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Intergeneric hybrids between *Enarthrocarpus lyratus*, a wild species, and crop brassicas

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Summary. Attempts were made to produce intergeneric hybrids between *Enarthrocarpus lyratus*, a wild species, and several species of crop brassicas: *B. campestris*, *B. nigra*, *B. oleracea*, *B. juncea*, *B. napus* and *B. carinata*. Hybrids using *E. lyratus* as female parent were realized by means of embryo rescue in four combinations – *E. lyratus* × *B. campestris*, *E. lyratus* × *B. oleracea*, *E. lyratus* × *B. napus* and *E. lyratus* × *B. carinata*. Reciprocal crosses showed strong pre-fertilization barriers and yielded no hybrids except in one combination – *B. juncea* × *E. lyratus* – in which a single hybrid could be realized. All of the hybrids were multiplied in vitro through the multiplication of axillary shoots. Morphological and cytological studies confirmed hybridity. All hybrids were completely pollen sterile except for *E. lyratus* × *B. carinata*, which showed 2% pollen fertility. Attempts to double the chromosome number through the in vitro application of colchicine to axillary meristems of F_1 hybrids were successful in only one hybrid, *E. lyratus* × *B. oleracea*. Cytological studies of the hybrids indicated the presence of a partial homology between the genomes of *E. lyratus* and crop brassicas. Backcross progenies were raised from all of the five F_1 hybrids to develop male-sterile alloplasmic lines.

Key words: Crop brassicas – *Enarthrocarpus lyratus* – Intergeneric hybrids – Embryo rescue

Introduction

Many wild allies of crop brassicas in the *Brassica* coenospecies group are potential donors of desirable nuclear/organelle-encoded characters. These include resistance to fungal pathogens and cytoplasmic male sterility. Brassica species, which are an important source of edible oil and vegetables, are susceptible to several diseases such as alternaria blight, white rust and black leg, which cause severe losses in yield. Thus, wide hybridization to introgress genes conferring resistance to fungal pathogens from wild relatives to crop species and also to develop alloplasmic male-sterile lines for hybrid seed production is an attractive approach.

Enarthrocarpus lyratus, a weedy species endemic to the Mediterranean region, possesses resistance to white rust and alternaria blight. Hybridization between *Enarthrocarpus* and crop brassicas has been unsuccessful due to the occurrence of crossability barriers; even embryo culture has not been successful in obtaining hybrids from these crosses (Harberd 1976). In recent years, various in vitro methods such as ovary culture (Matsuzawa and Sarashima 1986; Takahata 1990; Takahata and Takeda 1990), ovule culture (Mohapatra and Bajaj 1984) and the sequential culture of ovaries and ovules (Nanda Kumar et al. 1988; Agnihotri et al. 1990) have successfully been employed in wide hybridization in brassicas.

We have crossed *E. lyratus* with six species of crop brassicas, *B. campestris*, *B. nigra*, *B. oleracea*, *B. juncea*, *B. napus* and *B. carinata*, using these different approaches. The present paper reports details on the crossability barriers, the efficacy of different in vitro methods – ovary, ovule and sequential culture – in realizing the hybrids and the morphology and cytology of the hybrids and induced amphidiploids.

Materials and methods

Plants of *Enarthrocarpus lyratus* (Forsk.) DC. (2n = 20 EnEn), *B. campestris* L. ssp. *oleifera* var 'brown sarson' cv 'Pusa Ka-

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lyani' (2n = 20, AA), *B. nigra* (L.) Koch 1C 257 (2n = 16, BB), *B. oleracea* L. var 'alboglabra' (2n = 18, CC), *B. juncea* (L.) Czern. cv 'Pusa Bold' (2n = 36, AABB), *B. napus* L. – 706 (2n = 38, AACC) and *B. carinata* A.Br. (2n = 34, BBCC) were grown under field conditions. Reciprocal crosses were carried out between *Enarthrocarpus* and the crop species. The aniline blue fluorescence method (Linskens and Esser 1957) was used to study pollen germination and pollen-tube growth in pollinated pistils.

In vitro methods

Pollinated ovaries were excised 4–6 days after pollination (DAP), surface sterilized in freshly prepared mercuric chloride (0.1%) and rinsed twice with sterilized distilled water; two to four ovaries were then cultured in a separate culture tube containing MS (Murashige and Skoog 1962) medium supplemented with casein hydrolysate (CH, 400 mg/l).

For ovule culture, the pollinated ovaries (10–15 DAP) were surface sterilized and then the ovules were excised aseptically; five to ten ovules were cultured in each culture tube.

For sequential culture, pollinated ovaries (4–6 DAP) were cultured as described above. The cultured ovaries were dissected 5–15 days later, and those ovules showing enlargement were cultured on a fresh medium.

Multiplication of hybrids

Seedlings obtained from cultured ovules were used for multiplication. The shoot tips were cultured on MS medium containing BAP (0.5 mg/l) for induction of multiple shoots. Rooting of shoots was induced on MS medium containing NAA (0.1 mg/l). The plantlets were transferred to pots containing garden soil and grown under field conditions.

Induction of amphiploidy and cytology

An aqueous solution of colchicine (0.1%) was applied by means of small cotton plugs to the axillary meristems of single node segments cultured on MS medium containing 0.5 mg/l BAP. Colchiploid shoots, produced in vitro, were rooted and subsequently transferred to soil. For cytological studies flower buds were fixed in Carnoys solution and anthers were squashed in 1% acetocarmine. Pollen fertility was studied using acetocarmine staining.

Results

Pollen germination was good in those crosses where *E. lyratus* was used as the female parent. Many pollen tubes grew through the stigma (Fig. 1A) and style (Fig. 1B)

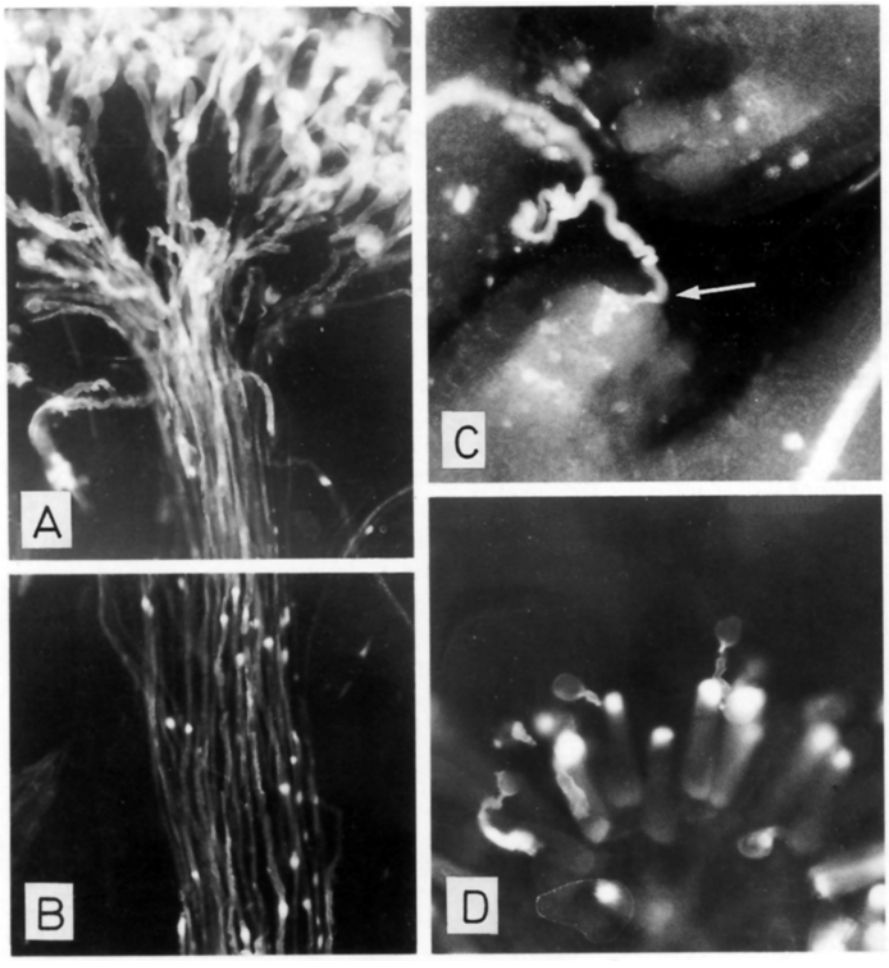

Fig. 1A–D. Aniline blue fluorescence of pollinated pistils in intergeneric crosses. **A–C** *E. lyratus* × *B. oleracea*: **A** part of the stigma showing profuse pollen germination and pollen tubes in the stigma and upper part of the style; **B** part of the style with many pollen tubes; **C** a part of the ovary showing pollen tube entry into the ovule (**arrow**). **D** *B. oleracea* × *E. lyratus*. A part of the stigma region. Pollen tubes have entered the papillae, but show swelling at their tips. Many of the papillae have developed callose at the tip

and entered the ovary. Many of the pollen tubes showed swelling and the deposition of thick callose plugs. Pollen tube entry into the ovules (Fig. 1C) was frequently observed.

In the reciprocal crosses, in which *E. lyratus* was the pollen parent, very few pollen grains germinated, and most of the pollen tubes failed to enter the papillae. A few of the pollen tubes which did enter the papillae showed swelling of the tube tip (Fig. 1D). Many of the stigmatic papillae developed callose at their tips. None of the pollen tubes were observed in the style.

No hybrid seeds were obtained in the field pollinations in any of the combinations. Although pollinated pistils showed some initial enlargement, they dried up in about 2 weeks when left on the plant.

Ovary culture

Ovary culture was not effective in realizing hybrid seeds in any of the crosses attempted. Although ovary culture yielded some seeds in two crosses – *B. napus* × *E. lyratus* and *B. carinata* × *E. lyratus* – all the plants raised from these seeds turned out to be matromorphs.

Ovule culture

Ovules excised 10–15 days after pollination were cultured on MS medium containing CH. The younger ovules that were excised 10 DAP showed a tendency to proliferate and form callus; they did not yield any hybrids. When the excision of ovules was delayed up to 15 DAP, all of the ovules had degenerated, and no healthy ovule was realized for culture in many of the combinations. Some healthy ovules were realized (in ovaries excised 15 DAP) in seven of the crosses and these were cultured (Table 1). Ovule culture yielded hybrids only in two of the crosses, *E. lyratus* × *B. oleracea* and *E. lyratus* × *B. carinata*. In other crosses all of the ovules turned brown and dried up within 10–15 days after culture.

Sequential culture

A majority of the cultured ovaries dried up without developing into fruits. About 10–15% remained fresh and showed the enlargement of a few ovules; these were excised and cultured (Table 2). The number of enlarged ovules were more in ovaries cultured for five days when compared to those cultured for 10 days; when culture of ovaries was extended for 15 days, all the ovules had collapsed and no healthy ovules were realized for culture. Sequential culture yielded hybrids in 5 combinations. In all successful crosses, hybrids were realized from only those ovules which were excised from ovaries cultured for 5 days.

In vitro multiplication of hybrids

The hybrid seedlings of all of the five successful crosses were multiplied through in vitro culture of shoot tips. About four to six new shoots from pre-existing axillary buds developed from cultured shoot tips on BAP medium in 4 weeks. A few cultured shoot tips in all of the hybrids showed in vitro flowering after 2–3 subcultures. Shoots of all the hybrids were successfully rooted on NAA medium; the seedlings were transferred to soil and grown to the flowering stage.

Induction of amphidiploidy

Of the 543 nodal segments (from the five successful F_1 hybrids) treated with 0.1% colchicine, nine nodal segments from two of the crosses, *E. lyratus* × *B. campestris* and *E. lyratus* × *B. oleracea*, produced slow growing shoots with thick, dark green leaves from the axils in 20–25 days. The shoots were rooted on NAA medium and subsequently transferred to soil. Only two amphidiploids of the cross *E. lyratus* × *B. oleracea* established and flowered; others died before they reached the flowering stage.

Table 1. Responses of cultured ovules of intergeneric crosses

Cross	Number of pollinated ovaries used for ovule culture	Number of enlarged ovules realized and cultured	Number of ovules germinated	Number of hybrids obtained
E. lyratus × *B. campestris*	69	0	0	0
B. campestris × *E. lyratus*	72	0	0	0
E. lyratus × *B. nigra*	58	43	0	0
B. nigra × *E. lyratus*	63	0	0	0
E. lyratus × *B. oleracea*	43	47	5	5
B. oleracea × *E. lyratus*	57	12	0	0
E. lyratus × *B. juncea*	60	76	0	0
B. juncea × *E. lyratus*	74	6	0	0
E. lyratus × *B. napus*	49	0	0	0
B. napus × *E. lyratus*	51	9	0	0
E. lyratus × *B. carinata*	54	32	1	1
B. carinata × *E. lyratus*	47	0	0	0

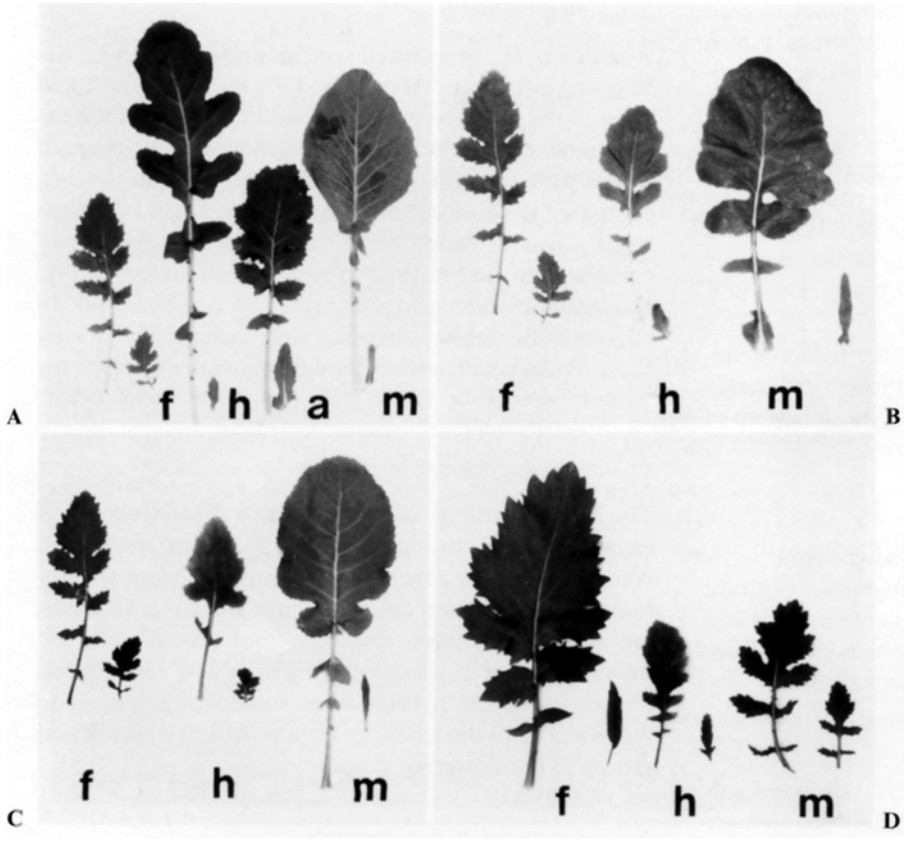

Fig. 2A–D. Leaves of the female parent (*f*), F_1 hybrid (*h*), amphidiploid (*a*) (in Fig. 2A only) and male parent (*m*) of intergeneric crosses. In each, larger one (*left*) is lower leaf and smaller one (*right*) is upper leaf. **A** *E. lyratus* × *B. oleracea*; **B** *E. lyratus* × *B. campestris*; **C** *E. lyratus* × *B. carinata*; **D** *B. juncea* × *E. lyratus*

Table 2. Results of sequential culture in intergeneric crosses

Cross	Number of ovaries cultured[a]	Number of enlarged ovules realized and cultured	Number of seedlings obtained	Number of hybrids obtained
E. lyratus × *B. campestris*	95	34	3	3
B. campestris × *E. lyratus*	259	117	0	0
E. lyratus × *B. nigra*	169	75	0	0
B. nigra × *E. lyratus*	69	12	0	0
E. lyratus × *B. oleracea*	196	136	25	25
B. oleracea × *E. lyratus*	65	57	0	0
E. lyratus × *B. juncea*	247	77	2	0
B. juncea × *E. lyratus*	155	48	1	1
E. lyratus × *B. napus*	167	87	3	3
B. napus × *E. lyratus*	157	34	0	0
E. lyratus × *B. carinata*	168	93	4	4
B. carinata × *E. lyratus*	71	20	0	0

[a] Ovaries were dissected 4–6 DAP

Morphology

The hybrids were intermediate to their respective parents in many of the morphological characters such as leaf shape (Fig. 2A–D), inflorescence and flower characters. Some of the characters were distinctive of the male or the female parent. The details of the morphological characters of the parents and the hybrids are given in Table 3.

Synthetic amphidiploids of *E. lyratus* × *B. oleracea* were also similar to the F_1 hybrid in morphological characters. However, a few characters of the amphidiploid were different from those of the F_1 hybrid. For example, the plant was green and glaucescent in the F_1 hybrid, while it was green and non-glaucescent in the amphidiploid. The flower was yellow in the F_1 hybrid, while it was white in the amphidiploid. The fruit of the am-

Table 3. Morphological characters of the parents and the hybrids

Character	Female parent	F_1 hybrid	Male parent
E. lyratus × *B. oleracea*			
Stem	40–50 cm, green, non-glaucescent, lower branches procumbent	60–70 cm, green, glaucescent, branches erect	100–125 cm, green, glaucescent branches erect
Leaves	Upper leaves petiolate, lyrate pinnatisect with runcinate lobes	Upper leaves petiolate, oblong with dentate margin	Upper leaves sessile, oblong, entire
Inflorescence	Flowers bracteate almost to the apex of the inflorescence	Same as in female parent	Flowers ebracteate
Flowers	Yellow with violet veins	Yellow	White
E. lyratus × *B. campestris*			
Stem	40–50 cm, green, non-glaucescent, lower branches procumbent	45–55 cm, green, non-glaucescent, lower branches procumbent	125–150 cm, green glaucescent branches erect
Leaves	Upper leaves petiolate, lyrate pinnatisect with runcinate lobes	Upper leaves petiolate, intermediate	Upper leaves sessile, with auricle claspings, lanceolate, entire
Inflorescence	Flowers bracteate almost to the apex of the inflorescence	Same as in female parent	Flowers ebracteate
Flowers	Normal	Most of the buds abscise before opening. Some petals modified into thin filaments, pistils curved	Normal
E. lyratus × *B. carinata*			
Stem	40–50 cm, lower branches procumbent	40–50 cm, lower branches procumbent	125–150 cm, branches erect
Leaves	Upper leaves petiolate, lyrate pinnatisect with runcinate lobes	Upper leaves petiolate, lyrate pinnatisect with dentate lobes	Upper leaves petiolate, oblanceolate, entire
Inflorescence	Flowers bracteate almost to the apex of the inflorescence	Same as in female parent	Flowers ebracteate
Flowers	Yellow with violet veins	Yellow	Light yellow
B. juncea × *E. lyratus*			
Stem	125–150 cm, branches erect	45–55 cm, lower branches procumbent	40–50 cm, lower branches procumbent
Leaves	Upper leaves lanceolate, subentire	Upper leaves intermediate	Upper leaves lyrate, pinnatisect with runcinate lobes
Inflorescence	Flowers ebracteate	Same as in the male parent	Flowers bracteate almost to the apex of the inflorescence
Flowers	Bright yellow	Yellow	Yellow with violet veins
E. lyratus × *B. napus*			
Stem	40–50 cm, green non-glaucescent, branches procumbent	50–60 cm, green glaucescent, branches ascending	125–150 cm, green glaucescent, branches ascending
Leaves	Leaves petiolate, lyrate pinnatisect with runcinate lobes	Lower and middle leaves petiolate, upper leaves sessile, intermediate in shape	Middle and upper leaves sessile with auricle claspings; middle leaves lyrate pinnatifid, upper leaves subentire, lanceolate
Inflorescence	Flowers bracteate almost to the apex of the inflorescence	Very few flowers at the lower part of the inflorescence are bracteate	Flowers ebracteate
Flowers	Yellow with violet veins	Yellow	Yellow

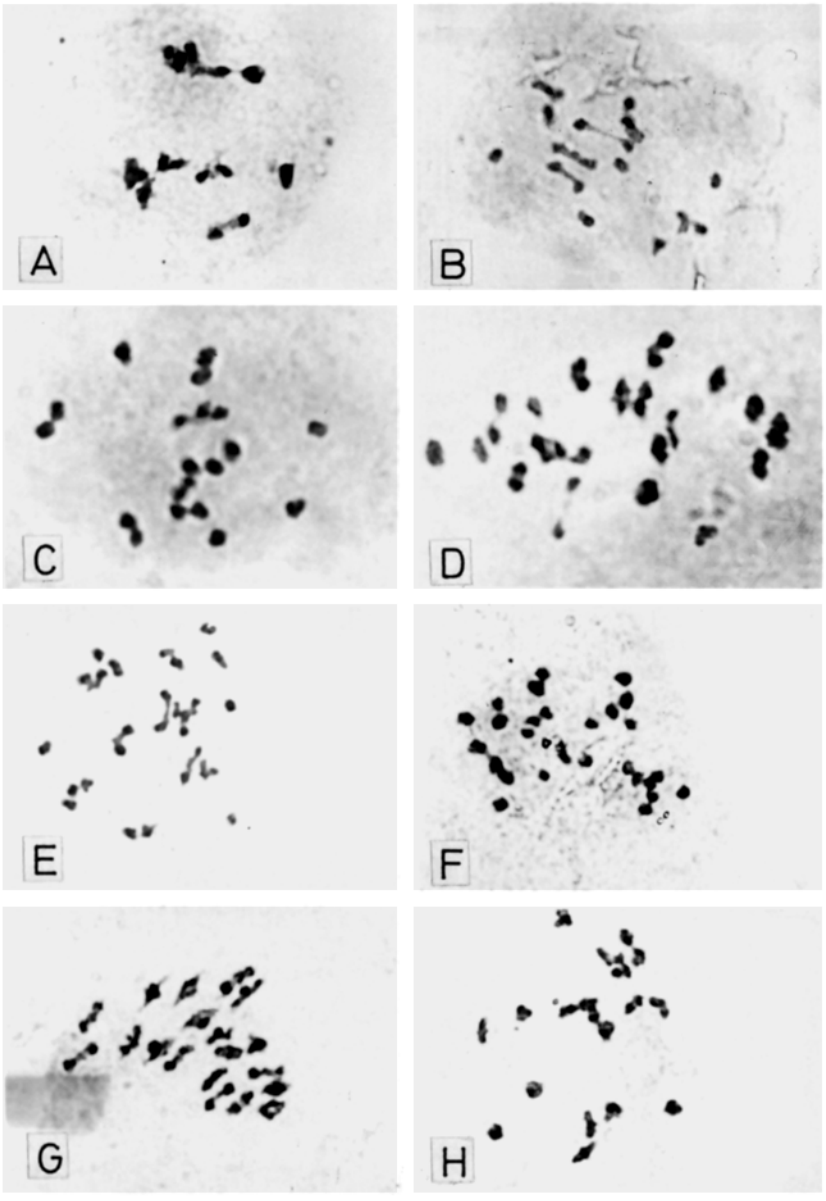

Fig. 3A–H. Metaphase I of meiosis in *E. lyratus* and in F_1 hybrids. **A** *E. lyratus* 6 II + 2 IV; **B** *E. lyratus* × *B. oleracea* 8 I + 4 II + 1 III; **C** *E. lyratus* × *B. campestris* 6 I + 4 II + 2 III; **D** *E. lyratus* × *B. carinata* 8 I + 13 II + 1 III; **E** *B. juncea* × *E. lyratus* 14 I + 7 II; **F** *E. lyratus* × *B. napus* 10 I + 6 II + 1 III + 1 IV; **G** and **H** *E. lyratus* × *B. oleracea*-induced amphidiploid: **G** 19 II; **H** 11 II + 1 IV + 2 VI

phidiploid was of the shattering type, and the seeds were brown and round.

Cytology and fertility

E. lyratus showed, in addition to the expected ten bivalents at metaphase I, 2 quadrivalents in 36% of the PMCs (Fig. 3A). Meiosis in the intergeneric hybrids was highly disorganized. There was a preponderance of univalents (Fig. 3E) and a significant number of bivalents (Table 4). Also, all of the hybrids had higher associations – tri- and quadri-valents (Fig. 3 B, C, D and F). One pentavalent each in *B. juncea* × *E. lyratus*, *E. lyratus* × *B. napus* was also recorded. Anaphase I and subsequent stages were highly irregular, a typical characteristic of wide hybrids, which resulted in the formation of sterile pollen. Only one F_1 hybrid *E. lyratus* × *B. carinata* showed 2% pollen fertility.

Induced amphidiploid *E. lyratus* × *B. oleracea* (2n = 38, EnEnCC) showed 19 bivalents at M_1 in a majority of cells in generation A_1 (Fig. 3G). Higher associations (Table 4) were also observed (Fig. 3H). These plants showed 82% pollen fertility. Nearly 20% of the flowers set fruits and seeds when allowed to open pollinate.

F_1 hybrids as well as induced amphidiploids were backcrossed with crop species, and a considerable number of BC_1 seeds were realized in all of the hybrids.

Table 4. Metaphase pairing of the F_1 hybrids and amphidiploids

Cross	Somatic chromosome number	Genome	Number of PMCs studied	Mean chromsome associations (range in parenthesis)					
				I	II	III	IV	V	VI
E. lyratus × *B. oleracea*	19	EnC	42	11.42 (6–9)	3.14 (0–6)	0.40 (0–2)	0	0	0
E. lyratus × *B. campestris*	20	EnA	58	13.65 (6–20)	2.20 (0–5)	0.62 (0–3)	0.02 (0–1)	0	0
E. lyratus × *B. carinata*	37	EnEnBC	39	8.49 (4–13)	12.89 (10–16)	0.35 (0–2)	0.48 (0–2)	0	0
B. juncea × *E. lyratus*	28	EnAB	70	12.89 (6–20)	6.04 (1–10)	0.47 (0–3)	0.31 (0–2)	0.07 (0–1)	0
E. lyratus × *B. napus*	29	EnAC	78	12.67 (3–19)	6.10 (3–12)	0.74 (0–2)	0.47 (0–2)	0	0
Amphidiploid									
E. lyratus × *B. oleracea*	38	EnEnCC	58	0	15.13 (7–19)	0.03 (0–1)	1.45 (0–6)	0.03 (0–2)	0.27 (0–2)

Discussion

The studies reported here confirm the earlier investigation of Harberd (1976) that unilateral incompatibility operates between *E. lyratus* and crop brassicas. The barriers to hybridization were largely postfertilization in crosses in which *E. lyratus* was used as the female parent, whereas the reciprocal crosses showed strong pre-fertilization barriers. Embryo rescue was essential even in crosses involving *E. lyratus* as the female parent as revealed by the failure to realize hybrid seeds through field pollinations. Ovary culture was not successful in any of the crosses. Ovule culture was successful in only two combinations: *E. lyratus* × *B. oleracea* and *E. lyratus* × *B. carinata*. The sequential culture method was superior to the culture of only ovaries or ovules and yielded hybrids in as many as five combinations; this was the only successful method in three of the crosses, *E. lyratus* × *B. campestris*, *E. lyratus* × *B. napus* and *B. juncea* × *E. lyratus*. Surprisingly, sequential culture was successful in realizing hybrids in the cross *B. juncea* × *E. lyratus* in which pollen germination was poor and pollen-tube growth was arrested in the stigma. Apparently, a few pollen tubes in at least some of the pistils were able to grow through the stigma and style and effect fertilization.

In the sequential culture method, the cultured ovaries showed enlargement of some ovules when these were dissected after 5–10 days of culture. However, most of the enlarged ovules showed degeneration when ovary culture was extended to 15 days. For the successful rescue of the hybrid embryos, it was therefore necessary to culture the enlarged ovules within 5 days after from culture of the ovaries. This may explain the superiority of sequential culture over ovary culture. Delourme et al. (1989) also suggested that the success rate of ovary culture could be improved when cultured ovaries are dissected and developing embryos are sub-cultured instead of letting them grow inside the ovaries.

As has been reported in many other wide hybrids (Nanda Kumar et al. 1988; Batra et al. 1989; Agnihotri et al. 1990) all five hybrids produced in the present investigations were multiplied through culture of shoot tips. This has been very useful in raising a large number of hybrid plants, particularly in difficult combinations, for cytological studies and for restoring fertility through the induction of colchiploidy. Plantlets obtained in vitro could be successfully transferred to soil.

Hybridity in all five combinations was confirmed by means of morphological and cytological studies. Hybrids were largely pollen sterile. Attempts were made to induce colchiploidy to restore fertility. However, it was successful only in one combination *E. lyratus* × *B. oleracea*.

Meiosis in hybrids was characteristic of wide hybrids with a preponderance of univalents. Bivalents were frequently recorded. Higher associations, tri- and quadrivalents with the occasional penta- and hexa-valents, were also observed. Chromosome homology between various genomes in Brassica has been thoroughly investigated (Prakash and Hinata 1980). Based on chromosome pairing in haploids of Brassica genomes and the hybrids between them, the extent of auto- and allo-syndesis has been studied. It was concluded that *B. campestris*, *B. nigra* and *B. oleracea* genomes can form 1 III + 2 II, 2 II, and 3 II, respectively, as a result of autosyndesis (Armstrong and Keller 1981, 1982; Prakash and Hinata 1980; Mizushima 1980). It may well be that *E. lyratus* also forms three autopairs based on $x=6$ basic number for the subtribe. The formation of bivalents may primarily be due to autosyndesis although allosyndesis cannot be ruled out. The occurrence of multivalents clearly re-

flects intergenomic affinity to a considerable extent. In view of these facts, we suggest that at least two trivalents are of an allosyndetic origin in *E. lyratus* × *B. oleracea* and *E. lyratus* × *B. campestris* hybrids. Similarly, in hybrids between digenomic species (*B. carinata*, *B. juncea* and *B. napus*) and *E. lyratus*, the presence of quadri- and penta-valents suggests the occurrence of intergenomic pairing. We, therefore, propose a partial homologous relationship between *Enarthrocarpus* and *Brassica* genomes. This affinity is also expressed in induced alloploid *E. lyratus* × *B. oleracea* (2n = 36, EnEnCC), which forms, besides 19 bivalents, higher associations such as quadri-, penta- and hexa-valents in generation A_1.

The hybrid *E. lyratus* × *B. carinata* was found to have 37 chromosomes against the expected number of 2n = 27. This hybrid seems to possess two genomes of *E. lyratus* and one genome of *B. carinata*, and probably arose as a result of fertilization of unreduced eggs in *E. lyratus*. This phenomenon is known to occur in Brassiceae at a relatively high frequency (Delourme et al. 1989; Ripley et al. 1990).

The intergeneric hybrids with various genomic constituents and the induced fertile amphidiploid, *E. lyratus* × *B. oleracea*, will serve as a bridge for cytoplasmic substitutions in crop species and also for the introgression of genes. A good amount of homology between *Brassica* and *Enarthrocarpus* genomes would facilitate gene transfer across generic barriers.

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