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"Erussica", the intergeneric fertile somatic hybrid developed through protoplast fusion between *Eruca sativa* Lam. and *Brassica juncea* (L.) Czern.

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Summary. Hypocotyl calli-derived protoplasts of two cultivars of *Brassica juncea* (2n = 36), a major oil-seed crop, were fused with normal as well as γ-irradiated mesophyll protoplasts of *Eruca sativa* (2n = 22). The irradiation of the *Eruca* fusion partner increased the plating efficiency as well as the morphogenic potentiality of the fusion products over the normal fusion. Fertile plants could be regenerated from such fusion products. Analysis of 63 out of 181 plants regenerated showed that, indeed, 11 somatic hybrids (2n = 58) and 10 partial somatic hybrids (chromosome number ranged between 50 and 56) had been obtained. Pollen viability (0% – 82.9%) and seed set (0% – 50%) of the hybrids indicated them to be useful for future studies.

Key words: Intergeneric somatic hybridization – *Brassica* + *Eruca* – Somatic hybrid – Partial somatic hybrid

Introduction

A number of wild relatives of oil-seed crop *Brassica* carry useful genetic traits. Attempts to expand the pool of accessible genes by overcoming natural isolating mechanisms between these relatives and oil-yielding *Brassica* crops through sexual crossing had been initiated almost six decades back. In spite of such attempts, the genetic resources existing in the exotic germ plasms remained largely unused. The main reason for the underuse of this natural resource is the difficulty in obtaining intergeneric sexual hybrids. With the advent of techniques such as somatic cell fusion, which is a logical extension of gametic cell fusion but with most of the sexual incompatibility barriers bypassed, the chances to achieve a breakthrough have improved. In fact, several intergeneric somatic hybrid plants have already been produced in Cruciferae (Gleba and Hoffmann 1980; Toriyama et al. 1987a, b; Chatterjee et al. 1988; Fahleson et al. 1988).

Brassica juncea (L.) Czern. is the major oil-yielding crop species of the Indian subcontinent. Its relative, *Eruca sativa* Lam., is believed to contain some desirable agronomic traits, particularly aphid and drought tolerance (Tsunoda et al. 1980). It has been considered desirable, from the plant breeder's point of view, to evolve hybrids between the two plant types. In the absence of any past record of successful production of intergeneric sexual hybrids, it was considered a prerequisite to attempt the synthesis of parasexual intergeneric hybrids through protoplast fusion of these two plant types. The conditions for the standardization of methodologies of protoplast culture and fusion had already been fulfilled by us (Chatterjee et al. 1985, 1988; Sikdar et al. 1987). The present investigation describes our success in recovering fertile intergeneric somatic hybrids between *Brassica juncea* and *Eruca sativa* via protoplast fusion.

Materials and methods

Experimental strategy

The following experimental steps were adopted to generate plants from the fusion products between protoplasts of *B. juncea* and *E. sativa* (1) During fusion, four times as many *Eruca* protoplasts were kept as *B. juncea* protoplasts. This permitted as many as ca. 70% *B. juncea* protoplasts to be fused with those of *Eruca*. In addition, the density of *Eruca* protoplasts while plating was kept high (1×10^5 protoplasts/ml), so that the homokaryon *Eruca* protoplasts could not regenerate in such a situation. (2) The microcolonies developed after fusion were kept under specific cultural conditions when plant regeneration of *B. juncea* protoplast-derived calli or of its fused products was

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only possible through organogenesis. (3) In another set of experiments, *Eruca* protoplasts were alternatively γ-irradiated at LD_{50} and LD_{80} levels before fusion with *B. juncea*. Such fused protoplasts were likely to generate the following consequences: (a) production of cybrids if the nucleus of *Eruca* was totally inactivated; (b) production of partial somatic hybrids as the result of chromosome destabilization through irradiation; (c) promotion of morphogenic potentiality of the fused cells due to elimination of inhibitory factor(s). (4) Selection of novel plant types as the result of fusion was initially carried out on the basis of morphological features of the whole plants. Subsequently, the chromosomal status, pattern of enzyme isoforms of esterase, and isoelectric focusing pattern of RuBP carboxylase were investigated.

Plant materials and preparation of sources for protoplasts

Seeds of two varieties of *Brassica juncea* (L.) Czern. (2n = 36), i.e., cv T-59 and cv B-85 (obtained from Dr. S. Ghosh, Bidhan Chandra Krishi Viswa Vidyalayay, Kalyani), and *Eruca sativa* Lam. (2n = 22) (obtained from Dr. P. R. Kumar, All India Coordinated Program for Rape and Mustard, ICAR, New Delhi), formed the experimental material. Leaves from aseptically grown shoot tips of *E. sativa* and hypocotyl calli of *B. juncea* were used for protoplast isolation. Handling of the seed culture and apical shoot-tip culture of *E. sativa* was carried out according to the method outlined already by Sikdar et al. (1987). Induction of hypocotyl calli of *B. juncea* was carried out according to Chatterjee et al. (1988).

Protoplast isolation

First and second nodal leaves of 3-week-old *E. sativa* and 10-day-old hypocotyl calli of *B. juncea* were used as the source of protoplasts. Mesophyll protoplasts of *E. sativa* and hypocotyl callus protoplasts of *B. juncea* were isolated according to Sikdar et al. (1987) and Chatterjee et al. (1988), respectively.

γ-Irradiation

Purified protoplasts of *E. sativa* suspended in 0.6 *M* mannitol + 14 m*M* $CaCl_2$. $2H_2O$ solution were exposed to varying doses of γ-irradiation from a ^{60}Co source. The LD_{50} and LD_{80} of γ-irradiation were estimated as 22.5 kr and 41 kr, respectively, on the basis of the ability of the protoplasts to divide, measured by their plating efficiency.

Protoplast fusion, culture, and plant regeneration

Protoplast fusion was carried out between protoplasts from hypocotyl calli of each of the two cultivars of *B. juncea* and from mesophyll protoplasts of *E. sativa*. Hypocotyl callus-derived protoplasts of *B. juncea* cv T-59 and cv B-85 are hereafter referred to as Bj T-59 and Bj B-85, respectively, and mesophyll protoplasts of *E. sativa* as Es. The γ-irradiated mesophyll protoplasts of *E. sativa* at LD_{50} and LD_{80} are referred to as Es 50 and Es 80, respectively. Thus, for each of the cultivars of *B. juncea*, the following set of protoplast fusion experiments were carried out: Bj + Es; Bj + Es 50; Bj + Es 80; Bj self-fusion; Es self-fusion; Es 50 self-fusion; Es 80 self-fusion. The protoplasts were suspended at 1×10^6/ml density. In the case of intergeneric fusions, protoplasts of two origins, i.e., Es and Bj, were mixed at a ratio of 4:1. The procedures for protoplast fusion, culture of the fused products, and regeneration of plants were similar to that of Chatterjee et al. (1988).

Selection of putative hybrids

Regenerated plants that resembled neither of the parents in morphological characters such as growth pattern, leaf thickness, leaf shape, and nature of trichome were selected initially as putative somatic hybrids. After multiplication in culture of each line, five regenerants in each case were potted and grown under outdoor conditions for further analysis.

Confirmation of hybrid character by chromosome analysis

Putative hybrids were confirmed as somatic hybrids by studying their meiotic chromosomes. Pollen mother cells from 45-day-old in vitro-grown plants were smeared with 1% aceto-carmine and studied.

Analysis of enzyme isoforms

Additional confirmation of somatic hybrids was carried out by analyzing the enzyme isoforms for esterase (E.C. 3.1.1.2), following the procedure described by Chatterjee et al. (1988).

Analysis of ribulose biphosphate carboxylase (RuBP Case)

Isoelectric focusing of RuBP Case of somatic hybrids was carried out according to the method of Cammaerts and Jacobs (1980), with broad-range ampholine (pH 3.5–10) at a constant voltage of 500 V for $12 \times 12 \times 0.2$ cm^3 gel. The running time was 30 h at 15 °C.

Analysis of pollen viability and hybrid fertility

Pollen viability of hybrid plants was carried out according to Sundberg et al. (1987). For each hybrid plant, flowers were self-pollinated and backcrossed with both the parents separately. The number of siliquas obtained was recorded, and the number of placenta/siliqua was counted for ten randomly selected siliquas of each hybrid plant. The fertility percent of the hybrids was determined as number of seeds set per siliqua × 100/number of placenta produced per siliqua.

Morphological characterization of potted hybrid plants

Plant height of the hybrids was determined during seed development. Critical observations on leaf shape, leaf thickness, amount of trichomes, flower size, sizes of sepal, petal, stamen, gynoecium, siliqua, seeds, and color of seeds were made.

Results

Table 1 represents the results of plant regeneration from fusion of protoplasts between *Brassica juncea* and *Eruca sativa*. Maximum plating efficiency was attained in cases of self-fusion of protoplasts of *B. juncea*. The plating efficiency of Bj + Es was lower compared to Bj self-fusion. However, the plating efficiency improved when irradiated protoplasts of *E. sativa* were used as one of the fusion partners compared to the use of nonirradiated Es. Regeneration of plants could be achieved within 5 weeks after fusion through organogenesis. The morphogenic potentiality of the calli raised from the Bj + Es 50 fusion experiment was more than twofold, and it was more than three-or fourfold higher than that of the calli raised from the Bj T-59/B-85 self-fusion and Bj + Es fusion experiments, respectively. Almost similar results were obtained with Bj + Es 80, but with less efficiency than with Bj + Es 50. Of the total 181 plants regenerated, 107 exhib-

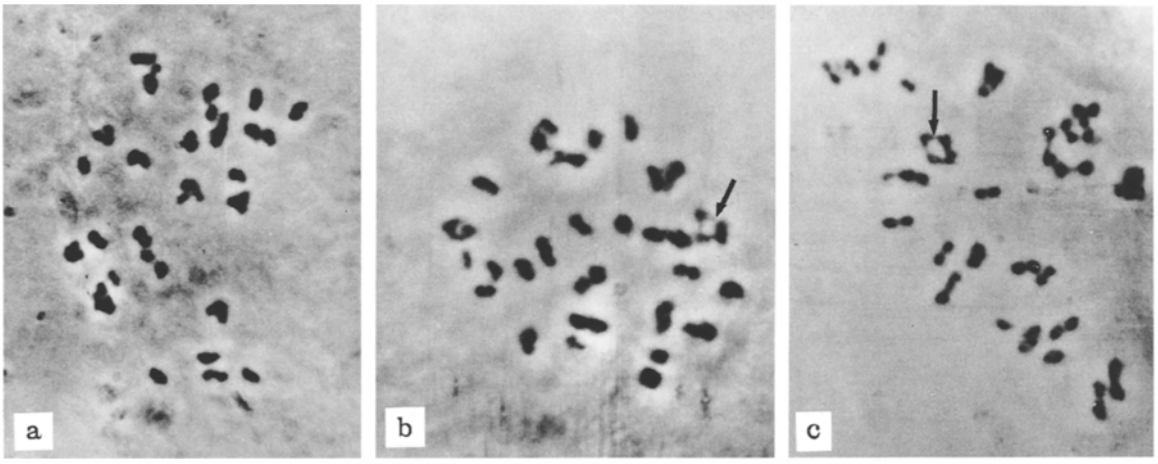

Fig. 1a–c. Light micrograms of: **a** metaphase I of line no. E 2/5 showing 29 bivalents (2n = 58); **b** metaphase I of line no. E 1/30 showing 27 bivalents and 1 quadrivalent (2n = 58); **c** metaphase I of line no. E 2/108 showing 26 bivalents and 1 quadrivalent (2n = 56). *Arrow* indicates quadrivalent

Table 1. Results of intergeneric protoplast fusion between *B. juncea* and *E. sativa*

Fusion partners	Approximate no. of protoplasts used in fusion	Total no. of calli developed	Plating efficiency	No. of plants regenerated from different calli	% of plant regeneration	Nature of regenerated plants		
						Plants similar to either of the parents		Plants not similar to either of the parents
						Brassica-resembling	*Eruca*-resembling	
Bj T-59+Es	$3.2\times10^5+12.8\times10^5$	164	0.010	12	7.31	4	–	8
Bj T-59+Es 50	$1.6\times10^5+6.4\times10^5$	220	0.028	52	23.63	15	–	37
Bj T-59+Es 80	$1.6\times10^5+6.4\times10^5$	113	0.014	20	17.7	4	–	16
Bj T-59 self-fusion	4×10^5	154	0.039	16	10.19	16	–	–
Bj B-85+Es	$3.2\times10^5+12.8\times10^5$	178	0.011	10	5.61	4	–	6
Bj B-85+Es 50	$1.6\times10^5+6.4\times10^5$	196	0.025	43	21.93	14	–	29
Bj B-85+Es 80	$1.6\times10^5+6.4\times10^5$	98	0.012	16	16.31	5	–	11
Bj B-85 self-fusion	4×10^5	132	0.033	12	9.09	12	–	–
Es self-fusion	4×10^5	6	0.002	–	–	–	–	–
Es 50 self-fusion	4×10^5	–	–	–	–	–	–	–
Es 80 self-fusion	4×10^5	–	–	–	–	–	–	–

ited altered morphological characters such as stunted growth, thick leaf, and intermediate hairiness, while the rest exhibited *Brassica* phenotype. Not a single plant with *Eruca* phenotype regenerated from any of the intergeneric fusion experiments.

Chromosome analysis

Chromosome analysis of the 63 plants revealed that 11 somatic hybrids (SH) could be recovered. Such hybrids contained $1_{IV}+27_{II}$ as the predominant chromosome configurational feature (Fig. 1a and b) at diakinesis (Table 2). No univalents could be seen at diakinesis of these somatic hybrids. Ten other plants were classified as partial somatic hybrids (PSH). The chromosome number of these plants ranged from 50 to 56 (Fig. 1c). In no case could chromosome numbers be found in the regenerated plant lines that were higher than the summation of the diploid chromosome number of both the parents. In some hybrids (Table 2) two quadrivalents were found at early diakinesis. It was also determined that none of the somatic hybrids had originated as the result of fusion between Bj+Es 80.

Morphology

Table 3 depicts some of the morphological features of the hybrid plants. Morphologically, all the pot-grown hybrid plants were smaller (average length 30.4 cm, range 22.6–37.8 cm) than both the parents (Fig. 2a). The leaves were smaller (Fig. 2b) and thicker than each of the parents. The number of trichomes was smaller than the *B. juncea*

Table 2. Showing meiotic chromosome configuration, esterase banding pattern, pollen viability, and seed set of different hybrid lines raised through protoplast fusion between *B. juncea* and *E. sativa*

Hybrid line no.	Fusion partners	Meiotic chromosome configuration at diakinesis	2n	Classification	Pollen viability (%)	Seed set (%)		
						Selfed	× *B. juncea*	× *E. sativa*
E 1/2	Bj T-59+Es 50	28_{II}	56	PSH	10.2	0.3	0.2	–
E 2/5	Bj B-85+Es	29_{II}	58	SH	70.3	37.5	32.1	–
E 2/9	Bj B-85+Es	$1_{IV}+27_{II}$	58	SH	29.3	6.1	10.8	–
E 2/24	Bj B-85+Es 50	27_{II}	54	PSH	–	–	0.03	–
E 1/30	Bj T-59+Es	$1_{IV}+27_{II}$	58	SH	67.7	31.3	33.0	–
E 2/36	Bj B-85+Es 50	$1_{IV}+27_{II}$	58	SH	12.0	1.3	1.8	–
E 1/45	Bj T-59+Es	$2_{IV}+25_{II}$	58	SH	29.2	7.5	8.6	–
E 1/51	Bj T-59+Es	27_{II}	54	PSH	22.4	5.1	8.4	–
E 2/63	Bj B-85+Es	$2_{IV}+25_{II}$	58	SH	61.9	25.0	30.2	–
E 1/71	Bj T-59+Es 50	25_{II}	50	PSH	2.0	0.01	0.03	–
E 1/79	Bj T-59+Es 50	$1_{IV}+25_{II}$	54	PSH	6.6	0.2	0.1	–
E 1/82	Bj T-59+Es	29_{II}	58	SH	82.9	50.0	39.4	–
E 2/92	Bj B-85+Es	$1_{IV}+27_{II}$	58	SH	39.1	15.6	17.8	–
E 1/100	Bj T-59+Es 50	$1_{IV}+24_{II}$	52	PSH	–	–	0.02	–
E 1/101	Bj T-59+Es 50	27_{II}	54	PSH	7.1	1.1	1.9	–
E 2/108	Bj B-85+Es 50	$1_{IV}+26_{II}$	56	PSH	7.2	1.4	1.7	–
E 2/111	Bj B-85+Es 50	29_{II}	58	SH	12.8	2.1	3.0	–
E 1/119	Bj T-59+Es	$1_{IV}+26_{II}$	56	PSH	38.4	11.5	16.0	–
E 2/131	Bj B-85+Es 50	$2_{IV}+24_{II}$	56	PSH	9.6	0.09	0.1	–
E 1/135	Bj T-59+Es 50	$1_{IV}+27_{II}$	58	SH	11.2	0.2	0.2	
E 1/143	Bj T-59+Es	29_{II}	58	SH	76.4	34.3	37.2	–
Parental materials:								
B. juncea cv T-59		18_{II}	36		98.0	68.4	84.2	–
B. juncea cv B-85		18_{II}	36		96.4	66.6	83.3	–
E. sativa		11_{II}	22		95.9	70.0	–	80.0

SH – somatic hybrid
PSH – partial somatic hybrid

parent but greater than the *Eruca* parent. The flowers were larger than *B. juncea* but smaller than *Eruca* (Fig. 2c and d). The flowers resembled more those of *B. juncea*. Sepals, petals, and anthers were intermediate in shape, whereas the stigma was larger than both the parents (Fig. 2e).

Some of the partial somatic hybrids contained abnormal growth of gynoecium, petal, and stamen (Table 3). Some bore smaller flowers and the stigma protruded out before the maturity of the flower. The siliquas were mostly intermediate in size (Fig. 2f). The seeds were round in shape (Fig. 2g), either smaller than both the parents or intermediate in size. All the seeds of the hybrids were reddish brown in color, unlike the dark-brown color of *B. juncea* or yellowish brown of *E. sativa*.

Analysis of pollen fertility and seed set

The pollen fertility of the parental lines ranged from 95.9% to 98%, while in the hybrid lines the pollen viability was between 0% and 82.9% (Table 2). Hybrid lines originating from Bj+Es 50 fusion showed low pollen fertility (0%–12.8%).

It is evident from Table 2 that 50% seed set on selfing could maximally be achieved in the hybrid line no. E 1/82, whereas in line nos. E 2/24 and E 1/100 no seed set was recorded. Seed fertility was found to be lower (0%–2.1%) in the hybrid lines originating from Bj+Es 50 fusion compared to Bj+Es fusion. When the hybrids were backcrossed with *B. juncea* pollen, capacity to set seed improved in most cases, but backcrossing with *E. sativa* pollen yielded no seed setting.

Analysis of enzyme isoforms of esterase

Esterase isozyme analysis of the somatic hybrids revealed that the genes of both the parental nuclei had coded (Fig. 3a). However, some of the partial somatic hybrids exhibited an intermediate banding pattern, where certain characteristic bands of *Eruca* were absent.

RuBP carboxylase analysis

E. sativa showed two nuclear-coded bands (SS) that have the same isoelectric focusing point of the upper two of the three nuclear-coded bands of *B. juncea*. Hence, its hybrids could not be distinguished from those of

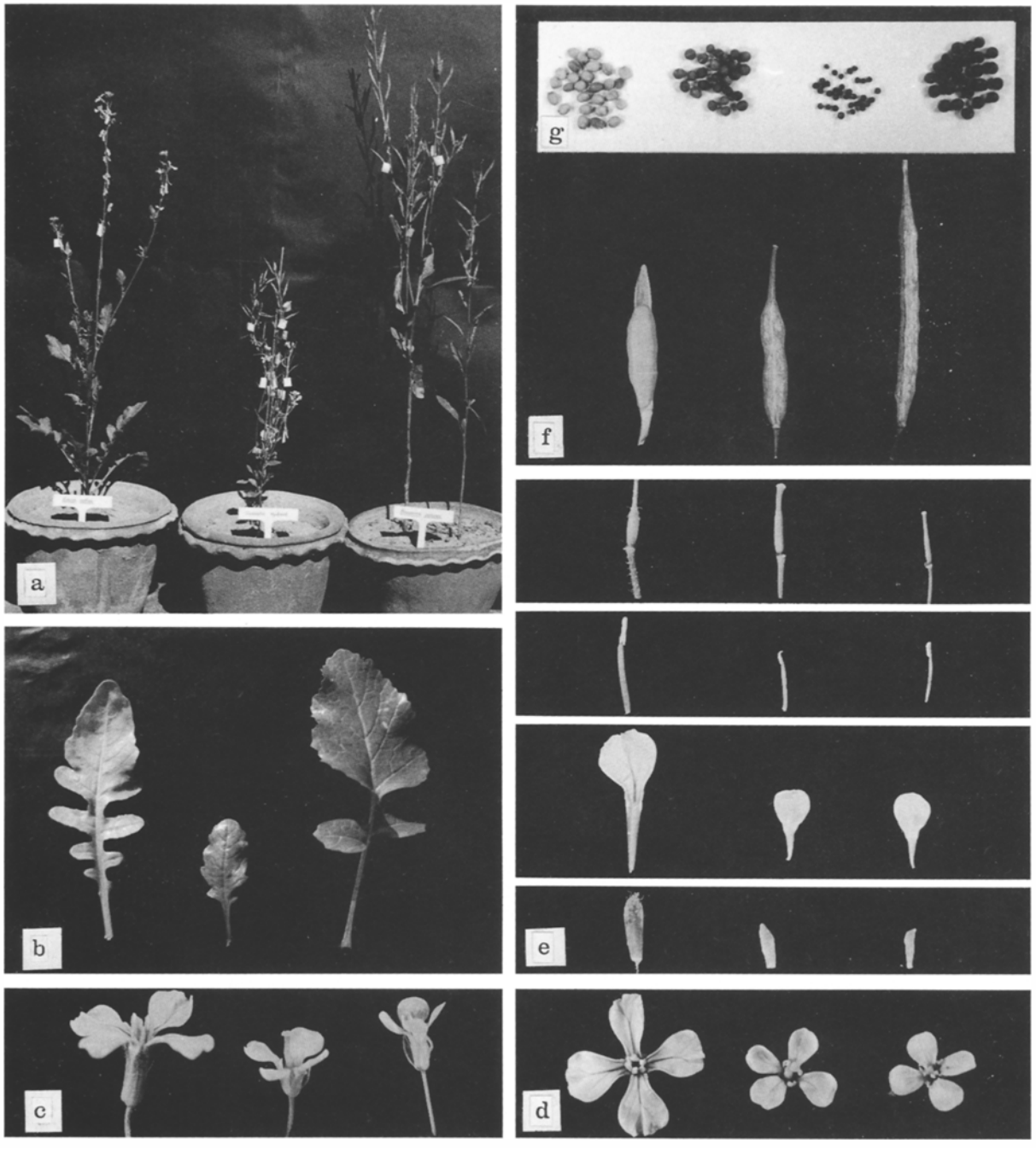

Fig. 2a–g. Morphological features of whole pot-grown plants (**a**), leaves (**b**), side view (**c**), and top view (**d**) of flowers, floral parts (**e**), fruits (**f**), and seeds (**g**) of *B. juncea* cv T-59 (*right*), *E. sativa* (*left*) and their somatic hybrid E 1/45 (*middle*)

B. juncea (Fig. 3b). In the somatic hybrids, however, the plastid type could not be unambiguously identified by its large subunit (LS) characteristics (Fig. 3b).

Discussion

The cultural procedure and the medium used for regeneration of plants from the fused protoplasts were similar to those we used earlier in somatic hybridization between *B. juncea* and *Diplotaxis muralis* (Chatterjee et al. 1988). The use of increased regeneration capability (Schieder 1982; Austin et al. 1985; Chatterjee et al. 1988) and morphological characters (Adams and Quiros 1985; Kinsara et al. 1986; Chatterjee et al. 1988) as markers for the selection of putative hybrids worked well. The experimental strategy set out for selection of intergeneric somatic hybrids between *B. juncea* and *E. sativa* has also yielded results.

Table 3. Morphological features of different plant parts of the hybrids

Hybrid line no.	Plant height (cm)	Leaf morphology			Flower morphology					Size of siliqua (i)
		Leaf size (a)	Thickness (b)	Trichome (c)	Sepal (d)	Petal (e)	Anther (f)	Stigma (g)	Any abnormality (h)	
E 1/2	35.0	+	+++	++	+++	++	+	++	Stamens-(5+1 staminode)	++
E 2/5	29.5	+	+++	++	+++	+++	++	++	–	+++
E 2/9	25.1	+	+++	++	+++	+++	++	+++	Stigma protruded out before maturity	+++
E 2/24	37.8	+	+++	++	++	++	+	++	Gynoecium distorted	–
E 1/30	31.0	+	+++	++	+++	+++	++	++	–	+++
E 2/36	22.6	+	+++	++	+++	+++	++	++	–	+++
E 1/45	24.4	+	+++	++	+++	+++	++	+++	–	+++
E 1/51	34.5	+	+++	+++	+++	+++	++	++	Stamens-(5+2 staminode	+++
E 2/63	35.3	+	+++	++	+++	+++	++	++	–	+++
E 1/71	26.6	+	++	+++	+	+	+	+	Flowers very small, stigma protruded out before maturity	++
E 1/79	23.9	+	++	+++	++	++	+	++	Petals – 5	++
E 1/82	31.8	+	+++	++	+++	+++	++	++	–	+++
E 2/92	30.0	+	+++	++	+++	+++	++	++	–	+++
E 1/100	28.2	+	++	+++	++	++	++	+	Gynoecium distorted	–
E 1/101	25.6	+	++	++	++	+++	+	++	–	+++
E 2/108	35.6	+	+++	++	+	+	+	+	Flowers small, stamens-5	++
E 2/111	32.8	+	+++	++	+++	+++	++	++	Stigma protruded out before maturity	+++
E 1/119	27.7	+	+++	++	+++	+++	++	++	–	+++
E 2/131	29.9	+	++	++	+++	+++	++	++	Stigma protruded out before maturity and gynoecium distorted	++
E 1/135	34.5	+	+++	++	+++	+++	++	++	Stigma protruded out before maturity	+++
E 1/143	36.3	+	+++	++	+++	+++	++	++	–	+++
Parental materials:										
B. juncea cv T-59	88.4	+++	++	+++	++	++	+	+	–	++++
B. juncea cv B-85	79.8	+++	++	+++	++	++	+	+	–	++++
E. sativa	64.2	+++	++	+	++++	++++	+++	+	–	++

– Denotes no abnormality for h and no siliqua formation for i
\+ Denotes 3–4 cm or a, 0.5–0.7/sq cm for c, 0.3–0.4 cm for d, 0.5–0.7 cm for e, 0.2–0.3 cm for f and 0.1–0.12 cm for g
++ Denotes 0.11–0.14 cm for b, 1.2–1.5/sq cm for c, 0.6–0.8 cm for d, 1.1–1.5 cm for e, 0.3–0.4 cm for f, 0.18–0.2 cm for g and 2.5–3 cm for i
+++ Denotes 10–12 cm for a, 0.16–0.2 cm for b, 1.7–2/sq cm for c, 0.9–1 cm for d, 1.6–1.8 cm for e, 0.5–0.7 cm for f, 0.22–0.23 cm for g and 3.5–4 cm for i
++++ Denotes 1.2–1.5 cm for d, 2–2.4 cm for e and 5–6 cm for i

At diakinesis the chromosome configuration between the AB and the E genome (Prakash and Hinata 1980) of the somatic hybrids did not create a situation where auto- and allosyndetic characteristics could be ideally judged. Nevertheless, the appearance of one to two (observed at early diakinesis) quadrivalent(s) indicated that the possibility for genetic crossing-over to reconstruct the *B. juncea* genome exists. Additionally, generation of partial somatic hybrids through destabilization of *Eruca* chromosomes as the result of γ-irradiation has been shown to be feasible. That chromosome destabilization can be induced through irradiation has been shown earlier (Dudits et al. 1980; Chatterjee et al. 1988). Such partial somatic hybrids also open up the possibility for chromosome addition lines of *B. juncea* with chromosomes of *Eruca* genome. In the present reconstituted plant lines,

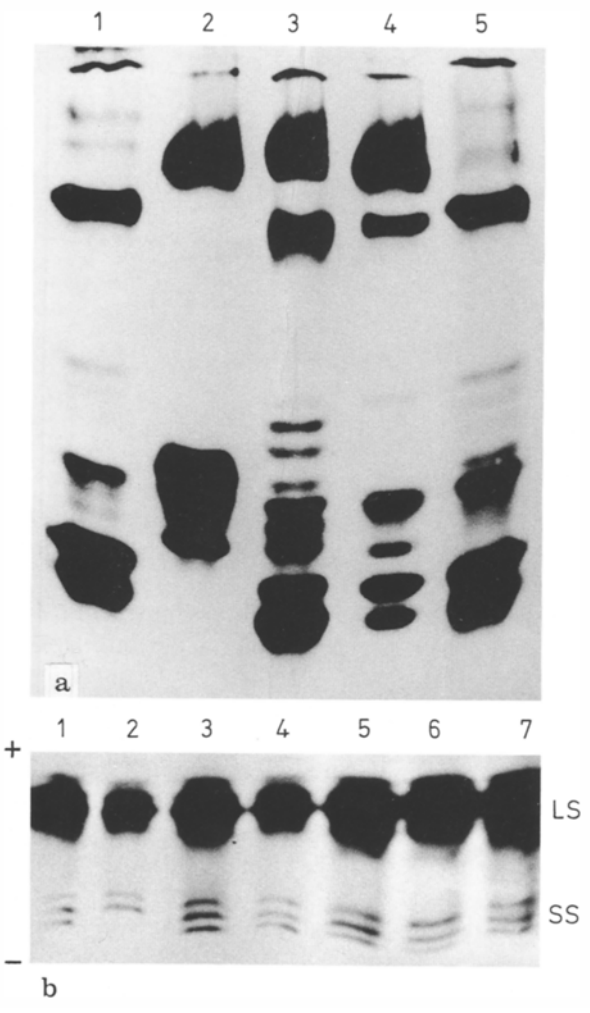

Fig. 3. **a** Esterase isozyme pattern of: *1 B. juncea* cv T-59; *2 E. sativa; 3* SH (line no. E 1/30); *4* SH (line no. E 2/5); *5 B. juncea* cv B-85. **b** Isoelectric focusing pattern of RuBP Case of *B. juncea, E. sativa,* and their somatic hybrids; *1 B. juncea* cv T-59; *2 E. sativa; 3* SH (line no. E 1/30); *4* SH (line no. E 1/143); *5* SH (line no. E 2/5); *6* SH (line no. E 1/45); *7 B. juncea* cv B-85

mutations, which remained unexpressed, may additionally be present in the *Eruca* genome as the result of irradiation. They may show up in subsequent generations. Thus, the significance of the present effort will rest on the behavior of the reconstituted plants and the genetic variability they might have acquired through this genetic introgression process. Our future studies will be directed towards such an assessment.

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