Characterizing intergeneric regenerants of protoplast fusion between *Hyoscyamus muticus* (Egyptian henbane) and *Atropa belladonna* (Indian sagangur)

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In the absence of a selection system, protoplast fusion between *Hyoscyamus muticus* L. and *Atropa belladonna* L. was obtained. The conditions for obtaining such a fusion were determined. The regenerants obtained subsequent to fusion were characterized based on morphology and cytological analysis. The regenerants exhibited phenotypes that were intermediate or resembling the parental types.

HYOSCYAMUS MUTICUS L. was introduced into India from Egypt for its cultivation and medicinal use[1]. Combining some characteristics of this species, viz. alkaloid content and arid habitat in a plant like *Atropa belladonna* (a sexually incompatible distant relative) is an essential step in the improvement of these medicinally important plants. Production of somatic hybrids was reported between sexually incompatible plant species specifically for obtaining higher content of secondary metabolites[2]. Besides somatic hybridization the improvement of plant bearing tropane group of alkaloids was reported through protoclonal variation[3]. Protoplast fusion between *A. belladonna* and *H. muticus* was attempted and the analysis of a few of the fusion products is reported in the present paper.

Seed materials of *A. belladonna* L. ($2n = 72$) and *H. muticus* L. ($2n = 28$) were obtained from CIMAP experimental farm. Callus cultures were established from leaf explants on MS[4] medium supplemented with α-naphthaleneacetic acid (NAA, 2 mg/l) and benzylaminopurine (BAP, 0.5 mg/l) from *in vitro* grown plants. Suspension cultures from both the plants were established using the same medium in liquid[5]. Protoplasts were isolated from 3-month-old suspension cultures. Parental protoplasts (1×10^6 protoplasts/ml of each parent) were mixed in a centrifuge tube, centrifuged for 5 min at $30 \times g$ and the supernatant removed. One ml of fusion solution containing 25% PEG (mol. wt. 3500), 9% mannitol, 2.36% $Ca(NO_3)_2 \cdot 4H_2O$ was added slowly along the wall of the centrifuge tube and then incubated for 0–10 min at 30–32°C. PEG was diluted out slowly with a high-pH Ca^{2+} solution (0.74% $CaCl_2 \cdot 2H_2O$, 0.375% glycine, 10% mannitol, pH 10.5). The duration of incubation in the fusogen or dilutant was varied to determine the optimum fusion frequency.

The protoplasts were then further diluted with a stabilizer solution (0.74% $CaCl_2 \cdot 2H_2O$, 0.75% glycine, 10% mannitol in CPW salts[6], pH 5.8) for at least 1 h. Finally, the settled protoplasts were washed with protoplast culture medium (PCM). Estimation of fusion frequency was done as per Kinsara *et al.*[7] using fluorescence labels FITC (fluorescein isothiocyanate), RITC (rhodamine isothiocyanate)[6], before culture of protoplasts.

After fusion, the heterokaryons along with the parental protoplasts were cultured at an optimum culture density (1×10^5 protoplasts/ml) in a standardized PCM (MS + 3% sucrose (w/v), 9% mannitol (w/v), 1 mg/l NAA, 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l BAP)[5].

Chromosomal counts in the actively growing healthy root tips (1–2 mm) of plants regenerated *in vitro*, hybrid callus and parental seedlings were determined. The roots or calli were pretreated in a saturated solution of *p*-dichlorobenzene (PDB) for 4 h (initially for 5 min in a deep freezer and subsequently at 7°C). The tissue of *A. belladonna* was pretreated in water for 24 h at 2°C. All the tissues were fixed in 1 : 3 :: acetic acid : absolute alcohol. The samples were stained with a mixture (1 : 9) of 1 N HCl and 2% aceto-orceine (dissolved in 45% glacial acetic acid).

Protoplast fusion between *H. muticus* and *A. belladonna* was confirmed on the basis of differential staining by FITC and RITC. The frequency of fusion of protoplasts from *H. muticus* and *A. belladonna* is shown in Table 1. The frequency of the heterokaryon formation with PEG alone is low (0–0.9%). Heterokaryon formation was 2.6% when the parental protoplasts were incubated in high Ca^{2+}/high pH solution for 20 min. However, this frequency could be marginally increased to 3.0% if the parental protoplasts were incubated in PEG for 5 min earlier. Also bursting of protoplasts was noticed to be lower in this treatment than with PEG alone. Thereafter,

Table 1. The effect of the interaction of PEG and ionic solution (high pH/Ca^{2+}) on heterokaryon formation* between protoplasts of *A. belladonna* labelled with FITC and *H. muticus* labelled with RITC

Incubation time (min) in PEG	Incubation time (min) in high pH/Ca^{2+}					
	0	15	20	25	30	45
0	0	0.05	2 6	0.96	0 06	— (B)
2	0 08	0.32	0.9	0.12	0 001	— (B)
4	0 06	0.25	1 86	0.08	0 015	— (B)
5	0.01	0.52	3 0	0.12	0 001	— (B)
8	0 09	0 1	0 08	0 03	0 (B)	— (B)
10	0 02	0.09	0.07	0.06	0 (B)	—

*Fusion frequency % (Percentage of heterokaryons per total number of protoplasts).
B = Bursting of protoplast.
Observations were taken after 1 h of fusion process was over under UV using a fluorescence inverted microscope.
A minimum of 25 microscopic fields were observed to arrive at the fusion frequency.

incubating the protoplasts for further time periods in either of the solutions did not increase the fusion frequency. Plating efficiency of parental protoplasts was to the tune of $56 \pm 2\%$ and $47 \pm 1.5\%$ in case of *H. muticus* and *A. belladonna*, respectively. However, when protoplasts were cultured after fusion at same culture density as parents (1×10^5 protoplasts/ml), the plating efficiency was observed to be $68 \pm 2\%$. Fast-growing colonies were visible within 4–5 weeks of culture. From 50 fast-growing colonies 15 callus lines were obtained. These callus lines were then transferred to a semisolid agar (0.8%) MS regeneration medium containing 3% sucrose 0.1 mg/l NAA and 0.5 mg/l BAP. Regenerated shoots developed into complete plantlets when the shoots were transferred to rooting medium containing half-strength MS nutrients without phytohormones. The regenerated plants were selected visually on the basis of the morphology, pattern and plant regeneration frequency. Calli obtained after fusion were highly compact and green compared to parental calli, which were fragile and yellowish green in nature. There was significant difference in the regeneration potential of hybrid callus compared to parental protoplast callus. The regeneration frequency from protoplast-derived calli of *H. muticus* and *A. belladonna* protoplasts was 80% and 60%, respectively. However, the regeneration frequency of callus obtained after fusion was extremely high (100%), i.e. whole of the callus would transform into regenerants. Similar screening for somatic fusion product was reported in other plant species where no selection system was available[8–11].

Amongst the different callus lines studied, plants regenerated from four selected calli were peculiar as some regenerants showed intermediate phenotype (Figure 1). From four such callus lines, 15 plants showing intermediate phenotypes were obtained and 9 of these plants were transferred to glasshouse. These plants flowered within 9–10 weeks of transfer to soil. The flower morphology in such plants was different from the parental types. The flowers had larger petal

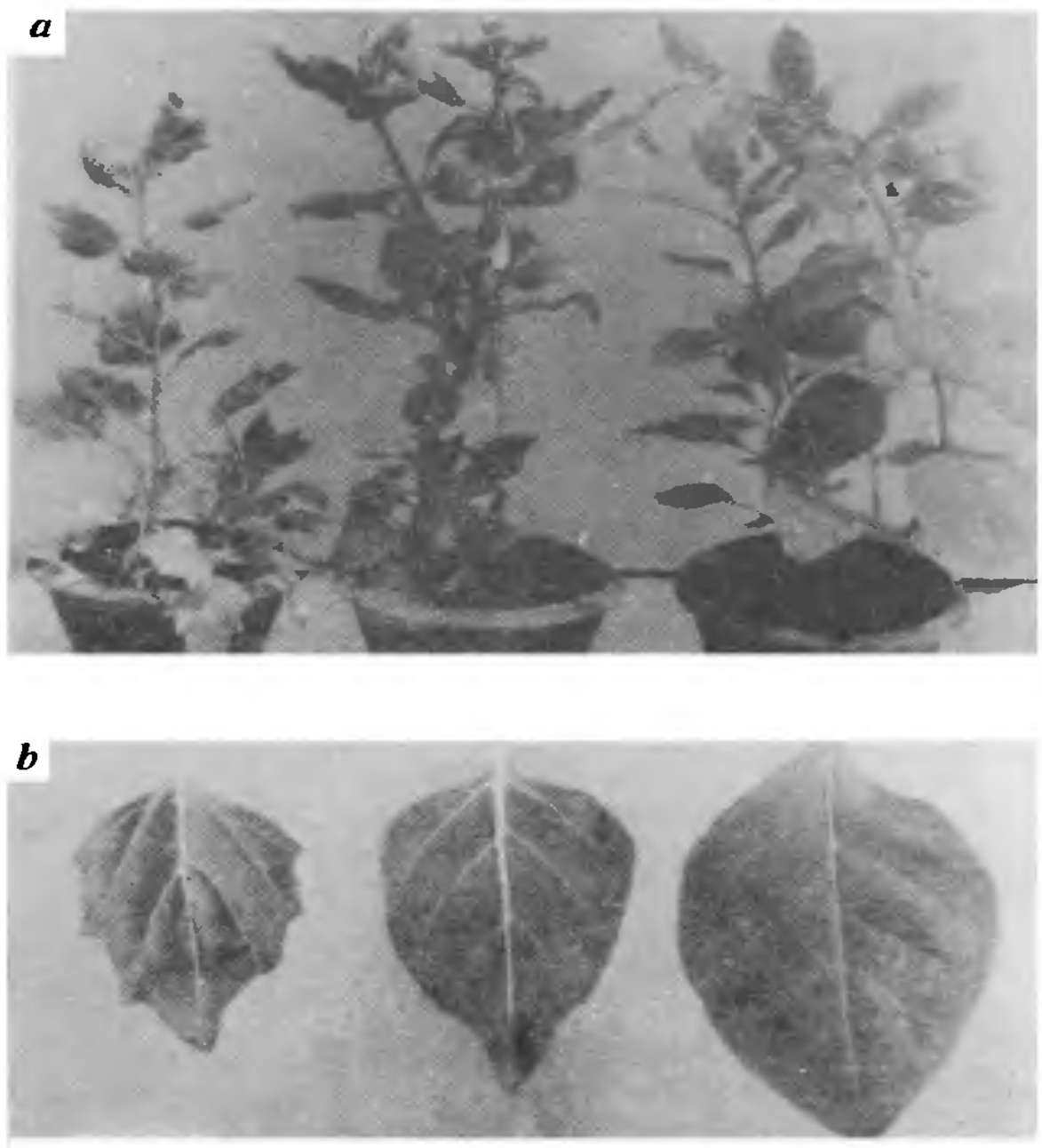

Figure 1. Morphological variation amongst fusion-derived regenerants as compared to parents. *a*, *H. muticus* (left), *A. belladonna* (right) and fusion-derived regenerant (middle); *b*, Leaf morphology of *H. muticus* (left), *A belladonna* (right) and fusion-derived regenerant (middle).

Table 2. Chromosome analysis of the calli obtained after fusion between *A. belladonna* ($2n = 72$) and *H. muticus* ($2n = 28$) and their regenerants

Replicate no.	Callus lines				Roots* of regenerated plants		
					Symmetric phenotype	Asymmetric phenotype	
	1	2	3	4		*A. belladonna*	*H. muticus*
1	110	65	105	92	100	81	40
2	105	87	140	95	105	86	56
3	102	86	130	101	102	70	30
4	107	83	125	107	103	56	25
5	85	82	97	100	100	96	28
6	—	120	100	—	103	100	42
7	—	100	—	—	100	53	24
8					108	62	28
9					104	72	32
10					—	73	38
11					—	75	28
12					—	82	39
13					—	72	28
14					—	—	28

*A minimum of 10 samples for each tissue were investigated for chromosome analysis.

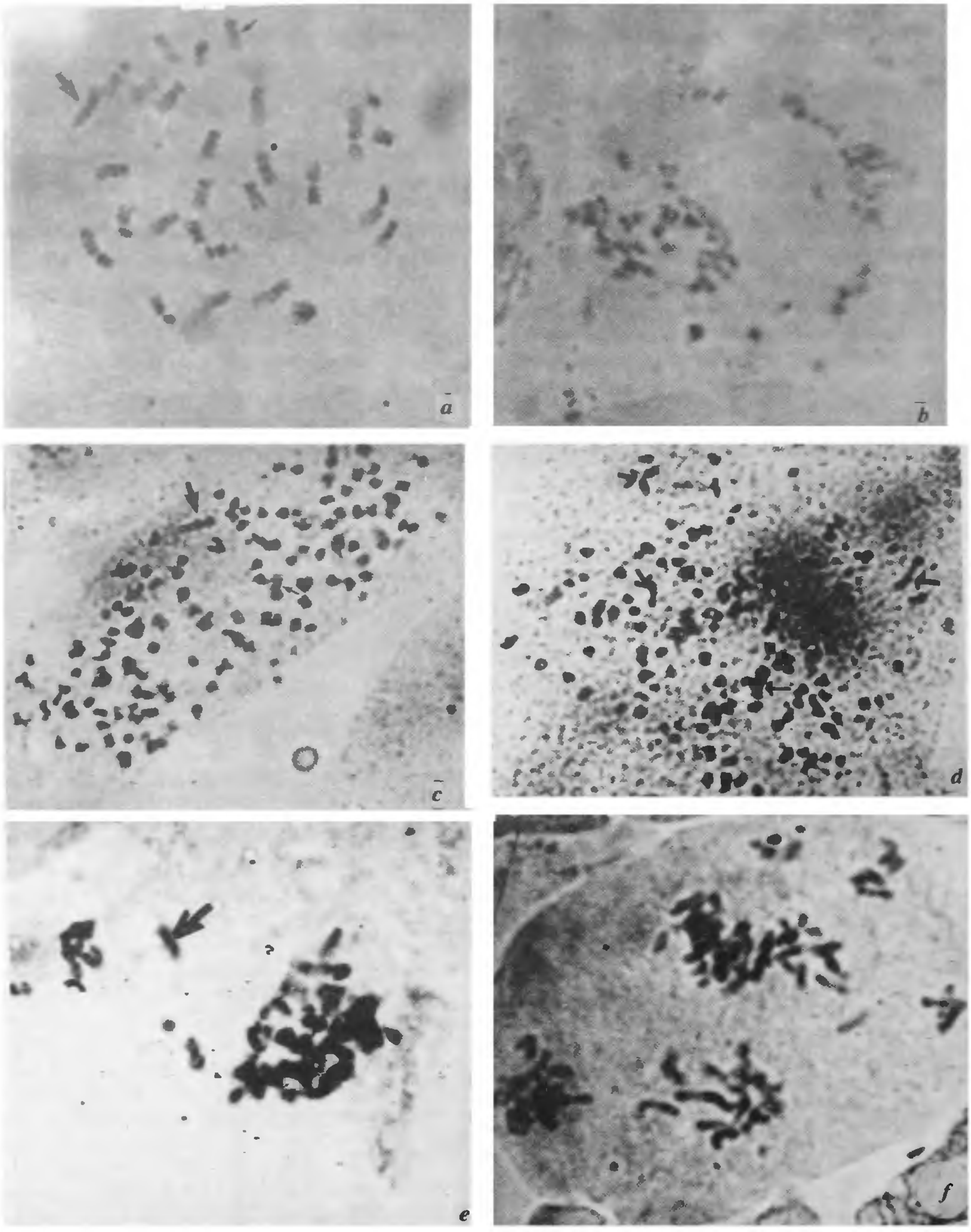

Figure 2. Somatic chromosome analysis of the parents and putative asymmetric hybrids between *H. muticus* and *A belladonna*. *a,b*, Metaphase chromosome of *H. muticus* ($2n = 28$) and *A. belladonna* ($2n = 72$). *c,d*, Chromosomes of fusion-derived regenerated plants ($2n = 100, 109$), majority of chromosomes are smaller *belladonna* type with few *muticus* type (arrow). *e*, Unequal anaphase separation (disjunction) with laggard chromosome (arrow). *f*, Multipolarity of chromosome during anaphase separation.

size compared to parents. They also differ in number of petals (6) and stamens (6) compared to parents (5). The shape and colour of anther was changed to heart-shaped white compared to rod-shaped purple in the parents.

These four selected callus lines in subsequent culture passage gave rise to two types of regenerants resembling *H. muticus* and *A. belladonna*. The regenerants included 12 asymmetric hybrids resembling *A. belladonna* and 17 resembling *H. muticus*. In a similar finding both *Arabiodopsis* and *Brassica* plants were found to regenerate from the same hybrid callus during the culture passage[12].

Table 2 shows mitotic chromosome analysis of selected calli and regenerated shoots showing intermediate phenotype (symmetric hybrids) or resembling the parental types (asymmetric hybrids). In the hybrid callus the chromosome number ranged from 83 to 140. Few regenerants which possessed intermediate phenotype showed chromosome numbers between 100 and 108. Of the asymmetric hybrid plants, those resembling *A. belladonna* possessed 56 to 100 chromosomes and those resembling *H. muticus* was 24–56. The cytological analysis of the chromosomes for the plants resembling *A. belladonna* showed a few larger-sized chromosomes of *H. muticus* (Figure 2 *c*, *d*). The *H. muticus* type of regenerants showed the presence of smaller-sized chromosomes of *A. belladonna*. This could have resulted in the production of asymmetric hybrids. Cytological anomalies such as elimination of chromosomes, unequal separation of chromosomes with the presence of laggards and multipolar orientation of chromosomes during anaphase were observed when the calli were observed after several subcultures (Figure 2 *e*, *f*).

The chromosome elimination subsequent to somatic hybridization was found to occur in most of the fusion studies. Chromosome elimination was observed in fusion involving *Arabiodopsis thaliana* and *Brassica campestris*[13]. In a study on the fusion between *A. belladonna* and *N. chinensis* the elimination of parental chromosomes has been observed. In most of the cases species-specific elimination takes place[14–16]. Similar findings were also obtained in studies on somatic hybridization between *A. belladonna* and *Datura innoxia*[17]. The phenomenon of chromosome elimination in the present study can be exploited for raising asymmetric hybrids and may be used for limited gene transfer as has been reported earlier with other crops[18]. The present work indicates the possibility of producing an intergeneric somatic hybrid between two medicinally important plants in the absence of a selection system.

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ACKNOWLEDGEMENTS. The work was supported by Council of Scientific and Industrial Research (CSIR), New Delhi. Laboratory facility by Director, CIMAP, Lucknow is acknowledged.

Received 30 May 1994, revised accepted 18 July 1995

Mechanism of wound healing induced by chitosan in streptozotocin diabetic rats

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Diabetes mellitus is a common metabolic disorder associated with many pathological conditions. Prolonged or incomplete wound healing is one among them. This is due to poor blood circulation, alterations in circulating constituents, abnormal platelet aggregation and decreased fibrinolytic activity. The aim of the present study was to envisage the role of chitosan in wound healing and to investigate its effect in normalizing the aggregation pattern of platelets in diabetes. At very low concentrations, chitosan has no effect on platelet aggregation, while high concentrations can aggregate the platelets. Chitosan is not capable of reversing the increased rate of aggregation in diabetic platelets. The number of platelets which may affect the normal clotting process were also significantly lower in diabetic rats. Chitosan was found to be a good wound-healing agent, even though it does not reverse the altered platelet aggregation. Chitosan may be enhancing wound healing by some other mechanism independent of its effect on platelet aggregation.

DIABETES mellitus is caused by decreased secretion of insulin by the β cells of Islets of Langerhans, lack of