Production of Disomic Haploids by Anther Culture of a Series of Trisomic Plants in *Nicotiana sylvestris*

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In order to investigate the genic imbalance caused by addition of an extra chromosome to normal diploid and haploid complements, an experiment was carried out on the production of trisomic ($2n+1$) and disomic haploid ($n+1$) plants in *Nicotiana sylvestris* ($n=x=12$). F_1 plants between triploid and diploid were backcrossed with the diploid plants. By the examination of chromosome number, 61 trisomic plants were selected from the B_1 progenies. These trisomic plants were grouped into 12 types by the leaf shape and flower morphology. The anther culture was performed using the 12 types of trisomic plants. The frequencies of androgenetic plantlet production varied greatly among the types of trisomic plants. This result indicates that the variety of the extra chromosome in the trisomic plants effects the frequency of plantlet production. In this anther culture, disomic haploids were obtained from 7 types of trisomic plants. No disomic haploid was obtained in the other 5 trisomic types. Besides the production of euploids and disomic haploids, several kinds of polyploids and mixoploids were also obtained. The frequencies of androgenetic plants derived from the pollen grains with 12 chromosomes were apparently higher than those of plants derived from the pollen grains with 13 chromosomes, except for 2 trisomic types, B 220 and Q 480, in which the frequencies of plants with and without the extra chromosomes were almost the same. These 2 trisomic types tended to produce at a high frequency the androgenetic plants with no extra chromosome in early stages of buds, and those with the extra chromosomes in late stages of buds. This was assumed to be due to unsynchronized development of pollen grains with and without the extra chromosome. Among the disomic haploids obtained, 5 types of plants developed flower organ. The flower of disomic haploid derived from B 220 trisomic plant was conspicuously small and malformed, while the corresponding trisomic plant produced normal and remarkably larger flowers than the normal diploid. The mechanism of reversed effects caused by the addition of the same extra chromosome on diploid and haploid level, however, was not clarified in this study.

KEY WORDS : *Nicotiana sylvestris*, anther culture, genic imbalance, trisomic plant, disomic haploid.

Introduction

Recently an anther culture technique has been developed for the production of various aneuhaploids in plant species. MATTINGLY and COLLINS (1974) succeeded in the production of 6 types of nullihaploids ($n-1$) by the anther culture of a monosomic series in *Nicotiana tabacum*. One of the authors already reported the induction of 12 types of disomic haploids ($n+1$) by the anther culture of various aneuploids in *N. tabacum* and investigated flower morphologies affected by an extra chromosome (NIIZEKI 1977, NIIZEKI *et al.* 1977). In this investigation, however, a strict analysis of genic imbalance induced by the extra chromosome was not performed, because *N. tabacum* is considered to have been evolved by allopolyploidy (CLAUSEN and GOODSPEED 1924). Duplicated genes may be maintained for some characters of this species and mask some portions of genic imbalance. Therefore, the diploid species which has an urgenome should be used in

order to avoid the masking effects by the genic duplication.

This paper reports the production of a series of trisomic plants and that of disomic haploid plants by the anther culture of the trisomic plants in a diploid species, *N. sylvestris* ($n=x=12$). Although a trisomic series of *N. sylvestris* is already reported by GOODSPEED and AVERY (1939), we produced our own trisomic series in the same species; the 12 trisomic types being identified by leaf shape and flower morphology.

Materials and Methods

Production of trisomic plants

In a preliminary investigation, 3 triploid plants, besides haploid and diploid plants of *N. sylvestris* were obtained by the anther culture of diploid plants (NIIZEKI *et al.* 1982). By crossing the triploid with the diploid plants, viable F_1 seeds were obtained. Chromosome numbers of 78 F_1 plants were determined, and the most of them were found to be aneuploid showing variable chromosome numbers from $2n=24$ to 32. The plants with 25 to 28 chromosomes were backcrossed with the diploid plants. By examining the chromosome number, 61 trisomic plants were selected among 266 B_1 plants. Besides the trisomic plants, 157 normal diploids and several aneuploids such as those having 23 or 26 chromosomes were obtained. One aneuploid plant had 25 chromosomes and a fragment chromosome.

The trisomic plants obtained were grouped into 12 types by leaf shape and flower morphology.

Anther culture of trisomic plants

Anther culture was attempted using the plants of 12 trisomic types. Most anthers used for the culture contained immature pollen grains from uninucleate to early binucleate stages. The medium used was the basic medium of NITSCH and HITSCH (1969) supplemented with 0.1mg/l indole-3-acetic acid (IAA), 0.1mg/l kinetin and 3g/l activated carbon. All anther cultures were kept at $26\pm0.5^\circ\text{C}$ under fluorescent light.

Androgenetic plantlets produced by the anther culture were transplanted onto the medium without growth regulators and activated carbon. After 1 to 2 weeks of the culture, most plantlets proliferated many roots, some of which were sampled for the chromosome count. The sampled root tips were pretreated in 0.002 M 8-hydroxyquinoline for 4 hours, and then fixed with alcohol-acetic acid solution (3:1). They were macerated in 5% pectinase solution for 2 hours and then stained with alcoholic hydrochloric acid-carmin (SNOW 1963). The squashing method of root tips was used for cytogenetical examination.

Results and Discussion

Classification of trisomic plants

One of the authors reported in *N. tabacum* that the vegetative characters such as plant height, leaf number, leaf size and leaf shape were highly variable in different environments, comparing to which the flower morphology was considerably stable (NIIZEKI 1977). Therefore, in this study the classification of trisomic plants was first performed tentatively by observing leaf shape. Then in the flowering season, a final classification

Table 1. Trisomic plants grouped into 12 types by comparing sizes of various floral parts

Plant type	Floral parts*								Number of plants
	1 mm	2 mm	3 mm	4 mm	5 mm	6 mm	7 mm	8 mm	
Diploid	92.2	15.2	6.0	8.0	19.0	79.8	78.2	76.6	
Trisomic									
A210	91.2	14.8	5.2	6.4	18.0	72.2	72.2	68.2	5
B211	85.8	15.6	5.2	6.4	16.6	75.8	74.6	71.8	2
B220	118.6	16.6	6.8	9.0	21.2	99.4	105.6	98.6	3
E242	110.4	16.0	5.8	8.2	19.0	91.8	88.4	83.2	2
H272	84.2	13.8	5.0	6.6	16.8	70.8	74.6	69.0	5
K323	79.2	16.0	5.6	7.0	15.2	71.2	67.2	65.4	7
M343	96.2	17.0	5.4	7.0	19.2	80.4	80.8	78.0	6
Q480	97.0	15.2	6.0	8.2	19.0	81.6	81.2	79.4	15
R412	92.4	15.8	6.0	8.6	19.2	75.6	78.0	75.8	10
R415	97.0	16.0	6.0	7.8	19.6	87.8	90.0	80.0	2
S132	74.2	11.8	6.0	7.2	18.8	63.8	62.0	58.6	3
T164	72.8	15.0	5.0	7.4	15.2	66.4	64.6	62.0	1
									61 (Total)
D**	3.9	1.4	0.9	1.0	2.7	7.1	4.3	6.0	

* Floral parts, 1 to 8 are illustrated in Fig.1. Their values indicate the means of 5 flowers from a single plant in each trisomic type.

** D value of Tukey test at 5% level.

Fig.1. Various part of a flower measured. 1 to 8 correspond to those in Table 1.

Table 2. Production of androgenetic plantlets by anther culture of diploid and trisomic plants

Plant type	No. of anthers cultured	No. of anthers forming plantlets	% of anthers forming plantlets
Diploid	227	25	11.0
Trisomic			
A210	222	15	6.8
B211	219	35	16.0
B220	216	34	15.7
E242	194	8	4.1
H272	198	9	4.5
K323	213	4	1.9
M343	234	5	2.1
Q480	233	17	7.3
R412	242	25	10.3
R415	233	11	4.7
S132	232	32	13.8
T164	233	26	11.2

was carried out, based on the sizes of 8 floral parts in each of five flowers of all 61 trisomic plants (Fig. 1). Thus, the classification of a complete series of trisomics was accomplished (Table 1). The result showed that all parts of the flower of the type B220 trisomic were significantly larger than those of the normal diploid. Furthermore, plant height and leaf size of this type were apparently larger than those of the normal diploid. The various parts of the flower of other trisomic types were larger or smaller than those of the normal diploid.

Anther culture of trisomic plants

Frequencies of the androgenetic plantlet production by the anther culture using plants of 12 trisomic types are shown in Table 2. The number of cultured anthers was around 200 in every trisomic type. The frequencies of plantlet production varied greatly among the 12 trisomic types. The highest and the lowest frequencies were 16.0% in B211 and 1.9% in K323, respectively. This result indicates that the kind of the extra chromosomes in the trisomics apparently affects the frequency of plantlet production by the anther culture. In normal diploids, the frequency of plantlet production was 11.0%. Four

Table 3. Chromosome number of androgenetic progenies induced by anther culture of diploid and trisomic plants

Plant type	Number of androgenetic progenies											Total
	Chromosome number									Mixoploid		
	12	13	24	26	36	39	48	65	72	12+24 12+24 +	13+26	
Diploid	43 (89.6)		3 (6.3)		2 (4.2)							48
Trisomic												
A 210	22 (53.7)		14 (34.1)		5 (12.2)							41
B 211	64 (18.0)		9 (11.4)		5 (6.3)		1 (1.3)					79
B 220	41 (36.9)	34 (30.6)	9 (8.1)	16 (14.4)	3 (2.7)	7 (6.3)				1 (0.9)		111
E 242	32 (74.4)		8 (18.6)	1 (2.3)	2 (4.7)							43
H 272	4 (33.3)	4 (33.3)			3 (25.0)					1 (8.3)		12
K 323	62 (86.1)	3 (4.2)	5 (6.9)		1 (1.4)				1 (1.4)			72
M 343	16 (88.9)		2 (11.1)									18
Q 480	22 (36.1)	21 (34.4)	4 (6.6)	4 (6.6)	7 (11.5)	1 (1.6)		1 (1.6)			1 (1.6)	61
R 412	54 (72.0)	5 (6.7)	9 (12.0)		5 (6.7)		1 (1.3)				1 (1.3)	75
R 415	18 (72.0)		4 (16.0)	1 (4.0)	1 (4.0)					1 (4.0)		25
S 132	44 (72.1)	7 (11.5)	4 (6.6)	1 (1.6)	2 (3.3)		1 (1.6)			1 (1.6)	1 (1.6)	61
T 164	66 (75.0)	4 (4.5)	13 (14.8)		2 (2.3)		1 (1.1)			2 (2.3)		88
Total*	445 (64.9)	78 (11.4)	81 (11.8)	23 (3.4)	36 (5.2)	8 (1.1)	4 (0.6)	1 (0.1)	1 (0.1)	6 (0.9)	3 (0.4)	686

() Percentage.

* Total number of androgenetic progenies originated only from trisomic plants.

trisomic types, B211, B220, S132 and T164, showed almost the same or slightly higher frequencies of plantlet production than normal diploids. The other trisomic types showed lower frequencies than the diploid.

Chromosome number of androgenetic plants produced by anther culture

The chromosome number was determined in 686 plants produced by the anther culture of trisomic and diploid plants (Table 3). Disomic haploids were obtained from the plants of 7 trisomic types, B220, H272, K323, Q480, R412, S132 and T164. No disomic haploid was obtained in the other 5 trisomic types. Besides the production of euploids and disomic haploids, several kinds of eu- and aneupolyploids were produced. The polyploid production by the anther culture of *Datura innoxia* is mainly explained by the nucleus fusion or endoreduplication in the early stages of embryoid formation from pollen grains (SUNDERLAND 1974, SUNDERLAND *et al.* 1974). Mixoploids such as those with 12 and 24 or 13 and 26 chromosomes were obtained in this study. They might be originated from the chromosomal duplication occurred in some parts of embryoid tissue in considerably late developmental stages. The number of plants derived from pollen grains with 12 and 13 chromosomes in each trisomic type is shown in Table 4. Androgenetic plant production from pollen grains with 12 and 13 chromosomes is expected to be of the same frequency, if no univalent elimination takes place. The frequency of plants derived from the pollen grains with 12 chromosomes, however, was higher than those derived from the pollen grains with 13 chromosomes, except for B220 and Q480 in which the frequencies of plants derived from the pollen grains with 12 and 13 chromosomes were almost the same. The reasons why no or very few plants with the extra chromosomes were produced from the

Table 4. Transmission of extra chromosomes to androgenetic progenies by anther culture of trisomic plants

Type of trisomics	No. of euploids* (no extra chromosome)	No. of aneuploids* (with extra chromosomes)	% of aneuploids
A210	41	0	0
B211	79	0	0
B220	54	75	51.4
E242	42	1	2.3
H272	8	4	33.3
K323	69	3	4.2
M343	18	0	0
Q480	33	28	45.9
R412	69	6	8.0
R415	24	1	4.0
S132	52	9	14.8
T164	84	4	4.5
Total	573	113	16.4

* Including various polyploids and mixoploids.

most trisomic types are assumed as follows: (1) The pollen grains with the extra chromosome are not formed or are formed only in a low frequency because of elimination of the extra chromosome due to irregular meiosis, and (2) though the pollen grains with and without the extra chromosome are formed at the same frequency, the pollen grains having the extra chromosome do not possess a complete totipotency to form viable plantlets or die in the early stages of proembryoids. Because a previous report (NIIZEKI *et al.* 1982) showed that the megaspores with various extra chromosomes were certainly produced in high frequency, there may be no reason to believe the occurrence of frequent extra chromosome elimination only in microspore formation. Therefore, it is reasonably

assumed that no or rare occurrence of plants with the extra chromosomes is due to incomplete totipotency of pollen grains or abnormal development induced by the genic imbalance resulting in death of proembryoids.

Discrepancy between bud development stages suitable for producing androgenetic plants with and without extra chromosomes

In two types of trisomic plants, B220 and Q480, the extra chromosomes were transferred to the androgenetic progenies at high frequencies. This may be caused by the complete totipotency and normal development of the embryoid.

Fig. 2. Relationship between the length of buds used for anther culture and the frequency of androgenetic plants originated from pollen grains with or without an extra chromosome. (1) Q 480. (2) B 220.

--- and — Plants originated from pollen grains with and without an extra chromosome, respectively.

% Number of androgenetic plants at a certain bud length $\times 100$ / Total number of androgenetic plants with or without the extra chromosomes.

Fig. 3. Some pollen grains after the first pollen mitosis accumulated starch grains while the other did not in the same anther of a B 220 trisomic plant.

complete totipotency and normal development of the embryoid. Fig. 2 indicates the relationship between bud length and frequency of the androgenetic plants with or without the extra chromosomes in the anther culture of these 2 trisomic types. In both types, there was a tendency that high frequency of androgenetic plants without the extra chromosome was observed in early stages of buds, and high frequency of plants with the extra chromosomes in late stages of buds. Suitable stages of pollen grains for the anther culture in *Nicotiana* species is reported to be from the uninucleate to early binucleate stages (ENGVILD 1974, NITSCH 1970, SUNDERLAND and WICKS 1971). If the pollen grains with and without extra chromosome develop synchronously, the discrepancy in the stage suitable for the induction of androgenetic plants may not be observed between the 2 kinds of pollen grains. In the B 220 and Q 480 trisomics, the microscopic observation of immature pollen grains after the first pollen mitosis revealed that in the same anther some pollen grains accumulated starch grains and the others did not (Fig. 3). The starch grains in *Nicotiana* species were observed in a little advanced stage of pollen

grains after the first pollen mitosis (NITSCH 1970, SUNDERLAND 1974). Therefore, the synchronized pollen development presumably did not occur between the 2 kinds of pollen grains, and the development might be delayed in the pollen grains with the extra chromosome. Thus, the discrepancy of the stage suitable for androgenetic plant development between the pollen grains with or without an extra chromosome might be due to their unsynchronized development. Because of rare occurrence of plants with the extra chromosomes, it could not be tested whether a similar discrepancy is present or not in the other trisomic types.

Flower morphology of disomic haploids

The 7 types of disomic haploids were transplanted to pots and grown in a greenhouse. The vegetative growth of the all disomic haploids was very slow as compared with the trisomic plants, and only 5 types of disomic haploids derived from the B 220, K 323, Q 480, S 132 and R 412 trisomics developed flower organs. A detailed analysis of flower morphology as performed in the trisomic plants was not possible, because flowers of some disomic haploids were malformed. By the arbitrary observation, the length of flowers in disomic haploids derived from K 323, Q 480, S 132 and R 412 was almost the same as or slightly shorter than those of normal haploids (Fig. 4). The flower of disomic haploid derived from B 220, however, was conspicuously small and severely malformed, while the flower of B 220 trisomic plant was normal, and remarkably larger than the normal diploid. This means that some extra chromosomes affect the flower size of the trisomic plant positively and that of the disomic haploid negatively. Clarification of the mechanism leading to the reversed effects of genic imbalance by addition of the same extra chromosome at the diploid and haploid levels was not made in the present study.

Fig. 4. Comparison between flowers of normal diploid, trisomic plant, normal haploid and disomic haploid.

- 1 Q 480. 2 B 220.
 A Normal diploid.
 B Trisomic plant.
 C Normal haploid.
 D Disomic haploid.

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Nicotiana sylvestris の三染色体植物シリーズの 薬培養による二染色体半数体の育成

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正常二倍体および半数体に余剰染色体が添加された場合の遺伝子平衡の乱れを比較検討するため、単一ゲノムを持つ *Nicotiana sylvestris* ($n=x=12$) の三染色体植物およびその薬培養による二染色体半数体の育成を試みた。

3倍体と2倍体の交雑 F_1 に2倍体を戻交雑し、その子孫の中から61個体の三染色体植物を選抜した。得られた三染色体植物は葉形および花器形態によって12型の完全シリーズに分類することが出来た。

薬培養による花粉起源植物の出現頻度は三染色体植物の型の間で大きな変異を示した。このことは余剰染色体の種類が花粉起源植物の出現頻度に大きく影響することを示唆する。薬培養によって二染色体半数体が得られたのは7つの型の三染色体植物からであり、残りの5つの型からは得られなかった。また正常半数体や二染色体半数体の他に多くの倍数体や混数体が得られた。一般に12染色体(n)を持つ正常半数性花粉起源の植物の出現頻度は $13(n+1)$ 染色体を持つ花粉起源のものより明らかに高い傾向を示した。しかし2つの型、B220とQ480の三染色体植物からは余剰染色体を持つ植物と持たない植物がほぼ同頻度で出現した。またこの2つの型では花蕾の早い時期のものからは余剰染色体を持たない植物、遅い時期のものからは余剰染色体を持つ植物が高頻度で出現する傾向であった。これは12本と13本の染色体を持つ花粉が葯内で同調的に発育しないためであることを示唆する。

得られた二染色体半数体のうち生殖生長期に達したのは5型のみであり、その花器は正常半数体とほぼ同じ大きさか、あるいはやや小さく、奇形花も出現した。しかしB220の三染色体植物の花形は正常で2倍体より著しく大きいのに対し、このB220由来の二染色体半数体は著しい奇形を示しごく小さいものであった。このことは同一余剰染色体が2倍体および半数体レベルで遺伝子平衡の乱れの方が大きく異なることを意味している。