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ANTHER CULTURES OF FOUR DIPLOID *NICOTIANA* SPECIES AND CHROMOSOME NUMBERS OF REGENERATED PLANTS[1]

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Abstract

Response of immature anthers of *Nicotiana alata, N. otophora, N. plumbaginifolia,* and *N. sylvestris* to *in vitro* culture was investigated. The media used all contained the same mineral salts but differed in the presence of organic substances and other additives. The effect of cold pretreatment on anther response was also studied. Anthers of *N. alata* were totally nonresponsive, whereas those of the other species produced embryos or callus through proliferation of the microspores. Beneficial effects of cold pretreatment were observed in anther cultures of *N. plumbaginifolia* and *N. sylvestris.* Auxin and cytokinin were essential for the induction of microspore development in *N. plumbaginifolia.* In addition to haploid, higher ploidy levels up to 4*n* also occurred in microspore-derived plants of the three *Nicotiana* species. The variation in ploidy level in *N. otophora* and *N. plumbaginifolia* was geometric (*n*, 2*n*, 4*n*), whereas that in *N. sylvestris* was characterized by the presence of triploids at a frequency intermediate that of diploids and tetraploids. No aneuploid plants were observed.

Key words: Anther culture; haploid; polyploid; *Nicotiana* spp.; androgenesis.

Introduction

Nicotiana alata, N. otophora, N. plumbaginifolia, and *N. sylvestris* are diploid species with 24 or fewer chromosomes. Production of haploid plants through anther culture and regeneration of plants from mesophyll protoplasts of these species have been reported. For these reasons they have been considered to be suitable candidates for model species in somatic cell genetics research (Bourgin *et al.*, 1979). Recently, several biochemical mutants were isolated from protoplast cultures of haploid *N. plumbaginifolia* (Maliga, 1982) and considerable efforts were made to improve the efficiency of protoplast culture of *N. sylvestris* (Durand, 1979; Negrutiu

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and Mousseau, 1980; Negrutiu and Muller, 1981; Muller *et al.*, 1983). However, except for *N. sylvestris*, the information on haploid production in these species is rather scanty. In this paper we report results in anther cultures of the four species and chromosome numbers of the plants regenerated from microspores of *N. otophora*, *N. plumbaginifolia*, and *N. sylvestris*.

Materials and Methods

Seeds of *N. alata* Link and Otto ($2n=18$), *N. otophora* Grisebach ($2n=24$), *N. plumbaginifolia* Viviani ($2n=20$), and *N. sylvestris* Spegazzini and Comes ($2n=24$) were kindly provided by Dr. V. A. Sisson of the Tobacco Research Laboratory, U. S. Department of Agriculture, Oxford, North Carolina. Seedlings were planted in 22-cm clay pots in March and April and subsequently maintained in a greenhouse of National Taiwan University in Taipei with the day and night temperatures at approximately 25°C and 20°C, respectively.

Three different media, designated "liquid", "AC", and "hormone", were used for anther culture. They all contained the major salts (half strength), iron (full strength), and minor salts (full strength) of Murashige and Skoog (1962) but differed in organic substances and other additives (Table 1). The liquid medium was dispensed in 3 ml aliquots to 60×15 mm glass Petri dishes, and the agar (AC and hormone) media in 10 ml aliquots to 120×22 mm culture tubes.

Flower buds with the corolla just extended beyond the tips of the calyx, in which the microspores were at the late unicellular to early bicellular stages, were

Table 1. *Organic constituents and other additives of the media used for anther culture of* ***Nicotiana*** *species*

Constituents	Medium		
	Liquid	AC	Hormone
		(mg/l)	
myo-Inositol	5,000	100	100
L-serine	100	—	—
L-glutamine	800	—	—
Thiamine • HCl	—	0.4	0.4
NAA	—	—	0.1
Kinetin	—	—	0.2
Sucrose	20,000	20,000	20,000
Agar	—	8,000	8,000
Activated charcoal	—	3,000	—
pH	5.7	5.7	5.7

excised. Some buds were cultured immediately after excision, others were chilled at 9°C for 7 to 9 days (Sunderland and Roberts, 1979) before anther culture was employed. Flower buds with the calyx removed were surface-sterilized in 70% ethanol for 1 min, followed by 3 min in 5% calcium hypochlorite, and then rinsed thoroughly in sterile distilled water. Under aseptic conditions, the corolla were removed and the anthers were dissected. Twenty-five anthers from five flower buds of *N. plumbaginifolia* and 15 anthers from three flower buds of the other species were inoculated into each dish containing liquid medium, and five anthers from one flower bud of each species were inoculated into each tube containing AC or hormone medium. Anther cultures were incubated at 25°C with 16 h illumination. Callus and embryos were transferred to Murashige and Skoog's medium without growth substances for differentiation and further growth.

Root tips were collected from anther-derived plants grown in culture medium. They were treated with 0.002 M 8-hydroxyquinoline at 20°C for 3 h, fixed in ethanol-glacial acetic acid (3:1) overnight, stained in Feulgen reagent for 1 h, and then treated with 5% pectinase for 1 h. In the preparation of slides for chromosome counts, root tips were squashed in a drop of 45% acetic acid.

Results

A total of 755 chilled and unchilled anthers of *N. alata* was cultured on different media, but none of them produced embryos or callus.

Two accessions of *N. otophora*, 38B and 38C, were used for anther culture. Of these, 38C was studied more extensively. Results obtained from this accession showed that embryos could be produced from anthers cultured on all media, with the frequency of responding anthers ranging from 0.7% on the liquid medium to 10.5% on the AC medium. Pretreatment of the flower buds with low temperature did not improve the response. The number of embryos per responding anther was generally low, approximately 1 to 2 in anthers cultured on the liquid medium and less than 10 in anthers cultured on the hormone medium. Most embryos grew into plants upon transferred to medium devoid of growth substances. Chromosome numbers of the plants are shown in Table 2 and Figs. 1 and 2. It can be seen that 38B tended to give rise to more diploids and tetraploids than did 38C, which produced predominantly haploids. One plant from 38C was a mixoploid with root tips containing both haploid and diploid cells.

In the cultures of *N. plumbaginifolia* only the hormone medium was found to be effective. The frequency of responding anthers for the two accessions, 43B and 43C, tested was 22.5% and 18.4%, respectively, following cold pretreatment. Direct embryogenesis was the usual pathway to plant formation, but in the presence of NAA and kinetin in culture medium dedifferentiation occurred and many embryos

Table 2. *Ploidy levels of plants derived from anther culture of three* ***Nicotiana*** *species*

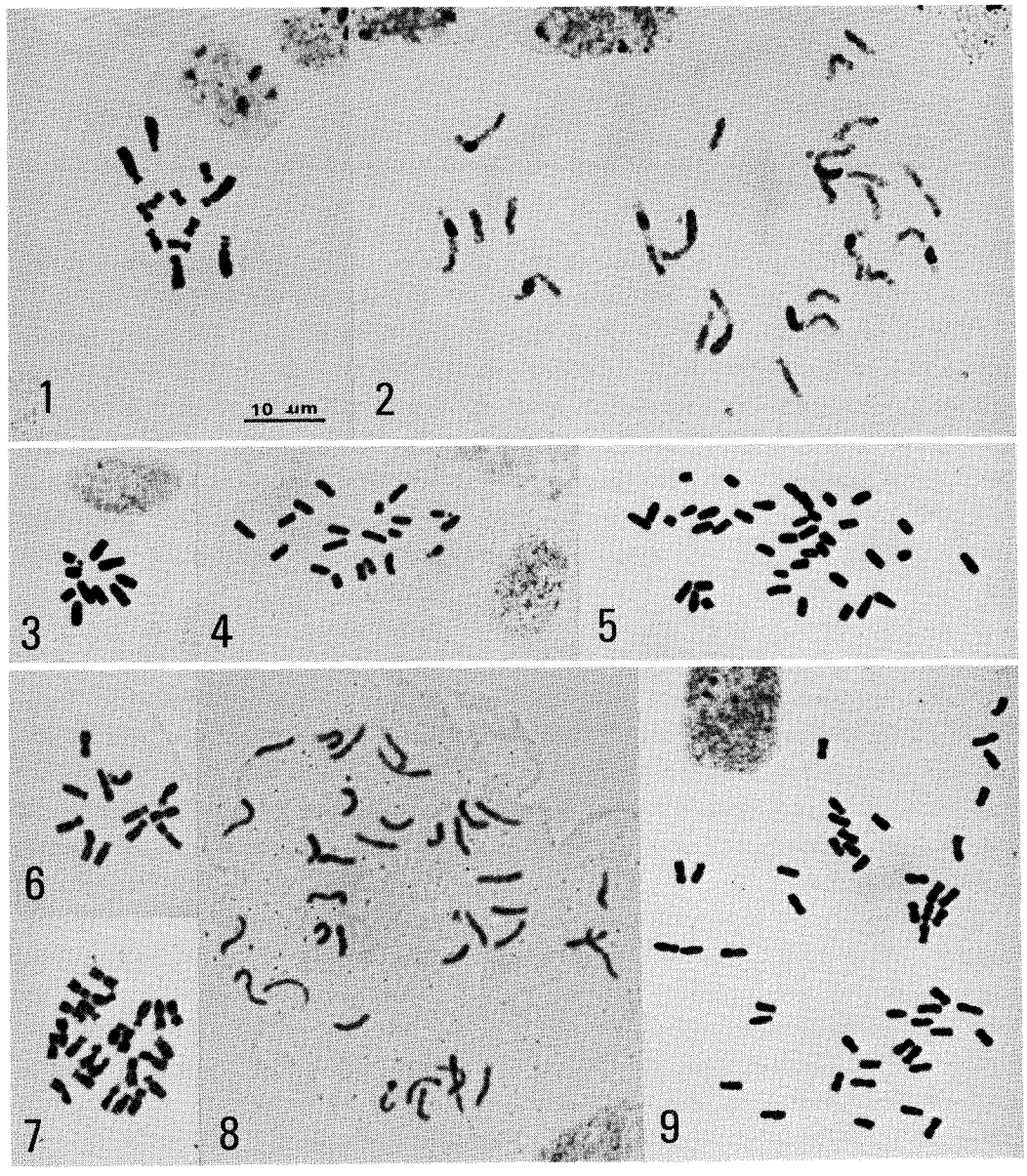
Species	Acc. no.	No. plants	Ploidy level				
			n	2*n*	3*n*	4*n*	*n*/2*n*
N. otophora	38B	19	9	8	0	2	0
N. otophora	38C	18	13	4	0	0	1
N. plumbaginifolia	43B	25	5	13	0	7	0
N. plumbaginifolia	43C	11	10	1	0	0	0
N. sylvestris	56A	106	79	21	3	2	1

developed into callus tissues instead of plants. Callus gave rise to shoots and roots on medium devoid of growth substances. As in *N. otophora*, plants from the two accessions differed in ploidy level (Table 2). Plants derived from 43C were predominantly haploid, whereas those from 43B showed *n*, 2*n*, and 4*n* chromosomes (Figs. 3–5). No aneuploid and mixoploid plants were observed, however.

In *N. sylvestris*, the presence of NAA and kinetin in culture medium induced callus formation from the filament of anther, and once this occurred development of the microspores seemed impaired. Of the media without growth substances, liquid was found to be more effective than agar medium supplemented with activated charcoal. While on liquid medium 13.3% anthers of unchilled buds and 32.0% anthers of chilled buds produced embryos, the frequencies of embryogenic anthers on AC medium were only 2.6% and 15.9% for unchilled and chilled buds, respectively. The number of embryos per responding anther was also increased following cold pretreatment, for in some anthers cultured on the liquid medium more than one hundred well-developed embryos and plantlets could be observed. One hundred and six plants were randomly selected for chromosome counts and the results are summarized in Table 2 and Figs. 6 to 9. Anther-derived plants of *N. sylvestris* differed from those of *N. otophora* and *N. plumbaginifolia* in the presence of a small fraction (2.8%) of triploids.

Discussion

In this study haploid plants of *N. otophora*, *N. plumbaginifolia*, and *N. sylvestris* were successfully raised by culture of chilled and unchilled anthers on various media. The data indicate that the culture method and medium suited to one species may not be applied to the other species. For example, cold pretreatment was beneficial to anther response in *N. sylvestris* and *N. plumbaginifolia* but showed no positive effect on embryo and plant yields in anther culture of *N. otophora*. NAA and kinetin were essential for induction of androgenesis in *N. plumbaginifolia* but



Figs. 1-9. Chromosome numbers in root-tip cells of anther-derived plants of three *Nicotiana* species. Fig. 1. ***N. otophora***, metaphase showing 12 chromosomes. Fig. 2. ***N. otophora***, late prophase showing 24 chromosomes. Fig. 3. ***N. plumbaginifolia***, metaphase showing 10 chromosomes. Fig. 4. ***N. plumbaginifolia***, metaphase showing 20 chromosomes. Fig. 5. ***N. plumbaginifolia***, metaphase showing 40 chromosomes. Fig. 6. ***N. sylvestris***, metaphase showing 12 chromosomes. Fig. 7. ***N. sylvestris***, metaphase showing 24 chromosomes. Fig. 8. ***N. sylvestris***, late prophase showing 36 chromosomes. Fig. 9. ***N. sylvestris***, metaphase showing 48 chromosomes.

Table 3. *Optimum conditions for anther culture of three* ***Nicotiana*** *species*

Species	Culture medium	Cold pretreatment	% responding anthers	Pathway to androgenesis
N. otophora	AC	−	10.5	Embryo
N. plumbaginifolia	Hormone	+	20.5*	Embryo, Callus
N. sylvestris	Liquid	+	32.1	Embryo

* Data from two accessions.

inhibitory to microspore development in *N. sylvestris*. The optimum culture conditions for these species and responses of the anthers are summarized in Table 3.

The results of this study confirm that androgenesis in most *Nicotiana* species follows the pathway of embryo formation (Nitsch, 1969). The shift of development from embryo to callus in anther culture of *N. plumbaginifolia* may be attributed to presence of 0.1 mg/l NAA in the culture medium. By culturing anthers of this species on a medium containing approximately the same concentration (10^{-6} M) of kinetin as we used but a weaker auxin (IAA, 10^{-6} M), Tran Thanh Van and Trinh (1980) showed that plantlets arise directly from microspores without the intervention of a callus phase.

Euploidy appears to be the only type of chromosome variation occurring in anther-derived plants of the three *Nicotiana* species. This conclusion is in accordance with the results previously reported for *N. sylvestris* (Tomes and Collins, 1976; McComb and McComb, 1977) and *N. plumbaginifolia* (Tran Thanh Van and Trinh, 1980) but at variance with the finding in *N. otophora*. In the latter species Collins *et al.* (1972) observed an unusual high frequency of aneuploid cells in anther-derived haploid plants. This controversy cannot be satisfactorily explained until more work is done.

The presence of a geometric series of ploidies (n, $2n$, $4n$) in anther-derived plants of *N. otophora* and *N. plumbaginifolia* indicates that endomitosis and/or endoreduplication are probably the causes for chromosome changes observed in these species. The remarkable difference in ploidy level between the two accessions of both species suggests that the phenomena of endomitosis and endoreduplication may be under genetic control. Anther-derived plants of *N. sylvestris* are characterized by the occurrence of triploids at a frequency intermediate between that of diploids and tetraploids. As has been pointed out by McComb and McComb (1977), this would imply that nuclear fusion at a very early stage of microspore development is mainly responsible for the ploidy level changes.

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四種二元體於草的花藥培養及再生植株染色體的數目

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Nicotiana alata, *N. otophora*, *N. plumbaginifolia* 及 *N. sylvestris* 是二元體的野生菸草。爲了開發這四種植物做爲研究高等植物體細胞遺傳材料的可行性，我們曾嘗試以花藥培養的技術誘導單元體植株。在適當的培養條件下，*N. otophora*, *N. plumbaginifolia* 及 *N. sylvestris* 花藥內的花粉都可經由胚或癒合組織的途徑發育成植株。檢查花粉植株的染色體數目，發現除單元體外，尙有不同比率的二元體、多元體及混雜體。