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Increasing the transmission rate of the extra chromosome in a trisomic *Nicotiana sylvestris* line by modifying the means of pollination

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Summary. Trisomics of primary trisomic line B220 of *Nicotiana sylvestris*, which contain an extra chromosome shown to be a satellite chromosome, can be readily identified by their larger flower and leaf sizes. In seed-propagated species, the low transmission of the extra chromosome has prevented such plants from becoming agriculturally useful cultivars. In line B220, the transfer of the extra chromosome in $2n \times 2n+1$ crosses was very low (13.5%), although n and $n+1$ pollen grains were produced in equal quantities, as was confirmed by anther culture. This was due to the delayed development of $n+1$ pollen grains, which are not at full maturity at the time of anthesis. The transfer of the extra chromosome in $2n \times 2n+1$ crosses was increased by a 1 day delay in pollination and also by pollination of small pollen grains selected through nylon meshes. The delayed pollination increased the frequency of trisomics by 9%, whereas pollen selected by using 30 and 25 μm nylon meshes induced an extremely high transfer of the extra chromosome, namely 51.9% and 70.4%, respectively. The observed frequencies of trisomics and tetrasomics in artificial selfing of $2n+1$ plants with selected small pollen grains were lower than those expected from the data of reciprocal crosses between 2n and $2n+1$ plants. This discrepancy seems to indicate a disadvantage of the $n+1$ pollen in fertilization due to the longer style of the trisomics relative to that of the diploids.

Key words: Extra chromosome – Primary trisomics – *Nicotiana sylvestris* – Pollen sizing – Delayed pollination

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Introduction

Aneuploids have been particularly useful for studying the genetics of many plant species, and in *Datura*, maize, tomato, barley, wheat and tobacco they have been extensively investigated. Among the anueploids, trisomic plants of many species have been the most extensively studied, one reason being that they are readily detectable by their altered morphological, histological and physiological traits. Compared to normal diploid plants, trisomics generally have a reduced viability and vigour, although this is not always true: the primary trisomic line, B220, in *Nicotiana sylvestris* has larger flowers and leaves than the normal diploids (Niizeki et al. 1984). This B220 trisomic line seems to be the same line as the trisomic "Enlarged" described by Goodspeed and Avery (1939). If such trisomic lines were to be found in major crop species they could become very useful. In those species in which vegetative propagation is the normal means of multiplication, some cultivars are known to be aneuploid. Many varieties of hyacinth are aneuploids and trisomic (Darlington and Mather 1944). In species in which seeds are the means of propagation, the low transmission of an extra chromosome in the trisomics has prevented them from becoming agriculturally useful. In trisomics, the ability of pollen grains with an extra chromosome to compete with normal pollen grains in fertilization may be impaired because the former mature later than the latter. In barley trisomics, pollen grains containing the extra chromosome are immature when the anthers shed their pollen while normal pollen grains are fully mature at that time and are able to effect fertilization (Ramage 1965). This observation is supported by studies of Tsuchiya (1960), who found that pollen grains of barley trisomics can be divided into two groups according to size. Presumably, in the small sized group are

pollen grains having the extra chromosomes: they are not full size at anthesis due to immaturity. We report here the possibility of increasing the transfer of an extra chromosome in trisomic plants of *N. sylvestris* by controlling the time of pollination and also by pollinating with small pollen grains selected by using nylon meshes.

Materials and methods

Production of trisomics and comparison with normal diploids

In a preliminary examination, three triploid plants, in addition to haploid and diploid plants of *N. sylvestris* (2n = 24) were obtained by culturing the anthers of diploid plants. By crossing the triploids with pollen from the diploids, viable F_1 seeds were obtained. The chromosome numbers of 78 F_1 plants were determined, and the majority were found to be aneuploid with chromosome numbers ranging from 2n = 24 to 2n = 32. Plants with 25–28 chromosomes were backcrossed to the diploids. By examining the chromosome numbers, 61 trisomics were selected from among 266 B_1 plants. The trisomics obtained were grouped into 12 types by flower morphology. Details on the trisomics have been presented in a previous paper (Niizeki et al. 1984). Among these trisomics, we found one trisomic, B220, which had flowers and leaves much larger than those of the normal diploid. Plants of this trisomic line were compared in detail with the diploids for several flower parts, plant height, leaf number, leaf width and length of the largest leaf. Ten plants each of the B220 and diploids were used.

Anther culture of trisomic B220

Anther culture was attempted using the trisomics. Most of the anthers cultured contained immature pollen grains in the uninucleate to the early binucleate stages. The basic medium of Nitsch and Nitsch (1969) supplemented with 0.1 mg/l indole-3-acetic acid (IAA), 0.1 mg/l kinetin and 3 g/l activated carbon was used. The anther cultures were kept at 26° ± 0.5 °C under fluorescent light.

Androgenetic plantlets produced by anther culture were transplanted onto medium without growth regulators and activated carbon. After 1–2 weeks of culture, most plantlets proliferated many roots; some of these were sampled in order to determine the chromosome number. The root tips were pretreated in 0.002 *M* 8-hydroxyquinoline for 2 h, fixed with alcohol-acetic acid (3:1), macerated in 2% pectinase for 10 min and then stained with alcoholic acid-carmine (Snow 1963). The squashing method was used on the root tips for cytogenetical examination.

Pollination of the diploids using pollen grains from B220 plants at various stages of floral bud and flower development

Pollen grains of B220 plants at various stages of bud and flower development – from bud lengths of 40 mm to flowers 3 days after anthesis – were used to pollinate normal diploids that had been emasculated 1–3 days before anthesis. The reciprocal crosses between B220 and the diploid were also carried out on the day of anthesis. Natural self-pollination of B220 plants was also performed. The chromosome number of the progenies was determined by the same method as given above.

Pollination by small and large pollen grains

Pollen grains were collected from ten flowers of B220 on the day of anthesis and dispersed in approximately 2 ml of a 0%, 5%, 10% and 20% sucrose solution in a centrifuge tube. The solution containing the pollen was then maintained at room temperature or centrifuged at 80 *g* for 5 min. Both precipitated pollen grains and pollen grains in the supernatant were used to pollinate emasculated flowers of normal diploids. At the same time, the diameters of the pollen grains in the sediment and supernatant were measured microscopically. In addition, two sizes of small pollen grains, less than 30 and 25 μm, taken from flowers on the day of anthesis, were selected by nylon meshes and used to pollinate emasculated flowers of normal diploids and B220 plants.

Results and Discussion

Compared to normal diploids, B220 trisomics had significantly larger floral parts and a longer leaf (Table 1 and Fig. 1) indicating that the extra chromosome increased both flower and leaf size. Plant height, however, was almost the same, and leaf number was slightly less than that found in the normal diploid. In the B220 trisomics, the extra chromosome was determined to be a satellite chromosome (Fig. 2). In a previous paper (Niizeki et al. 1984), this extra chromosome was shown to be transferred to androgenetic progenies produced by anther culture at a frequency of about 50%. Thus, the extra chromosome is not eliminated during meiosis and an equal quantity of pollen is produced with and without the extra chromosome. Figure 3 shows the relationship between trisomic B220 floral bud length at the time when the anthers were taken for culture and the frequency of the resulting androgenetic plants with or without the extra chromosome. These buds were between 16 and 39 mm in length and contained pollen grains at the uninucleate to the early binucleate stages. Almost all the androgenetic plants produced from pollen of the early bud stages did not possess the extra chromosome, where-

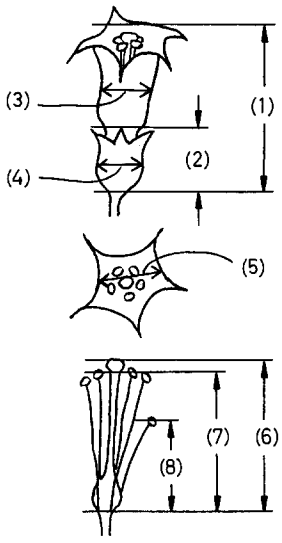

Fig. 1. Various floral parts that were measured. Numbers *1–8* correspond to the morphological characters given in Table 1

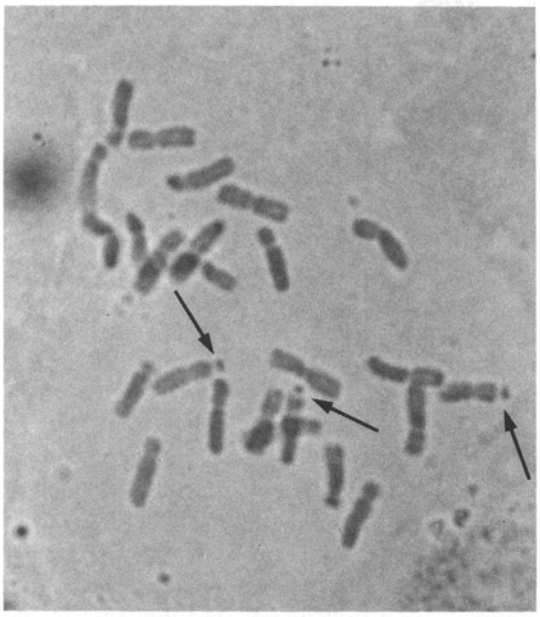

Fig. 2. Chromosomes of the B220 trisomic plant. Three satellite chromosomes are indicated by *arrows*

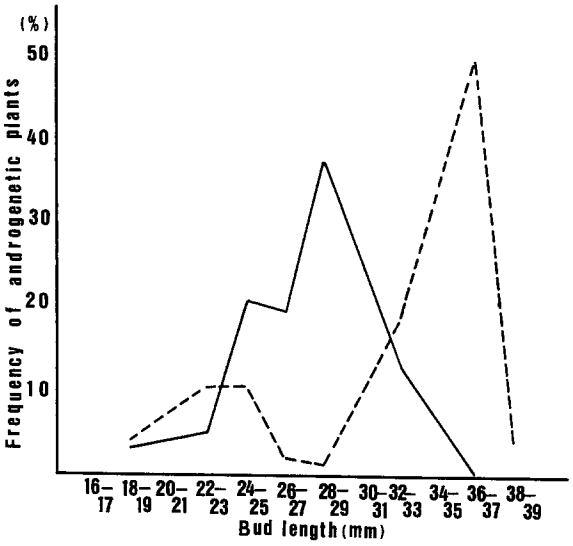

Fig. 3. Relationship between the length of the B220 flower bud used for anther culture and the frequency of androgenetic plants developed from pollen grains with or without an extra chromosome. - - - - and ——: plants originating from pollen grains with and without an extra chromosome, respectively

Table 1. Comparison between a B220 trisomic line (a) and a diploid line (b) for various morphological characters. Ten plants were observed for all characters

Character[a]	Mean ± S.D.	*t* value	*P*
Floral parts (mm)			
1	a 120.80 ± 3.05 b 97.10 ± 1.37	22.57	$P < 0.001$
2	a 18.00 ± 0.94 b 14.60 ± 0.97	8.09	$P < 0.001$
3	a 7.20 ± 0.63 b 6.00 ± 0.00	6.31	$P < 0.001$
4	a 9.40 ± 0.70 b 7.70 ± 0.48	6.53	$P < 0.001$
5	a 21.50 ± 1.27 b 20.20 ± 1.14	2.45	$0.02 < P < 0.05$
6	a 98.90 ± 2.38 b 83.50 ± 0.97	19.01	$P < 0.001$
7	a 110.30 ± 3.27 b 83.40 + 1.07	24.90	$P < 0.001$
8	a 102.10 ± 3.07 b 79.40 ± 0.97	22.47	$P < 0.001$
No. of leaves	a 17.00 ± 0.67 b 17.80 ± 0.79	2.50	$0.02 < P < 0.05$
Leaf length (cm)[b]	a 33.75 ± 2.55 b 30.45 ± 2.01	3.23	$0.01 < P < 0.02$
Leaf width (cm)[b]	a 14.95 ± 1.44 b 14.25 ± 0.79	1.37	$0.2 < P < 0.3$
Plant height (cm)	a 120.00 ± 3.33 b 118.10 ± 5.40	0.95	$0.3 < P < 0.4$

[a] Floral parts nos. 1–8 are illustrated in Fig. 1
[b] The largest leaf of an individual plant was used for these measurements

Table 2. Relationship between flower bud length and developmental stage of pollen grains in B220 trisomics

Bud length (mm)	Developmental stage of pollen grains	Remark
–10	In meiosis	
10–25	Uninucleate	
25–40	Early binucleate	All pollen grains without starch grains
40–70	Ditto	Some pollen grains with starch grains
70–90	Ditto	All pollen grains with starch grains

as a large number of the androgenetic plants produced from the pollen of the late bud stages had the extra chromosome. If pollen grains with and without the extra chromosome develop at the same rate, the frequencies of both types of regenerants should be the same in each size class of buds from which the anthers were dissected. In the B220 trisomics, microscopical observation of immature pollen grains after the first pollen mitosis revealed that in the same anther some pollen grains accumulated starch grains and others did not (Table 2). In *Nicotiana* species, starch grains have been observed in the advanced stages of pollen development after the first pollen mitosis (Nitsch 1970; Sunderland 1974). In the present trisomics, synchronized pollen development presumably did not oc-

cur between the two kinds of pollen grains – the development of pollen grains having the extra chromosome being delayed.

The frequency at which the extra chromosome was transferred was determined by hand pollinating diploid plants with B220 pollen collected from floral buds at various developmental stages, and 0, 1, 2 and 3 days after anthesis (Fig. 4). When the pollen grains from floral buds 40–70 mm in length were used, a small number of viable seeds were produced. These floral buds contained pollen grains in the early binucleate stage (Table 2). Some of the pollen grains germinated at 25 °C on agar medium containing 10% sucrose indicating their potential for fertilization. The pollen grains with the extra chromosome, however, rarely fertilized the ovule of the diploids. Pollen grains obtained from buds less than 40 mm in length were in the uninucleate stage and did not produce any viable seeds. The number of trisomics increased with an increase in the floral but length and reached a maximum frequency of 22.5% 1 day after anthesis (compared to 13.5% obtained on the day of anthesis). Thus, relative to normal pollen, the development of pollen grains having the extra chromosome is delayed, and they appear to reach full maturity 1 day after anthesis. The frequency of trisomics decreased greatly on the second and third days after anthesis.

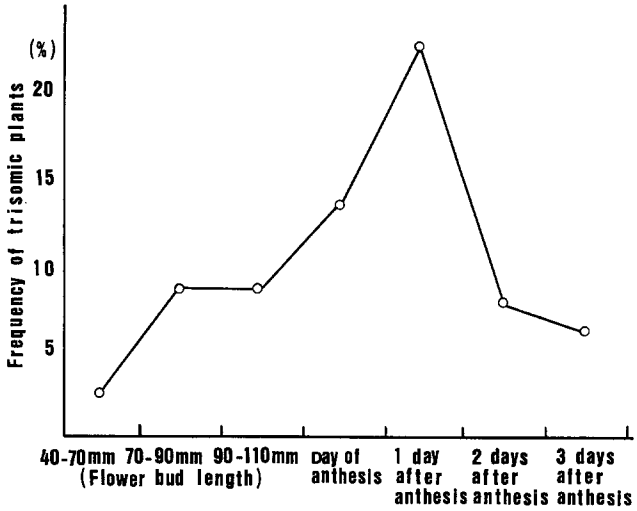

Fig. 4. Frequency of trisomics in $2n \times 2n+1$ crosses using pollen grains from various flower bud stages, and 0, 1, 2 and 3 days after anthesis of B220 trisomic plants. At each stage the chromosome number in 60–70 plants was examined

In order to produce more trisomics, pollen grains obtained from B220 plants on the day of anthesis were centrifuged or naturally sedimented in water or sucrose solutions. The pollen grains in the sediment and supernatant were used separately to pollinate the emasculated flowers of the diploids (Table 3). The mean size of the pollen grains in the sediment was 32.1 μm; that of the supernatant was 29.5 μm (Fig. 5). The latter probably contained many immature pollen grains having an extra chromosome. When pollen grains from the sediment were used to pollinate diploid plants, they sometimes produced viable seeds, 9.8% of the seedlings obtained being trisomic; pollen grains from the supernatant did not produce any viable seeds.

As an alternative way to size the pollen grains, we used 30 and 25 μm nylon meshes. The pollen grains passing through 30 and 25 μm nylon meshes gave 51.9% and 70.4% trisomics, respectively (Table 4). Apparently, the frequency of pollen grains having an extra chromosome is larger among the smaller pollen grains. Selective pollination with the smaller pollen grains resulted in an extremely high frequency of trisomics.

Table 3. Seed setting of normal diploids crossed with pollen grains of B220 fractionated by natural sedimentation (1 *g*) or centrifugation (80 *g*)

Solvent	Gravity (*g*)	Time (min)	Fraction of pollen[a]	No. of crosses	No. capsules with seeds	Capsules with seeds (%)
Distilled water	1	5	a	38	2	5.2
			b	38	0	0
Distilled water	80	5	a	24	1	4.1
			b	24	0	0
5% sucrose	80	5	a	4	0	0
			b	4	0	0
10% sucrose	1	5	a	26	6	23.0
			b	26	0	0
10% sucrose	80	5	a	6	0	0
			b	8	0	0
20% sucrose	80	5	a	12	2	16.6
			b	12	0	0

[a] a: sediment; b: supernatant

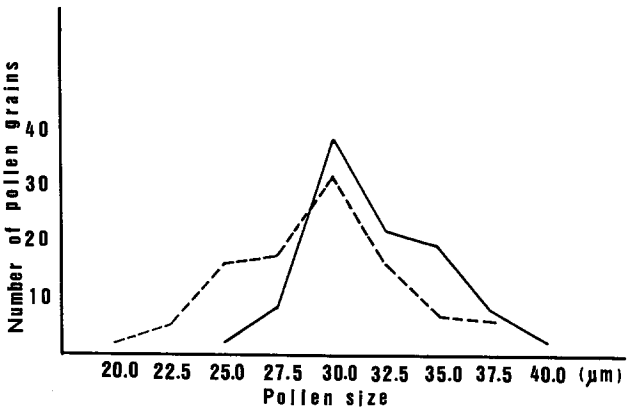

Fig. 5. Frequencies of pollen grains of different sizes in the sediment (——) and in the supernatant (- - - -). Separation by centrifugation (80 *g*) for 5 min in distilled water. Mean size of pollen grains in sediment, 32.1 μm; mean size of pollen grains in supernatant, 29.5 μm. The observed means are significantly different ($t = 5.95$; $P < 0.001$)

Table 4. Frequencies of diploid (2n) and trisomic plants (2n + 1) from the cross 2n (♀) × 2n + 1 (♂)

Pollen grains used	Frequency (%)		No. plants examined cytologically
	2n	2n + 1	
Unselected pollen grains; on the day of anthesis	86.5	13.5	74
Unselected pollen grains; 1 day after anthesis	77.5	22.5	71
Pollen grains passed through 30 μm nylon meshes	48.1	51.9	54
Pollen grains passed through 25 μm nylon meshes	29.6	70.4	54

The cross, B220 × normal diploid, produced 32.1% trisomics on the day of anthesis when performed whereas the reciprocal cross produced only 13.5%. This fact indicates that the extra chromosome is transferred via the ovule at a higher frequency than via the pollen. From the frequencies of trisomics produced in reciprocal crosses between diploid and B220 plants, it is possible to estimate the frequencies of diploids, trisomics and tetrasomics obtained from the B220 plant crossed with B220 pollen grains at different stages. When flowers of B220 plants were crossed with pollen grains from those of B220 plants 1 day after anthesis, the frequency of trisomic offspring was 36.2%, which was close to the estimated frequency of 39.7% (Table 5); natural self-fertilization of these plants resulted in 31.0% trisomics indicating that artificially delayed pollination of the trisomics will produce trisomic offspring at a higher frequency than natural self-fertilization. On the contrary, the frequencies of trisomics and tetrasomics produced using small pollen grains selected by 30 and 25 μm nylon meshes were more or less lower than the expected ones. This result suggests that the immature, small pollen grains are at a disadvantage in fertilization when pollinated to the longer style of the trisomics.

Buchholz and Blakeslee (1930) found that in some trisomic lines of *Datura*, the pollen tube containing the extra chromosome grows slower than that of normal pollen grains. Based on this result, Buchholz et al. (1932) succeeded in obtaining 75% or more trisomic offspring by cutting off a lower portion of a style when normal pollen tubes reached it and replacing it with the upper portion of the same style or grafting this portion onto a style of a different flower. This kind of operation is, however, very laborious, and it is difficult to obtain reproducible results. On the other hand, sizing pollen grains using nylon meshes is very simple, and gives triso-

Table 5. Observed and expected frequencies of three classes of progeny from 2n + 1 plants pollinated with different categories of pollen grains from the same plants

Pollen grains used		No. of plants[a]				χ^2 value
		2n	2n + 1	2n + 2	Total	
Unselected pollen grains; on the day of anthesis	Obs.	39 (67.2)	18 (31.0)	1 (1.7)	58	1.90
	Exp.[b]	34 (58.6)	22 (37.9)	2 (3.4)	58	$0.3 < P < 0.5$
Unselected pollen grains; 1 day after anthesis	Obs.	36 (62.1)	21 (36.2)	1 (1.7)	58	3.22
	Exp.	31 (53.4)	23 (39.7)	4 (6.9)	58	$0.1 < P < 0.2$
Pollen grains passed through 30 μm nylon meshes	Obs.	29 (48.3)	28 (46.7)	3 (5.0)	60	9.08
	Exp.	20 (33.3)	30 (50.0)	10 (16.7)	60	$0.01 < P < 0.02$
Pollen grains passed through 25 μm nylon meshes	Obs.	27 (43.5)	30 (48.4)	5 (8.0)	62	25.53
	Exp.	12 (20.1)	36 (57.3)	14 (22.6)	62	$P < 0.001$

[a] The percentage is given in parentheses

[b] Expected frequency was calculated from the frequencies of 2n and 2n + 1 plants in the crosses, 2n + 1 × 2n on the day of anthesis and 2n × 2n + 1, in which four different categories of pollen grains were used

mic seeds at a frequency as high as approximately 70%. In this case, however, collection of a large amount of small pollen grains is necessary in order to obtain high seed set.

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