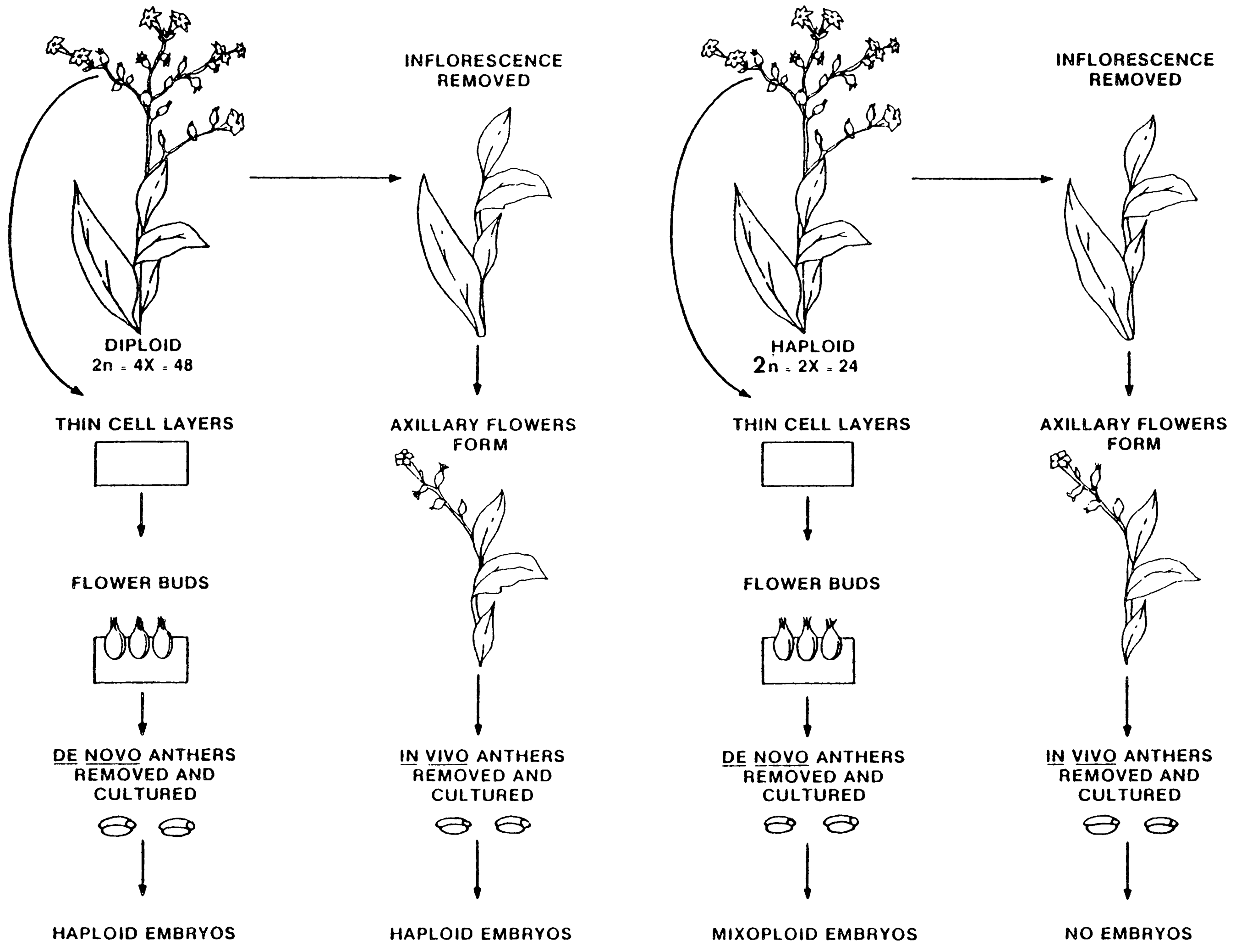


tilled water, epidermal and subepidermal sections (thin cell layers; TCLs) approximately 2 x 10mm were removed from the peduncles and cultured on Murashige and Skoog (1962) basal medium with 100 mg/l myo-inositol, 1 uM IBA, 1 uM kinetin, 0.4 mg/l thiamine, 30 g/l glucose, and 8.0 g/l agar. Four days after TCLs were excised from a plant, the plant's inflorescence was removed to promote axillary flower formation.

Several plants which originated from vegetative buds on haploid and diploid TCLs were grown in the greenhouse to flowering. At anthesis, measurements were taken on flower size, number of pistils, and number of anthers. These data were compared with similar measurements on seed-sown diploid plants and anther-derived haploid plants. Means are based on 50 observations/treatment. Height and leaf size were not compared as the two sets of plants were grown at different times and under different environmental conditions.

When anthers reached late tetrad/premitotic microspore stage, they were aseptically removed from TCL-derived and *in vivo* buds and cultured (Fig. 2). One anther from each flower bud was squashed, stained with aceto-carmine, and microspores were examined to ensure that the anthers were at the appropriate stage of development. At that time, the numbers of anthers

Figure 2. Diagram of procedures to obtain haploid and mixoploid embryos from *Nicotiana tabacum* 'Samsun'.



INFLORESCENCE REMOVED
INFLORESCENCE REMOVED
DIPLOID
2n = 4X = 48
HAPLOID
2n = 2X = 24
THIN CELL LAYERS
AXILLARY FLOWERS FORM
THIN CELL LAYERS
AXILLARY FLOWERS FORM
FLOWER BUDS
FLOWER BUDS
DE NOVO ANTHERS REMOVED AND CULTURED
IN VIVO ANTHERS REMOVED AND CULTURED
DE NOVO ANTHERS REMOVED AND CULTURED
IN VIVO ANTHERS REMOVED AND CULTURED
HAPLOID EMBRYOS
HAPLOID EMBRYOS
MIXOPLOID EMBRYOS
NO EMBRYOS

and pistils per flower were counted, the size of the bud and anthers were measured, the number of flower buds per TCL was counted, and the time from TCL excision to anther culture was recorded.

Anthers were tested for androgenetic potential on 2 media. The first (Tran Thanh Van, 1977) consisted of Murashige and Skoog (1962) macro- and micronutrients, 30 g/l sucrose, 0.4 mg/l thiamine, 100 mg/l myo-inositol, 10 g/l agar, 1 uM kinetin, and 1 uM IAA (filter sterilized). This 'TCL medium' was dispensed at the rate of 10 ml per 60 X 15mm disposable petri dish. The second medium (activated charcoal bilayer; AC bilayer) was a nutrient bilayer consisting of 8 ml of an agar-solidified (0.8%) basal Nitsch (1969) medium with 0.5% activated charcoal (Johansson et al., 1982) and 2 ml of the same liquid medium without activated charcoal placed on top. All the anthers from a single bud were cultured in the same petri dish. A minimum of 120 anthers/treatment were tested on the TCL medium and were incubated under continuous light (60-75 uE s^{-1} m^{-2}) at 25-27°C. A minimum of 200 anthers per treatment was cultured on the AC bilayer and incubated under 16 hour photoperiod (54 uE s^{-1} m^{-2}) at 25°C. After 8 weeks incubation, the number of anthers which produced embryos and the number of embryos per anther were counted under a dissecting microscope.

Data were analyzed separately for each medium by SAS ANOVA (Council and Helwig, 1982).

Anthers at various stages from all 4 types of flowers were fixed in 3 parts ethanol to 1 part acetic acid. The anthers were squashed, stained with acetic orcein, and their contents examined under an Olympus B061 microscope. Microsporogenesis in de novo and in vivo haploid flowers was compared at metaphase/anaphase I and at the tetrad stage. The ratio of dyads and tetrads was estimated by scoring the frequency of each in a sample of at least 3000 pollen mother cells from both de novo and in vivo haploid flowers.

Embryos which formed from anthers of both de novo and in vivo flowers were subcultured onto a half strength semi-solid (0.8g/l agar) Murashige and Skoog (1962) basal medium. After root formation, anther-derived plants were transferred to the greenhouse. Actively growing root tips were excised, treated with 0.1% colchicine for 2 hours at room temperature, fixed in 3 parts ethanol to 1 part acetic acid for 24 hours at 5°C, macerated in 0.5N HCl at 60°C for 5 minutes, and stained in leuco-basic fuchsin for 2 hours. Root tips were then squashed in acetic orcein for chromosome counts.

RESULTS

Flower characteristics of diploid 'Samsun' plants which originated from seed and haploid 'Samsun' plants which originated via anther culture were not statistically different respectively, from those of diploid and haploid plants which developed from vegetative buds on TCLs (Table 1). The corolla length for both types of haploid plants was smaller than for diploids, but the numbers of anthers (5) and pistils (1) per flower were constant.

De novo haploid flowers required less time in culture to reach the microspore stage than *de novo* diploid flowers (Table 2). Also, the number of flowers per TCL was greater for haploids than diploids.

The *de novo* flowers of both haploid and diploid plants differed significantly from *in vivo* flowers of haploids and diploids. At the time of anther culture, the mean bud and anther sizes of both *de novo* flowers were significantly smaller than for *in vivo* flowers. (Table 2). The number of pistils and anthers per flower for the *in vivo* plants was constant at 1 and 5, respectively. The number of anthers for the *de novo* haploid

Table 1. Comparisons of flowers on diploid and haploid plants from various origins.

Flower source	Corolla length [z] (cm)	Anther number	Pistil number
Seed-sown diploids	4.5-5.0 a[y]	5	1
Anther-derived haploids	3.5-4.0 b	5	1
TCL[x] diploid	4.5-5.0 a	5	1
TCL haploids	3.5-4.0 b	5	1

[z] at anthesis

[y] Mean separation within columns by Student Newman-Keulls' procedure, 5% level.

[x] Thin Cell Layer

Table 2. Comparisons of *de novo* and *in vivo* flowers of haploid and diploid *Nicotiana tabacum*.

Flower source	Number of anthers examined	Mean bud length (mm)	Mean # anthers per bud	Mean anther length (mm)	Mean # pistils per bud	Days to *de novo* bud formation	Flowers per TCL
de novo haploids	425	4.8 b[z]	6.0 a	1.5 b	1.2 a	26.7 b	12.1 a
in vivo haploids	330	11.0 a	5.0 c	2.9 a	1.0 b	------	------
de novo diploids	380	4.8 b	5.6 b	1.4 b	1.2 a	32.9 a	9.8 b
in vivo diploids	350	11.3 a	5.0 c	3.0 a	1.0 b	------	------

[z] Mean separation within columns by Student Newman-Keulls' procedure, 5% level.

and diploid flowers ranged from 5 to 10 and 5 to 8, respectively. The number of pistils ranged from 1 to 4 in de novo haploid flowers and from 1 to 3 in de novo diploid flowers (Table 2). Flower morphology of in vivo haploids did not differ significantly from those of in vivo diploids.

Anthers cultured on the TCL medium did not respond as well as those cultured on the AC bilayer medium with respect to both the percentage of in vivo diploid anthers that responded and the number of embryos produced per responding anther (Table 3). At least 10% of the de novo haploid and diploid anthers cultured on the Nitsch bilayer produced embryos; no anthers from in vivo haploid plants produced embryos on either media. From a total of 21 embryos derived from de novo haploid anthers only 2 survived longer than 3 weeks. The abnormal features of these embryos likely affected their survival (Fig. 3). Normal survival of embryos from in vivo and de novo diploid anthers was observed.

The 2 surviving embryos formed from de novo haploid flowers callused at their bases and formed multiple shoots when subcultured, regardless of medium. Roots formed only after several subcultures. Chromosome counts in root tips revealed a mixoploid nature; the number of chromosomes per mitotic cell in one

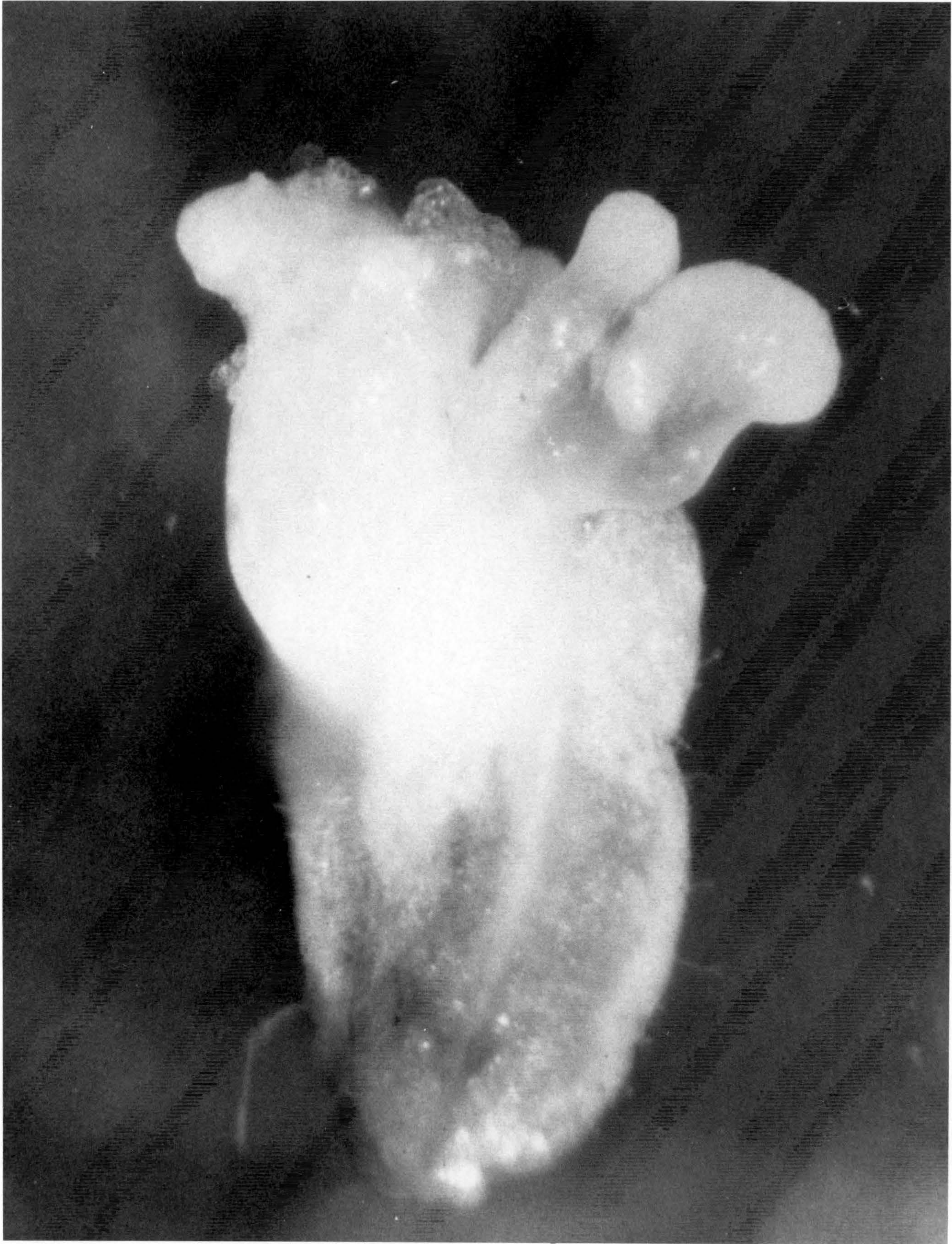
Table 3. Androgenetic response of anthers from *de novo* and and *in vivo* flowers of haploid and diploid *Nicotiana tabacum* 'Samsun'.

Anther source	Per cent anthers responding/plate	Mean # plantlets/ responding anther
Thin cell layer medium		
de novo haploids	1.7 b[z]	1.0 bc
de novo diploids	0.0 b	---
in vivo haploids	0.0 b	---
in vivo diploids	0.9 b	1.0 bc
control[Y]	52.0 a	3.7 ab
Nitsch bilayer medium		
de novo haploids	10.9 b	1.0 bc
de novo diploids	10.5 b	4.1 ab
in vivo haploids	0.0 b	---
in vivo diploids	52.0 a	6.2 a

[z]Mean separation within columns by Student Newman-Keulls' test, 5% level.

[Y]*in vivo* diploids on Nitsch bilayer medium

Figure 3. Abnormal embryos arising from *de novo* haploid flower anther.



root varied from 3 to 32 (Table 4; Fig. 4).

Similar meiotic abnormalities for de novo and in vivo haploids were observed throughout microsporogenesis. These included univalents (Figs. 5A & D), empty tetrads, and deformed pollen (Figs. 5C & F). Dyad frequency after telophase II (Figs. 5B & E) was greater for de novo than for in vivo haploids (Table 5). The meiotic process for in vivo and de novo diploids followed the normal stages (Fig. 6).

DISCUSSION

Due to chromosomal changes, plants derived from tissue cultures can often be atypical, especially when regenerated from callus tissue (Meins, 1983). When de novo vegetative shoots were removed from TCLs of both haploid and diploid plants and grown to anthesis in the greenhouse, normal plants with floral characteristics similar to anther-derived haploids or seed-sown diploids resulted. De novo flowers of TCLs from haploid and diploid plants, however, differ significantly from in vivo flowers. Recent work has demonstrated that the morphology of in vivo and in vitro flowers depends on the dominant proportion of nuclear genome of one of the two parental species (Trinh and Tran Thanh Van, 1983). Even though size differences

Table 4. Number of mitotic cells observed with various chromosome numbers in diploid, haploid, and mixoploid tobacco.

Source plant	Chromosome number								
	0-5	6-10	11-15	16-20	21-25	26-30	31-35	36-40	over 41
Seed-sown diploids									44
Anther-derived haploids					53				
Anther-derived mixoploids from haploid TCLs[z]	5	22	45	54	13	5	5		1

[z] Thin cell layers.

Figure 4. Root squash preparations from mixoploid plants. A. n=13 B. n=24 C. n=32 D. n= greater than 42.

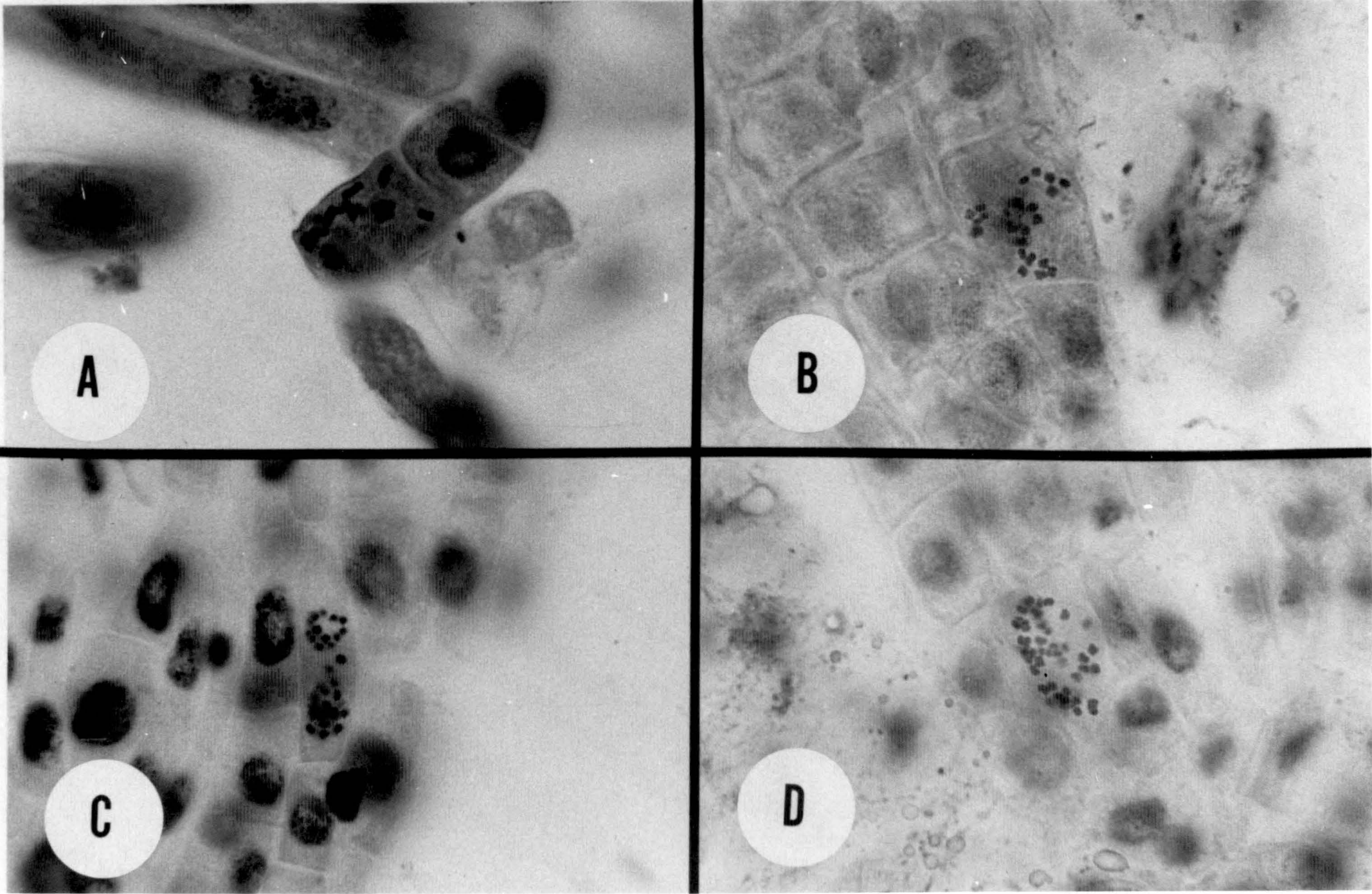

A
B
C
D

Figure 5. Meiotic stages of *in vivo* (A-C) and *de novo* (D-F) haploid plants. A.& D. univalents present at meta-anaphase I. B.& E. tetrads and dyads. C.& F. abnormal pollen grains.

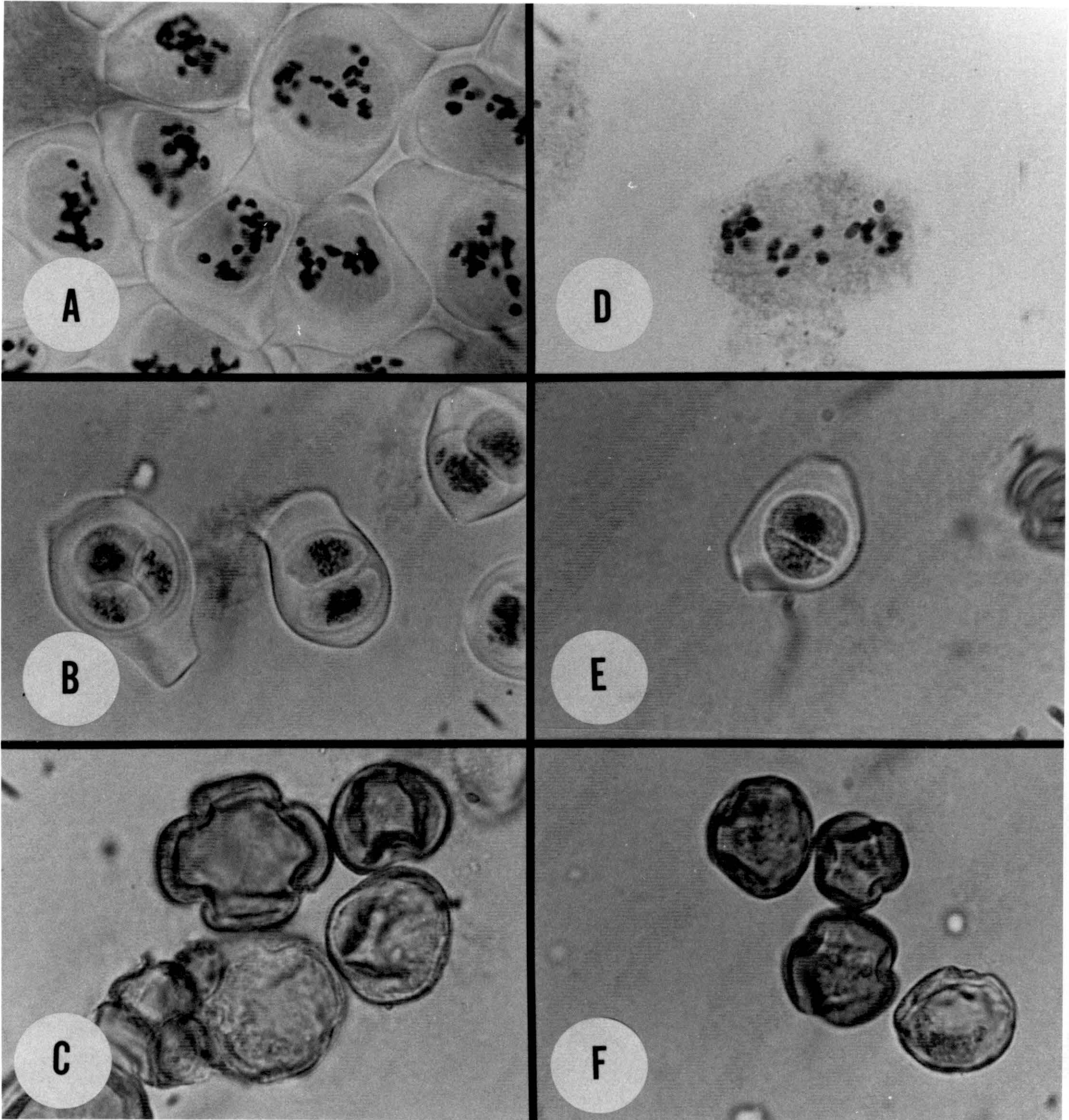
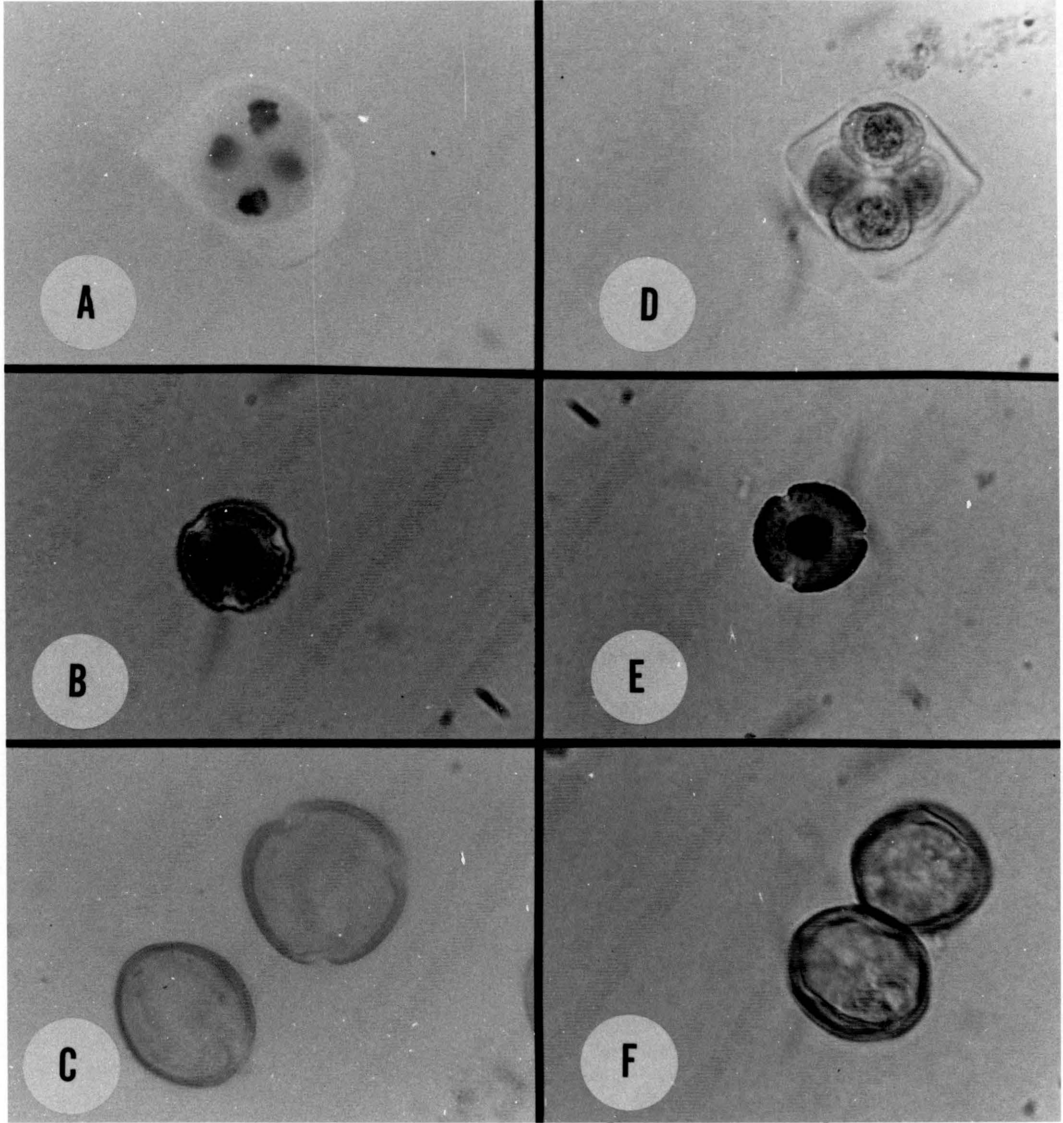

A
D
B
E
C
F

Table 5. Comparison of dyad and tetrad formation in *de novo* and *in vivo* haploid flowers.

	% Dyads	% Tetrads
De novo haploids	44.8 a[z]	55.2 b
In vivo haploids	30.4 b	69.6 a

[z]Mean separation within columns by Student Newman-Keulls' procedure, 5% level.

Figure 6. Meiotic stages of *in vivo* (A-C) and *de novo* (D-F) diploid plants. A.& D. tetrads. B.& E. microspores. C.& F. normal pollen grains.


A
D
B
E
C
F

can be related togenotype and environment, variation for number of floral organs indicates some relaxation of control during meristem development in vitro.

In addition to the morphological differences from in vivo flowers, de novo flowers of haploid TCLs differed from diploid TCLs. The haploid TCLs formed more flowers in a shorter period of time than did the diploid TCLs (Table 2). The greater number of flowers formed on haploid TCLs may be a function of smaller cell size in haploids, and hence the culture of a greater number of potential meristematic centers per TCL than for an equal-sized diploid explant. Earlier development of haploid buds could be a function of shorter cell cycle for haploid cells due to decreased DNA content; cell division cycle time following protoplast isolation have been demonstrated to increase with increasing ploidy level (Berlyn, 1983).

Although the anthers from de novo haploid flowers responded to culture, there was no response from the in vivo haploid anthers on either medium. Embryogenesis and regeneration of "hypohaploid" plants from anthers cultured on the TCL medium has been reported (Tran Thanh Van, 1977); however, we had little success with this medium. More than 50% of the anthers from in vivo diploid plants cultured simultaneously on AC bilayer

medium responded by yielding embryos. This demonstrates that the lack of androgenesis on TCL medium was a function of medium composition rather than innate qualities of the anthers.

The high response with *in vivo* diploid anthers on AC bilayer has been demonstrated by others (Johansson et al., 1982; Veilleux and Booze-Daniels, 1984). Theoretically, the liquid portion allows somatic anther tissue and embryogenic spores to overcome the inhibitory effects of components in the anther wall on spore germination (Sunderland and Roberts, 1977). The presence of activated charcoal in the solid medium also promotes pollen germination by absorbing inhibitory factors (Johansson, 1983; Kohlenbach and Wernicke, 1978). The minimal androgenetic success on TCL medium may have been due to the auxin and cytokinin present; Bajaj (1983) has reported that for *Nicotiana* , growth regulators are not needed for induction, and Nitsch (1969) stated that a mixture of auxin and cytokinin leads to callus formation rather than embryogenesis in anther cultures of *Nicotiana*.

The mixoploid nature of plants which were regenerated from cultured anthers of *de novo* haploid buds indicates microspore rather than somatic origin. "Hypohaploids" of similar origin were described by Tran Thanh Van (1977) with chromosome numbers

per mitotic cell ranging from 3 to 24; our chromosome counts were often greater than 24 in number (Table 4; Figs. 4C & D). Hence, "hypohaploid" does not properly describe our plants. In addition, the variegated leaves and flowers described by Tran Thanh Van and Trinh (1978) were not exhibited in our material.

Mixoploidy is not unexpected in plants regenerated from haploid cell and tissue cultures because of their susceptibility to changes in ploidy level during cell proliferation and growth *in vitro* (Hu et al., 1982). Hypotheses regarding production of mixoploids have included disturbed meiosis, atypical mitosis, endomitosis, the presence of lagging chromosomes, irregular spindles, the formation of restitution nuclei at the first meiotic division, or a cytomixic phenomena such as the exchange and fusion of nuclei of neighboring cells. Indirect embryogenesis, ie. from anther-derived callus, has resulted in mixoploid regenerants of wheat (DeBuyser and Henry, 1980). However, direct embryogenesis from microspores is typical of *Nicotiana* . In our case, the 2 embryos which gave rise to the mixoploids, had been derived from anther culture of *de novo* haploid anthers and were apparently direct embryos; however, due to the callus formation over 12 weeks in culture before multiple shoot regeneration, the resulting plantlets may be

classified as indirect embryos. This may explain the increased chromosome numbers of the cells compared with earlier described hypohaploids of similar origin (Meins, 1983; Torrey, 1967).

The reasons for differences in response of _in vivo_ and _de novo_ haploid anthers remains puzzling. Meiotic analysis revealed a similar ontogeny of the 2 anther types. At metaphase I and anaphase I univalent chromosomes were common (Figs. 5A & D). The second meiotic division was followed by tetrad and dyad formation and various kinds of pollen abnormalities, similar in both types of haploid anthers. These stages are quite different from diploid microsporogenesis (Figs. 6 A-F) in which association at meta-anaphase I, tetrad formation after meiosis II (Figs. 6A & D), and finally normal pollen regularly occur (Figs. 6C & F).

Tran Thanh Van and Trinh (1978) compared the univalents, bivalents, and pseudomultivalents at meta-anaphase I of meiosis in both _in vitro_ and _in vivo_ haploid anthers. They found a significantly higher frequency of bivalents and pseudomultivalents in _de novo_ versus _in vivo_ meiosis. The higher response of _in vitro_ anthers in culture was attributed to this more regular meiosis _in vitro_. In our observations, associations at

meta-anaphase were impossible to determine unequivocally. In studies with haploids of *Nicotiana otophora* and *Nicotiana tabacum*, Collins and Sadasivaiah (1972) observed that associations are usually partial foldbacks with very little pairing between non-homologous chromosomes. The number of bivalents in haploids has been used to predict the mode of origin of various species (Kimber and Riley, 1963) but various *Nicotiana* haploids studied have shown a great deal of variation in pairing at metaphase I (Magoon and Khanna, 1963). It is clear that there is no general agreement on the amount of association that can be attributed to pairing. This line of investigation was abandoned in the present study.

The higher dyad frequency for *de novo* haploid anthers may be related to their response to anther culture. The formation of dyads may result from various types of nuclear restitution such as the suppression of meiosis II, abnormal orientation of the spindle axes at meiosis II, or simply from imperfect chromatid separation at anaphase II (Collins et al., 1972). Functional unreduced microspores could result from any of the above. Embryogenesis of such microspores would be expected to yield diploid rather than mixoploid anther-derived plants, however. Also, *in vivo* anthers would have been expected to exhibit some androgenesis, even though their dyad frequency was less. At

present we cannot offer a satisfactory explanation for the increased androgenic response of *in vitro* over *in vivo* haploid anthers.

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