PREFERENTIAL RECOVERY OF SOMATIC HYBRIDS FROM PROTOPLAST FUSION OF TWO *NICOTIANA* SPECIES IN THE ABSENCE OF ARTIFICIAL SELECTION

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Abstract: Mesophyll protoplasts of *Nicotiana plumbaginifolia* (PP) and *N.sylvestris* (SS) were mixed at equal density, treated with polyethylene glycol (PEG) and then cultured in medium with no selection pressure against parental cells. Cytological, morphological, and biochemical studies showed that about 78% of plants regenerated from protoplast fusion were somatic hybrids. The ploidy levels of somatic hybrids varied, and the frequencies of plants with PPSS, PPPPSS, and PPPPSSSS genome constitutions were 84.8%, 8.7%, and 6.5%, respectively. Aneuploid numbers and chromosome structural changes were common in somatic hybrids. The successful recovery of somatic hybrids in the absence of artificial selection is attributed to a high viability of the fusion products following PEG treatment and a fast rate of growth and development of hybrid cells during *in vitro* culture.

INTRODUCTION

Somatic hybridization can overcome barriers to sexual crosses and therefore is a useful tool for basic research and crop improvement. Because the frequency of heteroplasmic fusion is usually low following the induction of protoplast fusion, selection of hybrid cells becomes an essential step in somatic hybridization. Among the various methods of selection developed, complementation of biochemical markers selectable at the cellular level appears to be the most effective one. Unfortunately, such markers are not available in most major crops (for a review see Harms, 1985).

Recently, several reports indicate that somatic hybrids, both interspecific and intergeneric, can be obtained without selection pressure (Liao, 1986; Chae and Choi, 1987; Toriyama *et al.*, 1987; Lee and Chen, 1990; Lin and Chen, 1990). These findings are of practical importance, because the major obstacle in somatic hybridization, selection of hybrid cells, could be bypassed in many cases.

The reasons for the successful recovery of somatic hybrids in the absence of deliberate selection are not clear. Several investigators observed that the calli of some interspecific hybrids were more vigorous and grew faster than those of their parents (Smith *et al.*, 1976; Schieder, 1978, 1980; O'Connell and Hanson, 1987). Our previous study showed that successful recovery of somatic hybrids of *Nicotiana sylvestris* and *N. otophora* was due to differential responses of parental protoplasts to polyethylene glycol (PEG) treatment and culture, and to genetic

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complementation in the hybrids (Lee and Chen, 1990).

Efforts were made in this study to further evaluate the notion that vigor of somatic hybrids is the result of genetic complementation, using protoplasts of *N. sylvestris* and *N. plumbaginifolia* as the fusion partners.

MATERIALS AND METHODS

Plant material

Seeds of *Nicotiana plumbaginifolia* Viviani (PP) and *N. sylvestris* Spegazzini & Comes (SS) were provided by the U.S. Department of Agriculture, Beltsville, Maryland. They were sterilized for 1 min in 70% ethanol and followed by 10 min in 1% sodium hypochlorite. After thoroughly washed in sterile distilled water, seeds were germinated on agar plates containing Murashige and Skoog (1962) inorganic salts and 3% sucrose. The plants were maintained and propagated *in vitro* as described by Negrutiu and Mousseau (1980).

Protoplast isolation, fusion, and culture

Protoplasts of the two *Nicotiana* species were isolated separately as described by Huang and Chen (1988). They were washed once with W5 solution (Medgyesy *et al.*, 1980) and resuspended in the same solution at a density of 2×10^5/ml. Protoplast suspensions of the two species were mixed at equal volumes. The protoplast mixtures were treated with or without PEG and cultured as described by Lee and Chen (1990). No selection pressure was applied in either case.

Chromosome determination

Somatic chromosomes were prepared from root-tip cells of *in vitro* cultured plants. Excised roots were treated with 0.002 M 8-hydroxyquinoline at 18 to 20°C for 2.5 h, fixed in ethanol-acetic acid (3:1) for overnight, hydrolyzed with 1 N HC1 at 60°C for 7 min, stained in leuco basic fuchsin for 1 h, and treated with 1% pectinase for 1 h. Root tips were squashed in 45% acetic acid.

Separation of esterase isozymes

Regenerated plants were grown for about four or five weeks. Leaves (1 g) were removed from plants and were ground in a chilled mortar in 1 ml enzyme extraction medium (0.1 M potassium phosphate, pH 7.5; 1 mM Na_2-EDTA; 1% triton X-100; and 0.1% 2-mercaptoethanol). Samples were centrifuged at 12,000 rpm for 15 min at 4°C. One volume of supernatant and 4 volumes of sample buffer (30% glycerol; 12.5% 2-mercaptoethanol; 31.25% Tris-phosphate, pH 6.7; 33 mg/100 ml bromophenol blue) were mixed well, before centrifugation at 12,000 rpm for 5 min at 4°C. Supernatants were collected and used for isozyme analysis on electrophoresis.

Electrode buffer contained 6 g/l Tris and 28.8 g/l glycine. The separation gel and the stacking gel were as described by Kakefuda and Duke (1984). After

electrophoresis, gels were stained for esterase activity according to the procedure described by Vallejos (1983).

RESULTS

Development of protoplasts

In both control and PEG-treated experiments, development of protoplasts was asynchronous. At the time of plating (10 days after culture initiation), some protoplasts had grown into colonies of approximately 20 cells while others remained undivided. Colonies became visible 1 week after plating, and after another week the most advanced colonies had reached the size of 2 mm in diameter.

Calli of 2 mm in diameter were selected and transferred to an auxin-free medium for plant regeneration. Shoots began to emerge about 10 days after transfer, and they continued to emerge until approximately 50 days. One piece of callus usually produced several shoots, but only two to three were cut off for root formation.

Karyotypes of parental species

N. plumbaginifolia has 5 pairs of large, 3 pairs of medium, and 2 pairs of small chromosomes; all of them were telocentric or acrocentric and one pair possesses a satellite (Fig. 1A). On the other hand, the chromosomes of *N. sylvestris* (12 pairs) are rather uniform in size and are either metacentric or submetacentric; two pairs have a satellite (Fig. 1B). Thus, karyotypes of these two species differ markedly, and the differences could be used as a basis to determine genome constitutions of plants regenerated from protoplast fusion.

Genome constitutions of regenerated plants

In the control experiment (without PEG treatment), 50 calli differentiated into *N. sylvestris* plants and eight differentiated into *N. plumbaginifolia* plants; however, no hybrid plants were obtained. When the times of shoot emergence from calli of these species were compared, it appeared that plant regeneration occurred earlier in *N. sylvestris* than in *N. plumbaginifolia* (Fig. 2). About 70% of regenerated plants were diploid and 30% were tetraploid. No apparant structural aberrations of chromosomes were evident.

In the PEG-treated experiment, 45 calli differentiated into hybrid plants (Fig. 1C, D), 11 into *N. plumbaginifolia* plants, and only 2 into *N. sylvestris* plants. As shown in Fig. 2, plants regenerated during the early periods of culture (36-55 days) were almost exclusively somatic hybrid while those regenerated at the last period of culture (66-75 days) were solely *N. plumbaginifolia.* When compared with the control, it appeared that hybrid plants regenerated earlier than both parents.

Genome constitutions and chromosome numbers of the hybrids are shown in Table 1. Plants derived from one single piece of callus usually had the same genome constitution or related constitutions, with one being doubled from the other. Aneuploid numbers and structural abnormalities such as deficiency were observed in somatic hybrids but not in parental plants.

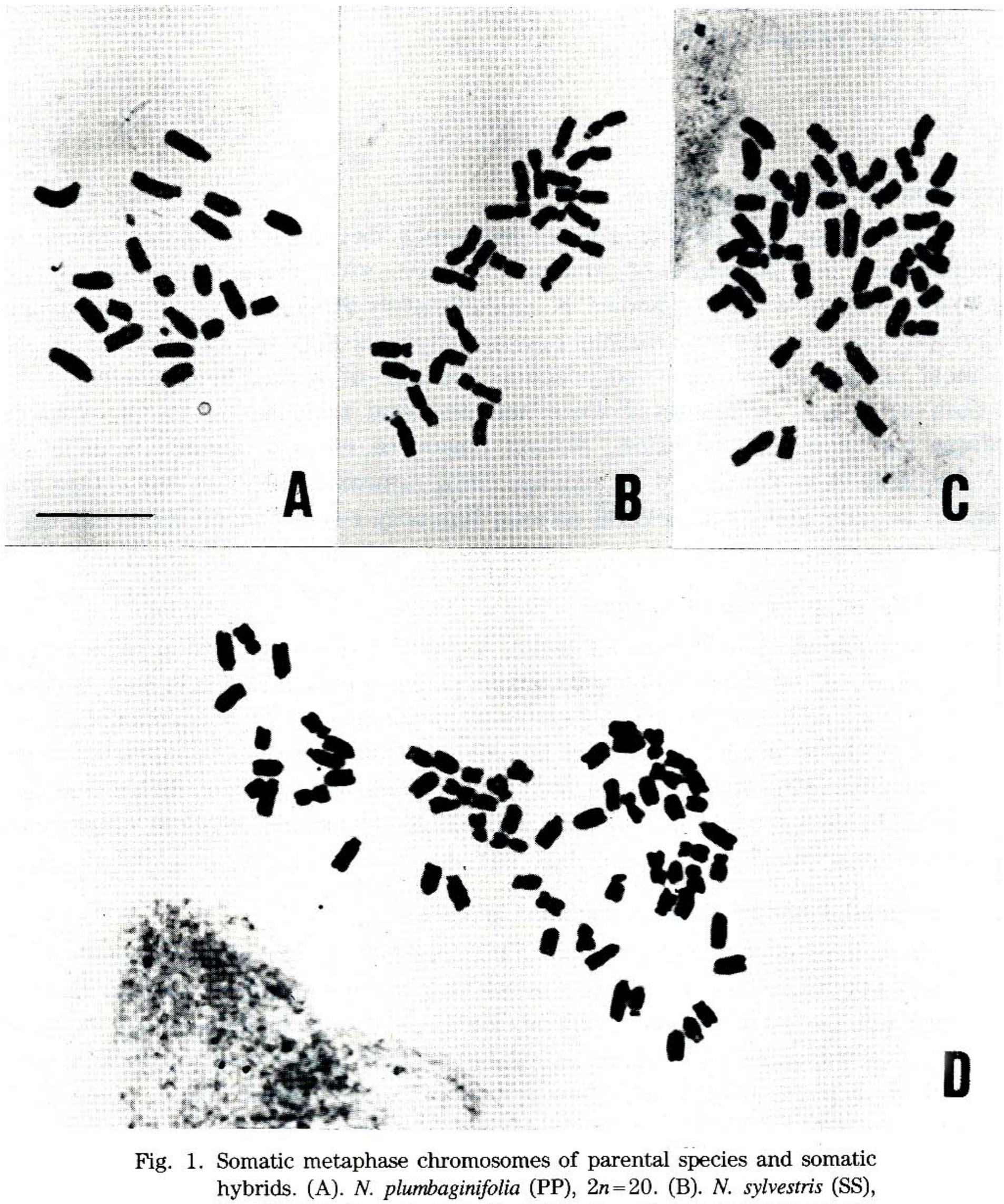

Fig. 1. Somatic metaphase chromosomes of parental species and somatic hybrids. (A). *N. plumbaginifolia* (PP), $2n=20$. (B). *N. sylvestris* (SS), $2n=24$. (C) Somatic hybrid PPSS, $2n=44$. (D) Somatic hybrid PPPPSS, $2n=64$. Bar = 10 μm.

Morphology of somatic hybrid plants

A few of the cytologically identified somatic hybrids were transplanted to pots and raised to the flowering stage. Plants of the somatic hybrids were taller than both parents (Fig. 3A). With respect to other morphological characteristics, such as shape of the leaves; size, shape, and color of the flower (Fig. 3B); and growth habit, the somatic hybrids were intermediate between and clearly distinctive from the two parents.

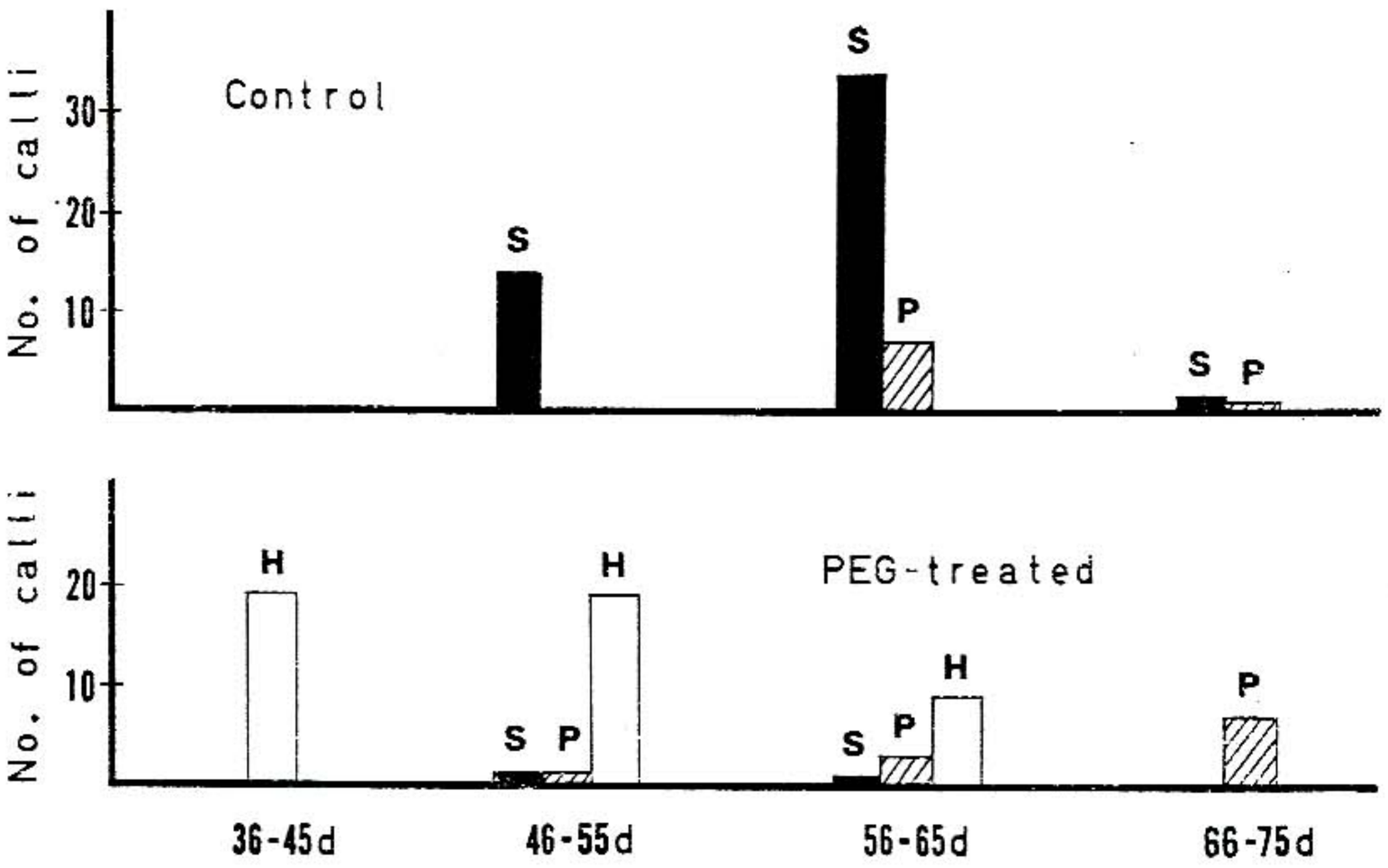

Fig. 2. Relationship between time of shoot emergence (days from beginning of culture) and frequency of calli producing parental and hybrid plants. P = *N. plumbaginifolia;* S = *N. sylvestris;* H = somatic hybrid.

Table 1. Genome constitutions and chromosome numbers of somatic hybrids of *N. plumbaginifolia* and *N. sylvestris*

Genome constitution	No. of calli[a]	Chromosome no. of somatic hybrid plant[b]
PPSS	39	37+1f[c](1), 41(2), 41+1f(1), 42(10), 42+1f(1), 43(7), 43+1f(1), 44(29), 44+1f(1)
PPPPSS	4	61+1f(1), 62(2), 63(2), 64(2)
PPPPSSSS	3	85(1), 86(2), 88(1)

[a]Two calli gave rise to both PPSS and PPPPSSSS plants, therefore, they were counted twice.
[b]The number of plants in each category is indicated in parentheses.
[c]f: fragment.

Aanalysis of esterase isozymes

The isozyme patterns of esterase from the two parents and eight somatic hybrids are shown in Fig.4. The two parents had one common and two species-specific bands. All somatic hybrids contained esterase bands of both parents.

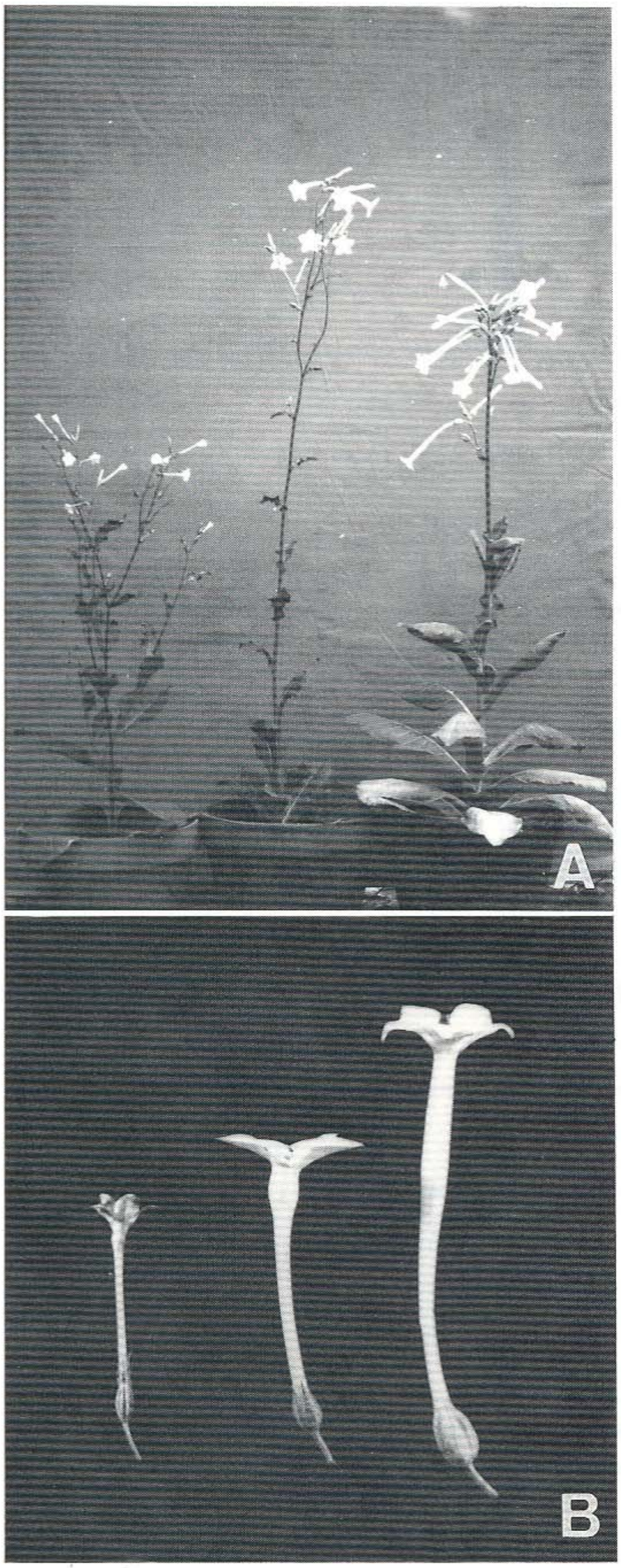

Fig. 3. Morphological characteristics of parental species and somatic hybrids PPSS. (A) Flowering plants of (left to right) *N. plumbaginifolia,* somatic hybrid, and *N. sylvestris*. (B) Flowers of (left to right) *N. plumbaginifolia,* somatic hybrid, and *N. sylvestris*.

DISCUSSION

The results of this study revealed that when protoplast mixtures of *N. plumbaginifolia* and *N. sylvestris* were treated with PEG and cultured under appropriate conditions, a great majority of plants recovered, especially those during the early periods of culture, were somatic hybrids. As our previous study (Lee and Chen, 1990) suggested, this may be the result of genetic complementation between two parents, since protoplasts of *N. sylvestris* are sensitive to PEG but develop rapidly whereas those of *N. plumbaginifolia* are more resistant to PEG but develop slowly. However, the results of the present study also indicate that the somatic hybrids develop faster than both parents (Fig. 2). This phenomenon has also been observed by other investigators in *Nicotiana* (Smith *et al.*, 1976; Liao, 1986), *Datura* (Schieder, 1978, 1980), and *Lycopersicon* (O'Connell and Hanson, 1987). Thus, vigor in growth and development of hybrid cells appears to be a common phenomenon in plant tissue culture, and in many cases can be used as a basis for isolation of somatic hybrids when selectable markers are not available.

The fact that a majority (84.8%) of hybrid calli differentiated into tetraploid plants with PPSS genome constitution indicates that most of the heteroplasmic fusions involve two protoplasts, one from each parent. However, two of these calli also produced octoploid (PPPPSSSS) plants in addition to tetraploids. The most likely explantation for these chimeric calli is that spontaneous chromosome

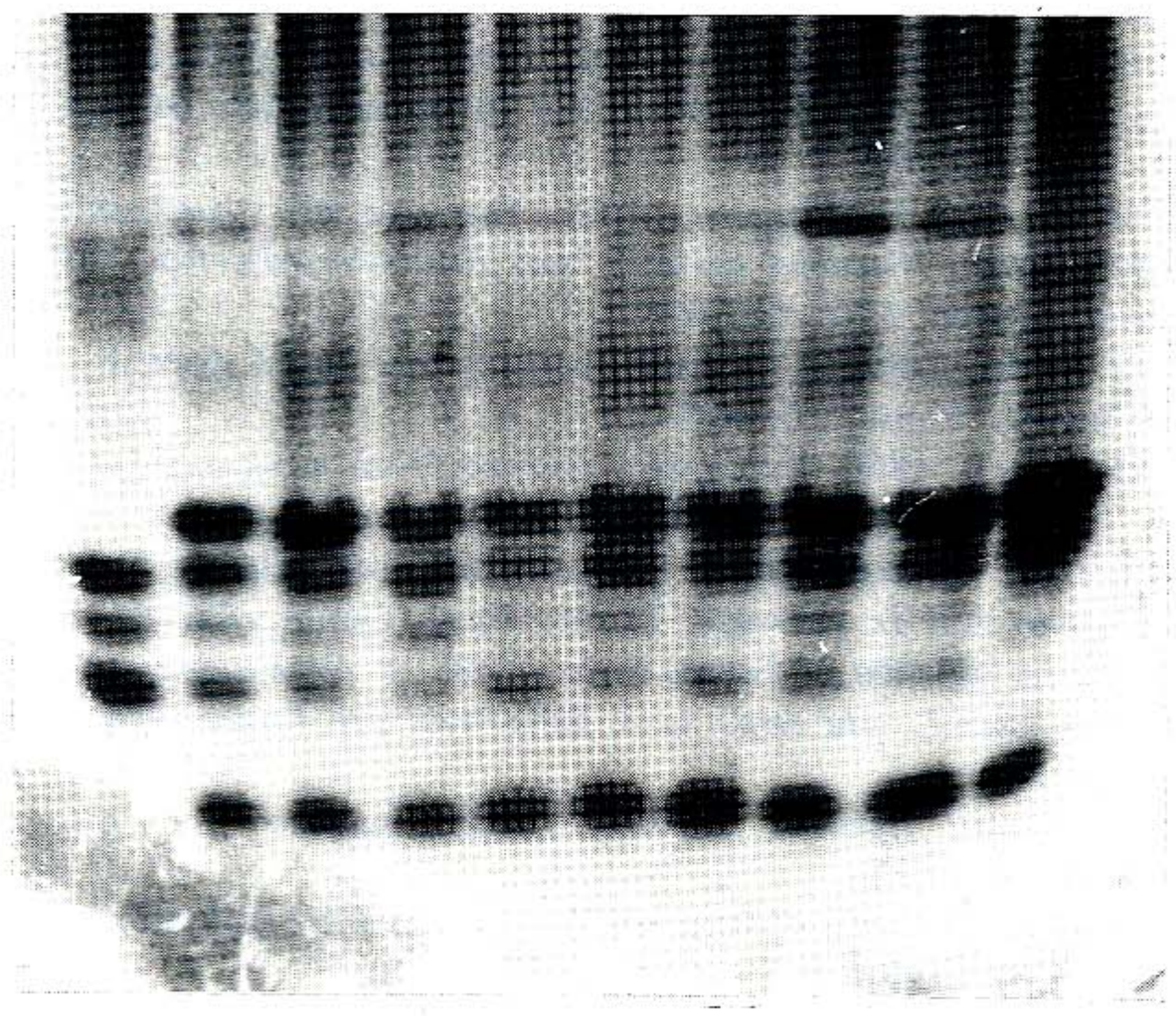

Fig. 4. Esterase isozyme patterns of *N. sylvestric* (lane 1), somatic hybrids (lanes 2-9), and *N. plumbaginifolia* (lane 10).

doubling of cells occurred in the tetraploid calli. Calli producing PPPPSS plants might originate from fusion of two *N. plumbaginifolia* protoplasts and one *N. sylvestris* protoplast. There are two possible explanations for the lack of PPSSSS calli. First, there were proportionally fewer *N. sylvestris* protoplasts in cultures after the PEG treatment, as protoplasts of this species were very sensitive to PEG. Second, plants with the genome constitution of PPSSSS may not be viable.

Variations in chromosome number and structure of somatic hybrids have been attributed to treatment of protoplast cultures with PEG (Smith *et al.*, 1976; Gleba and Evans, 1983). However, in this study we have shown that the variations were more common in somatic hybrids than in parental plants regenerated from protoplast fusion. This result suggests that hybridity may be, in large part, responsible for the occurrence of chromosome variations.

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在無人為篩選壓力下由原生質體融合得到兩種菸草的體細胞雜種

李 鳳 鳴　　陳 其 昌

摘　　要

分別分離兩種菸草 *Nicotiana plumbaginifolia* 與 *N. sylvestris* 葉肉原生質體，等量混合後利用 PEG 誘導融合，並培養於對二親本無篩選壓力之培養基中，在所得到的再生植株中，體細胞雜種約佔 78%，而各種染色體組組合之體細胞雜種，在雜種植株中所佔的比率分別爲 PPSS 84.8%, PPPPSS 8.7%, PPPPSSSS 6.5%。任一染色體組組合之雜種植株皆可觀察到染色體數目或構造上的變異，顯示此二種菸草染色體間存在相當程度的不相容性。再生植株經染色體鑑定、外型比較，及同功酵電泳圖譜分析的結果，發現早期分化所得到的植株全爲體細胞雜種，此現象當可視之爲雜種優勢。