INTERSPECIFIC HYBRIDIZATION AND CHARACTERIZATION OF HYBRIDS IN GENUS *Cicer* L.

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1993

TO

ONE WHO CARED

CERTIFICATE

This is to certify that the thesis entitled **"INTERSPECIFIC HYBRIDIZATION AND CHARACTERIZATION OF HYBRIDS IN GENUS *CICER* L."** submitted in partial fulfillment of the requirements for the degree of **"MASTER OF SCIENCE IN AGRICULTURE"** of Indira Gandhi Krishi Vishwa Vidyalaya, Raipur (M.P.) is a record of the bonafide research work done by Shri Suresh V Naik under our guidance and supervision. The subject of the thesis has been approved by the student Advisory Committee and the Director of Instructions.

No part of the thesis has been submitted for any other degree or diploma (Certificate, awards etc.) or has been published. All the assistance and help received during the course of the investigation have been duly acknowledged by him.

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(SURESH V. NAIK)

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Word(s)	Abbreviations
6-Benzylaminopurine	BAP
cultivar	cv.
Gibberellic acid	GA
hours	h
hectare	ha
Hours After Pollination	HAP
Indole-3-acetic acid	IAA
ICRISAT Chickpea Cultivar	ICCC
ICRISAT Chickpea Wild Accession	ICCW
Kinetin	Kn, KIN
kilo volts	kv
1-Napthalicacetic acid	NAA
nano meter	nm
Pollen Mother Cell	PMC
parts per million	ppm
volume per volume	v/v
Micro meter	µm

INTRODUCTION

CHAPTER I
INTRODUCTION

Chickpea (*Cicer arietinum* L), is the only cultivated species of the genus *Cicer*, belongs to the tribe *Cicereae* Alef. of family, Leguminosae. Considering area under production, it is the third most important food legume crop in the world after dry bean (*Phaseolus vulgaris* L.) and dry pea (*Pisum sativum* L.). Chickpeas are grown annually in an area of about 10.7 million ha. with a production of approximately 7.7 million tons and is an important source of protein particularly in the Indian subcontinent. India, Pakistan, Bangladesh and Nepal together account the 76% of world's chickpea production and 80% of area (FAO, 1992). Among the twelve major pulse crops grown in India, chickpea stands first with a contribution of 28% to total pulse area and 34% to total pulse production (FAO, 1992).

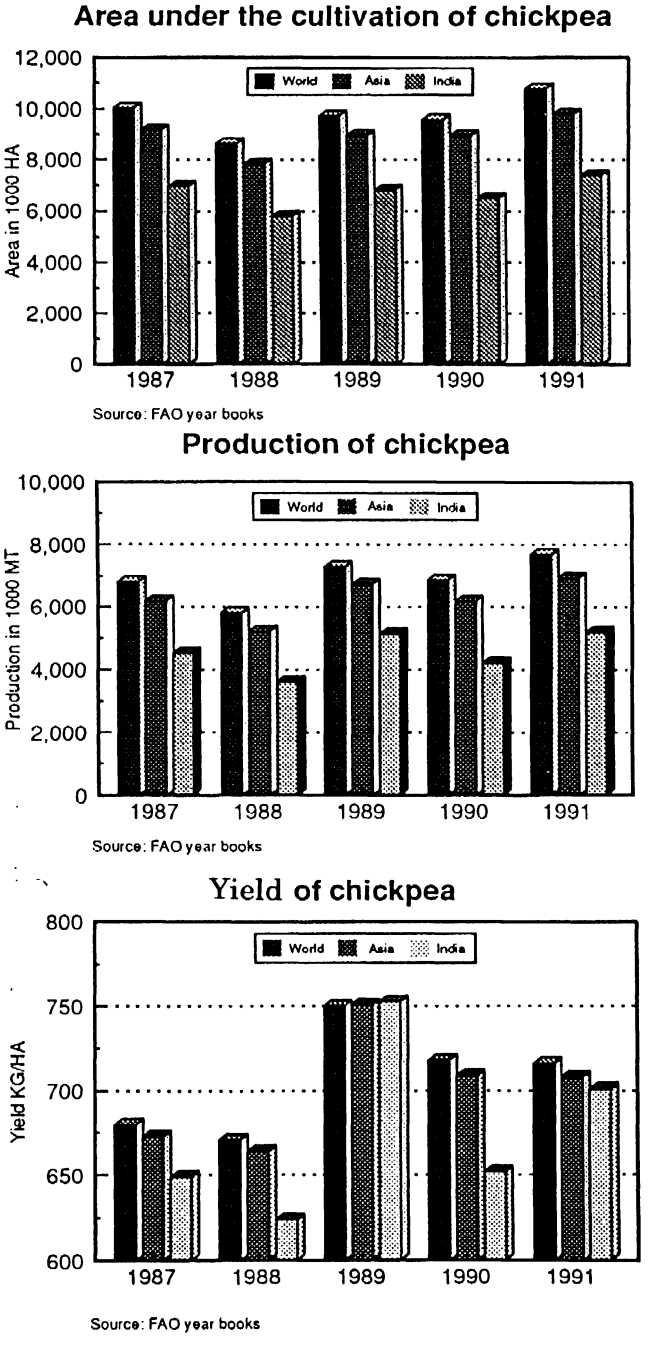


Figure 1: Comparative data of area, production and Yield of chickpea for world, asia and India

Increased yield, which is a major goal in chickpea breeding, can be achieved by breeding for various objectives including incorporation of resistance to diseases, insects and nematodes, as well as tolerance to environmental stresses such as cold, heat, drought and salt (Singh, 1987). More than 50 diseases causing pathogens have been reported on chickpea so far from different parts of the world (Nene, 1990), out of which some very serious diseases, in order of importance, are: Ascochyta blight, Fusarium wilt, Botrytis gray mould, Black root rot, Phytopthora root rot, and Pythium root rot. Ascochyta blight damage in Pakistan resulted in severe shortage of pulses and required $US 7.43 million worth of import in 1982/83 (Malik 1984). Fusarium wilt causing 10% loss in yield has been regular feature in chickpea growing states of India (Singh and Dahiya, 1973). Likewise an estimated annual loss of $US 1 million was reported in Pakistan (Sattar *et al.*, 1953). This disease is considered to be the main cause of failure for the winter crop in different parts of the world (Byth *et al.*, 1980) Botrytis gray mold was responsible for heavy losses in the Indo-Gangetic plains of India during 1979-82 (Grewal & Laha, 1983).

Most important insect pest of chickpea is pod borer (*Heliothis armigera* Hubner) which damages up to 20% of the crop (Reed *et al.*, 1987). Unfortunately, most of the genotypes selected for the resistance against pod borer from the germplasm have been found to be very susceptible to Fusarium wilt. Leaf miner is another important insect pest of chickpea. Kay (1979) indicated that crop losses caused by the leaf miner in USSR range from 10-40%. Cyst nematode infests about 24% of chickpea crop in major chickpea growing areas of Syria (Greco *et al.*, 1984). But no resistant source from the present germplasm collection has been found for the cyst nematode (Greco, 1987).

The use of resistant cultivars is the best method to minimize the loss, since the other methods are either impractical or not economically feasible. Identification of stable genetic source with tolerance or resistance to various root rots, wilt, and Ascochyta blight should be one of the major objectives in *Cicer* breeding. One of the main reasons for slow progress through breeding of chickpea is the efforts of breeders in the past with a narrow genetic base (Singh, 1987). Utilization of wild species in breeding program is one method designed to introduce additional germplasm into cultivated varieties (Stalker, 1980). Frey (1983) reported that an increase in biomass and yield potential by introduction of alien germplasm in advanced breeding lines, using wide hybridization in oats, barley, sorghum and pearl millet. Such an approach may be profitable in chickpea (Singh, 1987). Increasing numbers of breeding programs are utilizing wild related germplasm for crop improvement (Harlan, 1976).

When the germplasm collection of chickpea were utilized, scientists found that these collections of chickpea do not have the desired genes for some of the valuable characters, such as three or four flowers per peduncle or four or five seed per pod, but these traits are present in wild *Cicer* species (Singh, 1987). In last two decades, the situation has changed and now breeders have access to a large number of germplasm accessions of wild *Cicer* species (Malhotra *et al.*, 1987). When the annual wild species and chickpea cultivars were screened against the resistance for various biotic and abiotic stresses at ICARDA, Syria, sources of resistance were found for *Ascochyta* blight, leaf miner, seed beetle,

cyst nematode, and cold. Wild species are the only source of resistance so far to seed beetle and cyst nematode and they have higher levels of resistance than the cultivated species for *Ascochyta* blight, leaf miner, and cold (ICARDA, 1989). Several other earlier reports showed the presence of resistance in the wild *Cicer* species (**Table 1**).

The genus *Cicer* includes 42 known wild species including 9 annual ones, which are a valuable source of variability for various desirable traits available to breeders (van der Maesen, 1987). These wild genes can only be useful if they can be transferred to cultivated chickpea. The efforts required to transfer even a single gene from a wild to a cultivated species is often very extensive, and quantitative traits are even more difficult to transfer (Stalker, 1980).

Genetic relationships among the seven annual *Cicer* species depending upon their interspecific crossability has been reported by Ladizinsky and Adler (1976a, b), which is very useful in utilization of wild species for the transfer of desirable traits from wild to cultivated *Cicer*. The knowledge available for other *Cicer* species is meager, because interspecific hybrids are difficult to produce among many of them (Pundir and van der Maesen, 1983), and more efforts are needed on the crossability among the various wild *Cicer* species. Since most of the wild *Cicer* species are not cross compatible with *C. arietinum* (chickpea) or between themselves, concerted efforts are required to overcome barriers to interspecific hybridization (Malhotra *et al.*, 1987).

The wild annual *Cicer* species have greater potential as a source of utilizable germplasm than the perennial wild *Cicer* species, because the annual habit of plants are thought to have evolved from primitive perennial forms, and cultivated *Cicer* was supposed to have evolved from an annual wild

ıle 1 Reported sources of resistance in wild *Cicer* species to biotic and abiotic constraints.

ıecies	Observations	Source
reticulatum	Resistant to Ascochyta blight.	Reddy and Nene (1978) Singh *et al.* (1981a)
	Resistance to seed beetle	ICARDA (1989)
echinospermum	Tolerant to Ascochyta blight, leaf miner and cold	ICARDA (1989)
	Resistance to seed beetle	ICARDA (1989)
pinnatifidum	Resistant to Ascochyta blight	Sandhu (1980a.b) Singh *et al.* (1981a)
	Tolerant to cold	ICARDA (1989)
judaicum	Resistant to botrytis grey mould	Singh *et al.* (1982a)
	Resistant to Ascochyta blight	Sandhu *et al.* (1980a,b) Singh *et al.* (1981b)
	Resistance to leaf miner	ICARDA (1989)
	Tolerant to cold	ICARDA (1989)
bijugum	Tolerant to Ascochyta blight	Sandhu *et al.* (1981a) ICARDA (1989)
	Seed-size 9 g/100 seeds.	van der Maesen and Pundir (1984)
	Tolerant to leaf miner	ICARDA (1989)
	Resistance seed beetle	ICARDA (1989)
	Resistance to cold	ICARDA (1989)
	Resistance to cyst nematode	ICARDA (1989)
chorassanicum	Resistance to leaf miner	ICARDA (1989)
cuneatum	Tolerent to Ascochyta blight	Singh *et al.* (1981a) ICARDA (1989)
	Good vigour, 3 seeds	Singh *et al.* (1981a)
	Resistance to seed beetle	ICARDA (1989)
yamashitae	Tolerant to leaf miner	ICARDA (1989)

species. Considering insufficient availability of the information on the barriers of interspecific hybridization and interrelationship among various annual species of *Cicer*, present study was planned with following major objectives:

1. To produce interspecific hybrids between annual *Cicer* species
2. To evaluate pre-fertilization barriers to interspecific hybridization
3. To rescue interspecific hybrid embryos through *in vitro* methods
4. To characterize parents and hybrids using morphological, cytological, and scanning electron microscope studies of pollen grains and
5. To understand the interrelationships among the annual *Cicer* species.

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

2.1 TAXONOMY AND BIOSYSTEMATICS OF GENUS *Cicer*

Old world domesticated chickpea (*Cicer arietinum* L.) is one of the first grain legumes, grown mainly in central and west Asia, south Europe, Ethiopia and north Africa (van der Maesen, 1987). The genus *Cicer* L. is a member of the family Leguminosae and subfamily Papilionoideae. It was earlier classified in the tribe *Vicieae* Br. along with genera *Pisum, Lens, Vicia, Lathyrus, and Vavilovia* (Hutchinson, 1964; Gunn, 1969). Recent evidences using various lines of investigations viz., somatic chromosome number (Ramanujam, 1976), pollen grain ultra structure (Gapochka, 1974; Clarke and Kupicha, 1976), seed morphology and testa topography (Lersten and Gunn, 1981), disulfide-linked

subunits of legumin (Vairinhos and Murray, 1982, 1983), interphase nuclear structure (Patankar and Ranjekar, 1984), seedling morphology (Nozzolillo, 1985), and chemical structure of Isoflavinoids (Ingham, 1981), recommended the removal of *Cicer* from *Vicieae* and placement in the monogeneric tribe *Cicereae* Alef.(Kupicha, 1981).

The genus *Cicer* comprises 43 species, of which 9 are annual, 33 perennial and one, *C. laetum* Rass & Sharip., with unspecified status (van der Maesen, 1987). The nine annual species of the genus *Cicer* are: *C. arietinum* L., *C. reticulatum* Lad., *C. echinospermum* Davis, *C. pinnatifidum* J.& S., *C. judaicum* Boiss., *C. bijugum* Rich., *C. cuneatum* Rich., *C. chorassanicum* (Bge.) M.Pop., and *C. yamashitae* kit.. All annual species are diploid with 2n=16 chromosome number. *Cicer arietinum* is the only cultivated species in this genus.

Cicer arietinum exhibits marked variation in respect to seed size, seed coat colour, seed surface, cotyledon colour, flower colour, size of leaflets, height etc. Based on the size, shape and coloration of seeds and flower petals, chickpea is divided into two major types, *kabuli* and *desi*. *Kabuli* is characterized by large round seeds, thin, white or pale cream seed coat and white flower petals, whereas, *desi* refers to smaller, angular seeds with thick seed coat and differently pigmented seed coat, and petals. Both types are easily crossable with each other (Auckland and van der Maesen, 1980).

There are many agronomically desirable traits present in both annual and perennial wild *Cicer* species. Resistance to ascochyta blight, has been found in *Cicer judaicum, C. pinnatifidum, C. reticulatum, C. anatolicum and C. montbretti* (Sandhu, 1980a; 1980b; Singh *et al.*, 1981), while *C. bijugum, C. cuneatum* and *C. yamashitae* are reported as tolerant to ascochyta

blight. Singh *et al.* (1982), and Madhu and Bedi (1986) reported that *C. judaicum* and *C. pinnatifidum* possess resistance to botrytis grey mold. Nene and Haware (1980) reported that *C. judaicum* carries resistance to fusarium wilt. Tolerance to cold was found in *Cicer microphyllum* (van der Maesen and Pundir, 1984). The wild species also show genetic variability for plant habit and morphology, protein content and adaptation to stress environments such as drought and infertile habitats (van der Maesen and Pundir, 1984). *C. cuneatum* produces more than three seeds per pod (Singh *et al.*, 1981).

Despite wild *Cicer* spp. being valuable source of desirable traits for chickpea improvement (van der Maesen and Pundir, 1984), little evaluation work has been done so far on the wild species, because of their poor adaptability at ICARDA (Aleppo, Syria) and ICRISAT (Patancheru, India), where most accessions of these species are maintained (**Table 2**).

2.1.1 CYTOLOGICAL STUDIES IN GENUS *Cicer*

Cytogenetic information is very valuable to plant breeders for understanding the chromosome structure and behavior in a species, and is relevant in the manipulation of sets of chromosomes, individual chromosome(s) or chromosomal segment(s) in order to maximize achievements in crop improvement. In chickpea, there is an increasing amount of cytogenetical information being generated from research program. Dombrovsky-Sludsky (1927) was first to report mitotic studies in *C. arietinum*. Since then, many workers have investigated chromosome number and morphology of chickpea. It has now been well established that chickpea has sixteen chromosomes in its somatic cell (2n=16), although early studies sometimes indicated 2n=14 (**Table 3**).

Table 2 Wild *Cicer* species in Collection at ICRISAT and ICARDA

Species	Number of accessions at		Origin
	ICRISAT	ICARDA	
Annuals			
C. bijugum	5	2	Turkey
C. chorassanicum	3	3	Afghanistan
C. cuneatum	1	0	Ethiopia
C. echinospermum	4	0	Turkey
C. judaicum	4	3	Lebanon
C. pinnatifidum	6	5	Turkey
C. reticulatum	4	1	Turkey
C. yamashitae	3	1	Afghanistan
Perennials			
C. anatolicum	3	1	Turkey
C. floribundum	1	0	Turkey
C. microphyllum	8	0	India
C. montbretii	2	2	Turkey
C. pungens	9	6	Afghanistan
C. rechingeri	1	0	Afghanistan

Table 3 Chromosome number reported in wild *Cicer* species

Species	Chromosome number (2n)	References
Annuals		
C. reticulatum	16	Ladizinsky and Adler 1976a,b Sharma and Gupta 1983, 1986
C. echinospermum	16	Ladizinsky and Adler 1976a,b
C. pinnatifidum	16	Iyengar 1939 Mercy et al. 1974a Ladizinsky and Adler 1976b Sharma and Gupta 1983, 1986
C. judaicum	16	van der Maesen 1972 Ladizinsky and Adler 1976b Sharma and Gupta 1983, 1986
C. bijugum	16	van der Maesen 1972 Ladizinsky and Adler 1976b Sharma and Gupta 1983,1984,1986
C. chorassanicum	16	Podlech and Dieterla 1969 Sharma and Gupta 1983, 1986
C. cuneatum	16	van der Maesen 1972 Ladizinsky and Adler 1976b Sharma and Gupta 1983, 1986
C. yamashitae	16	Ahmad and Slinkard *In* van der Maesen 1987
Perennials		
C. anatolicum	14,16	van der Maesen 1972
C. canariense	24	Santos Guerra and Lewis 1986
C. floribundum	14	Contandriopoulos et al. 1972
C. heterophylum	16	Contandriopoulos et al. 1972
C. incisum	16	Contandriopoulos et al. 1972 van der Maesen 1972
C. isauricum	16	Contandriopoulos et al. 1972
C. soongaricum	14 16	Iyengar 1939 Mercy et al. 1974a

Dombrovsky-Sludsky (1927) reported the diploid chromosome number in chickpea as 2n=14. This was endorsed by Rao (1929), Frahm-Leliveld (1957), Singh (1964), and Furnkranz (1968). Dixit (1932b) observed that the diploid chromosome number in *desi* cultivar Type-22 was 14; however, the gigas mutant of that cultivar had 2n=16. Later, Thomas and Revell (1946) found there is no difference in chromosome number between normal and gigas type, both had 2n=16 chromosomes.

Dixit (1932a), who investigated mitosis and meiosis of two *desi* and two *kabuli* lines, reported that the diploid chromosome number in *desi* type was 14, while in *kabuli* type it was 16. Dixit (1932b) considered *kabuli* type, which he designated as *C. kabulicum*, with large white flowers and seeds, to have originated as a chromosomal mutation from a small, white seeded line. Cobley (1956) supported Dixit (1932a, 1932b) and observed that the haploid chromosome number in *desi* and *kabuli* types to be 7 and 8, respectively. However, subsequent workers, including Vyas and Mehrotra (1963) and Sharma (1983) reported that the chromosome number in both *desi* and *kabuli* types was invariably 2n=16. Iyengar (1939) studied somatic and meiotic divisions in 30 lines of chickpea. Although these lines were from different locations and varied phenotypically, in all cases the chromosome number was invariably 2n=16. This was confirmed by a majority of later reports (Ramanujam and Joshi, 1941; Ahmed *et al.*, 1952; Phadnis, 1970; Sohoo *et al.*, 1970; Deromedis and Ochoa, 1974; Mercy *et al.*, 1974a; Ladizinsky and Adler, 1976a, 1976b; Ahmed and Godward, 1980; Sharma and Gupta, 1982; and Lavania and Lavania, 1983)(**Table 3**).

2.1.2 KARYOMORPHOLOGICAL STUDIES IN GENUS *Cicer*

The chickpea karyotype that has emerged from cytological investigations (Gupta and Bahl, 1983) as quoted by Bahl (1987) "is:

1. A pair of very long chromosomes, distinctly satellited and submetacentric.
2. Six pairs of metacentric to submetacentric chromosomes.
3. A pair of very short metacentric chromosomes."

Dombrovsky-Sludsky (1927) reported that all chromosomes of *Cicer arietinum* were attached to the nucleolus. Later, based on cytological investigations on 30 lines of chickpea, Iyengar (1939) concluded that four chromosomes were attached to the nucleolus during prophase. However, a review of the studies, including those of Meenakshi and Subramaniam (1963a, b), Vyas and Malhotra (1963), Mercy *et al.* (1974a), Ahmed and Godward (1980), and Sharma and Gupta (1982), leaves little doubt that only two chromosomes are attached to the nucleolus.

Phadnis (1970) reported general homogeneity of chromosome complements among seven morphologically distinct cultivars of chickpea. However, he felt that variations among these cultivars with regards to the position of primary and secondary constrictions and the length of the chromosomes could be used as criteria for identifying the cultivars cytologically. Ahmed and Godward (1980) investigated three chickpea lines and reported small chromosomal differences among them with respect to length of chromosomes, and arm ratio. Kutarekar and Wanjari (1983) conducted karyomorphological studies on 12 chickpea lines which differed morphologically. Each line was characterized by its own karyotype. Total

chromatin length of haploid complement of the lines ranged from 25.5 to 35.7 μm.

Vyas and Malhotra (1963) measured chromosome length, arm ratio and chromatin length per cell in one *kabuli* and one *desi* cultivar of chickpea. They claimed that the two cultivars differed significantly in characters such as total chromatin length, the position of centromere, size of chromosomes and arm ratio. Other researchers, however, observed no significant differences in the two types of chickpea. On the basis of cytological studies on four cultivars, two each of *kabuli* and *desi* types, Kharkwal (1978) reported that, in general, there was a close similarity in the morphology of the chromosomes in both types of cultivars. Sharma (1983) subjected six chickpea lines, two *kabuli* and four *desi*, to karyotypic analysis using root tip mitosis, and observed no significant variation among different lines.

Almost all the cytological studies designed to show differences and similarities among the cultivars of chickpea, have reported a general homogeneity in respect to overall morphology of the chromosome complements. At the same time, small chromosomal differences recorded may be real, or may be partly due to artifacts of technique. Bahl (1987) postulated that the differences might be due to small chromosomal changes in different microclimates.

2.1.3 SATELLITED CHROMOSOMES IN GENUS *Cicer*

Chickpea is most often reported to have one pair of satellited chromosome, but there have been reports of two pairs. One pair of satellited chromosome was reported by Dombrovsky-Sludsky (1927), Meenakshi and Subramaniam (1960), Vyas and Mehrotra (1963), Mercy *et al.* (1974a), Ahmed and Godward (1980), Sharma and Gupta (1982) and Lavania and Lavania

(1983). The satellite was observed on the longest chromosome pair by many workers (Meenakshi and Subramaniam, 1962; Phadnis, 1970; Mercy *et al.*, 1974a; Ahmed and Godward, 1980; Kutarekar and Wanjari, 1983; Lavania and Lavania, 1983; Sharma, 1983). Phadnis (1970) observed that the longest pair of chromosomes had a satellite at the short arm, whereas, Sharma (1983) found a satellite attached to the long arm of the longest pair of chromosome.

Meenakshi and Subramaniam (1962) investigated different roots and cells within the same root of chickpea and observed an intergrade between two pairs of chromosomes with a normal satellite and others with tandem satellites,i.e, two satellites are attached to the same chromosome. Phadnis (1970) observed satellites in two pairs of chromosomes in cv. N 59 and in both arms of one chromosome pair in cv. NP 100. Double satellited chromosomes were first reported by Phadnis and Narkhede (1969). Kharkwal (1978) observed one and two pairs of satellited chromosomes in *kabuli* and *desi* types, respectively. Kutarekar and Wanjari (1983) investigated seven cultivars of chickpea and reported that two cultivars, i.e, N 59 and RS 11, had two pairs of satellite chromosomes while the remaining five had only one such pair (**Table 4**).

2.2 CYTOLOGICAL AND KARYOMORPHOLOGICAL STUDIES IN GENUS *Cicer*

Cytological information on different *Cicer* species, other than *C. arietinum*, is restricted only to chromosome count, with little information on karyotype or pachytene analysis. All *Cicer* species except *C. pungens, C. montbretii, C. soongaricum*, and *C. anatolicum* have 2n=16. The diploid chromosome number was reported to be 14 in *C. pungens*, both 16 and 24 in *C. montbretii*, and 14 and 16 in *C. soongaricum* and *C. anatolicum*, respectively.

From the foregoing account of chromosome number in different *Cicer* species, it is clear that the most common somatic chromosome number is 2n=2x=16. Deviation from 16 to 14 reported may be erroneous, as was shown in *C. arietinum* and *C. soongaricum*. Mercy *et al.* (1974a), based on their study on *Cicer arietinum, C. soongaricum*, and *C. pinnatifidum*, argued that 2n=14 chromosome plants would be rare, and such plants might not survive in nature. Thus the basic chromosome number for *Cicer* is probably x=8. According to Ramanujam (1976), this basic number does not agree with basic number (x=7) for the tribe *Vicieae* in which *Cicer* is placed, but would agree with tribe *Trifolieae*.

On the basis of interphase nuclear structure in Leguminosae, Patankar and Ranjekar (1984) supported Ramanujam on the question of placement of *Cicer* in tribe *Vicieae*. They observed that *Cicer* showed chromocentric structure with about 12% condensed chromatin. Earlier, Lersten and Gunn (1981) also supported transfer of *Cicer* from *Vicieae* to the monogeneric *Cicereae* on the basis of scanning electron microscopic studies.

Karyotypic studies conducted on *Cicer* species have brought out some interesting varietal and specific characteristics about chromosome morphology. Iyengar (1939) studied 2 lines of *C. soongaricum*, and 30 lines of *C. arietinum* and compared his observations with that of Avdulov's (1937) on *C. pinnatifidum*. Iyengar (1939) reported that, in the above three species, there were marked size differences between the chromosomes, the shortest pair being about one-third of the length of the longest pair. The chromosome complement of *C. soongaricum* was the largest and that of *C. pinnatifidum* was the smallest, however, *C. arietinum* falling in between. Later, Mercy *et al.* (1974a) confirmed the observations of Iyengar and reported the average chromosome length in *C. soongaricum*, *C. arietinum* and *C. pinnatifidum* to be 2.70, 2.68, and 2.48 µm,

respectively. According to their study, the chromosome complement of *C. arietinum* consists of two long, four medium, one short and one very short pair of chromosomes, whereas, that of *C. soongaricum* was made up of two long, three medium, two short and one very short pair of chromosomes. Based on chromosome measurements in three varieties of chickpea, Ahmad and Godward (1980) numbered chromosomes from 1 to 8 in order of decreasing size of the chromosomes, and observed a size ratio between pair one and pair eight.

2.3 MEIOTIC CHROMOSOME ASSOCIATION IN GENUS *Cicer*

Few meiotic studies on chickpea and related *Cicer* species have been conducted to determine the morphology and behavior of individual chromosomes. Iyengar (1939) and Thomas and Revell (1946) observed that meiosis in *C. arietinum* is characterized by extreme diffused stage in diplotene and secondary association in metaphase I. Based on his findings of secondary association, constant attachment of four chromosomes to the nucleolus and the presence of four satellites, Iyengar considered *C. arietinum* to be an allotetraploid with duplication of chromosomes that took place during evolution. From karyotypic studies on seven cultivars of chickpea, Phadnis (1970) found chickpea to be diploid and ruled out the possibility of this species being tetraploid. Mercy *et al.*, (1974a) who conducted meiotic studies on *C. arietinum*, *C. soongaricum*, and *C. pinnatifidum*, reported that meiosis with eight bivalents at metaphase I was normal in all three species. However, a precocious disjunction of one or two pairs of chromosomes at metaphase I was observed in *C. arietinum*. Ladizinsky and Adler (1976a, 1976b) studied meiosis in six *Cicer* species, viz., *C. arietinum*, *C. echinospermum*, *C. reticulatum*, *C. pinnatifidum*, *C. judaicum and C. bijugum*. Meiosis was observed to be regular with eight bivalents in all the species. The number of chiasmata per cell

was 14.2 in *Cicer reticulatum*, 11.1 in *C. echinospermum*, 10.0 in *C. bijugum*, 9.8 in *C. pinnatifidum* and 10.1 in *C. arietinum*.

Sharma (1983), and Sharma and Gupta (1983), studied chromosome association and chiasmata frequencies in *Cicer chorassanicum, C. cuneatum, C. arietinum, C. bijugum, C. judaicum, C. pinnatifidum*, and *C. reticulatum*. In all the seven species, meiosis was regular and no meiotic abnormality was observed, although precocious disjunction of one large bivalent was noticed in some species. Chiasma frequencies were found to be independent of chromosome length which was attributed partly to karyotypic asymmetry. Mean chiasma frequency per cell was 15.5 in *C. bijugum*, 14.0 in *C. cuneatum*, 13.0 in *C. chorassanicum* and *C. pinnatifidum*, 12.8 in *C. arietinum*, 12.4 in *C. judaicum* and 11.5 in *C. reticulatum*.

During the course of detailed pachytene analysis of four species, viz., *Cicer arietinum, C. bijugum, C. chorassanicum* and *C. reticulatum*, Sharma (1983) observed that the number of metacentric and submetacentric chromosomes in meiosis was different from that of mitosis. He attributed the differences in chromosomal observations during the two stages to the differential condensation of chromosomes and even of different arms of the same chromosome.

2.4 CHROMOSOME PAIRING IN INTERSPECIFIC *Cicer* HYBRIDS

Ladizinsky and Adler (1976a,b) studied meiotic chromosome associations in six interspecific hybrids of *Cicer* species. The interspecific hybrid of *C. arietinum X C. reticulatum* was easy to obtain, developed normally, had regular meiosis with 8 bivalents and was fully fertile. Similar results were also reported by Ahmad (1988). In contrast, the hybrid of *C. reticulatum X C. arietinum* (line 58F) showed a quadrivalent, an anaphase I bridge and a

fragment during meiosis, indicating that this *Cicer arietinum* cultivar differed from *C. reticulatum* by a translocation or a paracentric inversion (Ladizinsky and Adler 1976a). Ahmad (1988) cytologically observed two quadrivalents in the hybrid *C. arietinum X C. echinospermum*, indicating that these two species differed by reciprocal translocation. The quadrivalent was also observed in one pollen mother cell of the interspecific hybrid of *C. judaicum X C. pinnatifidum*. The reciprocal of this cross however, was characterized by normal bivalent associations with occasional univalent formation (Ahmad 1988). Univalent formation was lowest in the hybrid of *C. pinnatifidum X C. bijugum* and highest in *C. judaicum X C. pinnatifidum*. Thus, chromosome association data indicate a close chromosome homology among these three species. In all the three hybrids, the stigma and style grew out of the keel at anthesis, leaving the anthers inside, these hybrids were functionally sterile in spite of relatively high pollen fertility (30-50%). However, F_2 seeds were produced in these three interspecific hybrids following hand pollination (Ladizinsky and Adler 1976b). At meiosis, in the cross *C. judaicum X C. chorassanicum*, chiasma frequency was reduced resulting in the presence of many univalents. Univalents were seen as chromosomes that lagged at early anaphase I and then moved away from the two poles. They were reported to be lost or have formed micronuclei, resulting in unequal sized pollen nuclei.

2.5 INTERSPECIFIC HYBRIDIZATION IN GENUS *Cicer*

Mercy and Kakar (1975) were first to attempt interspecific crosses between the cultivated annual *Cicer* species *C. arietinum* and a perennial species *C. soongaricum*. Although a total of 4,200 pollinations were made, not a single hybrid seed was obtained. Ladizinsky and Adler (1976a, b) used seven annual *Cicer* species, including *C. arietinum*, and made 14 out of 21 possible one-way crosses to study the meiosis in interspecific hybrids. The six successful

interspecific crosses were : *C. arietinum X C. reticulatum, C. arietinum X C. judaicum, C. reticulatum X C. echinospermum, C. judaicum X C. pinnatifidum, C. judaicum X C. bijugum,* and *C. pinnatifidum X C. bijugum.* On the basis of this study, the seven species were classified in to three crossability groups:

Group I : *C. arietinum, C. reticulatum, C. echinospermum*

Group II : *C. pinnatifidum, C. judaicum, C. bijugum*

Group III : *C. cuneatum*

such that crosses were not successful between groups, but were successful within a group.

Pundir and van der Maesen (1983) attempted 23 of the possible 56 two-way crosses involving eight annual *Cicer* species (except *C. echinospermum*) and were successful in seven cross combinations. While five of these seven successful crosses involved the same species combination as reported by Ladizinsky and Adler (1976a, b), they reported two new crosses, viz., *C. judaicum X C. cuneatum* and *C. arietinum X C. cuneatum.* The former new hybrid was completely sterile, but the details of the latter hybrid were not discussed. However, they did not carry out any cytological analysis of the seven interspecific hybrids. Ahmad (1988) carried out crossing program involving all nine wild *Cicer* species including cultigen and reported two new crosses viz., *C. chorassanicum X C. pinnatifidum,* and *C. judaicum X C. chorassanicum.* The former albino hybrid did not survive long but the later one was studied cytologically. Successful hybridization between *C. arietinum* and *C. reticulatum* has also been reported by Jaiswal *et al.* (1984) and Singh *et al.* (1984).

2.6 BARRIERS TO INTERSPECIFIC HYBRIDIZATION IN THE GENUS *Cicer*

In the genus *Cicer*, interspecific hybridization is rather difficult to accomplish due to various pre-zygotic and post-zygotic barriers. Possible reasons for the failure of interspecific hybridization in plants are failure of pollen germination, pollen tubes may cease growth before reaching the ovary or they may not be guided to the micropyle and/or embryo sac after reaching the ovary (Stalker, 1980). After fertilization, problems due to "somatoplastic sterility", consisting of lack of or abnormal development of endosperm or embryo, may still occur (Cooper and Brink, 1940).

Information on barriers to interspecific hybridization has just started coming in. Interspecific hybridization involving many annual *Cicer* species has been attempted (Ladizinsky and Adler, 1976a,b; Pundir and van der Maesen, 1983; Mercy *et al.*, 1974b; Mercy and Kakar, 1975; Ahmad, 1988). Manipulations like removal of stigma or stigma along with a part of style, pollination at late bud stage or early pollination and delayed pollination were not found to be beneficial (Singh and Singh, 1989). Mercy and Kakar (1975) studied the cross between *C. arietinum X C. soongaricum* and reported that a low molecular weight labile compound in the stigmatic and styler tissue was implicated in the inhibition of *in vitro* pollen germination and pollen tube growth, and suggested a pre-fertilization interspecific crossability barrier for the entire *Cicer* genus. However, Ahmad (1988), made interspecific crosses involving nine annual *Cicer* species, including cultigen *C. arietinum*, and found that the barriers are of post-fertilization nature.

2.6.1 INTERSPECIFIC HYBRIDIZATION AND APPLICATION OF PLANT GROWTH REGULATORS

It is a well-recognized and accepted fact that, like other morphogenetic phenomena, the post-fertilization changes leading to fruit formation are also under the influence of plant growth regulators, either in a sequence or independently, or in combination (Nitsch, 1952). Knowledge on these aspects is limited to a very small number of taxa making it impossible to conceive a widely applicable hypothesis (Sastri, 1984).

Achievement of pear X apple hybrid due the application of plant growth regulators marked the first step (Crane and Marks, 1952; Brock, 1954) and stimulated a series of other investigations based on the same approach. ß-naphthoxyacetic acid applied to the stigma promoted successful germination of incompatible pollen in interspecific crosses of *Trifolium* (Evans and Denward, 1955). Incompatibility between *Phaseolus vulgaris* and *P. acutifolius* was overcome by applying a mixture of naphthalene acetamide and potassium gibberellate (Al Yasiri and Coyne, 1964). *Nicotiana repanda* was crossed with *N. tabacum* by applying a lanolin paste of Indole Acetic Acid (IAA) (Pittagelli and Stavely, 1975). Hybrid in cross *Corchorus capsularis X C. olitoris* was not obtained until 300 ppm of IAA was applied to the pedicels of flowers (Islam, 1964). Successful application of gibberellic acid (75 ppm) in *Hordeum vulgare X H. bulbosum* cross was demonstrated by Subrahmanyam and Kasha (1971), in a range of interspecific crosses in *Hordeum* (Subrahmanyam, 1979). Plant growth regulators, particularly gibberellin and kinetin, were successfully used in intersectional, interspecific crosses in genus *Arachis* (Singh *et al.*, 1980; Sastri and Moss, 1982; Sastri *et al.*, 1981, 1982).

Cytokinins are suggested to stimulate both the cell divisions and the assimilate demand in growing embryonic tissues (Burrows and Carr, 1970; Smith and van Staden, 1979). In developing *Lupinus albus* seeds, the endosperm is rich in cytokinin, and this led Davey and van Staden (1979) to suggest that this cytokinin was helpful in embryo growth. Bennici and Cionini (1979) also suggested that there was a cytokinin requirement by the young embryos of *Phaseolus coccineus*. It has also been shown that in interspecific crosses in *Phaseolus*, endosperm did not develop normally and had much lower levels of cytokinins than endosperms from self-pollinations (Nessling and Morris, 1979). Cytokinin levels seem to be critical for the normal embryo development. However, whether an exogenous supply of cytokinin in these crosses can prevent embryo degeneration and promote its growth, is a matter still to be investigated (Sastri, 1984).

These studies indicated that plant growth regulators have profitably been used in some interspecific incompatible crosses, however, it is not yet clear as to what is the precise role of the plant growth regulators in such investigations. There were suggestions that in instances of retarded pollen tube growth and pre-fertilization abscission of the flower, plant growth regulators maintain the flowers until the pollen tubes have grown long enough to discharge the male gametes in the vicinity of the female gametes; it is also suggested that plant growth regulators may stimulate the incompatible pollen tube growth in the pistil so that fertilization can take place before flower has abscised, but the hybrid zygote obtained in this way may not develop any further or may not develop fully. In those cases embryos from immature fruits have to be excised and cultured for raising the hybrid plants (Sastri, 1984).

2.6.2 EMBRYO RESCUE STUDIES

The basic premise upon which embryo rescue operations have been attempted in wide crosses is that the integrity of the hybrid genome is retained in the stalled embryo and that its potential to resume normal growth may be realized if it is supplied with nutrient substances that are known to promote growth. The pioneer work of Laibach (1925) leading to the successful culture of embryos from *Linum perenne X L. austriacum* hybrids to the seedling stage has made it possible to rescue progenies from wide crosses in a number of plants. Hybrid embryo rescue operations have been successfully mounted in nearly 70 interspecific and intergeneric crosses involving approximately 35 genera and 120 species (Collins and Grosser, 1984). However, wide hybridization trials in legumes deserve special mention because the prospects of raising a fertile hybrid and eventual release of hybrid cultivars have dramatically improved in these families by embryo culture methods. In legumes, hybrid plants have been obtained by embryo culture from crosses involving different species of *Trifolium* (Keim, 1953; Evans, 1962; Phillips *et al.*, 1982; 1992), *Melilotus* (Webster, 1955; Scholsser-Szigat, 1962), *Phaseolus* (Honma, 1955; Braak and Kooistra, 1975; Mok *et al.* 1978; Rabakoarihanta *et al.*, 1979; Advarez *et al.*, 1981; Shii *et al.*, 1982), *Lotus* (Grant *et al.*, 1962), *Medicago* (Fridriksson and Bolton, 1963), *Lathyrus* (Pecket and Selim, 1965), *Vigna* (Ahn and Hartmann, 1978a, b; Chen *et al.*, 1983; Gosal and Bajaj, 1983), *Arachis* (Sastri *et al.*, 1980; Bajaj *et al.*, 1982; Sastri and Moss, 1982; Mallikarjuna and Sastri, 1985; Moss *et al.*, 1985; Moss and Stalker, 1987; Moss *et al.*, 1988; Pattee *et al.*, 1988) and *Glycine* (Tilton and Russell, 1984; Coble and

Schapaugh, 1990). Culture of embryos excised from pre-cultured hybrid ovules facilitated recovery of plants from *Medicago sativa X M. rupestris* cross (McCoy, 1985). The reports on rescuing interspecific hybrids in genus *Cicer* are meager. Singh and Singh (1989) reported hybrid of the cross *C. arietinum X C. cuneatum* which was sterile.

2.7 POLLEN MORPHOLOGY

The importance of palynological studies has been clearly demonstrated by a number of workers. The pollen morphology has the advantage of being conservative and, hence, is of taxonomic value (Vishnu-Mittre and Sharma, 1962; Fergusan and Skvarla, 1981). Among the various well associated morphological units in plants, the reproductive units demand the maximum protection which is attained in pollen grains by encasing the germplasm with a wall having a unique structure and organization. Apart from its functional significance, the protective wall bears important characteristics that are of immense diagnostic and phylogenetic value (Nair, 1964). Pollen grain diameter is stable and highly heritable (0.82 heritability, by additive genes), and is controlled by the genotype of the mother plant (Kumar and Sarkar,1983). Biosystematics of the subfamily Papilionaceae was elucidated using palynological studies (Fergusan,1984). In studies of the genus *Nicotiana*, Pandey (1970) detected a correlation between pollen grain size and self-incompatibility. On the adaptive significance of the exine Heslop - Harrison (1976) concluded that the exine ornamentation indicates the adaptation to particular condition, and that differences in exine patterns between pollen of different genera is to be considered as one of the pre-fertilization barriers in wide crossing program. Size and the shape of the pollen, furrow and pore number and appearance of the exine patterning are some of the characteristics

commonly used for establishing inter-relationships among taxa (Goldy *et al.*,1984).

The first study of pollen morphology in *Cicer* species was carried out by Vishnu-Mittre and Sharma (1962). They studied *C. soongaricum* and reported it to be faintly reticulate, 3-zoni-colporoidate (three apertures arranged in a circular zone around the pollen), and subprolate shape with polar axis length of 28-30µm and equatorial diameter of 24-26µm.

Gapochka(1974) studied pollen morphology in *C. arietinum* and four perennial species, *C. anatolicum, C. flexosum , C. soongaricum* and *C. spinosum*, and reported pollen size in the range of 20-30µm. Pollen grains of all five *Cicer* species had simple apertures, and all excepting *C. arietinum*, had wide deep furrows, and syncolporate colpi.

Pollen grain morphology of six perennial species : *C. anatolicum, C. incisum, C. montbretii, C. spiroceras, C. tragacanthoides*, and *C. pungens* , and three annual species *C. arietinum, C. pinnatifidum*, and *C. chorassanicum* were studied by Clarke and Kupicha (1976). All seven species studied by them had broad ectoapertures with obtuse ends and very large endoapertures which occupied about half the length of polar axis. They reported that the colpus extensions enclose a triangular area at the apocolpium in *C. montbretti, C. pungens*, and *C. chorassanicum.*

Ahmad (1988) studied nine annual *Cicer* species *C. arietinum, C. reticulatum, C. echinospermum, C. pinnatifidum, C. judaicum, C. bijugum, C. cuneatum, C. chorassanicum*, and *C. yamashitae* and reported the genus *Cicer* to be stenopalynous, since all species studied have similar pollen morphology. Slight difference among *C. bijugum, C. chorassanicum*, and *C. yamashitae* were noticed.

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

The materials used in the present study included both cultivated and wild annual *Cicer* species. The basic material comprising of *Cicer arietinum* (cvs. Annigeri, GL-769, ICCC-42, K-850, and ICCC-32), *C. reticulatum* (ICCW-6 and ICCW-49), *C. echinospermum* (ICCW-44), *C. pinnatifidum* (ICCW-37), *C. judaicum* (ICCW-34 and ICCW-36), *C. bijugum* (ICCW-42), *C. chorassanicum* (ICCW-26), and *C. cuneatum* (ICCW-47) was used for different studies. The species and accessions included in this research program along with source and origin of each one are listed in **Table 5**. Seeds, and plants raised from them at ICRISAT, Patancheru (A.P.), India, were used for various aspects of this research investigation. All the laboratory studies were carried out in the Tissue

Table 5 Accessions, source and origin of the annual *Cicer* species used in this research program.

Species	Accession	Source	Origin
C. arietinum(Desi)	Annigeri	ICRISAT	India
	GL-769	ICRISAT	India
	K-850	ICRISAT	India
	ICCC-42	ICRISAT	India
C. arietinum(Kabuli)	ICCC-32	ICRISAT	India
C. reticulatum	ICCW-6	ICRISAT	Turkey
	ICCW-49	ICRISAT	Turkey
C. echinospermum	ICCW-44	ICRISAT	Turkey
C. pinnatifidum	ICCW-37	ICRISAT	Turkey
C. judaicum	ICCW-34	ICRISAT	Lebanon
	ICCW-36	ICRISAT	Lebanon
C. bijugum	ICCW-42	ICRISAT	Turkey
C. chorassanicum	ICCW 26	ICRISAT	Afghanistan
C. cuneatum	ICCW-47	ICRISAT	Ethiopia

Culture and Transformation Laboratory of ICRISAT. Where it was not possible to include all accessions of different species in the different experiments under study, representative accessions from each species were selected.

3.2 METHODS

Seeds were first sown in small pots with vermiculite and kept in an incubator for germination at 28 ± 1°C. Seeds of wild *Cicer* species were mechanically scarified prior to sowing to speed up the germination. The germinated seedlings were transferred to the field. The sowing time of chickpea and wild *Cicer* species was so coordinated that both started flowering together. Extra lights using 1000 W bulbs, were provided in the field of 20 X 20 meters, from 18:00 h to 06:00 h, to extend the photoperiod for wild species, which considerably reduced the time until the first flowers were formed.

3.2.1 CYTOLOGICAL STUDIES

Root tips and flower buds were collected for cytological studies for mitosis and meiosis, respectively.

3.2.1.1 SOMATIC KARYOTYPE

In the wild species, the seed coats were mechanically scarified and the seeds were surface sterilized for 10 min in 0.1% mercuric chloride solution. No scarification was required for the *desi* and *kabuli* type chickpea accessions, but they were surface sterilized as above. The seeds were germinated in the sterilized Petri plates on filter papers at 28 ± 1°C. Young, 1-1.5 cm long, rapidly growing roots were excised around 11:30 h and were prefixed in the saturated solution of 1-Bromonaphthalene solution for 3 h at room temperature, followed by transfer to Carnoy's (Carnoy, 1886) fluid II (1 part of glacial acetic acid: 3 parts of chloroform: 6 parts of 95% ethanol) for 24 h. These roots were then transferred into Farmers fixative (3 parts of 95% ethanol: 1 part of glacial acetic

acid) for 24 h. A few drops of ferric chloride ($FeCl_3$) were added to obtain better and enhanced staining. After fixation, the roots were stored in 70% ethanol at 4-10°C until analyzed.

Root tips were hydrolysed in 1N HCl solution for 20 min at 60°C, washed thoroughly with distilled water and were stained in 2% Feulgen (Feulgen and Rossenbeck, 1924) stain. Squashes were made in 2% acetocarmine solution. Somatic cells with well spread metaphase chromosomes were photographed. The length of individual chromosome was measured. The position of the primary constriction was characterized by the ratio between the long arm and short arm of the chromosome. The secondary constriction was not included in determining chromosome arm length. Chromosome terminology was based on arm length ratio as used by Sharma and Gupta (1982), viz., 1.00-1.25 as metacentric, 1.26-1.75 as submetacentric, and ≥1.76 as submetacentric/acrocentric. Considering the range and average of total chromatin length per chromosome, they were grouped as below (Kutarekar and Wanjari, 1983):

Group	Chromatin length per chromosome
i) Long (A)	4.5µm and above
ii) Medium (B)	3.00µm to 4.49µm
iii) Short (C)	2.25µm to 2.99µm
iv) Very short (D)	2.24µm and below

The centromeric index (**CI**) was determined by the following equation:

$$CI=\frac{P}{P+Q}x100$$

where "p" and "q" represent the lengths of short and long arms of chromosome, respectively. Karyological data were averaged over the eight most definitive cells.

3.2.1.2 MEIOTIC STUDIES

Flower buds of appropriate size were collected from greenhouse sown chickpea (*desi* and *kabuli*), other *Cicer* species and F_1 plants, and fixed at room temperature for 24 h in Carnoy's fixative (6 parts 95% ethanol: 3 parts chloroform: 1 part glacial acetic acid). Later, the buds were transferred to Farmers fixative (3 parts 95% ethanol: 1 part glacial acetic acid) for 24 h to which trace amount of ferric chloride ($FeCl_3$) was added to enhance staining.

Chromosome associations at metaphase I were studied by squashing individual anther in slightly warm 2% acetocarmine stain. All cytological observations were made on temporary slides. Photomicrographs were taken with a Zeiss research microscope fitted with a photographic attachment.

3.2.2 PRODUCTION OF HYBRIDS

Seven *Cicer* species viz., *C. arietinum, C. reticulatum, C. echinospermum, C. pinnatifidum, C. judaicum, C. chorassanicum* and *C. cuneatum*, were utilized for making interspecific crosses in all possible combinations. Interspecific reciprocal crosses were also attempted. As suggested by Pundir and van der Maesen (1983) enhanced photoperiod at the field conditions was used to hasten the flowering in the wild species, thus

bringing forward flowering dates and avoiding the high temperature in the field later in the season.

Emasculation was done either, in the morning (08:30 -10:00 h) to be followed by immediate pollination, or in the evening (15:00 -16:30 h) for pollination the next morning (09:00 -10:00 h). Emasculation was done at the "hooded bud" stage (Eshel, 1968), in which petals just protrude from the calyx and the anthers are at about half the height of the style. Pollen from fully expanded flowers in which anther dehiscence had already taken place, were used for pollination, which was carried out by gently pressing the keel (full of pollen) against the stigmas of the emasculated flowers.

Preliminary observations revealed that abscission of hybrid pods, was occurring 2-5 days after pollination leading to a very low pod setting. Keeping this problem in view, a mixture of growth regulators containing 75 mg/l gibberellic acid (GA3), 10 mg/l naphthalene acetic acid (NAA) 10mg/l kinetin (Kn) was applied to a cotton pad at the base of the pedicel of the flowers, about half an hour after pollination. This increased the pod set during the preliminary studies (**Table 6**). About one quarter of the developing pods were used for the purpose of embryo and ovule culture and the rest were left to directly form hybrid seeds, if any, under the field conditions.

3.2.2.1 EMBRYO CULTURE

Eight different culture media (**Table 7**) including four standard ones viz., MS (Murashige and Skoog, 1962), B5 (Gamborg, 1968), Nitsch (1951) and Whites (1963); and four modified media: MS/2, B5/2, and MS-2, B5-2 with vitamins and amino acids variations (Williams and De Llautour, 1980) were

Table 6 The effect of hormone treatment on the number of mature pod formation in the interspecific crossing in *Cicer*.

Crosses	Without hormone application			With hormone application		
	No. of pollinations	No. of pods formed	% of success	No. of pollinations	No. of pods formed	% of success
C. arietinum (Annigeri) X *C. reticulatum* (ICCW-49)	10	2	20	10	8	80
C. arietinum (ICCC-32) X *C. reticulatum* (ICCW-49)	10	1	10	10	6	60
C. arietinum (Annigeri) X *C. echinospermum* (ICCW 44)	10	1	10	10	4	40
C. arietinum (ICCC-32) X *C. echinospermum* (ICCW 44)	10	0	0	10	3	30

Table 7 Composition of different media used in Embryo/Ovule culture study in genus *Cicer*.

Name of media	Salts	Vitamins	Amino-acids	Hormones mg/l
MS	MS	MS	MS	2 BAP, 0.2 NAA
B_5	B_5	B_5	B_5	2 BAP, 0.2 NAA
Whites	Whites	Whites	Whites	2 BAP, 0.2 NAA
Nistche	Nistche	Nistche	Nistche	2 BAP, 0.2 NAA
MS/2	1/2 MS	MS	MS	2 BAP, 0.2 NAA
B_5/2	1/2 B_5	B_5	B_5	2 BAP, 0.2 NAA
MS-2	MS	TF-2	TF-2	2 BAP, 0.2 NAA
B_5-2	B_5	TF-2	TF-2	2 BAP, 0.2 NAA

used for culturing immature embryos (**Table 8**). The composition of four standard media are presented in **Table 9**. An additional amino acid L-glutamine [earlier reported to be an effective amino acid for the growth of excised embryos (Paris *et al.*, 1953; Rijven, 1955; Matsubara, 1964; Monnier, 1978)], was uniformly added to all the media. The combination and concentration of plant growth regulators used in the study was 2mg/l BAP and 0.2mg/l NAA. For ovule culture agar free liquid media were used. The ovule was supported by filter paper bridge. For embryo culture, pods six to eight DAP and for ovule culture pods 3-4 DAP, were surface sterilized with 0.1% mercuric chloride and then washed with three changes of sterilized distilled water. Sterilization and inoculations were performed under strict aseptic conditions in the laminar air flow cabinet.

Ovules were taken out of the sterilized pods and placed on the sterilized Petri dish having a drop of sterilized cold water. For ovule culture study, the ovules from the cross pollinated pod were directly placed over the filter paper wicks in the liquid media. For embryo culture study, the ovule was held steady with a needle inserted into the structural tissue near the funiculus and a cut was made across the back of the ovule, opposite to the funiculus. The ovule wall was lifted back as a flap and a second cut towards the hilum exposed the central embryo sac region. A gentle pressure from upper side of the embryo with the aid of needle detached the embryo suspensor from the maternal tissue and forced the embryo and surrounding endosperm out on the petri dish, which was then transferred to culture medium. The cultures were transported in the racks and kept in dark for 3 days and later transferred to a well illuminated culture room. A constant temperature of 25±3°C was provided.

Table 8 Formulations of culture media used in the embryo/ovule culture study.

NUTRIENTS (mg/l)	MS[1]	B_5[2]	Whites[3]	Nitsches[4]
MACRO-NUTRIENTS				
NH_4NO_3	1650	-	-	-
KNO_3	1900	2500	80	125
$CaCl_2.2H_2O$	440	150	-	-
$MgSO_4.7H_2O$	370	250	737	125
KH_2PO_4	170	-	-	125
$(NH_4)_2SO_4$	-	134	-	-
$NaH_2PO_4.H_2O$	-	150	19	-
$Ca(NO_3)_2.4H_2O$	-	-	288	500
KCl	-	-	65	-
Na_2SO_4	-	-	200	-
MICRO-NUTRIENTS				
KI	0.83	0.75	0.75	-
H_3BO_3	6.3	3.0	(1.5)	0.5
$MnSO_4.4H_2O$	22.3	-	6.65	3.0
$MnSO_4.H_2O$		10.0	-	-
$ZnSO_4.7H_2O$	8.6	2.0	2.67	0.5
$Na_2MoO_4.2H_2O$	0.25	0.25	-	0.025
MoO_3	-	-	(0.0001)	-
$CuSO_4.5H_2O$	0.025	0.025	(0.001)	0.025
$CoSO_4.6H_2O$	0.025	0.025	-	-
$Fe_2(SO_4)_3$	-	-	2.5	-
Na_2EDTA	37.3	37.3	-	-
$FeSO_4.7H_2O$	27.8	27.8	-	-
H_2SO_4	-	-	-	0.0005 ml
Ferric citrate	-	-	-	10
ORGANICS				
myo-inositol	100	100	-	100
Nicotinic acid	0.5	1.0	0.5	0.5
Thiamine HCl	0.5	1.0	0.1	1.25
Pyridoxine.HCl	-	-	-	0.8
Glycine	0.1	10.0	0.1	-
Sucrose	30,000	20,000	20,000	20,000
pH	5.7	5.5	5.5	5.7

[1] Murashige and Skoog (1962)
[2] Gamborge *et al.* (1968)
[3] Whites (1963)
[4] Nitsche (1951)

Table 9 Composition of Tf2[1] Vitamins and Amino acids

Amino acid and Vitamines	Composition mg/liter
Casein hydrolysate	500
Myo-inositole	100
Nicotinic acid	2.5
Pyridoxin HCL	2.5
Thiamine HCL	0.5
Ca pantothenate	0.25
Glycine	7.5

[1] Williams and de Lautour (1980)

3.2.3 POLLEN GERMINATION AND POLLEN TUBE GROWTH STUDIES

3.2.3.1 *In vitro*

Crosses with *Cicer arietinum* as one of the parents were used in this study. Flower buds, with undehisced anthers and receptive stigma, were brought to the laboratory and emasculated. Flower buds, plus a small section of attached peduncle, were placed on semi-solid agar culture medium (Bassiri *et al.*, 1987) in Petri plates. Fresh self or foreign pollen was then carefully placed on the stigmatic surface using a stereo microscope. The time of pollination was recorded and time-course time table was devised by sampling the pollinated flower buds at 4 h intervals for the first 34 h and thereafter at 24 h intervals until 72 h after pollination (HAP). Sampled flower buds were fixed immediately in a mixture of glacial acetic acid, 95% ethanol and formaldehyde (1:8:1, v/v) for 24 h and then stored in 70% ethanol at 4°C.

3.2.3.2 *In vivo*

Flowers were emasculated and pollinated as usual in the field. Plant growth regulators were applied to the base of pedicels of pollinated pistils during crossing to enhance crossed flower retainability, which otherwise is very low. Flower buds were collected at the same time intervals and fixed into the fixative same as with the *in vitro* study.

At least five pistils from each cross combination within each time interval from both *in vivo* and *in vitro* collected flowers, were studied. For analysis, pistils were hydrolysed in 1N potassium hydroxide (KOH) solution for

4 h at room temperature, rinsed thoroughly with distilled water and then transferred to the staining solution overnight at room temperature. The stain consisted of 2% aniline blue in 20% potassium phosphate (K_3PO_4). Individual pistils were then placed on the slide and squashed by applying slight pressure on the cover glass, and observed under a light microscope with UV-illumination at a wave length of 356nm (Martin, 1959). To permit clear distinction between pollen tubes and vascular strands which also fluoresce, UV-illumination was altered with ordinary light at short intervals as suggested by Tomar and Gottreich (1975).

The length of the style and the distance travelled by the pollen tubes in each sample were measured using an ocular micrometer. The proportional length of the style traversed by the pollen tube after a given time period (for the cases where pollen tube length exceeded half the style length) was used to estimate the time required for the pollen tube to reach the vicinity of the most proximal ovule and, thus, enter the ovule. Penetration of the pollen into the ovule through the micropyle and subsequent enlargement of the ovule were taken as an indication of fertilization.

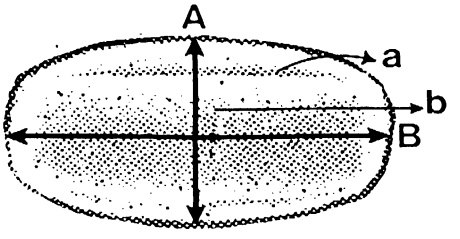
3.2.4 POLLEN MORPHOLOGY

Eight annual *Cicer* species comprising of one accession each of wild *Cicer* species and one cultivar each of *kabuli* and *desi* type of cultivated *Cicer arietinum* were used for pollen Scanning Electron Microscopic (SEM) studies. To characterize the interspecific hybrids obtained, samples of pollen were collected from F_1 plants and processed as per the method described by N. Padma, ICRISAT EM unit (personal communication). Mature pollens collected

from field grown plants were dried and stored in a desiccator. Dry pollen grains were uniformly mounted on SEM specimen stubs covered with double sided sticky tape and were uniformly coated with thin layer (200 nm) of gold in a sputter coating unit (model FD 500) and later observed and photographed using a Scanning Electron Microscope (JEOL- JSM 35 CF) at constant voltage of 15 kv.

Six pollen grains per specimen were measured for the following characteristics: Equatorial axis length (E), polar axis length (P), thickness of murri (T), mean diameter of lumina (D), and number of brochi per 10μm^2 of exine (N). The following size classes, based on the length of polar axis have been used in the present study:

Very small spores	< 10 μm
Small spores	10 - 25 μm
Medium size spores	25 - 50 μm
Large size spores	50 -100 μm
Very large spores	100-200 μm
Gigantic spores	>200 μm



EQUATORIAL VIEW

A. *Equatorial axis* (E)
B. *Polar axis* (P)

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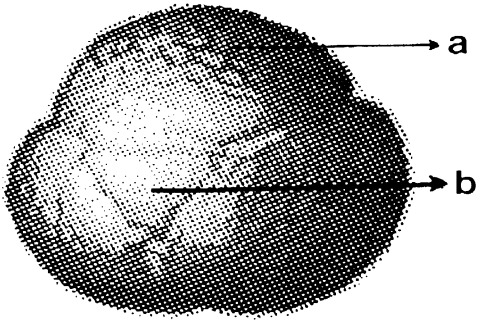
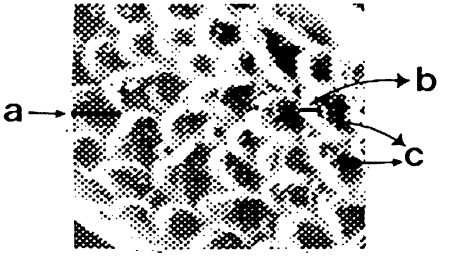
a. *Colpus (ectoaperture)*
b. *Mesocolpium*



POLAR VIEW

a. *Mesocolpium*
b. *Apocolpium*



EXINE ORNAMENTATION

a. *Diameter of Lumina* (D)
b. *Thickness of Murri* (T)
c. *Brochi* (N)

Figure 2: Description of pollen morphology

Following shape classes and relation between polar axis (P) and equatorial axis (E) of pollen grains when one of the aperture lies exactly at the center were used in present study:

Shape class	P/E ratio
Perbolate	< 4/8
Oblate	4/8 - 6/8
Suboblate	6/8 - 7/8
Oblate spheroidal	7/8 - 8/8
Prolate spheroidal	8/8 - 8/7
Subprolate	8/7 - 8/6
Prolate	8/6 - 8/4
Perprolate	> 8/4

The terminology used to describe the pollen grain morphology in present study was that of Erdtman (1966).

RESULTS

CHAPTER IV
RESULTS

4.1 CYTOLOGICAL STUDIES

The detailed cytological studies in terms of (i) chromosome number; (ii) chromosome length and total genomic length; (iii) relative chromosome length and; (v) arm ratio and centromeric index of five cultivars of *Cicer arietinum* and seven wild *Cicer* species (two accessions each of *C. reticulatum* and *C. judaicum*) were carried out.

4.1.1 KARYOMORPHOLOGICAL STUDIES IN GENUS *Cicer*

All the eight annual *Cicer* species studied, invariably showed a diploid somatic chromosome number of 16. However, they differed in karyotypic asymmetry, chromosome size and position of primary and secondary

constrictions. There were no significant differences in the chromosome morphology among accessions within a species. Accordingly, karyological data were collected from the ten most definitive cells observed and the average measurements were used to interpret the results. The data from chromosome measurements and other parameters estimated from these measurements are presented in **Tables 10** and **11**. The karyotypes and idiograms of the eight *Cicer* species are presented in **Plate 5** and **Figure 3** respectively.

Chromosome I : The total length of Chromosome I in different species of Cicer viz., *C. arietinum, C. reticulatum, C. echinospermum, C. pinnatifidum, C. judaicum, C. bijugum, C. chorassanicum* and *C. cuneatum* was found to be 4.6 µm, 5.4 µm, 6.6 µm, 4.7 µm, 3.8 µm, 4.5 µm, 3.1 µm, and 5.0 µm, respectively, which varied in the range of 3.1-6.6 µm, whereas, relative chromosome lengths recorded in the different species were 18%, 18%, 18%, 22%, 15%, 16%, 16%, and 16% respectively suggesting that Chromosome I has its contribution to the total genome between 15-22% . Accordingly the CI of chromosome I in Cicer spp. varied from 31.58-46.81%. In most of the species the secondary constriction was found on the long arm of this chromosome.

Chromosome II : The length of Chromosome II varied from 2.8-5.8 µm and its contribution to the length of total genome was 14-17%. *C. echinospermum* has largest and *C. chorassanicum* smallest chromosome II. Lengths of chromosome in other *Cicer* spp fall in between. The total length of this chromosome (relative chromosome length) in the different *Cicer* species were recorded as 4.1 µm(16%) in *C. arietinum*, 4.8 µm(16%) in *C. reticulatum*, 3.6 µm(17%) in *C. pinnatifidum*, 3.5 µm(14%) in *C. judaicum*, 4.4 µm(15%) in *C. bijugum* and 4.7 µm(15%) in *C. cuneatum*. The CI of chromosome II varies from 31.43-47.92% with *C. reticulatum* showing highest and *C. judaicum* smallest.

PLATE-5

Karyotype of the eight annual *Cicer* species.

PLATE-5

8 µm

C. arietinum (Annigeri)

C. reticulatum (ICCW-6)

C. echinospermum (ICCW-44)

C. pinnatifidum (ICCW-37)

C. judaicum (ICCW-34)

C. bijugum (ICCW-42)

C. chorassanicum (ICCW-26)

C. cuneatum (ICCW-47)

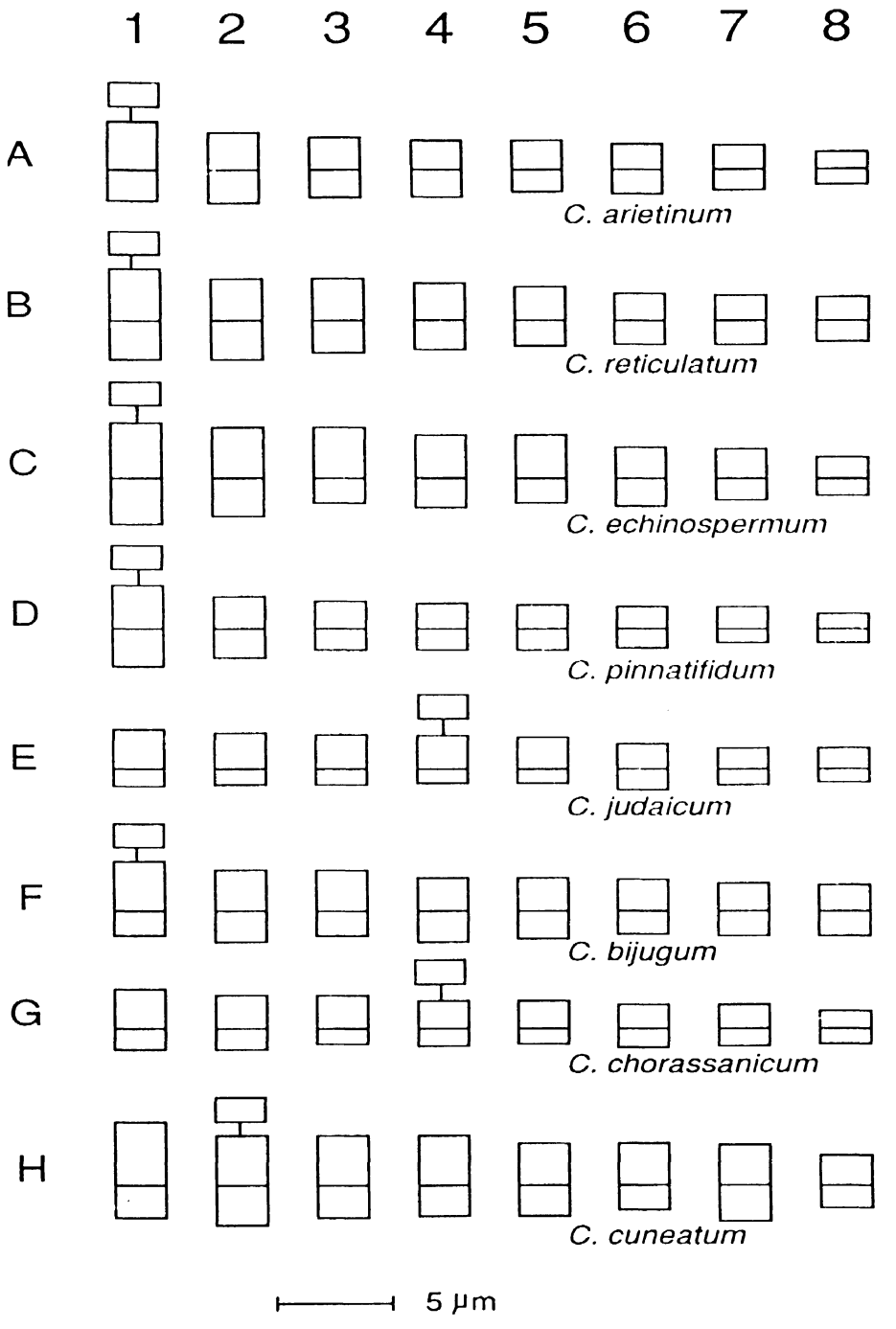


Figure 3: Karyotype of eight annual *Cicer* species

Table 10a Measurements of somatic choromosome of *C. arietinum*.

Chromosome No.	LA (µm)	SA (µm)	Total (µm)	AR	CI (%)	Chromosome Type (group)
I	2.8	1.8	4.6	1.55	39.13	SM(A)
II	2.2	1.9	4.1	1.15	46.34	M(B)
III	1.9	1.6	3.5	1.18	45.71	M(B)
IV	1.7	1.6	3.3	1.00	48.48	M(B)
V	1.7	1.3	3.0	1.30	43.33	SM(B)
VI	1.5	1.4	2.9	1.00	48.28	M(C)
VII	1.4	1.2	2.6	1.16	46.15	M(C)
VIII	1.0	0.9	1.9	1.10	47.37	M(D)
Total	14.2	11.7	25.9			

Table 10b Measurements of somatic chromosome of *C. reticulatum*.

Chromosome No.	LA (µm)	SA (µm)	Total (µm)	AR	CI (%)	Chromosome Type (group)
I	3.1	2.3	5.4	1.34	42.59	SM(A)
II	2.5	2.3	4.8	1.08	47.92	M(A)
III	2.5	1.9	4.4	1.31	43.18	SM(B)
IV	2.2	1.7	3.9	1.29	43.59	SM(B)
V	2.0	1.5	3.5	1.17	42.86	M(B)
VI	1.6	1.4	3.0	1.14	46.67	M(B)
VII	1.5	1.4	2.9	1.07	48.28	M(C)
VIII	1.4	1.2	2.6	1.16	46.15	M(C)
Total	16.8	13.7	30.5			

LA-Long arm; SA-Short arm; AR-Arm ratio; CI-Centromeric Index

Table 10c Measurements of somatic chromosome of *C. echinospermum*.

Chromosome No.	LA (μm)	SA (μm)	Total (μm)	AR	CI (%)	Chromosome Type (group)
I	3.6	3.0	6.6	1.20	45.45	M(A)
II	3.3	2.5	5.8	1.32	43.10	SM(A)
III	3.3	1.6	4.9	2.06	32.65	AC(A)
IV	2.8	1.9	4.7	1.47	40.43	SM(A)
V	2.8	1.6	4.4	1.75	36.36	SM(B)
VI	2.0	1.8	3.8	1.11	47.37	M(B)
VII	1.9	1.4	3.3	1.35	42.42	SM(B)
VIII	1.4	1.1	2.5	1.27	44.00	SM(C)
Total	21.1	14.9	36.0			

Table 10d Measurements of somatic chromosomes of *C. pinnatifidum*.

Chromosome No.	LA (μm)	SA (μm)	Total (μm)	AR	CI (%)	Chromosome Type (group)
I	2.5	2.2	4.7	1.13	46.81	M(A)
II	1.9	1.7	3.6	1.11	47.22	M(B)
III	1.6	1.2	2.8	1.33	42.86	SM(C)
IV	1.5	1.2	2.7	1.25	44.44	M(C)
V	1.4	1.2	2.6	1.16	46.15	M(C)
VI	1.4	1.1	2.4	1.18	45.83	M(C)
VII	1.3	0.8	2.1	1.60	38.10	SM(D)
VIII	0.9	0.8	1.7	1.12	47.06	M(D)
Total	12.5	10.2	22.6			

LA-Long arm; SA-Short arm; AR-Arm ratio; CI-Centromeric Index

Table 10e Measurements of somatic chromosome of *C. judaicum.*

Chromosome No.	LA (µm)	SA (µm)	Total (µm)	AR	CI (%)	Chromosome Type (group)
I	2.6	1.2	3.8	2.16	31.58	AC(B)
II	2.4	1.1	3.5	2.18	31.43	AC(B)
III	2.3	1.1	3.4	2.09	32.35	AC(B)
IV	2.2	1.0	3.2	2.20	31.25	AC(B)
V	2.1	1.0	3.1	2.10	32.26	AC(B)
VI	1.7	1.4	3.1	1.21	45.16	M(B)
VII	1.4	1.1	2.5	1.27	44.00	SM(C)
VIII	1.4	0.9	2..3	1.55	39.13	SM(C)
Total	16.1	8.8	24.9			

Table 10f Measurements of somatic chromosome of *C. bijugum.*

Chromosome No.	LA (µm)	SA (µm)	Total (µm)	AR	CI (%)	Chromosome Type (group)
I	3.0	1.5	4.5	2.00	33.33	AC(A)
II	2.5	1.9	4.4	1.31	43.18	SM(B)
III	2.5	1.5	4.0	1.66	37.50	SM(B)
IV	2.0	1.9	3.9	1.05	48.72	M(B)
V	2.0	1.7	3.7	1.17	45.95	M(B)
VI	1.9	1.4	3.3	1.35	42.42	SM(B)
VII	1.7	1.5	3.2	1.33	46.88	SM(B)
VIII	1.6	1.5	3.1	1.06	48.39	M(B)
Total	17.2	12.9	30.1			

LA-Long arm; SA-Short arm; AR-Arm ratio; CI-Centromeric Index

Table 10g Measurements of somatic chromosome of *C. chorassanicum*.

Chromosome No.	LA (µm)	SA (µm)	Total (µm)	AR	CI (%)	Chromosome Type (group)
I	2.0	1.1	3.1	1.81	35.48	AC(B)
II	1.7	1.1	2.8	1.54	39.29	SM(C)
III	1.7	0.8	2.5	2.12	32.00	AC(C)
IV	1.4	0.9	2.3	1.55	39.13	SM(D)
V	1.4	0.8	2.2	1.75	36.36	SM(D)
VI	1.2	1.0	2.2	1.20	45.45	M(D)
VII	1.2	0.9	2.1	1.33	42.86	SM(D)
VIII	0.9	0.8	1.7	1.12	47.06	M(D)
Total	11.5	7.4	18.9			

Table 10h Measurements of somatic chromosome of *C. cuneatum*.

Chromosome No.	LA (µm)	SA (µm)	Total (µm)	AR	CI (%)	Chromosome Type (group)
I	3.3	1.7	5.0	1.94	34.00	AC(A)
II	2.6	2.1	4.7	1.23	44.68	SM(A)
III	2.6	1.7	4.3	1.52	39.53	SM(B)
IV	2.6	1.6	4.2	1.62	38.10	SM(B)
V	2.2	1.9	4.1	1.15	46.34	M(B)
VI	2.2	1.6	3.8	1.37	42.11	SM(B)
VII	2.1	1.3	3.4	1.61	38.24	SM(B)
VIII	1.6	1.2	2.8	1.33	42.86	SM(C)
Total	19.2	13.1	32.3			

LA-Long arm; SA-Short arm; AR-Arm ratio; CI-Centromeric Index

Table 11 Chromosome size range (CSR), Mean chromosome length (CL), Mean haploid chromosome compliment length (HCCL) and karyotype formula of the eight annual *Cicer* species.

Species	CSR (µm)	CL (µm)	HCCL (µm)	Karyotype Formula
C. arietinum	1.9-4.6	3.24	25.9	$1_{SC}A^{SM}+3B^{M}+1B^{SM}+2C^{M}+1D^{M}$
C. reticulatum	2.6-5.4	3.81	30.5	$1_{SC}A^{SM}+1A^{M}+2B^{SM}+2B^{M}+2D^{M}$
C. echinospermum	2.5-6.6	4.50	36.0	$1_{SC}A^{M}+2A^{SM}+1A^{ACRO}+2B^{SM}+2B^{M}+1C^{SM}$
C. pinnatifidum	1.7-4.7	2.63	21.6	$1_{SC}A^{M}+1B^{M}+3C^{M}+1C^{SM}+1D^{SM}+1D^{M}$
C. judaicum	2.3-3.8	3.11	24.9	$1_{SC}B^{ACRO}+4B^{ACRO}+1B^{M}+2C^{SM}$
C. bijugum	3.1-4.5	3.63	29.0	$1_{SC}A^{ACRO}+4B^{SM}+3B^{M}$
C. chorassanicum	1.7-3.1	2.36	18.9	$1B^{ACRO}+1C^{ACRO}+1_{SC}C^{SM}+3D^{SM}+2D^{M}$
C. cuneatum	2.8-5.0	3.93	31.4	$1A^{ACRO}+1_{SC}A^{SM}+4B^{SM}+1B^{M}+1C^{SM}$

SC - Satellited chromosome

Chromosome III : The length of chromosome III was highest, 4.9 µm in *C. echinospermum* contributing 14% to total genomic length and smallest 2.5 µm in *C. chorassanicum* with 13% contribution to total genome. Lengths of chromosome III (and the contribution of this chromosome to the total genome) in other species was 3.5 µm(14%) in *C. arietinum*, 4.4 µm(14%) in *C. reticulatum*, 2.8 µm(13%) in *C. pinnatifidum*, 3.4 µm(14%) in *C. judaicum*, 4.0 µm(14%) in *C. bijugum* and 4.3 µm(14%) in *C. cuneatum*. The CI of chromosome III varied from 32% in case of *C. chorassanicum* to 45.71% in *C. arietinum*.

Chromosome IV : The CI of chromosome IV varied from 31.25% in case of *C. judaicum* to 48.48% in *C. arietinum*. The length of this chromosome was largest 4.7 µm in *C. echinospermum* and smallest 2.3 µm in *C. chorassanicum*. The lengths in other species were found to be 3.3 µm in *C. arietinum*, 3.9 µm in *C. reticulatum*, 2.7 µm in *C. pinnatifidum*, 3.2 µm in *C. judaicum*, 3.9 µm in *C. bijugum*, and 4.2 µm in *C. cuneatum*. The relative chromosome length was uniformly 1 3/4 in all species except in *C. chorassanicum* (12%) which indicates that the difference in the total length of chromosome was due to the differential condensation of chromatin at the time of observation.

Chromosome V : The length of chromosome V varied from 4.4 µm in *C. echinospermum* to 2.2 µm in *C. chorassanicum*. The percent contribution at this chromosome to the total genome was 13 in *C. bijugum* and *C. cuneatum*, 11 in *C. reticulatum*. Rest of the species had 12% contribution of this chromosome in total genome. The CI of chromosome V was highest 46.15% in *C. pinnatifidum* and smallest 32.26% in *C. judaicum*.

Chromosome VI : The relative chromosome length of the chromosome VI was 12% in *C. judaicum*, *C. chorassanicum*, and *C. cuneatum*. While in rest of the species it contributed 11% to the total genome. The length at this chromosome was recorded highest 3.8 µm in *C. echinospermum* and *C. cuneatum* and smallest 2.2 µm in *C. chorassanicum*. CI varied from 42.11-48.28%.

Chromosome VII : The length of chromosome VII varied from 3.4 µm in *C. cuneatum* to 2.1 µm in *C. chorassanicum*. The percent contribution of this chromosome to the total genome was 11 in *C. bijugum*, *C. chorassanicum* and *C. cuneatum*. Rest of the species had 10% contribution of this chromosome in total genome. The CI of chromosome VII varied from 38.10-48.28%.

Chromosome VIII : The length of chromosome VIII varied from 1.7-3.1 µm and percent contribution to total genome from 7-11, with *C. bijugum* having largest and *C. chorassanicum* and *C. pinnatifidum* with smallest chromosome VIII. Lengths of chromosome VIII in other species fall in between. The CI of chromosome VIII varied from 39.13% in *C. bijugum* to 48.39% in *C. judaicum*.

In *C. bijugum*, *C. judaicum*, *C. chorassanicum*, and *C. cuneatum* there was a graded change in size of the chromosome from I-VIII with the ratio of longest to shortest chromosome less than 2 suggesting that karyotypes of these species are symmetrical. Whereas the ratio of longest to shortest chromosome was found between 2-4 in *C. arietinum*, *C. reticulatum*, *C. echinospermum*, and *C. pinnatifidum*. Therefore, the karyotypes of these species is characterized assymtrical.

Genomic length and mean Chromosome length : When genomic length and mean chromosome length in different species of genus *Cicer* were studied, it became clear that the chromosomes in *Cicer* species are very small. *C. chorassanicum* had smallest genome with the total genomic length of

18.9 µm (mean chromosome length = 2.3 µm) whereas *C. echinospermum* had largest genome of 36 µm length (4.5 µm mean chromosome length). Rest of the species showed genomic length (and mean chromosome length) falling in between these two. *C. cuneatum*, *C. reticulatum*, *C. bijugum*, *C. arietinum*, *C. judaicum*, and *C. pinnatifidum* had total genomic lengths (and mean chromosome lengths) in descending order, 31.4 µm (3.9 µm), 30.5 µm (3.8 µm), 29 µm (3.6 µm), 25.9 µm (3.2 µm), 24.9 (3.1 µm), and 21.6 µm (2.6 µm) respectively.

4.1.1.1 KARYOTYPE OF *Cicer arietinum*

All the accessions of *desi* and *kabuli* type chickpea had the same diploid somatic chromosome number (2n=16). Furthermore, there were no apparent differences in chromosome morphology between these two type of chickpeas. The karyotypes consisted of five metacentric and three submetacentric chromosome pairs (**Tables 10a,11**).

The longest chromosome pair (number 1) was consistently satellited in the long arm and was submetacentric (**Figure 3A**). All five cultivated chickpea accessions analyzed had a somatic chromosome number of 2n=16.

The general picture of *C. arietinum* karyomorphology that emerged from various cytological studies is the presence of long (4.6µm) chromosome pair that is submetacentric and satellited, four pairs of medium sized metacentric to submetacentric chromosomes (2.9-4.1 µm), a pair of short metacentric chromosomes (2.6 µm) and a pair of very short metacentric chromosomes (1.9 µm).

4.1.1.2 KARYOTYPE OF THE WILD ANNUAL *Cicer* SPECIES

The karyotype of *C. reticulatum* was very similar to that of cultigen, *C. arietinum*. It consisted of five metacentric and three submetacentric chromosome pairs (**Tables 10b, 11**). Chromosome number 1 was always satellited in the long arm and submetacentric (**Plate 5, Figure 3B**).

The karyotype of the species *C. echinospermum* was found to be asymmetrical. There was a preponderance of submetacentric chromosomes with a corresponding decrease in the number of metacentric chromosomes and one pair of acrocentric chromosomes (**Table 10c, 11**). Thus, there were two metacentric and five submetacentric chromosome pairs and one pair of acrocentric chromosomes in this species, the longest chromosome pair was metacentric and satellited in the long arm (**Figure 3C**).

Cicer pinnatifidum was characterized by chromosomes that were intermediate in size and the karyotype was asymmetrical. There were seven metacentric and one submetacentric pairs of chromosome (**Table 10d, 11**). The longest chromosome pair was satellited (**Figure 3D**).

The karyotype of *C. judaicum* is symmetrical and consisted of five acrocentric pairs of chromosomes, two submetacentric and one metacentric pair of chromosomes (**Table 10e, 11**). The shortest chromosome pair (number 8) was submetacentric, and fourth acrocentric pair of chromosome was with secondary constriction (**Figure 3E**).

C. bijugum showed first large chromosome acrocentric and satellited pair (**Figure 3F**), four medium sized submetacentric chromosome pairs and three medium sized metacentric chromosome pairs. *C. chorassanicum* had two medium to small sized acrocentric chromosome pair (**Figure 3G**). Four small

to very small submetacentric chromosome pairs and two very small metacentric chromosome pairs. Fourth submetacentric, small chromosome pair was satellited. The chromosomes of *C. chorassanicum* (**Table 10G, 11**) were smaller than those of *C. bijugum* (**Table 10F,11**).

The karyotype of *C. cuneatum* consisted of one medium sized, metacentric chromosome pair, six submetacentric chromosome pairs out of which one was very small (number 8) and rest were medium sized pairs. The largest pair was found to be acrocentric. Second, large sized, submetacentric (**Figure 3H**) pair was satellited (**Table 10H, 11**).

4.2 CHROMOSOME PAIRING IN ANNUAL *Cicer* SPECIES

Meiosis was studied in five accessions of *C. arietinum*, two each of *C. reticulatum* and *C. judaicum*, and one each of remaining five wild *Cicer* species. Results indicated eight regular bivalents at metaphase I in all eight annual *Cicer* species studied (**Plate 6**).

Meiosis in all eight annual *Cicer* species was characterized by a diffuse prophase, making pachytene analysis very difficult. It was at diakinesis that bivalents could be first seen, but these remained highly condensed at metaphase I, making interpretation difficult.

4.3 INTERSPECIFIC HYBRIDIZATION AND CHROMOSOME PAIRING IN INTERSPECIFIC HYBRIDS

4.3.1 INTERSPECIFIC HYBRIDIZATION

Fifteen accessions (including five accessions of chickpea, four of *desi* and one of *kabuli*), representing the eight annual *Cicer* species, were used in the interspecific hybridization program. Interspecific hybridization was attempted

PLATE-6

Meiotic metaphase I configuration in the eight annual *Cicer* species.

Species	Accession No.	Ring II	Rod II	Chiasma Frequency
(A) *C. arietinum*	ICCC-42	3	5	11/11
(B) *C. reticulatum*	ICCW-49	3	5	11/11
(C) *C. echinospermum*	ICCW-44	2	6	10/10
(D) *C. pinnatifidum*	ICCW-37	4	4	12/12
(E) *C. judaicum*	ICCW-34	4	4	12/12
(F) *C. bijugum*	ICCW-42	4	4	12/12
(G) *C. chorassanicum*	ICCW-26	5	3	13/13
(H) *C. cuneatum*	ICCW-47	5	3	13/13

PLATE-6

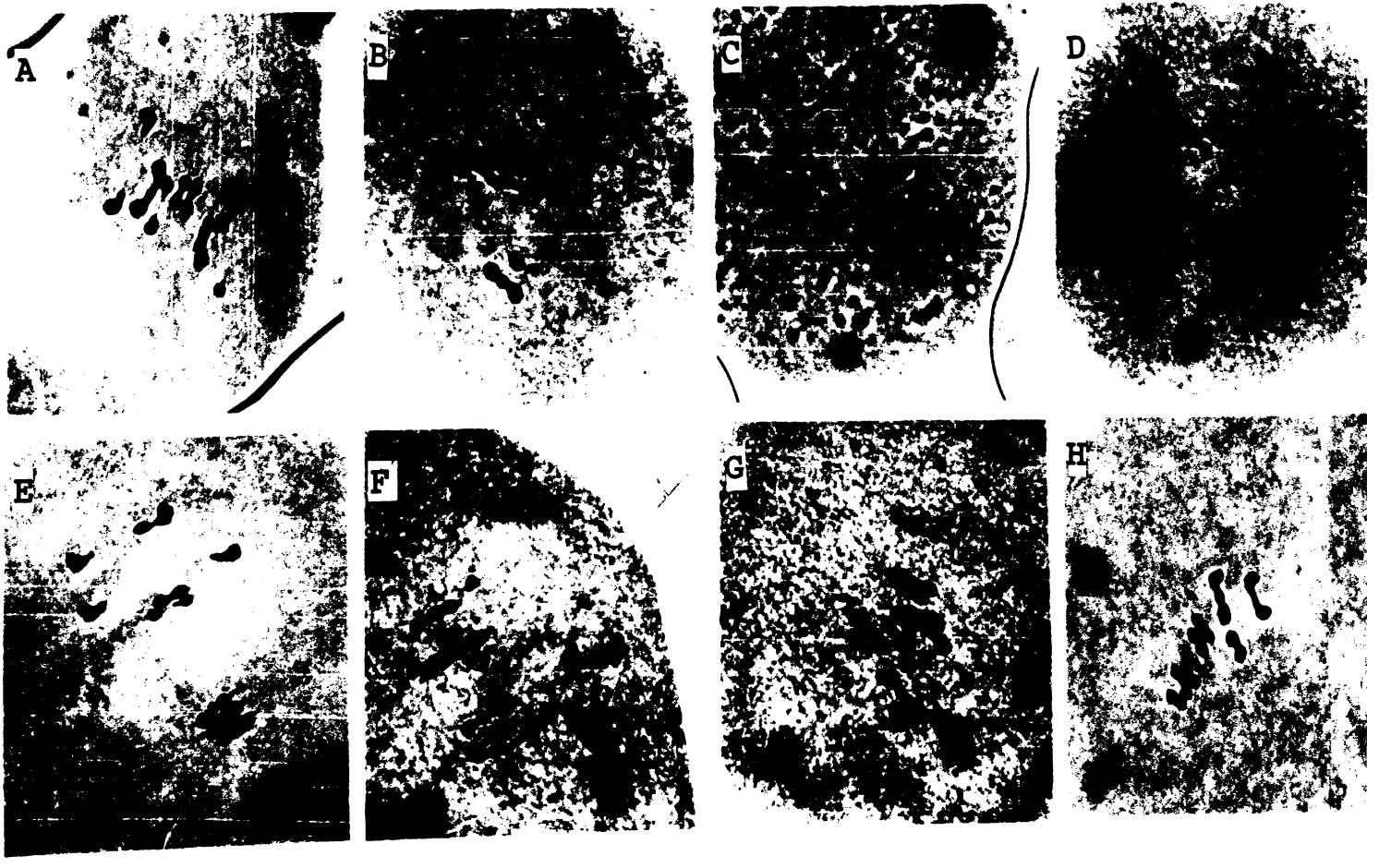


Table 12 Interspecific hybridization between *C. arietinum* and annual wild *Cicer* spp. Number of pollinations and number of seeds obtained (parenthesis).

Male parents Wild species	Female parent: *Cicer arietinum* cultivars				
	Annigeri	GL-769	K-850	ICCC-42	ICCC-32
C. reticulatum					
ICCW-49	80(25)	55(92)	105(66)	50(65)	82(104)
ICCW-6	75(47)[1]	80(127)	64(74)	45(53)	70(81)
C. echinospermum					
ICCW-44	40(25)	115(133)	135(50)	50(25)	77(63)
C. pinnatifidum					
ICCW-37	23(1)	155(1)	35(1)	10(1)	54(1)
C. judaicum					
ICCW-34	50(0)	90(0)	NA	60(0)	76(0)
ICCW-36	60(0)	89(0)	60(0)	NA	85(0)
C.bijugum					
ICCW-42	51(0)	140(0)	36(0)	15(0)	38(0)
C. chorassanicum					
ICCW-26	47(0)	114(0)	30(0)	25(0)	25(0)
C. cuneatum					
ICCW-47	45(0)	40(0)	40(0)	40(0)	42(0)

NA-Not Attempted,
[1] Three seeds obtained from reciprocal cross. Other reciprocal crosses did not yielded any seeds.

Table 13 **Interspecific hybridization studies in annual wild *Cicer* species. Number of pollinations and seeds obtained (parenthesis).**

Cross combination		Original cross	Reciprocal cross
Female	**Male**		
C. reticulatum	*C. echinospermum*	25(0)	10(0)
"	*C. pinnatifidum*	32(0)	12(0)
"	*C. judaicum*	11(0)	62(0)
"	*C. bijugum*	18(0)	13(0)
"	*C. chorassanicum*	9(0)	10(0)
"	*C. cuneatum*	14(0)	21(0)
C. echinospermum	*C. pinnatifidum*	13(0)	10(0)
"	*C. judaicum*	9(0)	35(0)
"	*C. bijugum*	8(0)	13(0)
"	*C. chorassanicum*	7(0)	3(0)
"	*C. cuneatum*	8(0)	45(0)
C. pinnatifidum	*C. judacum*	10(0)	9(0)
"	*C. bijugum*	10(0)	10(0)
"	*C. chorassanicum*	10(0)	25(5)
"	*C. cuneatum*	10(0)	40(0)
C. judaicum	*C. bijugum*	16(0)	10(0)
"	*C. chorassanicum*	10(0)	3(0)
"	*C. cuneatum*	10(0)	26(0)
C. bijugum	*C. chorassanicum*	10(0)	6(0)
"	*C. cuneatum*	13(0)	11(0)
C. chorassanicum	*C. cuneatum*	6(0)	30(0)

both between the cultivated chickpea and wild annual *Cicer* species, as well as among the wild annual *Cicer* species. A complete list of all interspecific crosses attempted, together with the success achieved, is presented in **Tables 12 and 13**.

A total of 3326 pollinations were made between *C. arietinum* and seven other wild annual *Cicer* species, including their reciprocals (**Table 12**). Three interspecific hybrids, viz., *C. arietinum X C. reticulatum*, *C. reticulatum X C. arietinum* and *C. arietinum X C. echinospermum*, which have been earlier reported, yielded 736, 3 and 296 hybrid seeds, respectively. Expansion and elongation of the pods after pollination was slow in majority of the crosses. The pods remained green and appeared healthy, but contained extremely small and shrivelled seeds. The application of solution of plant growth regulators to the pedicel at the time of pollination helped to increase the pod retainability on the plant. This increased the percentage of hybrid seed formation in compatible crosses, *C. arietinum X C. reticulatum* and *C. arietinum X C. echinospermum* (**Table 12, Table 6**), and resulted in a new hybrid, *C. arietinum X C. pinnatifidum* which has not been reported previously. This cross produced one seed with each of the accessions of the cultigen (**Table 12**).

More than 628 pollinations were made to produce hybrids between seven wild annual *Cicer* species (**Table 13**), but the only success obtained was in producing a hybrid *Cicer chorassanicum X C. pinnatifidum*.

4.3.2 EMBRYO RESCUE

Choice of suitable culture medium and knowledge of proper stage of the embryo to be cultured are prerequisites for raising hybrids through embryo rescue technique. Six to eight days old embryos of *C. arietinum*,

C. reticulatum and the hybrid between them were used to test the different culture media. Marked differences in the growth of embryos were observed on different media (**Table 14**). Out of 8 media tested, the B_5-2 medium was found to be best with respect to the self fertilized and hybrid embryos of *Cicer arietinum* and *C. reticulatum*, as 46%, 38%, and 20% of embryos respectively, were successfully rescued on this medium (**Table 14**). Even though the numbers of rescued hybrid embryos of *C. arietinum X C. reticulatum*, were much less than selfed embryos, B_5-2 gave better results as compared to the other media used in the study (**Table 14**) and was used for further rescuing the hybrids of other crosses. Twenty five excised embryos from each cross were cultured on B_5-2 medium but only few of them were successfully rescued (**Table 15**). All the crosses listed in **Table 12** and **Table 13** except reciprocals, were attempted for rescuing hybrid embryos but **Table 15** shows account of recovered crosses only. Embryos from the crosses, *C. arietinum X C. reticulatum*, *C. arietinum X C. echinospermum* and *C. arietinum X C. pinnatifidum*, were easily excised and cultured in the media when the pods were 6-8 days old and rescued successfully (**Plate 7,8**). In the remaining crosses it was noticed that the growth of the embryo was slow, therefore, it was difficult to excise 6-8 days old embryos and majority appeared to have aborted 6-8 DAP.

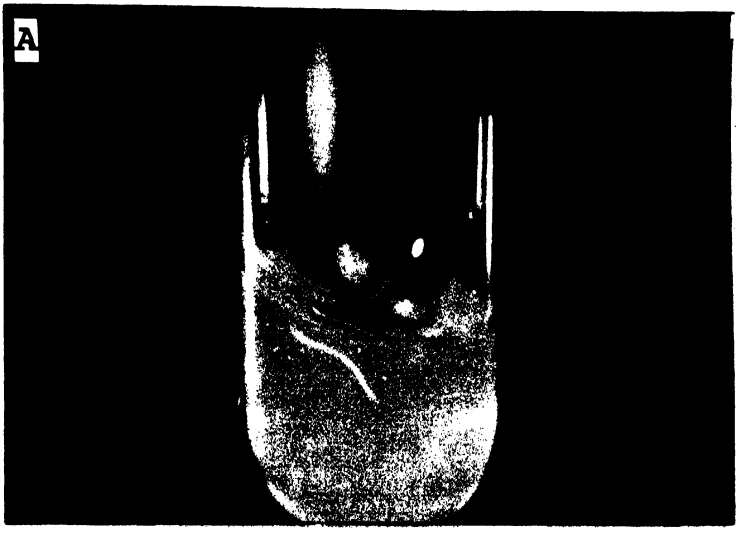
It was observed that some of the crosses failed because of the embryos tend to abort at late heart shape (3-4 days old) stage. When attempts were made to rescue these embryos before abortion, it was found that hybrid embryos excised before late heart-shaped stage do not grow on the artificial culture media. It may be either due to mechanical injury caused during excision of smaller sized embryo or it could be due to unsuitable osmotic pressure of medium.

PLATE-7

Embryo rescue of interspecific hybrids in genus *Cicer*.

(A) Growth of embryo 6 days after culture,
(B) Young plantlet 12 days after culture,
(C) Plantlet 15 days after culture.

PLATE-7



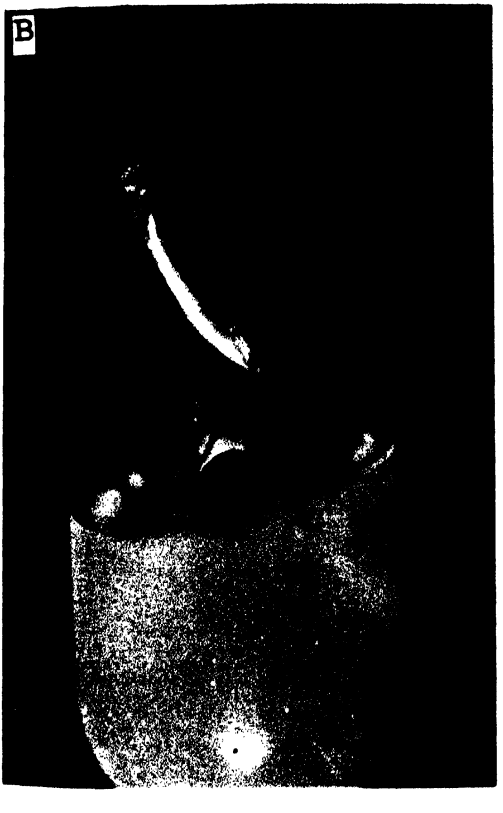


PLATE-8

Embryo rescue of interspecific hybrids in genus *Cicer*.

(A) Well grown plantlet in test tube 20 days after culture,
(B) Embryo cultured planlet subjected to hardening,
(C) Full grown embryo rescued plantlet prior to transfer to the glasshouse.

PLATE-8

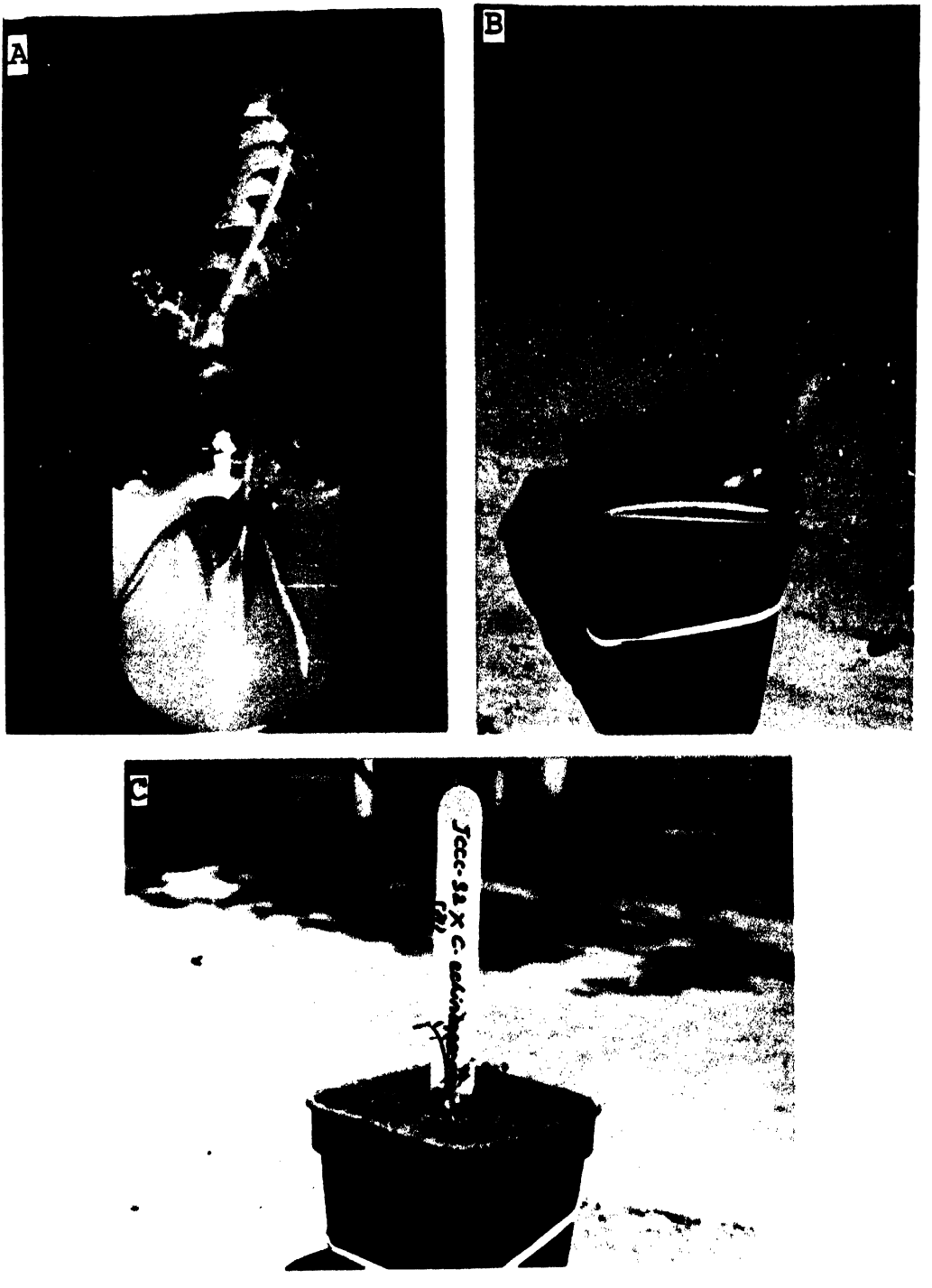


Table 14 Response of 17 days old embryos of *Cicer* species and their F_1 hybrid on different basal media.

S. No.	Media	*Cicer arietinum*			*Cicer reticulatum*			F_1 Hybrid		
		No. of Embryos Cultured	No. of Responding Embryos (%) 1 Week After Culture	No. of Responding Embryos (%) 2 Weeks After Culture	No. of Embryos Cultured	No. Responding Embryos (%) 1 week after Culture	No. of Responding Embryos (%) 2 Weeks After Culture	No. of Embryos Cultured	No. of Responding Embryos (%) 1 Week After Culture	No. of Responding Embryos (%) 2 Weeks After Culture
1.	MS	50	15 (30)	6 (12)	50	10 (20)	2 (4)	10	1 (10)	1 (10)
2.	B_5	50	17 (34)	5 (10)	50	12 (24)	0 (0)	10	1 (10)	0 (0)
3.	Nitchs	50	10 (20)	2 (4)	50	6 (12)	0 (0)	10	1 (10)	0 (0)
4.	Whites	50	8 (16)	0 (0)	50	5 (10)	0 (0)	10	0 (0)	0 (0)
5.	MS/2	50	22 (44)	9 (18)	50	17 (34)	4 (8)	10	1 (10)	1 (10)
6.	B_5/2	50	24 (48)	11 (22)	50	19 (38)	6 (12)	10	1 (10)	1 (10)
7.	MS-2	50	30 (60)	20 (40)	50	27 (54)	17 (34)	10	2 (20)	2 (20)
8.	B_5-2	50	35 (70)	23 (46)	50	32 (64)	19 (38)	10	2 (20)	2 (20)

Table 15 Response of The 6-8 Days Old Hybrid Embryos Cultured in B_5-2 Media.

Crossess	Number of embryos Cultured	Response in Culture After		Plants survived
		One Week (%)	Two Week (%)	(%)
C. arietiunm X C. reticulatum	25	5(20)	5(20)	4(16)
C. arietinum X C. echinospermum	25	5(20)	5(20)	3(12)
C. arietinum X C. pinnatifidum	25	3(12)	2(8)	1(4)

Table 16 Response of the 3-5 Days Old Ovules Cultured in B_5-2 Liquid Ovule Culture Media.

Crosses	Number of Ovules Cultured	Response In Culture After		
		3 days(%)	7 days(%)	10 days(%)
C. arietinum X C. judaicum	10	7(70)	2(20)	0(0)
C. arietinum X C. bijugum	10	6(60)	1(10)	0(0)
C. arietinum X C. chorassanicum	8	3(36)	0(0)	0(0)
C. arietinum X C. cuneatum	10	5(50)	0(0)	0(0)

To overcome these problems, ovules were cultured. Three to four days old ovules were cultured in the liquid ovule culture medium. Initial growth in the ovules was observed but they subsequently turned brown and ultimately embryos inside them died 10 days after culture (**Table 16**).

4.3.3 MORPHOLOGICAL AND CYTOLOGICAL OBSERVATIONS IN INTERSPECIFIC HYBRIDS OF *Cicer*

4.3.3.1 *C. arietinum X C. reticulatum*

These hybrids were obtained by crossing two accessions of *C. reticulatum* (ICCW-6 and ICCW-49) as the pollen parents to five *C. arietinum* lines, Annigeri, GL-769, K-850, ICCC-42 and ICCC-32, as the female parents (**Table 12**).

All F_1 hybrids were intermediate for morphological characters (**Plates 9,10, 13,14,15A,16A,17A,18A,19A**). Dominance of the purple flower color of *C. reticulatum* was clearly demonstrated in the hybrids involving the white flowered *C. arietinum* line, ICCC-32. This agrees with the results on genetics of flower color in the cultivated chickpea reported by van der Maesen (1972). Meiosis in all five hybrid plants was normal and eight bivalents were regularly formed (**Plate 20 A,B,F,G**). The interspecific hybrid showed normal chromosome and chromatid segregation and 93-96% pollen fertility. Pod formation was about 60% in hybrid where female parent was *desi* and 45% where female parent was *kabuli*.

4.3.3.2 *C. reticulatum X C. arietinum*

Intermediate morphological characters (**Plate 15B**) and abnormalities in the meiosis (**Plate 20C**) were observed in the hybrid of *C. reticulatum* (ICCW-6) X *C. arietinum* (Annigeri). The meiosis in hybrid showed occasional

PLATE-9

Plant morphology of parents and F_1 hybrid in genus *Cicer*

(A) *C. arietinum* (Annigeri)
(B) F_1 hybrid
(C) *C. reticulatum* (ICCW-6)

PLATE-9

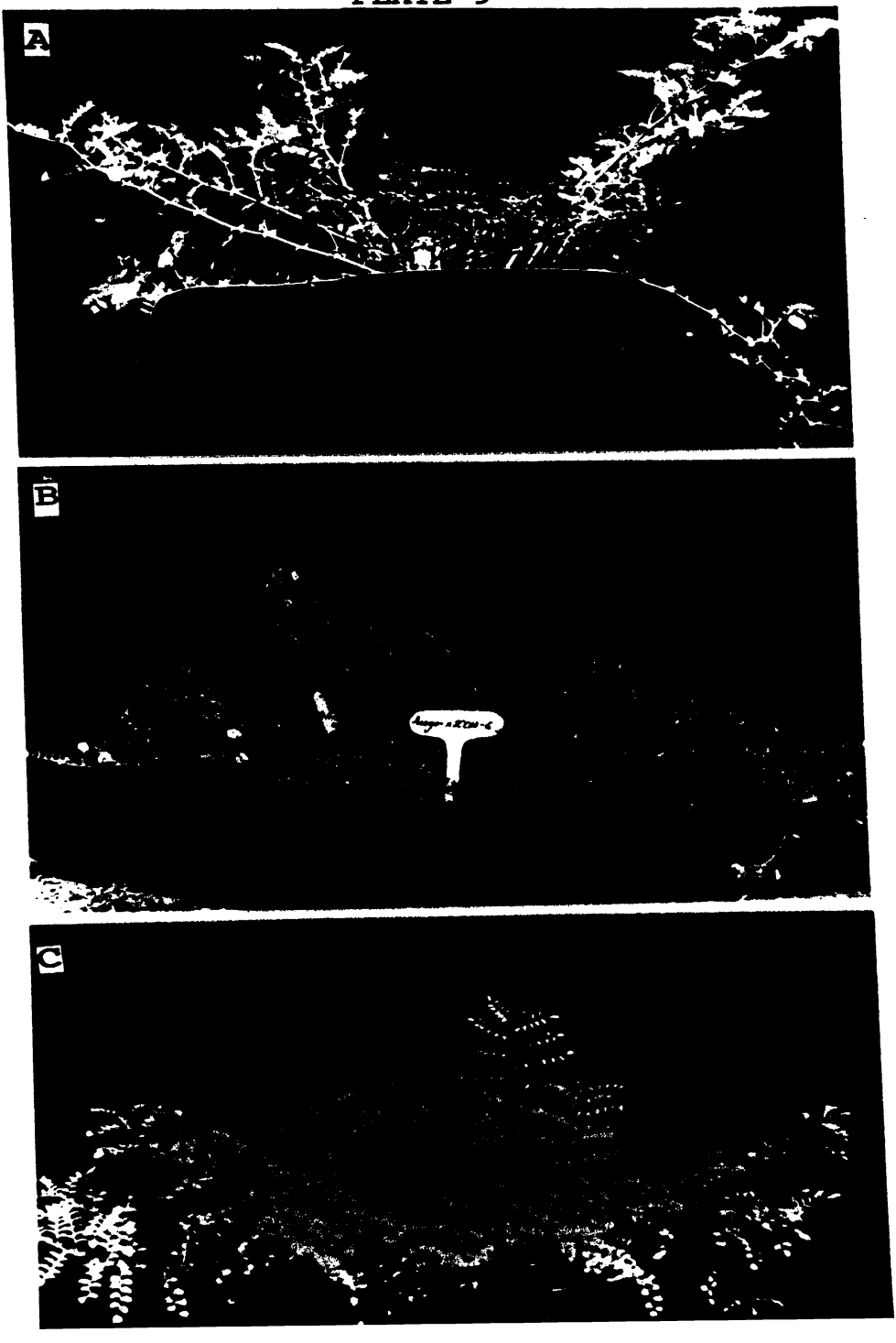


PLATE-10

Plant morphology of parents and F_1 hybrid in genus *Cicer*.

(A) *C. arietinum* (ICCC-32)
(B) F_1 hybrid
(C) *C. reticulatum* (ICCW-49)

PLATE-10

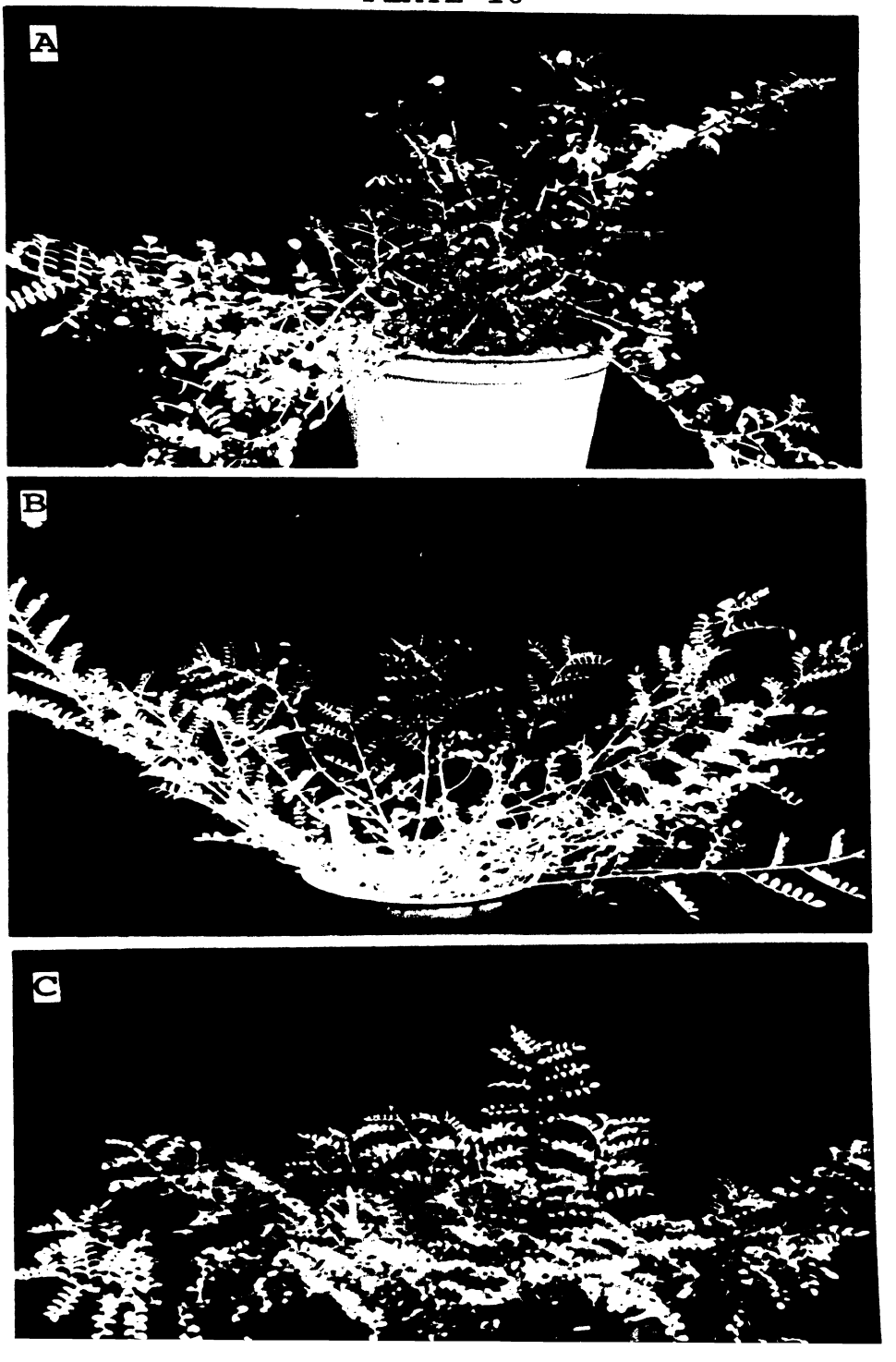


PLATE-11

Plant morphology of parents and F_1 hybrid in genus *Cicer*.

(A) *C. arietinum* (Annigeri)
(B) F_1 hybrid
(C) *C. echinospermum* (ICCW-44)

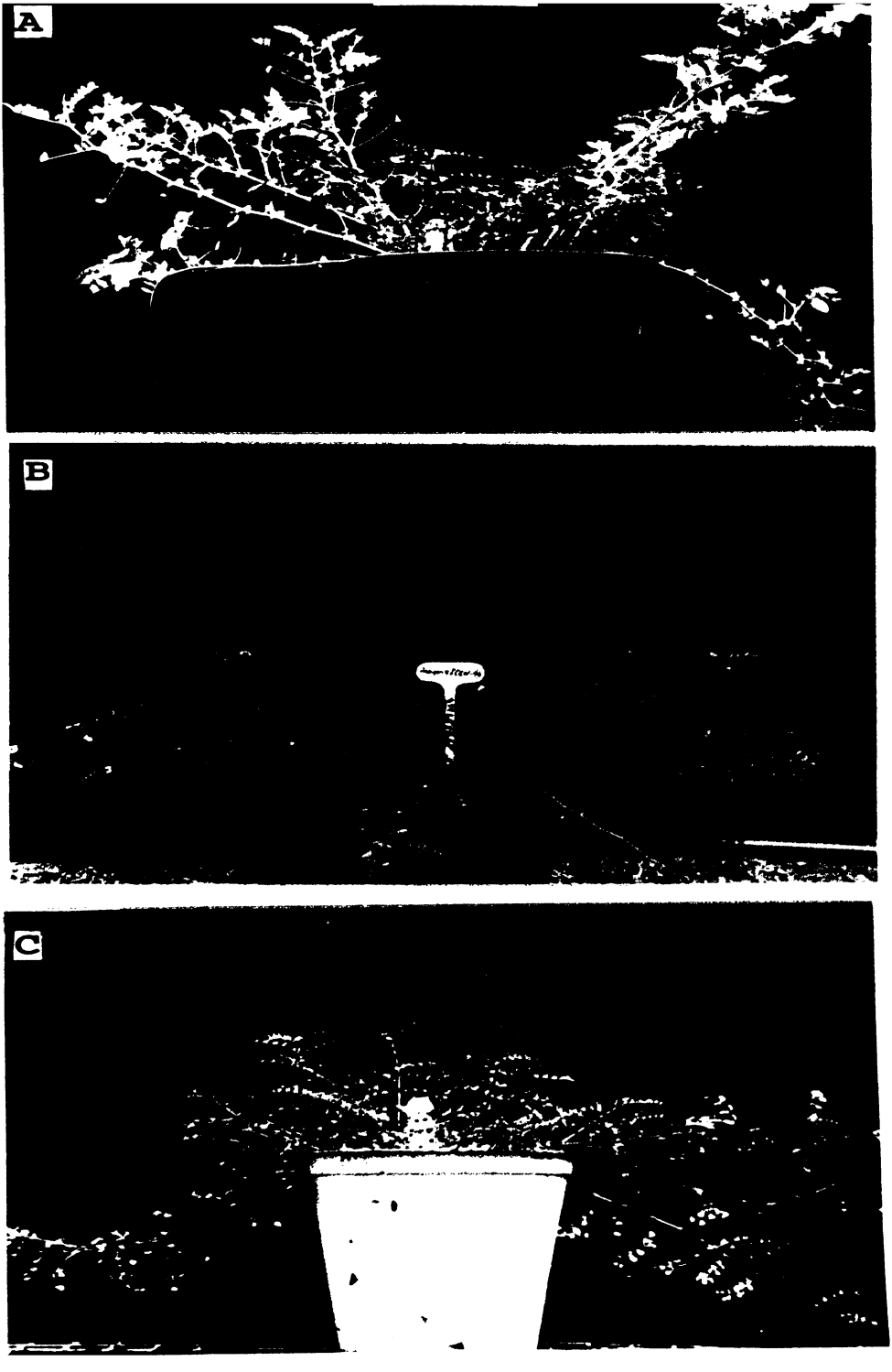

A
B
C

PLATE-12

Plant morphology of parents and F_1 hybrid in genus *Cicer*.

(A) *C. arietinum* (ICCC-32)
(B) F_1 hybrid
(C) *C. echinospermum* (ICCW-44)

PLATE-12

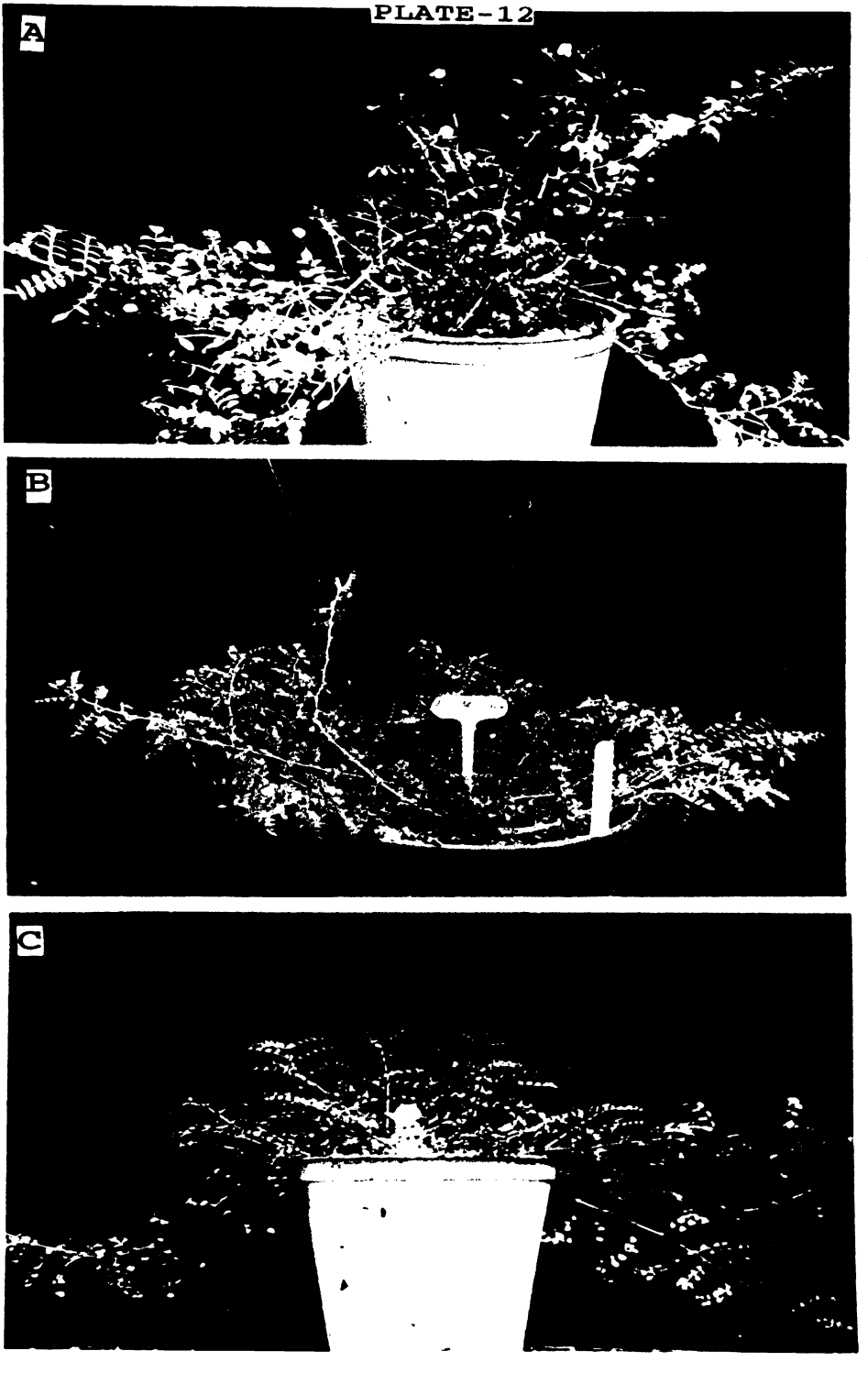
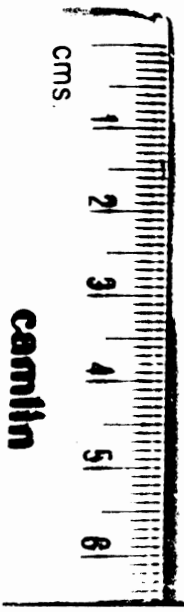


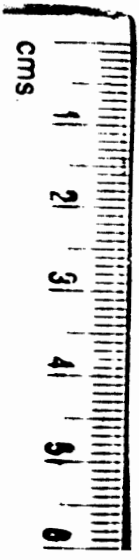
PLATE-13

Leaf morphology of parents and F_1 hybrid.

PLATE-13



C. arietinum ICCC-42 F_1 C. reticulatum ICCW-49



C. arietinum ICCC-42 F_1 C. echinospermum ICCW-44

PLATE-14

Leaf morphology of parents and F_1 hybrid.

PLATE-14

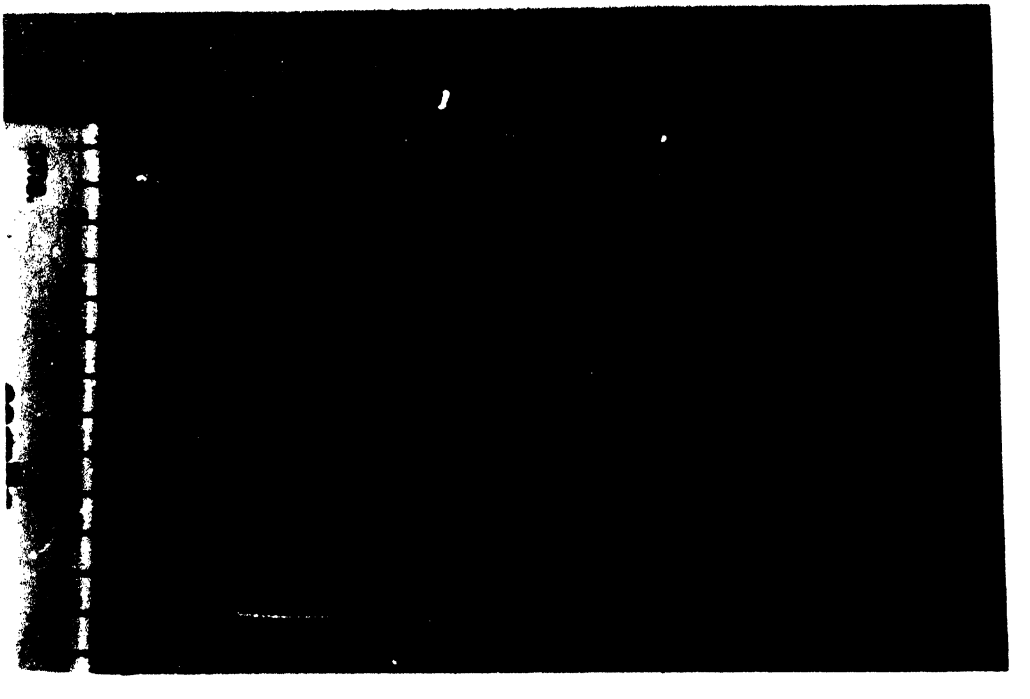


PLATE-15

Seed morphology of parents and interspecific F_1 hybrid in genus *Cicer*

(A) *C. arietinum X C. reticulatum*
(Annigeri, *desi*) (ICCW-6)

(C) *C. reticulatum X C. arietinum*
(ICCW-6) (Annigeri, *desi*)

(B) *C. arietinum X C. echinospermum*
(Annigeri, *desi*) (ICCW-44)

PLATE-15

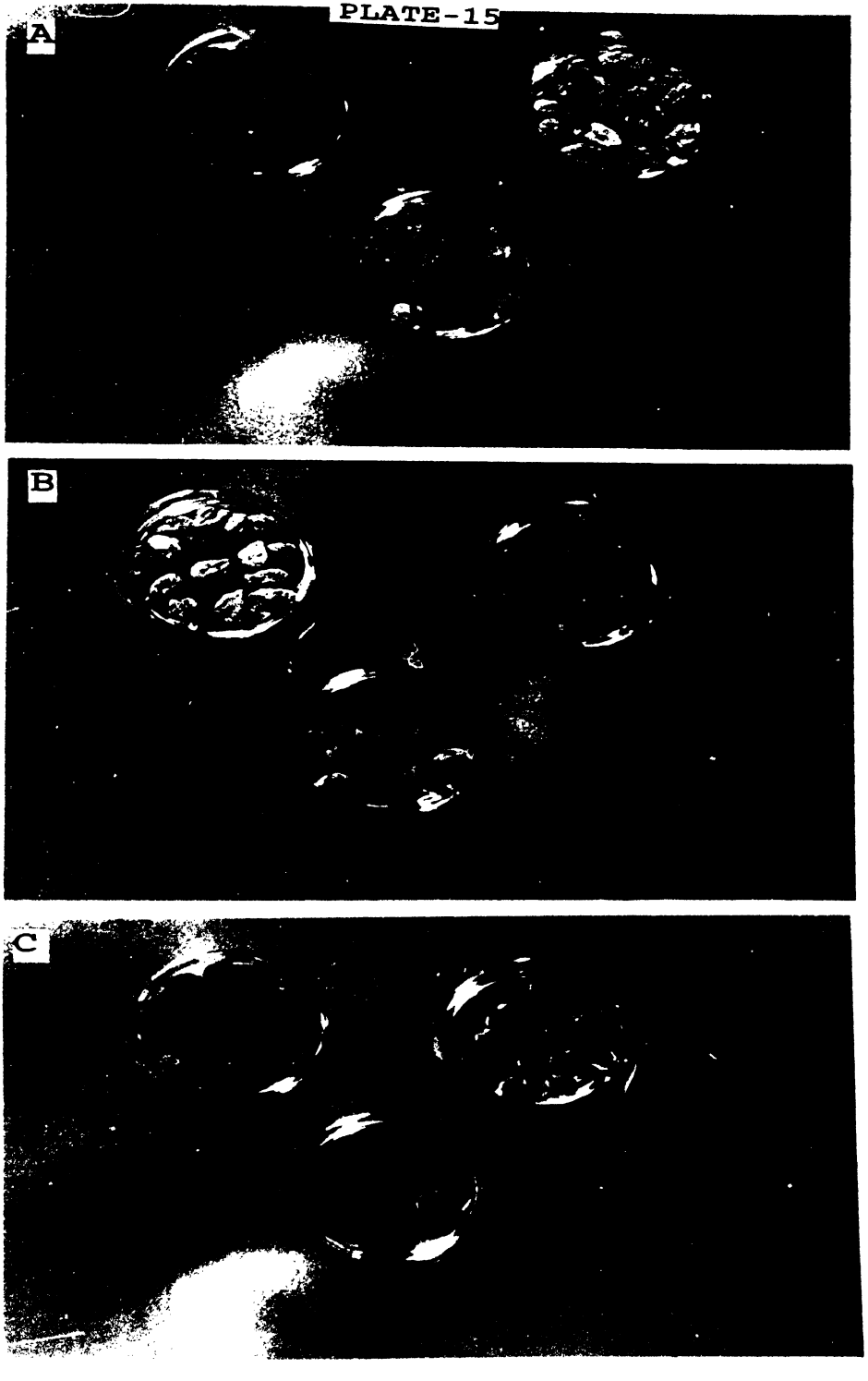


PLATE-16

Seed morphology of parents and interspecific F_1 hybrid in genus *Cicer*

(A) *C. arietinum X C. reticulatum*
(GL-769, *desi*) (ICCW-49)

(B) *C. arietinum X C. echinospermum*
(GL-769, *desi*) (ICCW-44)

PLATE-16

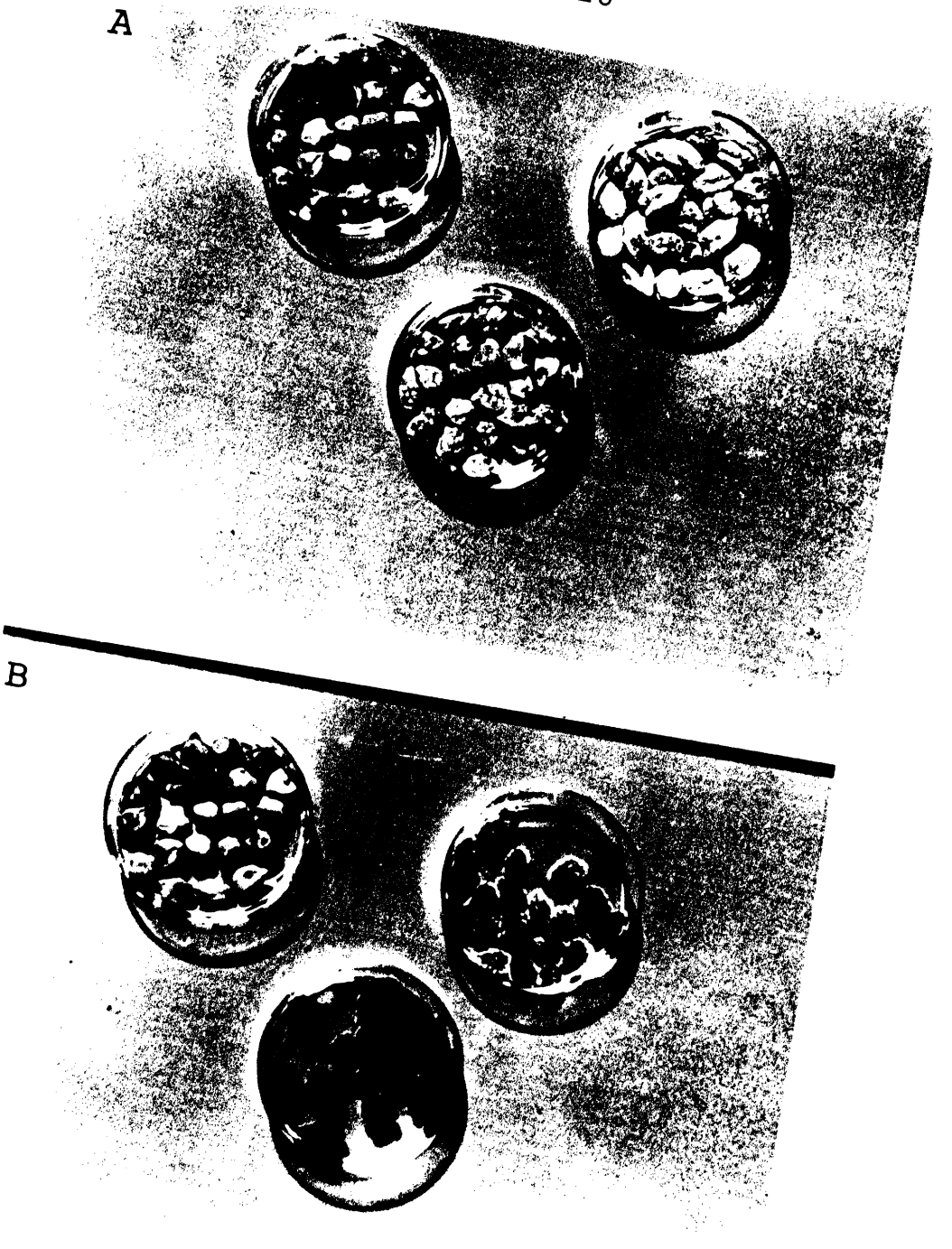


PLATE-17

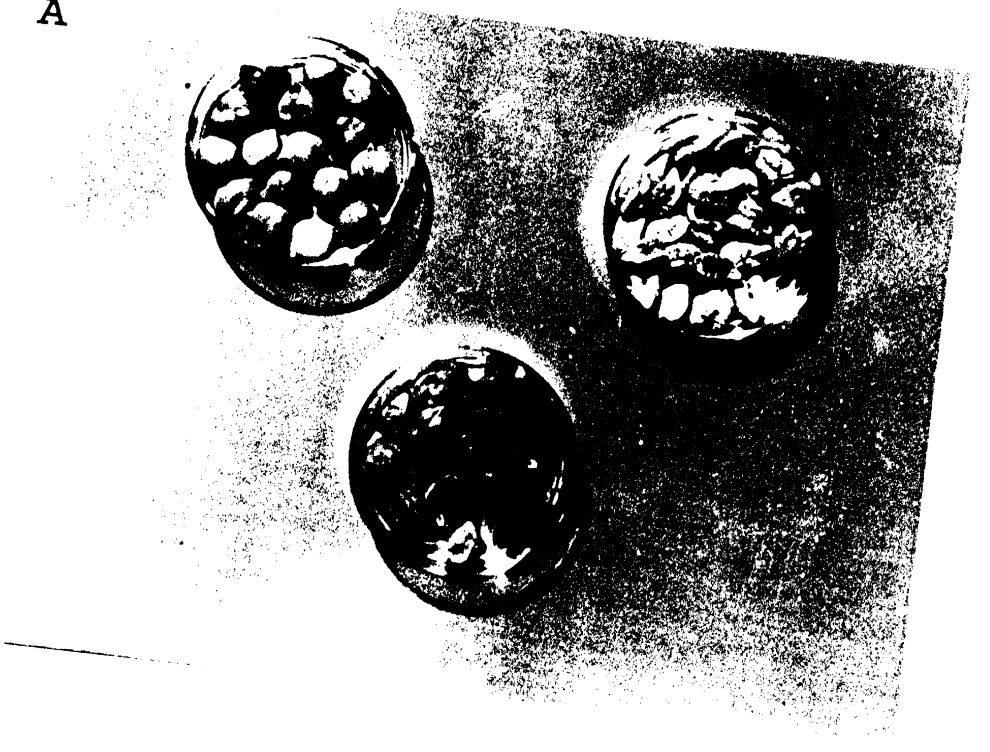
Seed morphology of parents and interspecific F_1 hybrid in genus *Cicer*

(A) *C. arietinum X C. reticulatum*
(K-850, *desi*) (ICCW-49)

(B) *C. arietinum X C. echinospermum*
(K-850, *desi*) (ICCW-44)

PLATE-17

A



B

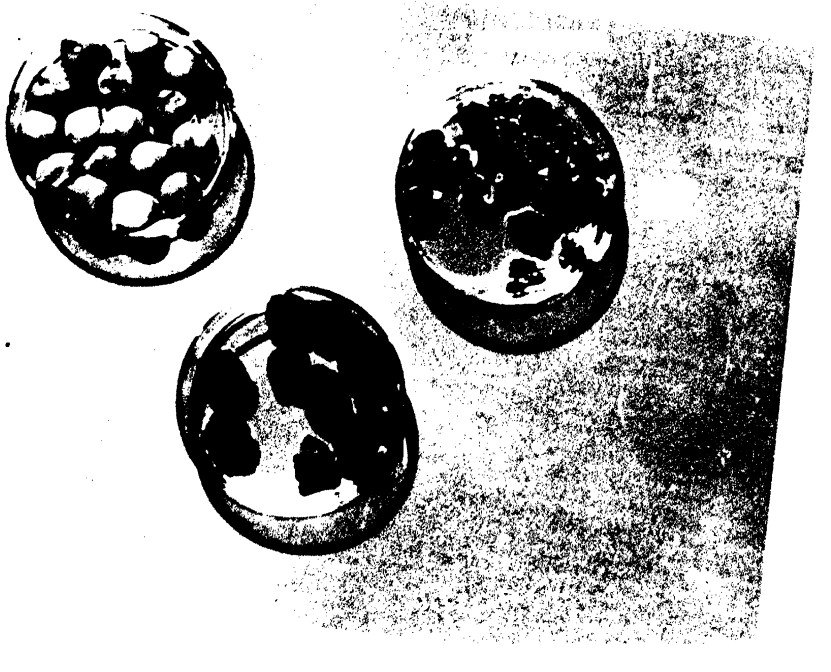


PLATE-18

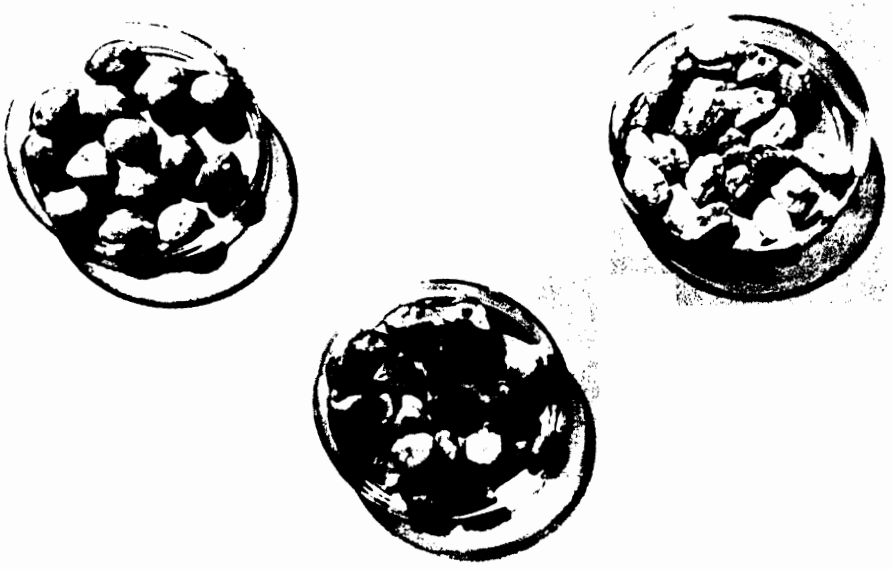
Seed morphology of parents and interspecific F_1 hybrid in genus *Cicer*

(A) *C. arietinum X C. reticulatum*
(ICCC-42, *desi*) (ICCW-6)

(B) *C. arietinum X C. echinospermum*
(ICCC-42, *desi*) (ICCW-44)

PLATE-18

A



B

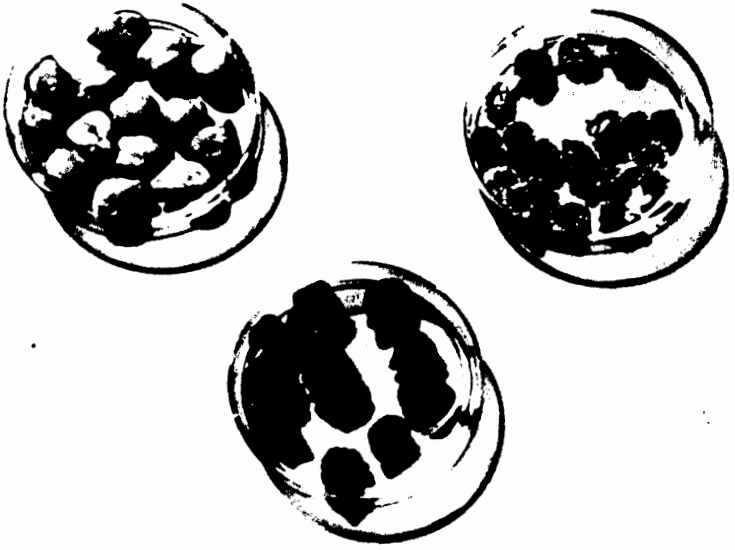


PLATE-19

Seed morphology of parents and interspecific F_1 hybrid in genus *Cicer*

(A) *C. arietinum X C. reticulatum*
(ICCC-32, *kabuli*) (ICCW-6)

(B) *C. arietinum X C. echinospermum*
(ICCC-32, *kabuli*) (ICCW-44)

PLATE-19

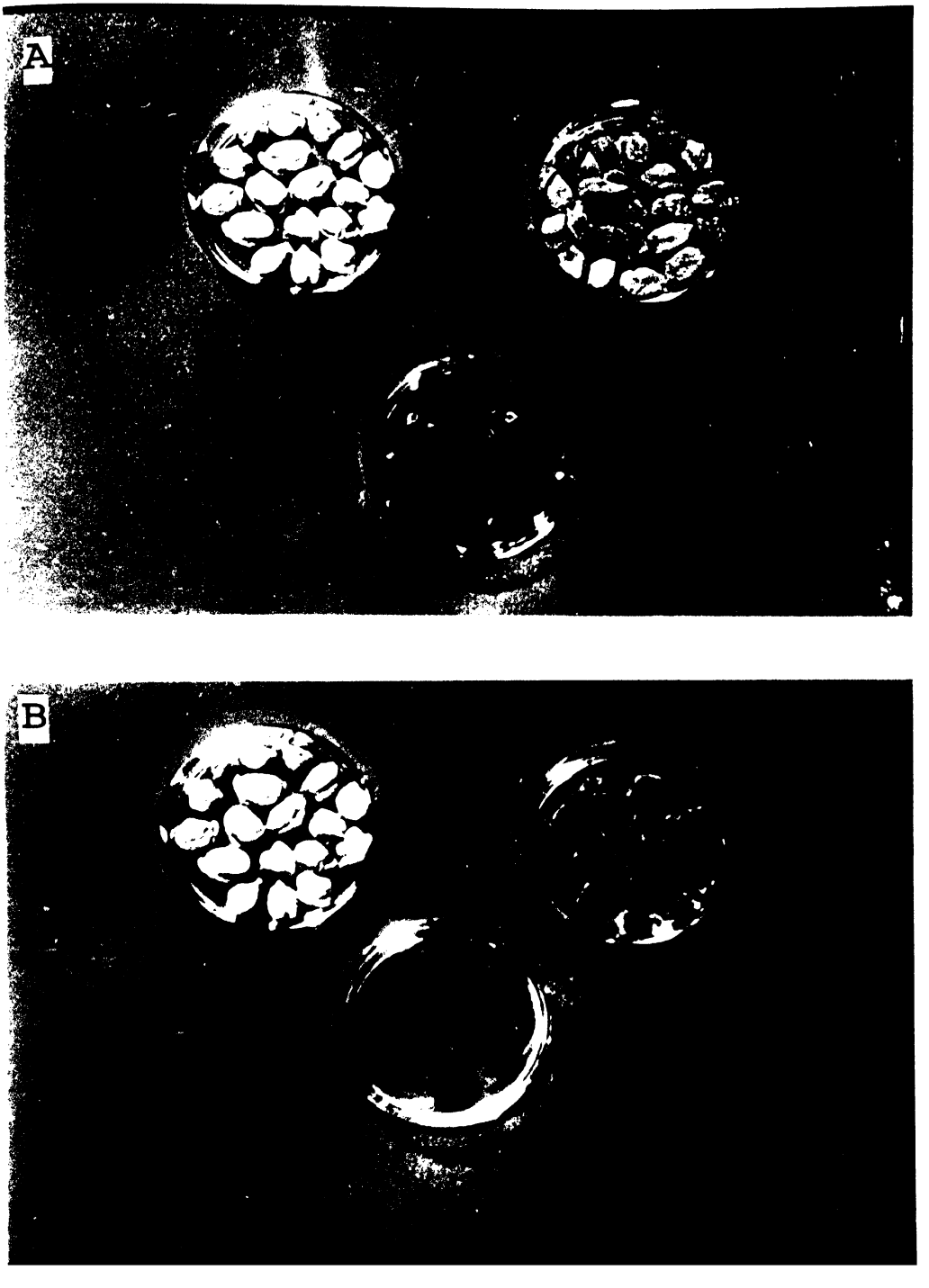
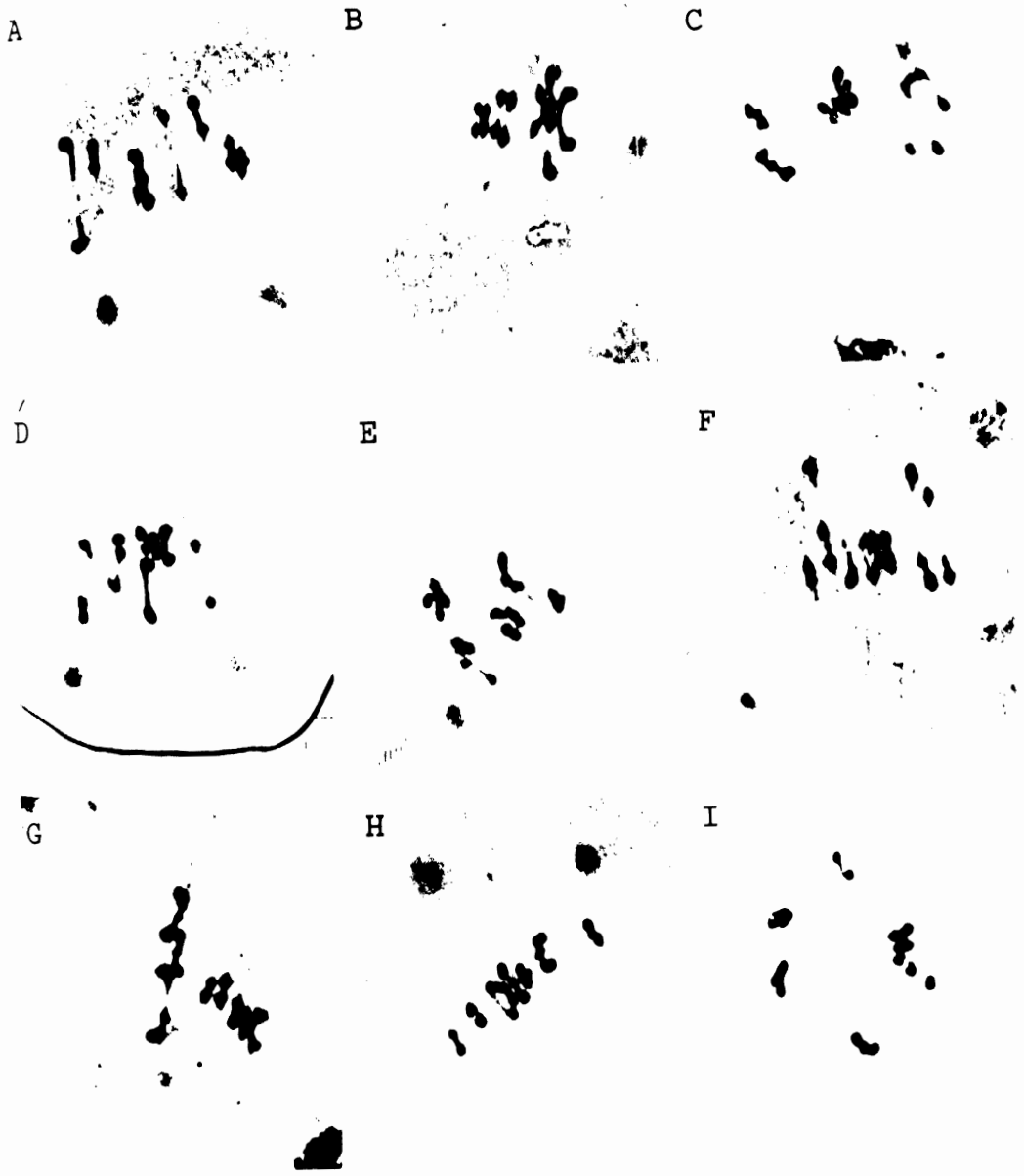


PLATE-20

Meiotic metaphase I configuration in the F_1 interspecific hybrids in annua *Cicer* species.

Cross	I	Ring II	Rod II	III	IV	Chiasma Frequency
(A) *C. arietinum* (Annigeri) X *C. reticulatum*(ICCW-6)	-	4	4	-	-	12/12
(B) *C. arietinum* (Annigeri) X *C. reticulatum*(ICCW-6)	-	2	6	-	-	10/10
(C) *C. reticulatum* (ICCW-6) X *C. arietinum* (Annigeri)	-	3	3	-	1	12/12
(D) *C. arietinum* (Annigeri) X *C. echinospermum* (ICCW-44)	-	3	3	-	1	12/12
(E) *C. arietinum* (Annigeri) X *C. echinospermum* (ICCW-44)	1	3	3	1	-	11/11
(F) *C. arietinum* (ICCC-32) X *C. reticulatum* (ICCW-49)	-	3	5	-	-	11/11
(G) *C. arietinum* (ICCC-32) X *C. reticulatum* (ICCW-49)	-	4	4	-	-	12/12
(H) *C. arietinum* (ICCC-32) X *C. echinospermum* (ICCW-44)	-	4	2	-	1	13/13
(I) *C. arietinum* (ICCC-32) X *C. echinospermum* (ICCW-44)	1	4	2	1	-	12/12

PLATE-20



formation of quadrivalent (in 50% of PMCs), though the chiasma frequency was approximately similar to the cross when *C. arietinum* was used as female parent. Pollen fertility was considerably reduced (55-60%) and pod set was 35-40%.

4.3.3.3 *C. arietinum X C. echinospermum*

Hybrid plants produced from the crosses of different cultivars of *C. arietinum* (Lines- Annigeri, K-850, GL-769, ICCC-42, and ICCC-32) with *C. echinospermum* (ICCW-44) were examined. The F_1 plants were vigorous and had a semi-erect growth habit with purple flowers, even in case when ICCC-32 (*kabuli*) with white flower color was used as female parent. The hybrid was intermediate for other morphological characters (**Plates 11, 12,13,14,15C,16B,17B,18B,19B**). The frequency of PMCs with a quadrivalents (**Plate 20 D,E,H,I**) and a trivalent and univalent was 72% and 21%, respectively.

The percent of pod set was observed to be high in case when *desi* accession of *C. arietinum* used as female parent (40% pod set), whereas, the pod set in case of *kabuli* accession was low (27% pod set). The seed coat structure was intermediate between those of the parental species.

4.3.3.4 *C. arietinum X C. pinnatifidum*

Hybrid seeds were produced for the first time between *C. arietinum X C. pinnatifidum* during the present study. However, only single seed from each cross using cultivars Annigeri, K-850, GL-769, ICCC-42 and ICCC-32 and *C. pinnatifidum* (ICCW-37) was produced (**Table 12**). Germination of the hybrid seed was normal but resulted in albino plants. The leaf morphology of

C. pinnatifidum was found to be dominating that of the hybrid plants (**Plate 22 A,B**), which confirms that it was not a selfed seed (**Plate 21**).

When the hybrid embryos from this cross was rescued by embryo culture technique, all the hybrid seedlings showed the same albino character and could not survive for more than 20-25 days. Therefore, no cytological characterization was possible in this hybrid derived from both field and through embryo rescue.

4.3.3.5 *C. chorassanicum X C. pinnatifidum*

Five hybrid seeds were produced when *C. chorassanicum* (ICCW-26) was used as female parent and *C. pinnatifidum* (ICCW-37) as male parent (**Table 13**). These hybrid seeds germinated normally, but the seedlings were albino, died 15-20 days after transplanting. Hence, no cytological characterization was possible for this interspecific hybrid. The leaf and seedling morphology was of intermediate character (**Plates 22 C,23**).

4.4 POLLEN GERMINATION AND POLLEN TUBE GROWTH STUDIES

4.4.1 SELFED *Cicer* SPECIES

The time taken between self pollination and fertilization in eight annual *Cicer* species under *in vitro* and *in vivo* conditions is summarized in **Table 17**. The time required for pollen tube to reach to micropyle after self pollination varied greatly in different *Cicer* species. Growth was fast in *C. pinnatifidum* in which the pollen tube took only 6.7 h *in vivo* and 8.9 h *in vitro* to reach its micropyle, whereas the longest periods of 23.6 and 34.7 h were required in *C. reticulatum*. It was observed in all the species studied that

PLATE-21

Plant morphology of parents and F_1 hybrid in genus *Cicer*.

(A) *C. arietinum* (ICCC-32)
(B) F_1 hybrid (albino)
(C) *C. pinnatifidum* (ICCW-37)

PLATE-21

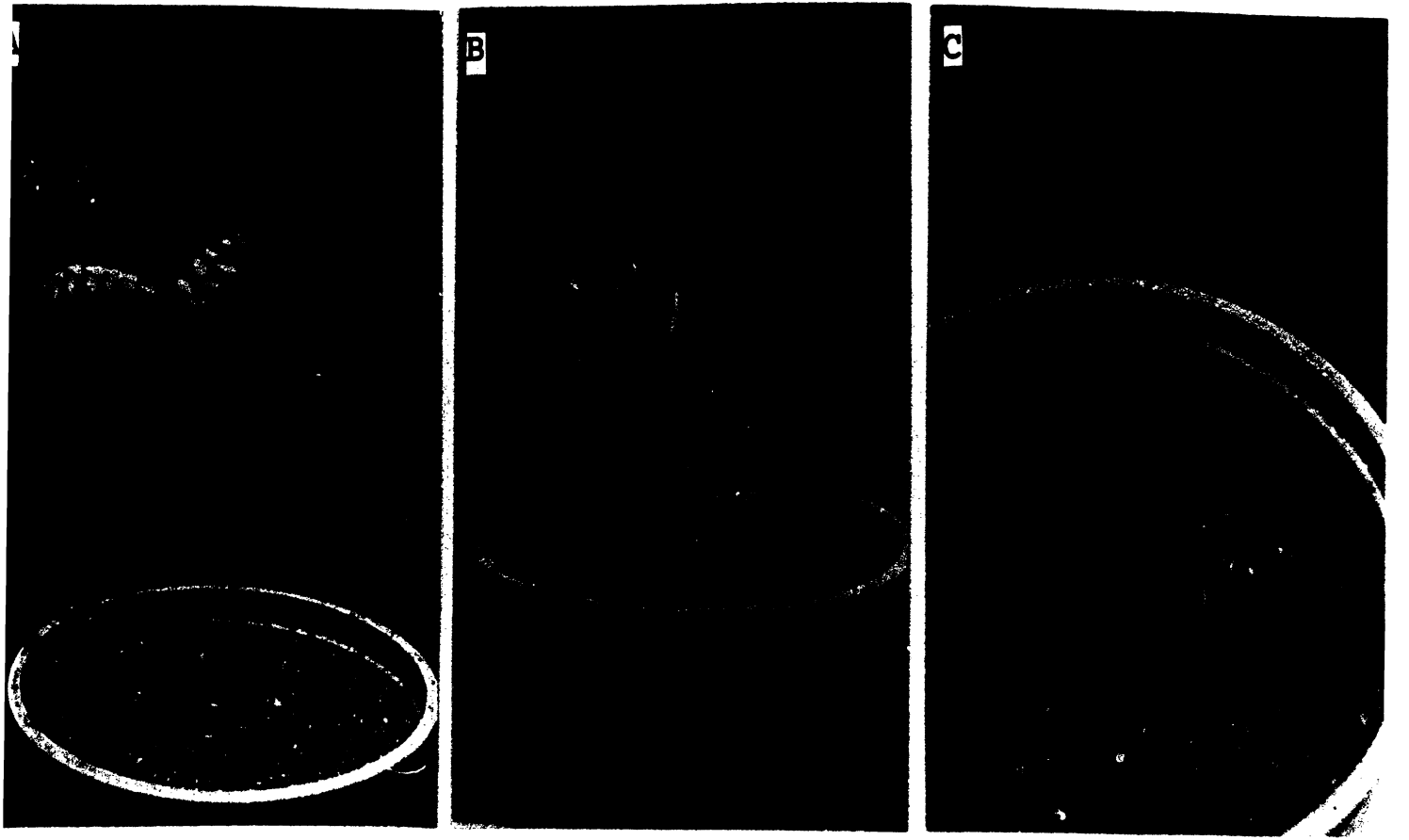


PLATE-22

Leaf morphology of parents and F_1 hybrid.

C. arietinum
GL-769

F_1

C. pinnatifidum
ICCW-37

C. arietinum
ICCC-32

F_1

C. pinnatifidum
ICCW-37

C. chorassanicum
ICCW-26

F_1

C. pinnatifidum
ICCW-37

PLATE-23

Plant morphology of the parents and F_1 hybrid

(A) *C. chorassanicum* (female)

(B) *C. pinnatifidum* (male)

(C) F_1 hybrid

PLATE-23

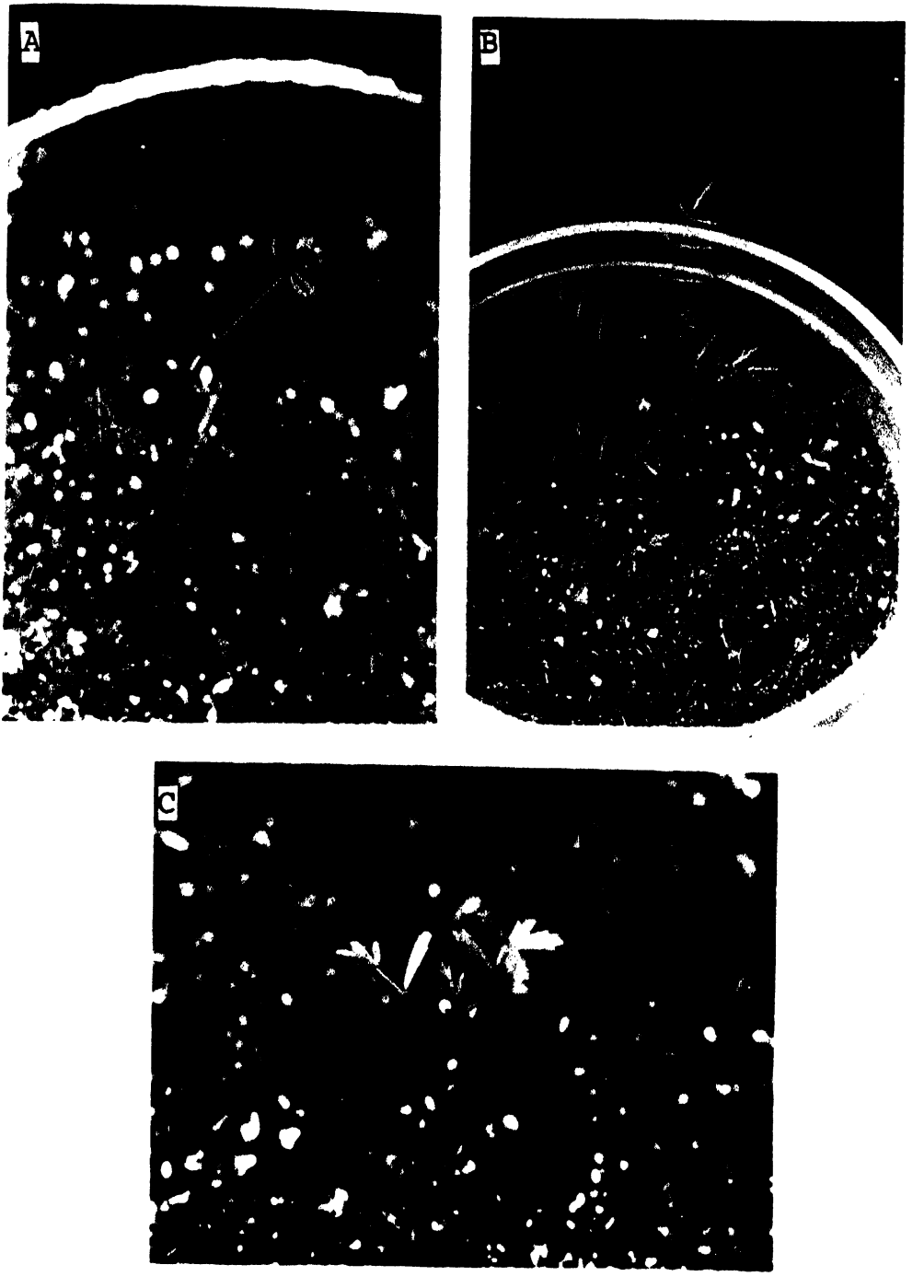


Table 17 Time taken for pollen tubes to reach micropyle after self pollination in annual *Cicer* species, *in-vivo* and *in-vitro*.

Species	Mean time taken (hrs)	
	In-vivo	*In-vitro*
C. pinnatifidum	6.7	8.9
C. chorassanicum	6.9	11.5
C. judaicum	7.8	11.9
C. echinospermum	8.3	13.2
C. cuneatum	11.5	16.8
C. bijugum	20.8	29.5
C. arietinum	22.9	34.1
C. reticulatum	23.6	34.7

the time required for pollen tubes to reach micropyle under *in vitro* condition was always more than the time took under *in vivo* condition.

The three species, *C. arietinum, C. reticulatum* and *C. echinospermum* have styles of approximately the same length, *C. echinospermum* required less than half the time for self fertilization than the other two species. Likewise, *C. pinnatifidum* and *C. judaicum* have styles of approximately the same length, yet the time required for self fertilization in *C. pinnatifidum* was much less than that required in *C. judaicum* (**Table 17**).

4.4.2 CROSSES BETWEEN CULTIVATED AND WILD ANNUAL *Cicer* SPECIES.

When the interspecific crosses were made using *C. arietinum* as female parent, the pollen of wild species germinated and penetrated the stigma after a short time in all cross combinations (**Plate 24A**). The time taken by pollen tubes to reach micropyle in interspecific crosses is presented in **Table 18**.

The pollen grains usually produced thick pollen tubes near the stigmatic surface at the time of germination (**Plate 24B**). However, as tubes grew down in the style, they became thinner and intensity of fluorescence also decreased. In few cases, pollen grains germinated on the style or ovary wall, but were unable to penetrate it. Instead, they usually formed short pollen tubes along the style or ovary wall, or the pollen tube twisted and folded back.

Pollen tubes could not be observed very clearly towards the end of the style because of the presence of glandular hairs (**Plate 24H**), but could be distinguished clearly in the ovary. Pollen tubes were characterized by irregularly spaced callose deposits. Sometimes callose was dense, filled up the pollen tube and was clearly visible (**Plate 24G**), while in other cases parts of the

PLATE-24

Pollen tube growth in interspecific crosses among annual *Cicer* species.

(A) Pollen started germination on the stigma in *C. arietinum X C. reticulatum*, 2hr after pollination (HAP),

(B) Pollen tube growth in *C. arietinum X C. echinospermum* 8 HAP, showing dense callose deposits,

(C) A pollen tube near the ovule after having formed a bulbous end in *C. arietinum X C. bijugum* (16 HAP),

(D) Callose deposits at the point of growth of the pollen tube in *C. bijugum X C. arietinum* (15 HAP),

(E) Twisted and curled pollen tube in *C. judaicum X C. arietinum* (10 HAP),

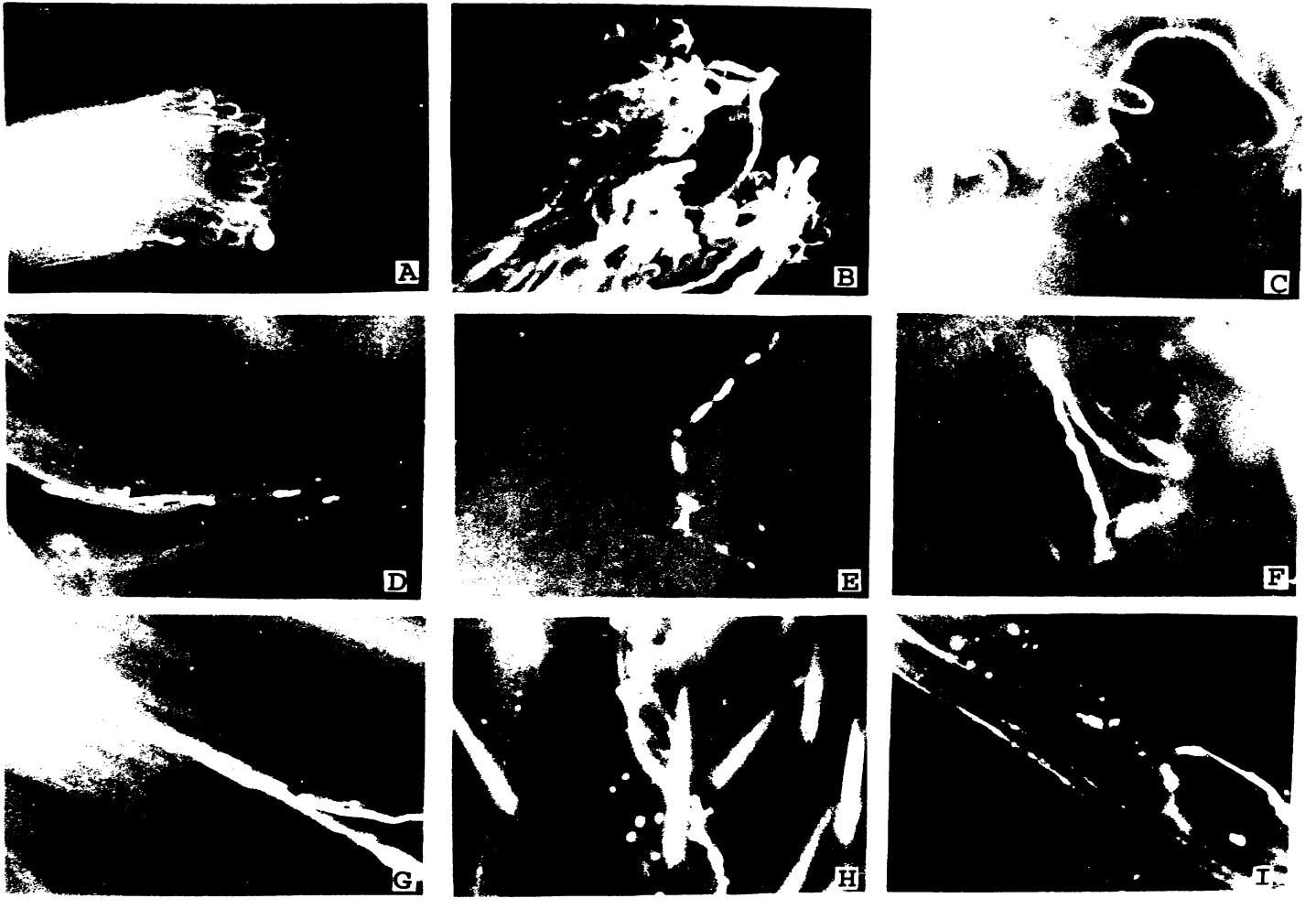
(F) Branched pollen tube in *C. arietinum X C. bijugum* (16 HAP),

(G) Whole tube filled with callose in *C. reticulatum C. arietinum* (13 HAP),

(H) Pollen tubes among the glandular hairs,

(I) Incomplete filling of callose in the tube showing the discontinuity in *C. pinnatifidum X C. Chorassanicum* (6 HAP).

PLATE-24



pollen tubes were devoid of callose and were not visible, giving the appearance of a discontinuous pollen tube (**Plate 24I**).

Time taken for pollen germination on stigma and pollen tube growth rates in style were similar in crosses involving different accessions of a given cross combination. Thus, data involving different accessions of a species were pooled. A great difference was observed between reciprocal crosses in the time required for pollen tubes to reach to micropyle. In all cases, except crosses involving *C. echinospermum* and *C. chorassanicum* as one of the parents, the time taken in reciprocal crosses was more in the same cross combination. The time required for fertilization varied considerably in different interspecific cross combinations, and ranged from lowest 8.2 h (*in vivo*) and 10.1 h (*in vitro*) in *C. arietinum* X *C. echinospermum* to highest up to 34.1 h (*in vivo*) and 45.2 h (*in vitro*) in *C. arietinum* X *C. reticulatum* (**Table 18**). Large differences in time taken by pollen grain to reach micropyle were observed in some reciprocal interspecific crosses. Time required for pollen tube to reach micropyle was almost double when *C. arietinum* was used as the male parent, rather than as female parent, in crosses with *C. judaicum*, *C. pinnatifidum* and *C. reticulatum* (**Table 18**).

Usually only one (**Plate 24C**), but occasionally two or three pollen tubes were observed near the ovules. Few abnormalities in pollen tube growth were also observed in these interspecific pollinations. The most common was the formation of bulbous portions in pollen tubes (**Plate 24D**). This bulbous structure, however, did not prevent further pollen tube growth as entry of the pollen tube into the ovule was observed despite this abnormality (**Plate 24C**). Other abnormalities such as curled and twisted pollen tubes (**Plate 24E**) and branched pollen tubes (**Plate 24F**) were observed.

Table 18 Time taken for pollen tubes to reach micropyle after cross pollination between *Cicer arietinum* and wild annual *Cicer* species, *In-vivo* and *In-vitro*.

CROSSES		MEAN TIME TAKEN (h)			
		Original Crosses		Reciprocal Crosses	
Female	Male	*In-vivo*	*In-vitro*	*In-vivo*	*In-vitro*
C. arietinum	*C. echinospermum*	8.2	10.1	9.1	12.6
C. arietinum	*C. cuneatum*	11.0	17.3	9.8	15.5
C. arietinum	*C. pinnatifidum*	11.9	18.9	7.8	9.5
C. arietinum	*C. chorassanicum*	13.2	19.7	19.8	31.2
C. arietinum	*C. bijugum*	15.3	22.1	10.2	17.1
C. arietinum	*C. judaicum*	17.2	26.8	10.5	18.0
C. arietinum	*C. reticulatum*	34.1	45.2	18.7	27.1

In spite of various abnormalities in pollen tube growth in different interspecific crosses and in their reciprocals, the pollen entered into the ovule in all interspecific crosses studied. Older ovules (72 HAP) appeared healthy and were developing in most of the crosses, indicating that some growth had occurred after fertilization, which showed that the barrier(s) to interspecific hybridization between the cultivated and the wild annual *Cicer* species were definitely of post-fertilization in nature.

4.5 POLLEN MORPHOLOGY

4.5.1 GENERAL FEATURES OF POLLEN OF *Cicer* SPECIES

The pollen of different species of *Cicer* were tricolporate (Amb type-fossaperturate). The shape of the pollen varied from oblate-spheroidal to prolate- depending upon the polar length to equatorial length (P/E) ratio, which ranged from 0.97-2.00 (**Table 19**). Outline of pollen in polar view was triangular with slightly convex mesocolpia and apertures were set in truncate corners, or more or less circular, whereas, in equatorial view it was circular to elliptic. The apertures were sometimes found to be projecting a little at the equator. Deep ectoapertures and colpi longer than the length of the polar axis. In some cases colpi end is extended, the extensions anastomosing to delimit a triangular apocolpium. Margins were very weakly thickened with coarsely granular membrane. Endoaperture pori, lolongate, with poorly defined margins. Exine ornamentation reticulate (polybrochate). The lumina varied in size, often smaller in center of the mesocolpium, but it was found absent in a broad imperforate band bordering the colpi. The lumina diameter varied from 0.70-2.14 µm. Apocolpia are microreticulate. Murri thickness was in the range of 0.41-0.93 µm. Size of pollen varies from small to medium.

Table 19 Morphological characterstics of pollen grains of eight annual *Cicer* species using classification and characters described by Erdtman (1966).

Species	Polar Axis P (µm)	Equatorial Axis E (µm)	P/E	Shape	Exine Ornamentation		
					Thickness Of Muri T (µm)	Mean Diameter of Lumina D (µm)	# of Brochi N
C. arietinum (ICCC-32)	33.1±1.5[1]	16.3±1.0	2.00±0.6	Prolate	0.72±0.02	1.01±0.06	64±5
C. arietinum (Annigeri)	31.7±1.6	18.8±1.0	1.69±0.2	Prolate	0.53±0.02	0.68±0.06	75±5
C. reticulatum (ICCW-49)	35.0±2.9	18.5±1.4	1.89±0.2	Prolate	0.52±0.02	0.82±0.06	79±5
C. echinospermum (ICCW-44)	33.8±1.1	17.0±1.3	1.99±0.3	Prolate	0.41±0.01	0.86±0.06	67±6
C. bijugum (ICCW-42)	23.8±0.7	19.0±1.3	1.25±0.1	Sub-Prolate	0.60±0.02	1.16±0.08	55±2
C. pinnatifidum (ICCW-37)	30.0±1.5	17.1±1.5	1.75±0.3	Prolate	0.64±0.01	2.10±0.10	42±6
C. judaicum (ICCW-36)	28.5±1.2	15.2±1.1	1.88±0.1	Prolate	0.46±0.04	1.20±0.10	64±4
C. cuneatum (ICCW-47)	30.9±1.1	17.6±2.0	1.76±0.2	Prolate	0.42±0.04	1.78±0.10	34±5
C. chorassanicum (ICCW-26)	21.9±2.5	22.5±2.5	0.97±0.4	Oblate-Shperoidal	0.93±0.06	2.14±0.13	35±6#

[1] Mean ± Standard Deviation

C. arietinum(A) cv. Annigeri (*desi*)- The pollen of *C. arietinum* cv. Annigeri was found to be of medium size with mean polar axis of 31.7±1.6 µm. Three prominent notches at poles gave pollen a triangular view (**Plate 25A**). Equatorial shape of the pollen was prolate with P/E ratio of about 1.69±0.2. Laterally convex mesocolpia, and deeply trilobed apocolpia were present. Ectoapertures were deep, and colpi was longer than the length of the polar axis, rather broad, widening from obtuse ends, with defined thin margins. Endoapertures pori were found with poorly defined margins. The murri was slightly thick (0.55±0.002 µm) with shallow lumina, which had an average diameter of 0.70±0.06 µm with approximate 78±5 brochi per 10 μm^2 (**Plate 28A**).

C. arietinum(B) ICCC-32 (*kabuli*)- The pollen of *C. arietinum* cv. ICCC-32 was of medium size (33.1±1.5 µm), slightly larger than Annigeri(*desi*) type pollen. They were found to be prolate in shape in equatorial view (P/E ratio=2.01±0.6) (**Plate 25B**), however, polar view of pollen was less triangular, more or less blunt. Ectoaperture was not deep but widened with an acute angle. Lacunae smaller than that of the *desi* type. Murri thickness was 0.72±0.02 µm, and with approximate 64±5 brochi per 10 μm^2. Diameter of lumina was 1.01±0.06 µm.

C. reticulatum- Pollen was medium sized (35.0±2.9 µm). It was circular in polar view (**Plate 25C**) and prolate in shape (**Plate 25D**). Colpi margins were microreticulate. Average lumina diameter was larger (0.82±0.06 µm)than that of *Cicer arietinum*. Number of brochi per 10 μm^2 was largest among all the species of *Cicer* studied (79±5) (**Plate 28B**).

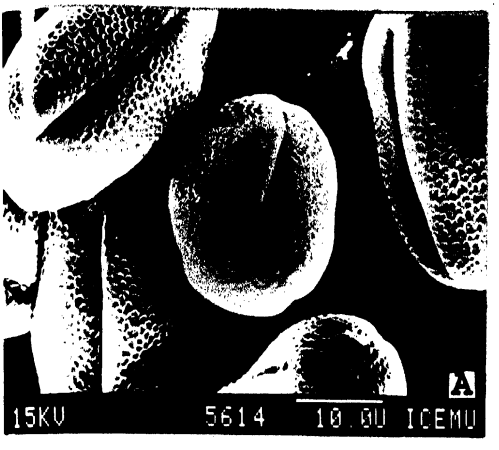
C. echinospermum- Pollen shape was prolate in equatorial view and the pollen was found to be of medium size (33.8±1.1 µm). It was triangular to trilobed in

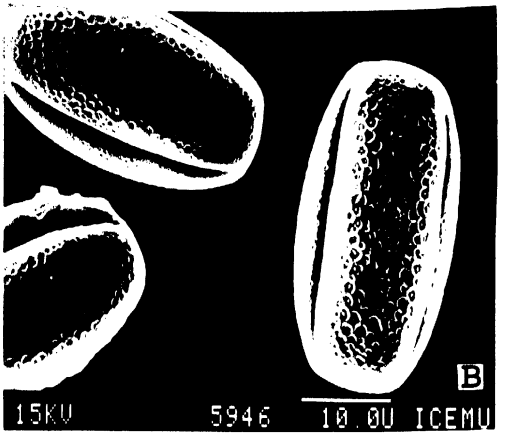
PLATE-25

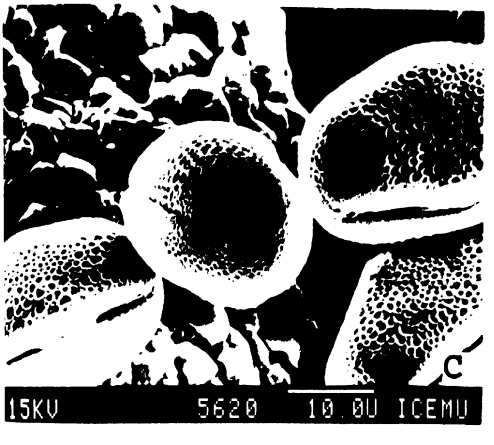
Scanning electron photomicrographs showing pollen grain morphology in annual *Cicer* species.

(A) Polar view of *C. arietinum* (*desi*),
(B) Equatorial view of *C. arietinum* (*kabuli*),
(C) Polar view of *C. reticulatum*,
(D) Equatorial view of *C. reticulatum*,
(E) Equatorial view of *C. pinnatifidum*,
(F) Polar view of *C. pinnatifidum*.

PLATE-25


A
15KV 5614 10.0U ICEMU


B
15KV 5946 10.0U ICEMU


C
15KV 5620 10.0U ICEMU


D
15KV 5618 10.0U ICEMU


E


F
15KV 5606 10.0U ICEMU

polar view (**Plate 26A**). Colpi furrows were deep with microreticulate margins. Colpi had a widening at polar end giving the apocolpium a projected appearance. Number of brochi per 10 μm² was 67±6 and lumina were shallow but granular (dia. approx.- 0.86±0.06 μm) surrounded by thin murri (0.41±0.01 μm) (**Plate 28C**).

C. pinnatifidum- Pollen of *C. pinnatifidum* was medium sized (30.0±1.5 μm), prolate in shape (**Plate 25E**), and circular in polar view (**Plate 25F**). Colpi margins at equator were wide at acute angle and were microreticulate. Deeper and wider lumina at mesocolpia (2.10±0.8 μm approx. dia.), surrounded by murri of 0.64±0.01 μm thickness. Brochi count was 42±6 per 10 μm² (**Plate 28D**).

C. judaicum- The pollen of *C. judaicum* was prolate spheroidal (**Plate 26B**) in shape (P/E ratio = 1.88±0.2), medium sized (28.5 μm polar axis length), and circular in polar view (**Plate 26C**). Lumina were deep perforated and granular with approximate diameter of 1.2±0.1 μm, surrounded by 0.46±0.04 μm thick murri. Brochi present were 64±4 per 10 μm² in number (**Plate 28E**).

C. bijugum- Pollen was sub-prolate in shape (**Plate 26D**) and small in size (23.8±0.7 μm) with weakly developed mesocolpia pouches. Colpi were fused (syncolporate) through narrow horn like channels delimiting small (3-4 μm) but prominent triangular apocolpia in polar view (**Plate 26E**). Large (approx. dia. 1.16±0.06 μm) and deep lumina with murri of 0.60±0.02 μm thickness. Brochi count was 55±3 per 10 μm² (**Plate 28F**).

C. chorassanicum- Pollen of this species was (21.9±2.5 μm) smallest of all the species studied (**Plate 26F**). Oblate spheroidal (**Plate 27A**) in shape (P/E ratio= 0.97±0.4). Broader at poles and equator, giving an overall spherical shape (**Plate 27B**). Colpi had the normal obtuse ends, but extended by two narrow

PLATE-26

Scanning electron photomicrographs showing pollen grain morphology in annual *Cicer* species.

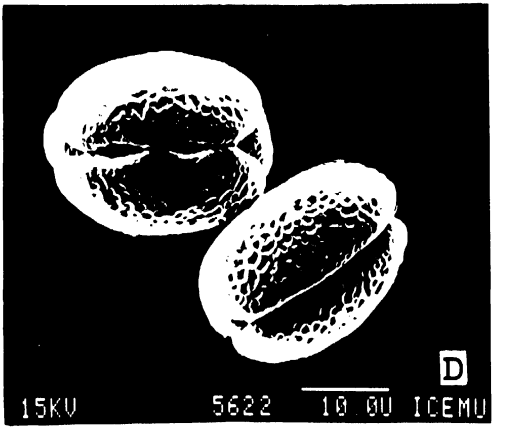
(A) Equatorial and polar views of *C. echinospermum*,
(B) polar view of *C. judaicum*,
(C) Equatorial view of *C. judaicum*,
(D) Equatorial view of *C. bijugum*,
(E) Polar view of *C. bijugum*,
(F) Cluster of *C. chorassanicum* pollen grains.

PLATE-26


A
15KV 5616 10.0U ICEMU


B
15KV 5609 10.0U ICEMU


C
15KV 5607 10.0U ICEMU


D
15KV 5622 10.0U ICEMU


E
15KV 5621 10.0U ICEMU


F

PLATE-27

Scanning electron photomicrographs showing pollen grain morphology in annual *Cicer* species.

(A) Equatorial view of *C. chorassanicum*,
(B) Polar view of *C. chorassanicum*,
(C) Polar view of *C. cuneatum*,
(D) Equatorial view of *C. cuneatum*,
(E) Exine ornamentation in *C. chorassanicum*,
(F) Exine ornamentation in *C. cuneatum*.

PLATE-27

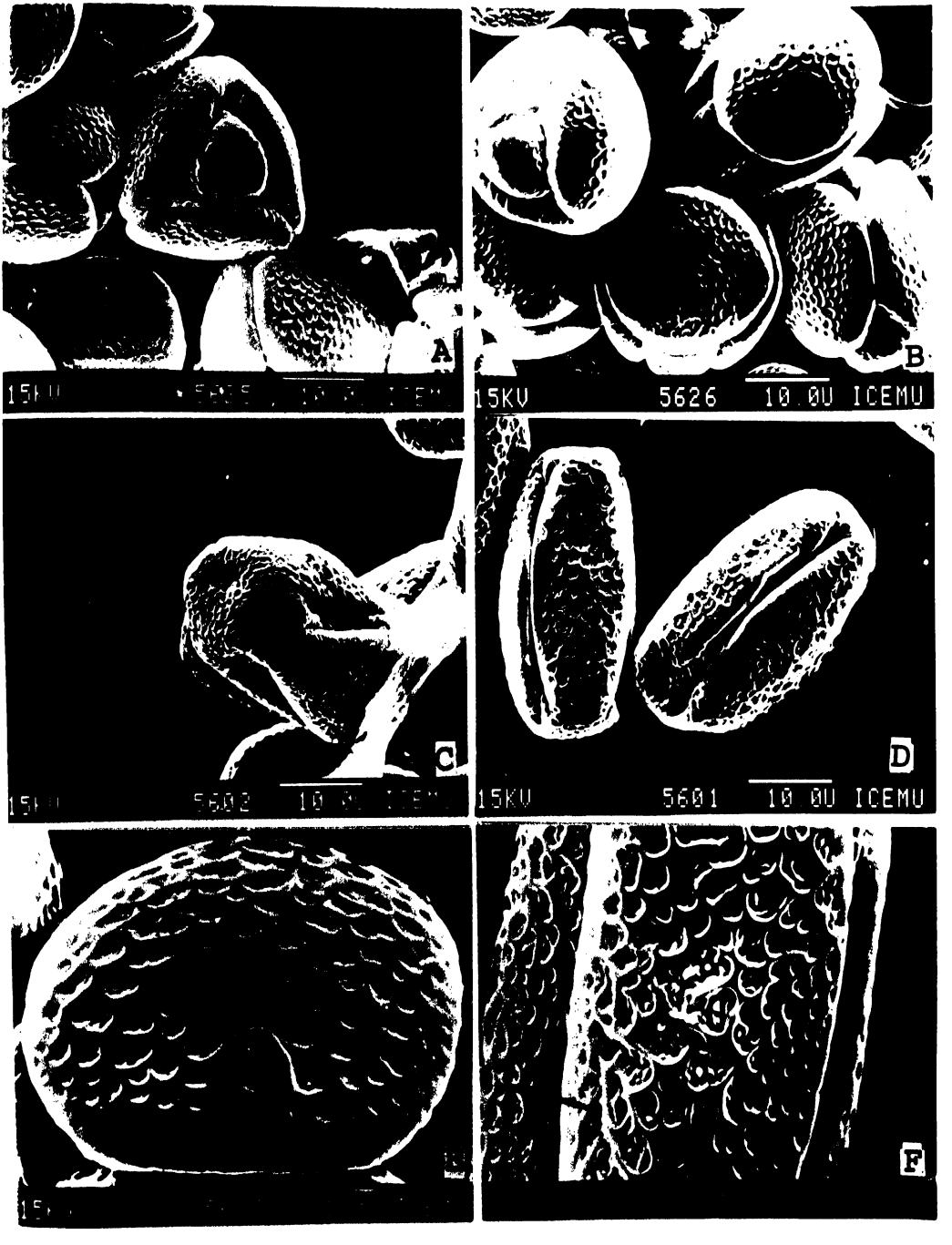
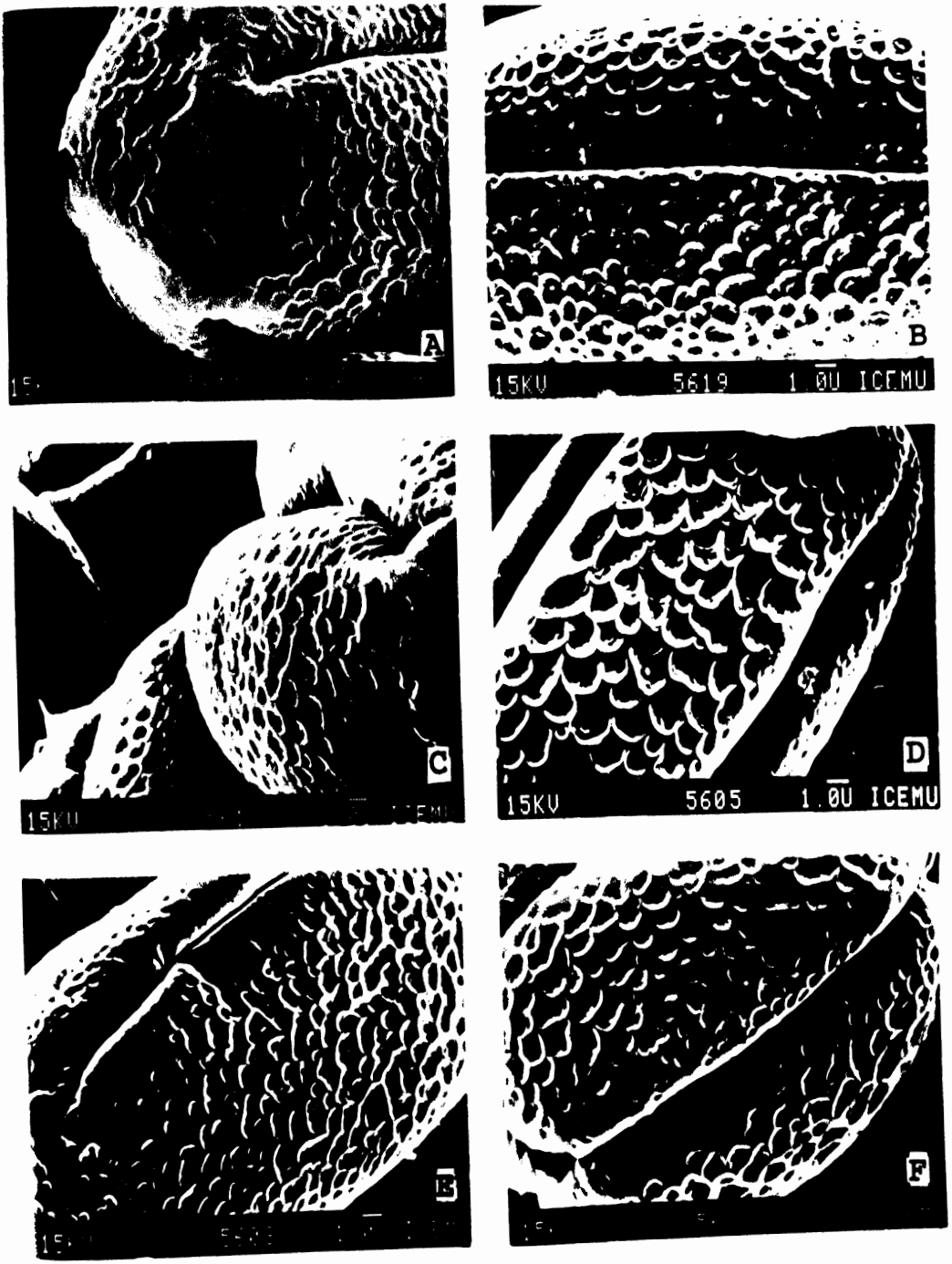


PLATE-28

Scanning electron photomicrographs showing pollen grain morphology in annual *Cicer* species.

(A) Exine ornamentation in *C. arietinum*,
(B) Exine ornamentation in *C. reticulatum*,
(C) Exine ornamentation in *C. echinospermum*,
(D) Exine ornamentation in *C. pinnatifidum*,
(E) Exine ornamentation in *C. judaicum*,
(F) Exine ornamentation in *C. bijugum*.

PLATE-28



horn like channels which link up with similar channels from an unusual, prominent large triangular apocolpia (≤10 µm) which was surrounded by colpus extensions (**Plate 27A**). Murri was very thick (0.93±0.06µm) surrounding the shallow lumina with a diameter of 2.14±0.13 µm. Brochi were 35±6 per 10 µm² in number (**Plate 27E**).

C. cuneatum- Medium sized (30.9 µm) pollen of *C. cuneatum* were found to be prolate in shape (P/E ratio = 1.76±0.2). Ectoaperture were very deep and widely furrowed (**Plate 27D**). Colpi were distinctly wide and deep at poles making apocolpium trilobed, rather irregular in shape (**Plate 27C**). Exine was deeply reticulate. Brochi were 34±5 per 10 µm² in number. Lumina was observed to be wide (approx. dia. 1.78±0.1 µm) and surrounded by 0.42 µm thick murri (**Plate 27F**).

4.5.2 POLLEN MORPHOLOGY OF INTERSPECIFIC HYBRIDS IN *Cicer* SPECIES

The overall morphology of the hybrid pollen was similar to the morphology of the normal pollen of the *Cicer* species (**Plates 29,30**), although individual differences were observed which were used to distinguish between the crosses (**Table 20**).

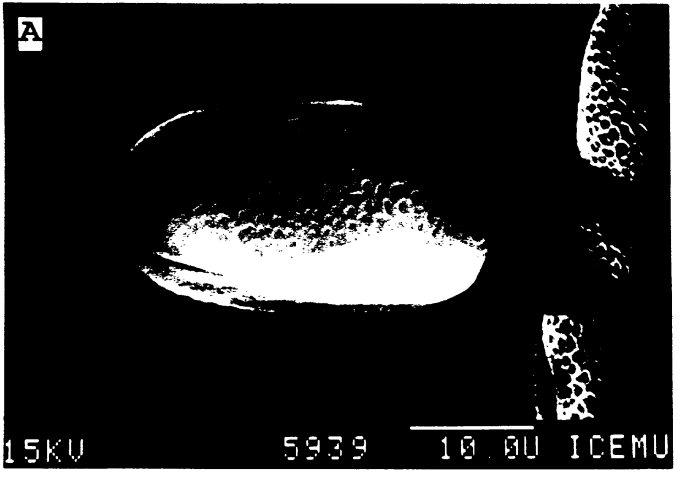
C. arietinum* (*Desi*) X *C. reticulatum: Pollen of this hybrid was medium in size (30.8±1.6 µm), smaller than *kabuli* cross. Poles blunt and circular (**Plate 29A**). Pollen prolate in shape (P/E ratio= 1.72±0.2) with slightly protruded apocolpium. Endoapertures pori with microreticulate colpi margins. Murri thickness of 0.61±0.02 µm with lumina diameter 0.48±0.13 µm, and number of brochi per 10 µm² were 77±6 (**Plate 30A**).

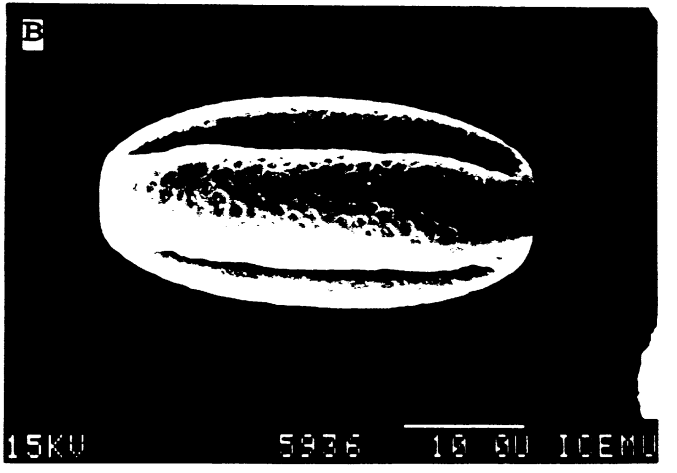
PLATE-29

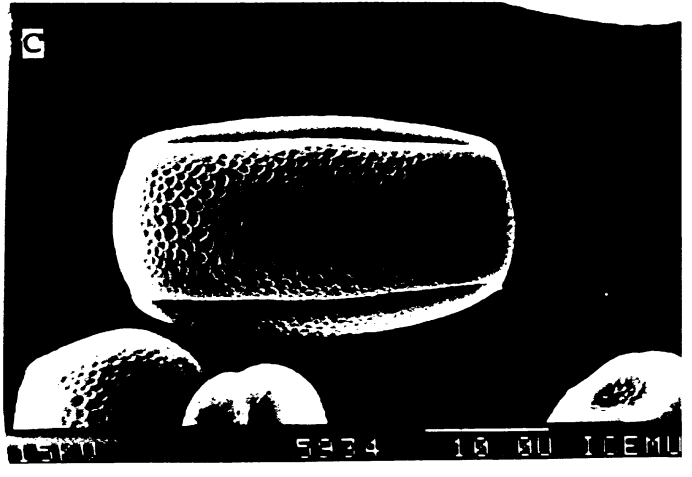
Scanning electron photomicrographs showing pollen grain morphology of F_1 interspecific hybrids in annual *Cicer* species.

(A) Equatorial view of *C. arietinum* (*desi*) X *C. reticulatum*,
(B) Equatorial view of *C. arietinum* (*desi*) X *C. echinospermum*,
(C) Equatorial view of *C. arietinum* (*kabuli*) X *C. reticulatum*,
(D) Equatorial view of *C. arietinum* (*kabuli*) X *C. echinospermum*.

PLATE-29


A
15KV
5939
10 0U
ICEMU


B
15KV
5936
10 0U
ICEMU


C
15KV
5934
10 0U
ICEMU

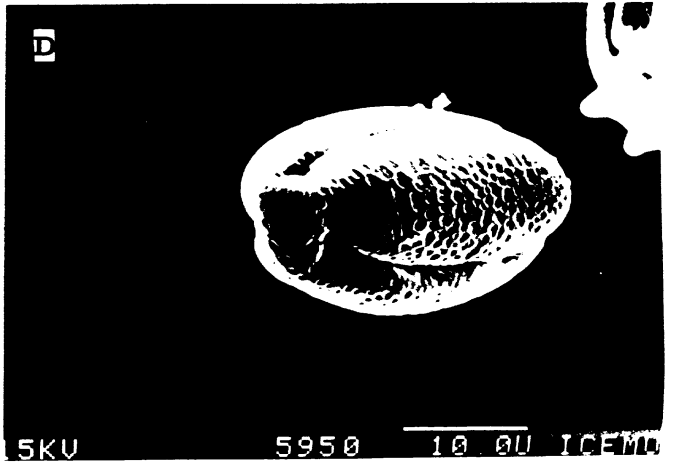

D
15KV
5950
10 0U
ICEMU

PLATE-30

Scanning electron photomicrographs showing pollen grain morphology of F_1 interspecific hybrids in annual *Cicer* species.

(A) Exine ornamentation of *C. arietinum* (*desi*) X *C. reticulatum*,
(B) Exine ornamentation of *C. arietinum* (*desi*) X *C. echinospermum*,
(C) Exine ornamentation of *C. arietinum* (*kabuli*) X *C. reticulatum*,
(D) Exine ornamentation of *C. arietinum* (*kabuli*) X *C. echinospermum*.

15KV
5935
1.0U ICEMU
C
15KV
B
15KV
A

Table 20 Morphological characterstics of pollen grains of interspecific hybrid between *Cicer* species using classification and characters described by Erdtman (1966).

Crosses	Polar Axis P (µm)	Equatorial Axis E (µm)	P/E	Shape	Exine Ornamentation		
					Thickness Of Murri T (µm)	Mean Diameter of Lumina D (µm)	# of Brochi N
C. arietinum (ICCC-32) X *C. reticulatum* (ICCW-49)	31.3±1.2[1]	19.5±1.5	1.61±0.2	Prolate	0.48±0.04	0.60±0.12	74±3
C. arietinum (Annigeri) X *C. reticulatum* (ICCW-49)	30.8±1.6	17.9±1.2	1.72±0.2	Prolate	0.61±0.02	0.48±0.13	77±6
C. arietinum (ICCC-32) X *C. echinospermum* (ICCW-44)	31.8±3.0	18.1±2.0	1.76±0.1	Prolate	0.65±0.04	0.91±0.09	66±4
C. arietinum (Annigeri) X *C. echinospermum* (ICCW-44)	33.1±1.0	19.0±1.2	1.74±0.1	Prolate	0.50±0.01	0.98±0.10	48±5

[1] Mean ± Standard Deviation

C. arietinum* (*Desi*) X *C. echinospermum: Pollen was medium sized (about 33.1±1.0 µm), prolate in shape (P/E ratio= 1.74±0.01), and triangular in polar view showing distinct triangular lobes (**Plate 29B**). Ectoaperture was deep. Murri thickness was 0.50±0.01 µm with 0.98±0.10 µm diameter of lumina, and number of brochi present were 48±5 per 10 µm² (**Plate 30B**).

C. arietinum* (ICCC-32, *Kabuli*) X *C. reticulatum: Pollen of this hybrid was of medium size (31.3±1.2 µm) and prolate in shape (P/E ratio= 1.61±0.2). Circular and blunt in polar view (**Plate 29C**). Apocolpium was slightly protruded. Ectoaperture was shallow which widens with acute angle. Pollen showed thin murri (0.48±0.04 µm), lumina with average diameter of 0.60±0.12 µm, and approximately 74±3 brochi per 10 µm² (**Plate 30C**).

C. arietinum* (ICCC-32, *kabuli*) X *C. echinospermum: Pollen was medium in size (31.8±3.0 µm) and prolate in shape (P/E ratio= 1.76±0.1). Triangular to trilobed in polar view (**Plate 29D**). It had deep colpi furrow and margins were microreticulate. Apocolpium projected at the polar ends. Number of brochi per 10 µm² was 66±4 with lumina diameter of approx.- 0.91±0.09 µm, surrounded by murri of 0.65±0.04 µm thickness (**Plate 30D**).

DISCUSSION

CHAPTER V
DISCUSSION

5.1 CYTOLOGICAL STUDIES

5.1.1 KARYOMORPHOLOGICAL STUDIES IN *C. arietinum*

In *C. arietinum* the longest chromosome pair (number 1) was consistently satellited in the long arm and was submetacentric. All five cultivated chickpea accessions analyzed had a somatic chromosome number of 2n=16. This is in agreement with numerous previous studies (Vyas and Mehrotra, 1963; Phadnis and Narkhede, 1969; Mercy *et al.*, 1974a, Sharma and Gupta, 1982; Lavania and Lavania, 1982,1983; Sharma and Gupta, 1982, 1983b, 1986; Mukherjee and Sharma, 1987; Ahmad, 1988). A diploid chromosome number of 14, 24, 32 or 33 was never observed which is in sharp contrast to

previous reports (Rau, 1929; Dixit, 1932a; Singh, 1964; Furnkranz, 1968; van der Maesen, 1972). Therefore, the present study proves that cultivated chickpea has a diploid chromosome number of 16. Research conducted here and elsewhere (Bahl, 1987; Ahmad, 1988) indicates that *Cicer* chromosomes are sticky and the primary constrictions are not clear and distinct following use of currently available pretreatments. This could explain the variation between the reports on the position of the primary constriction.

While the effect of pretreatments of root tips is to clear the cytoplasm, separate the middle lamella and to bring about scattering of chromosomes with clarification of constriction regions, different pretreatment agents behave differently and, thus different groups of organisms require different pretreatment agents to bring about the desired effect (Sharma and Sharma, 1980). In literature on *C. arietinum* karyomorphology, various pretreatments have been used, viz., no pretreatment (Vyas and Mehrotra, 1963; Phadnis, 1970; Kutarekar and Wanjari, 1983), 8-hydroxyquinoline (Mercy *et al.*, 1974a; Ahmad, and Godward, 1980), p-dichlorobenzene (Sharma and Gupta, 1982, 1986; Lavania and Lavania, 1983) and p-dichlorobenzene with aesculine (Mukherjee and Sharma, 1987). Recently, Ahmad (1988) in his study, gave 12 h of cold water pretreatment. Most of the above studies gave only diagrammatic sketches of the cultivated chickpea chromosomes, while only a few actual photomicrographs which often are of poor quality, that interpreting them would be difficult. Results from the present study indicates that the 1-bromonaphthalene gives better chromatin condensation and separation of constrictions than other pretreatment agents used in *C. arietinum*. This pretreatment has never been used in the genus *Cicer*. While a further improvement in the handling of chickpea chromosomes are needed, this pretreatment technique was adequate for the present objective.

Another reason for the discrepancies observed in most instances, the basis for classifying the various chromosome types was either not stated or, if it was stated, then it varied in different studies. Thus, when the same genotype was studied by more than one researcher, a different karyotype formula was proposed each time, viz., for genotypes N-59 (*desi*) and D-8 (*desi*) (Phadnis, 1970; Kutarekar and Wanjari, 1983), BG-203 (*desi*) (Lavania and Lavania, 1983; Mukherjee and Sharma, 1987) and C-214 (*desi*) (Sharma and Gupta, 1986; Mukherjee and Sharma, 1987). Nonetheless, the karyotype of the chickpea derived from the five accessions used in the present study, generally agrees with the previously published karyotypes. Differences in length of the total chromosome complement in different genotypes of chickpea, as observed in previous studies, could be real, although they may also be partly due to differences in chromatin condensation caused by different pretreatment agents as well as artifacts in the technique used. In all cases the position of secondary constriction was subterminal and it was on the longest chromosome. The smallest chromosome pair was never satellited, as had been reported by Iyengar (1939).

5.1.2 KARYOMORPHOLOGICAL STUDIES IN WILD *Cicer* SPECIES

Results from the present study were similar to earlier studies (Sharma and Gupta, 1986; Ahmad, 1988) on *C. reticulatum* except that Sharma and Gupta (1986) reported that chromosome 2 was acrocentric, while it was submetacentric in the present study and that of Ahmad (1988). It should be noted, however, that the size difference between these two chromosome pairs was not significant in the present study.

The karyotype of the species *C. echinospermum* was studied only once earlier (Ahmad, 1988). The difference observed between the present study and

that of Ahmad (1988) was the presence of two metacentric chromosomes instead of three, and presence of one acrocentric chromosome in the karyotype. The longest satellited pair was metacentric while it was reported submetacentric by Ahmad (1988).

The results of karyotype of *Cicer pinnatifidum* are in full agreement with those of Ahmad (1988). However, there is some discrepancy in location of secondary constriction, which was found to be in fifth chromosome pair. While Iyengar (1939) observed a satellite on the shortest chromosome of the complement, location of secondary constriction in present study was in agreement with Mercy *et al.* (1974a) and Sharma and Gupta (1986) who located it on the longest chromosome complement. It should be noted that chromosome number 5, which was found to be satellited in previous study (Ahmad, 1988), was not significantly different in length from the shortest chromosome. In addition, it is easy to misclassify *C. pinnatifidum* chromosomes owing to their small and regular intergrades of size. In this respect these results are closer to those of Mercy *et al.* (1974a) and Sharma and Gupta (1986), than those of either Iyengar (1939) or Ahmad (1988).

The karyotype of *C. judaicum* derived from the present study differed from that of Ahmad (1988) who described the karyotype consisting seven submetacentric chromosome pairs, one largest, acrocentric and one shortest, submetacentric, satellited chromosome and that of Sharma and Gupta (1986) who described the karyotype of this species as six metacentric and two submetacentric chromosome pairs with no satellited chromosome pair. It is difficult to comprehend the lack of satellited chromosome in *C. judaicum*, since in any eukaryotic genome, the nucleolar organizing region of satellited chromosomes carries genes for ribosomal RNA essential to normal functioning of ribosomes. Chromosome number 8 is very short and, as can be seen in the

report of Sharma and Gupta (1986). Their pretreatment produces highly condensed chromosomes compared to those in present study. These factors may have prevented Sharma and Gupta (1986) from viewing the satellited chromosomes in *C. judaicum*. Moreover, satellites can be observed clearly only in cells at early metaphase, since at later stages the constriction becomes indistinct.

The only karyotypic work done on *C. bijugum* and *C. chorassanicum* was by Ahmad (1988). Who reported that both species had two metacentric and six submetacentric, medium sized chromosomes. He was unable to locate the secondary constriction in either species. In present study, it was observed that the first large chromosome was acrocentric and satellited, and there were four medium sized submetacentric chromosome pairs and three medium sized metacentric chromosome pairs in *C. bijugum*. While *C. chorassanicum* had two medium to small sized acrocentric chromosome pairs, four small to very small submetacentric chromosome pairs and two very small metacentric chromosome pairs, fourth submetacentric chromosome pair was satellited. But the chromosomes of *C. chorassanicum* were smaller than those of *C. bijugum*.

The karyotype of *C. cuneatum* derived from present study differed considerably from Ahmad (1988), who found one metacentric and seven submetacentric chromosome pairs, and chromosome number 8 to be satellited. His description of the *C. cuneatum* karyotype disagreed with that of Sharma and Gupta (1986), for there was a major difference in the location of secondary constriction. Sharma and Gupta (1986) found it on the longest chromosome pair (chromosome I), while it was located on the shortest chromosome pair (chromosome VIII) in Ahmad's (1988) study and on chromosome II in present study. Due to small chromosome size differences, it is possible that the satellite could be located on any of the other smaller

chromosomes, but certainly not on the smallest chromosome (chromosome VIII). The different results in these studies are hard to explain, since *C. cuneatum* is represented only by a single accession in the world germplasm. Moreover, the seed material was obtained from the same source (ICRISAT) in all studies. In the present study, the longest and shortest chromosome pairs were not found to be satellited in any of the cells examined. It is, therefore, suggested that Sharma and Gupta's (1986) and Ahmad's (1988) findings were due to technical artifacts.

Considering all karyomorphological parameters of the eight annual *Cicer* species studied, the karyotype of cultivated chickpea resembled that of *C. reticulatum* and *C. echinospermum* more closely than any of the remaining *Cicer* species. The above mentioned three species also show a high degree of morphological resemblance. However, a closer look at the karyotypic formula indicates that *C. reticulatum* shows more karyotypic homology with the cultivated chickpea than does *C. echinospermum*. Thus, *C. reticulatum* is karyotypically the closest to *C. arietinum*, suggesting that it probably is the progenitor of the cultivated chickpea.

5.1.3 KARYOTYPIC ASYMMETRY IN ANNUAL *Cicer* SPECIES

A ***symmetrical*** karyotype is one, in which all the chromosomes are approximately of the same size, and have median (metacentric) or submedian (submetacentric) position of centromeres. Increasing ***asymmetry*** can occur either through a shift in centromere position from median to subterminal (acrocentric) or terminal (telocentric), or through the accumulation of differences in relative size between the chromosomes of the complement, thus making the karyotype more heterogeneous (Stebbins, 1971).

Stebbins (1971) classified karyotype symmetry based on four levels of asymmetry in arm ratio and three levels of asymmetry in ratio between the longest and the shortest chromosome. The same classification system, when applied to the eight annual *Cicer* species studied, indicated three groups of asymmetry in the genus *Cicer* (**Table 21**) against two reported by Ahmad (1987). The arm ratio proportion was taken as ±1.25 instead of <2:1. This gave a wider classification to the karyotype symmetry. The 2B type of asymmetry was the characteristic of three species, viz., *C. arietinum, C. reticulatum*, and *C. pinnatifidum*. While *C. echinospermum* grouped under 3B type of asymmetry. The 3A type of asymmetry was characteristic of the remaining four species. This was in contrast to Ahmad's (1987) report where he found these to group in 1A and 1B type of asymmetry. Symmetrical karyotypes are primitive characters in many genera and families (Stebbins, 1971). This is the case also in the genus *Cicer*, since 2B type includes the cultivated species which is the most highly evolved and has the most asymmetrical karyotype. While classifying the *Cicer* species, *C. pinnatifidum* was also found to be classified under 2B type along with *C. arietinum* which is not in agreement with Ahmad (1988) who classified it with *C. judaicum, C. bijugum, C. chorassanicum,* and *C. cuneatum*. This proves it to be less primitive than the remaining *Cicer* species. *C. echinospermum* is 3B type which is more asymmetrical than *C. arietinum* and *C. reticulatum*. This might be due to translocations or deletions in this species which placed it away from the 2B group (**Figure 4**).

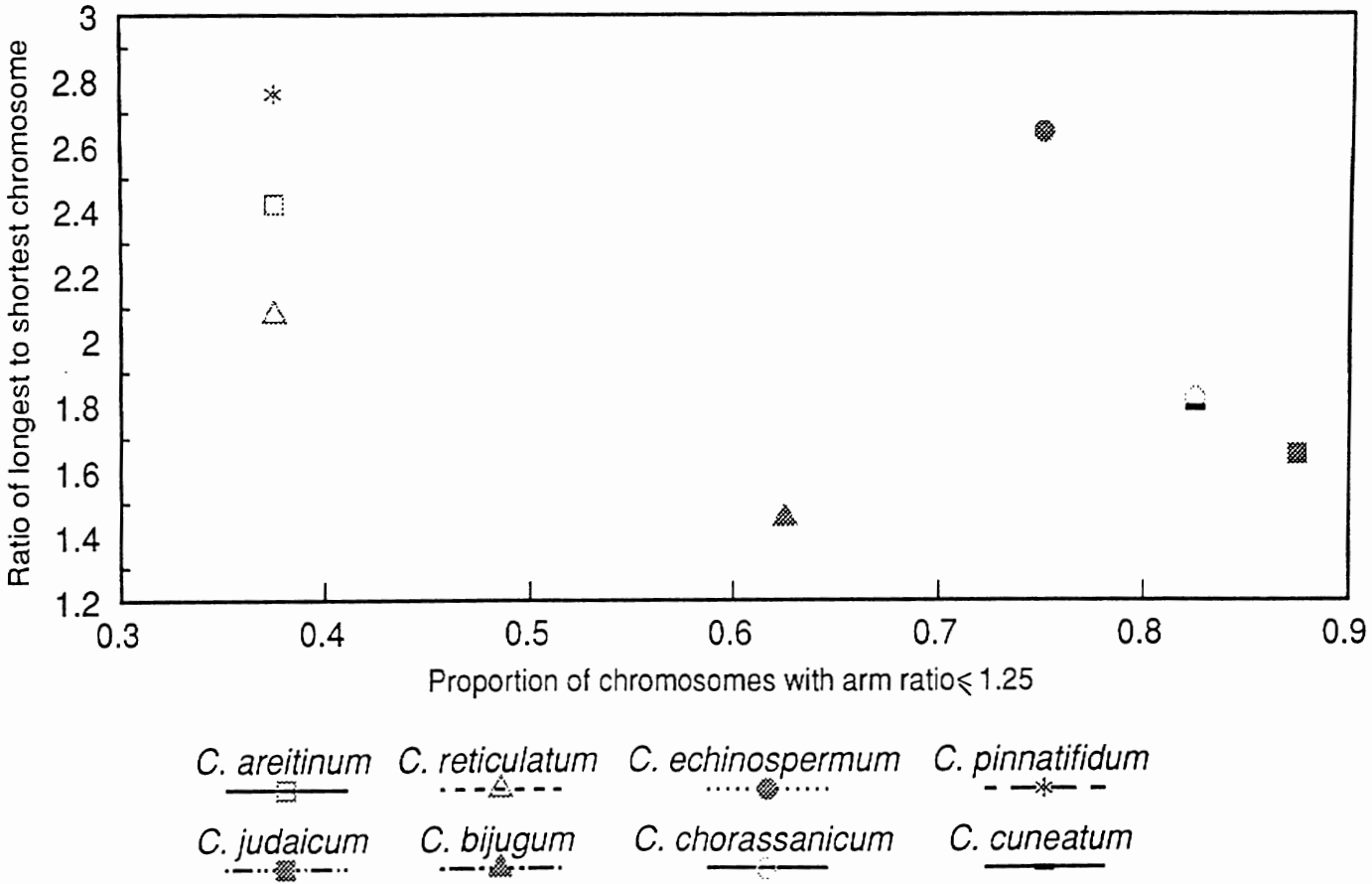
There is a predominant trend in flowering plants towards increasing asymmetry of the karyotype. This has been studied in *Crepis* and other genera of the Compositae, tribe *Cichorieae* (Stebbins, 1958). The karyotype of related species differ from each other with respect to both the length and the number of chromosome arms. In these examples, the chromosome number may remain

Table 21 Karyotype asymmetry in the eight annual *Cicer* species[1]

Ratio of Longest to shortest chromosome	Proportion of chromosome with arm ratio ≤ 1.25			
	0.0	0.01-0.5	0.51-0.99	1.0
< 2:1	1 A	2 A	3 A	4 A
	---	---	C. judaicum	---
	---	---	C. bijugum	---
	---	---	C. chorassanicum	---
	---	---	C. cuneatum	---
2:1-4:1	1 B	2 B	3 B	4 B
	---	C. arietinum	---	---
	---	C. reticulatum	---	---
	---	---	C. echinospermum	---
	---	C. pinnatifidum	---	---
> 4:1	1 C	2 C	3 C	4 C
	---	---	---	---

[1]Karyotype asymmetry classification according to Stebbins (1971).

Figure 4: Karyotype asymmetry in the eight *Cicer* species



constant while the relationships in size and form between different chromosomes may vary considerably from one karyotype to another. Such examples are best explained on the assumption of increasing karyotype asymmetry through pericentric inversions and unequal translocations. The most primitive and the most asymmetrical karyotype is found in the most advanced taxa and they have evolved from the taxa with symmetrical karyotype (Stebbins, 1971). Probably genus *Cicer* falls into this category.

The probable explanation for the evolution of symmetrical karyotype to asymmetrical karyotype is that natural selection would be expected to favor translocations and inversions which add genes to the cluster by transferring to its vicinity beneficial mutations that arise elsewhere in the complement. In this way, any chromosome arm that initially acquires an adaptive cluster consisting of a small number of genes to the cluster which would reinforce or render it more specialized, by the particular adaptation promoted by the cooperative action of these genes. On the other hand, chromosome arms which lacked such adaptive cluster would tend to become shorter through removal of genes from them whenever they increase their adaptive advantage by becoming linked to the genes belonging to an adaptive cluster. The evidence to support this hypothesis comes from the study by Stebbins (1958).

The development of karyotype asymmetry is associated with the entry into pioneer habitats, and often with the evolution of annual growth cycles (Stebbins, 1971). Additionally, which proves to be correct when we consider the available karyotypic information in genus *Cicer* this proves to be correct. By following the karyotypic information given by Ahmad (1989) for one of the wild perennial species, *Cicer anatolicum* showing a high degree of symmetry in that species proves that this perennial *Cicer* species has more symmetrical karyotype than annual ones.

5.2 INTERSPECIFIC HYBRIDIZATION AND CHROMOSOME PAIRING IN INTERSPECIFIC HYBRIDS IN *Cicer*.

5.2.1 INTERSPECIFIC HYBRIDIZATION

Seven *Cicer* species viz., *C. arietinum*, *C. reticulatum*, *C. echinospermum*, *C. pinnatifidum*, *C. judaicum*, *C. chorassanicum* and *C. cuneatum*, were utilized for making interspecific crosses in all possible combinations. Interspecific reciprocal crosses also were attempted.

5.2.1.1 *C. arietinum X C. reticulatum*

All F_1 hybrids of this cross showed a semi-erect habit. The hybrids involving ICCC-32 (*kabuli*) cultivar of *C. arietinum* as female parent was characterized by pink flower which it inherited from male parent *C. reticulatum*. Meiosis in all the hybrids of this cross was normal with regular formation of eight bivalents. Normal chromosome and chromatid segregation in this hybrid was proved by a high pollen (93-96%) and plant fertility.

Ladizinsky and Adler (1976a) also studied meiotic chromosome associations of the same hybrid, albeit with different genotypic combinations. Their results were generally in agreement with the results of the present study. However, Ladizinsky and Adler (1976a) found differences in meiosis of interspecific hybrids between the cultivated chickpea (Line 58 F) and *C. reticulatum* (Line 77), indicating that some chromosome repatterning had occurred in *C. arietinum*. But when a different chickpea cultivar, ICC-8928 (*desi*) was crossed with *C. reticulatum* accession ICCW-9, the hybrid formed eight regular bivalents (Jaiswal *et al.*, 1984). Ahmad (1988) also did not observe any repatterning among the *C. arietinum* lines used in the study. The extent of chromosome repatterning in *C. arietinum* is unknown. However, based on the

results the of present and previous studies (Ladizinsky and Adler, 1976a; Jaiswal *et al.*, 1984), it seems that repatterning probably occurs rarely in *C. arietinum*.

5.2.1.2 *C. reticulatum X C. arietinum*

The hybrid *C. reticulatum* (ICCW-6) X *C. arietinum* (Annigeri) showed intermediate morphological characters and abnormalities in the meiosis. As reported earlier (Ladizinsky and Adler, 1976b) meiosis in hybrid plants showed occasional formation of one quadrivalent, though the chiasma frequency was approximately similar to the cross when *C. arietinum* was taken as female parent. Pollen fertility was low (55-60%) and pod set was only 35-40%. The irregularities confirmed the views of Ladizinsky and Adler (1976b), that the parental lines differed by a translocation and a paracentric inversion. Also the effect of maternal cytoplasm on pairing can not be overruled.

5.2.1.3 *C. arietinum X C. echinospermum*

Hybrid plants from the cross between *C. arietinum* (cvs. Annigeri, K-850, GL-769, ICCC-42, and ICCC-32) and *C. echinospermum* (ICCW-44) were successfully produced and examined. The F_1 plants were vigorous had a semi-erect growth habit with purple flowers, even in a cross where ICCC-32 (*kabuli*) with white flower color was used as female parent. At meiosis univalents were occasionally observed. Seventy two percent of pollen mother cells (PMCs) had a quadrivalent and 21% trivalent and an univalent, indicating that the two parental species differed by a reciprocal translocation. Pollen stainability (which is more or less a measure of pollen fertility) of this hybrid was low, but not to the extent quoted by Ladizinsky and Adler, 1976b that it is practically sterile based on the data of selfed seed set. In the present study a high percent of seed set was obtained.

Harlan And De Wet (1971) placed *C. echinospermum* into secondary gene pool separated from *C. arietinum* and *C. reticulatum* and recommended that a large scale hybridization should be carried out to get good success in *C. arietinum X C. echinospermum* cross. This objective is fulfilled during present course of study and large number of seeds were obtained with each cultivar of *C. arietinum* when used as female parent.

5.2.1.4 *C. arietinum X C. pinnatifidum*

No hybrid seed was produced from this cross previously. However, one seed each using each cultivar (Annigeri, K-850, GL-769, ICCC-42 and ICCC-32) of *C. arietinum* X *C. pinnatifidum* (ICCW-37) was produced. Germination of the hybrid seeds was normal but they produced albino plants which survived only for 20-25 days after germination. Therefore, cytological characterization was not possible. The hybrids showed dominance of morphological characters from *C. pinnatifidum* which also confirmed that they were not selfed seeds. Cryptic structural changes in the chromosomes might be the cause of failure of the hybrid plants to survive or presence of a functional albino gene may be the cause of albinism in the hybrid.

5.2.1.5 *C. chorassanicum X C. pinnatifidum*

The formation of hybrid seed in this cross was earlier reported by Ahmad (1988). The results of the present study and earlier one were the same. Five hybrid seeds were produced when *C. chorassanicum* (ICCW-26) was used as female parent and *C. pinnatifidum* (ICCW-37) as male parent. These hybrid seeds germinated normally, but produced albino seedlings which died 15-20 days after transplanting. Fate of the hybrid plants produced in earlier study (Ahmad 1988) was the same. Therefore, no cytological characterization was possible for this interspecific hybrid.

5.3 POLLEN GERMINATION AND POLLEN TUBE GROWTH STUDIES.

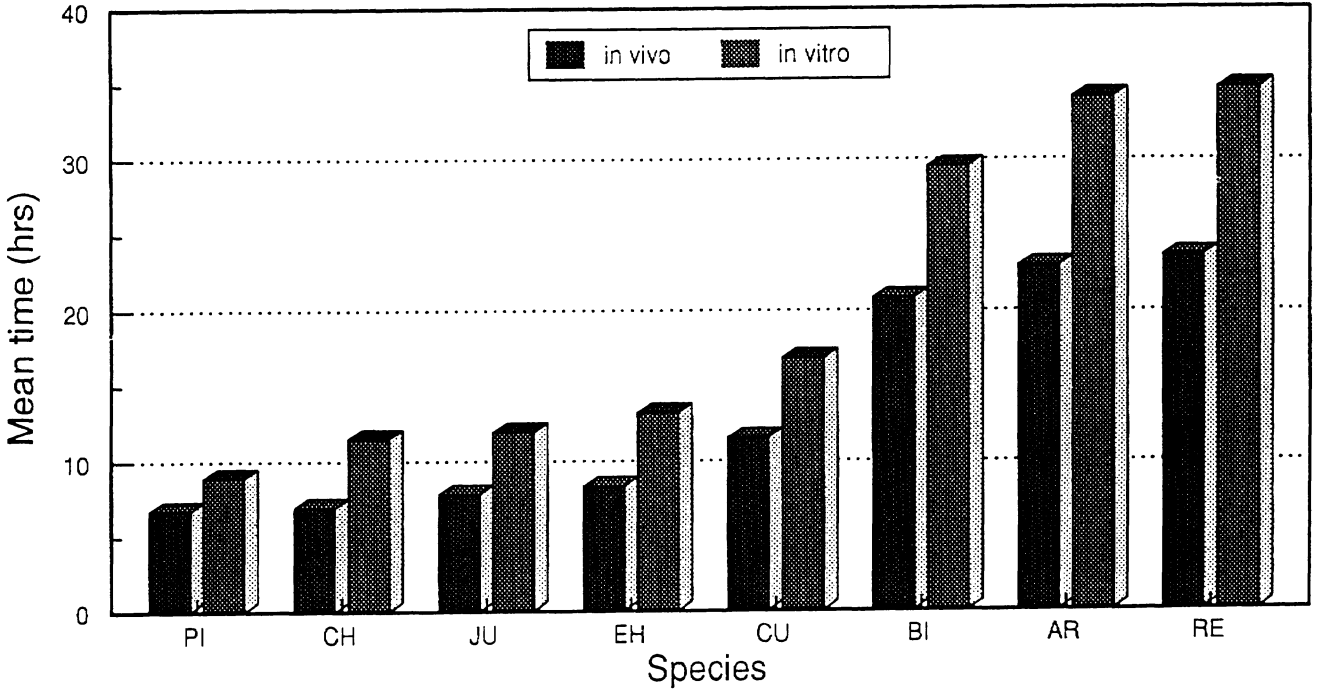
5.3.1 IN SELFED *Cicer* SPECIES

Penetration of the pollen into the ovule through the micropyle and subsequent enlargement of the ovule were taken as an indication of fertilization. This criterion for fertilization has been used by many workers (Sangduen *et al.*, 1983; Ramsay *et al.*, 1984; Bassiri *et al.*, 1987). When *C. arietinum* was self pollinated, the time required for a pollen tube to enter the ovule was 34.1 h and 22.9 h under *in vitro* and *in vivo* conditions, respectively. The difference of 10 h was also observed in earlier studies (Mercy *et al.*, 1974b). The difference noticed under two different conditions could be attributed to the nature of the studies (*in vitro* vs. *in vivo*) or it could be the effect of temperature on pollen tube growth. Growth of chickpea pollen tubes on nutrient medium increases until 35°C and then drops sharply (Jaiwal and Mehta 1983). The present *in vitro* study was under laboratory conditions at 25 ± 2°C which favors the faster pollen tube growth than it was reported by Jaiwal and Mehta (1983) but slower than its growth in *vivo* where the temperatures ranged from 28-36°C (**Figure 5**). The rise in temperature was also supported by extra lights provided to enhance the photoperiod to further the flowering in the wild *Cicer* species. The difference in the two studies may also be due to relative difference in receptivity of stigma under two study conditions.

5.3.2 IN INTERSPECIFIC CROSSES

There was no hinderance as such, observed in pollen germination and penetration of pollen tube on the stigma in all interspecific cross combinations between *C. arietinum* and the other wild annual *Cicer* species studied. Thus,

Figure 5: Time taken by pollen tubes after pollination to reach ovule in *Cicer* species.



AR- *C. arietinum* RE- *C. reticulatum* EH- *C. echinospermum* BI- *C. bijugum*

JU- *C. judaicum* PI- *C. pinnatifidum* CH- *C. chorassanicum* CU- *C. cuneatum*

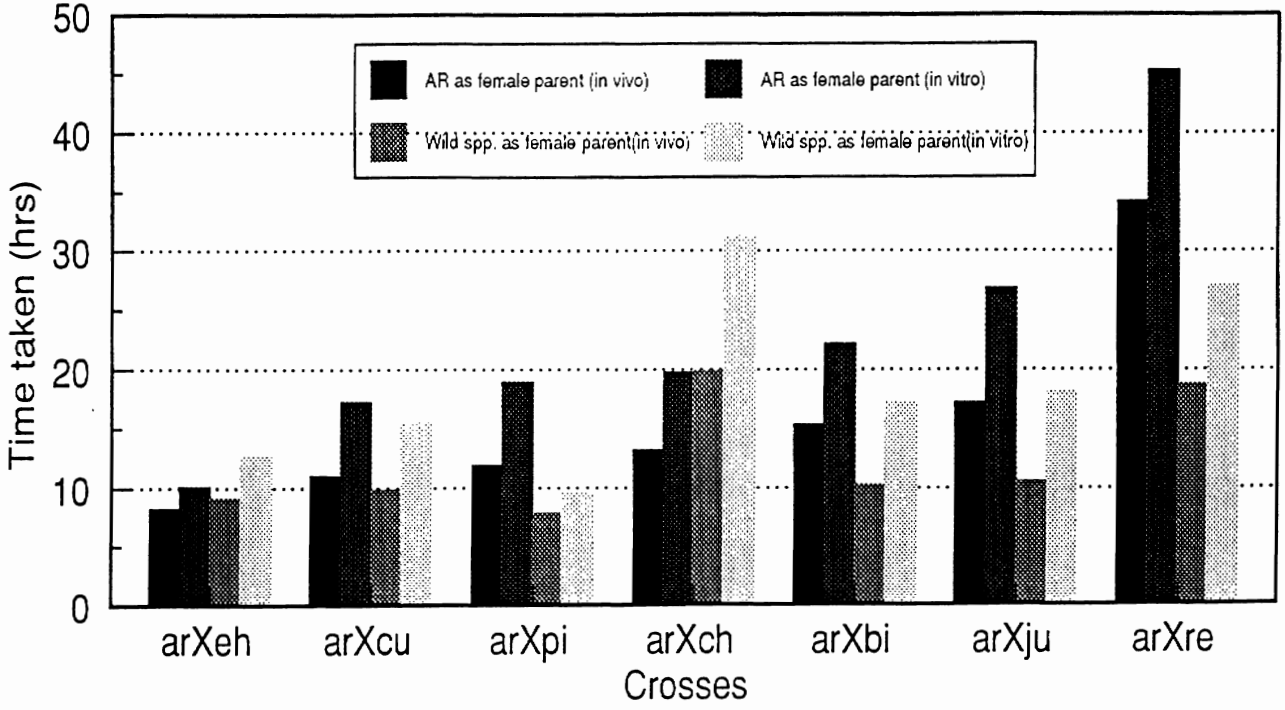
there was no indication of surface specificity in *Cicer* species, as reported in *Vicia faba* by Ramsay *et al.* (1984).

In contrast to the findings of Reger and James (1982) who reported pollen germination and penetration on the stigma as well as on the style and ovary in sorghum (*Sorghum bicolar* (L.) Moench), pearl millet (*Pennisetum americanum* (L.) Leck) and maize (*Zea mays* L.), the pollen germinated on the style or ovary wall were unable to penetrate it. Tilton and Russell (1983) reported pollen germination and fertilization in soybean, when pollen were directly applied to the excised gynoecium trimmed to expose the placenta and ovules. Although, this was not tried in present study, it is a good possibility to consider in *Cicer* species if there was a pre-fertilization barrier.

The pollen tubes were indistinguishable among the glandular hairs present towards the end of the style but they could be distinguished clearly near the ovary. Pollen tubes were characterized by irregularly spaced callose deposits and were in some respect similar to those of *Phaseolus* L. (Hawkins and Evans 1973). These results confirm the observations of Martin (1959) that the quantity and distribution of callose are quite variable.

The rate of growth of foreign pollen tube varied in different cross combinations, but it was noticed that there was no prefertilization barrier(s) as in all the cases the pollen tube grew as far as the ovary. The difference in the rate of pollen tube growth between reciprocal and normal crosses could be due to the difference in the styler tissue and its interaction with the pollen tube (**Figure 6**). However, this finding is in contrast to that of Mercy and Kakar (1975) who reported a strong pre-fertilization barrier in the form of failure of pollen germination and lack of penetration of pollen tubes in the styler tissues in the cross between *C. arietinum* and *C. soongaricum*. *C. soongaricum* is a

Figure 6: Time taken for the pollen tubes to reach ovule in the interspecific cross combinations in genus *Cicer*



AR- *C. arietinum* RE- *C. reticulatum* EH- *C. echinospermum* BI- *C. bijugum*

JU- *C. judaicum* PI- *C. pinnatifidum* CH- *C. chorassanicum* CU- *C. cuneatum*

perennial species and presumably, even more distantly related to *C. arietinum* than the annual *Cicer* species used in the present study. In the widest species used in present study, *C. cuneatum*, it was observed that pollen tubes were reaching the micropyle.

In cross combinations, where fertilization occurs relatively soon after pollination, reciprocal differences might not be important, but in crosses with delayed fertilization this could be relevant to success. If fertilization does not occur within certain time (24 to 72 h, depending on the species), the ovary turns yellowish brown, collapses and the flower drops. However, it is worth mentioning that during present study the application of solution of plant growth regulators at the base of pedicle which was pollinated by alien pollen, was found to delay the flower drop. This leads to the assumption that the fusion of the gametes does bring about biochemical changes in the ovary because of which flowers do not drop. Whether or not the slow pollen tube growth is the only factor rendering pistil to abort needs further investigation, but in many situations it is important to choose the right species as the female parent.

The reduction in number of pollen tubes occurred between the time they enter the stigma and the time they reach the ovules, probably due to gradual elimination of the less competent pollen tubes. A similar phenomenon was also observed earlier in self pollinated pistils of *C. arietinum* (Hawkins and Evans, 1973; Shivanna and Shivanna, 1983). It was observed in the cross wheat X rye that presence of crossability gene, Kr, the number of pollen tubes reaching the micropyle as high crossable genotypes had more pollen tubes than the low crossable ones (Jalani and Moss, 1980).

One of the common abnormalities observed in the pollen tube growth was the formation of bulbous portions in pollen tubes. This bulbous structure,

however, did not prevent further pollen tube growth, as entry of the pollen tube into the ovule was observed despite this abnormality. Some other abnormalities recorded were curled pollen tubes, twisted pollen tubes and branched pollen tubes. Similar abnormalities have also been reported in interspecific crosses in *Medicago* (Sangduen *et al.*, 1983) and also when sorghum was crossed with maize and pearl millet (Rager and James, 1982). In the present study, these abnormalities were not limited to any particular interspecific cross, but occurred randomly. The fact that these abnormalities were not a significant barrier to fertilization was demonstrated by their presence even in the crosses between *C. arietinum* and *C. reticulatum*. This cross is not only readily accomplished as intraspecific cross in *C. arietinum*, but also gives fertile stable hybrids (Ladizinsky and Adler, 1976a,b).

5.4 POLLEN MORPHOLOGY

5.4.1 POLLEN MORPHOLOGY OF *Cicer* SPECIES

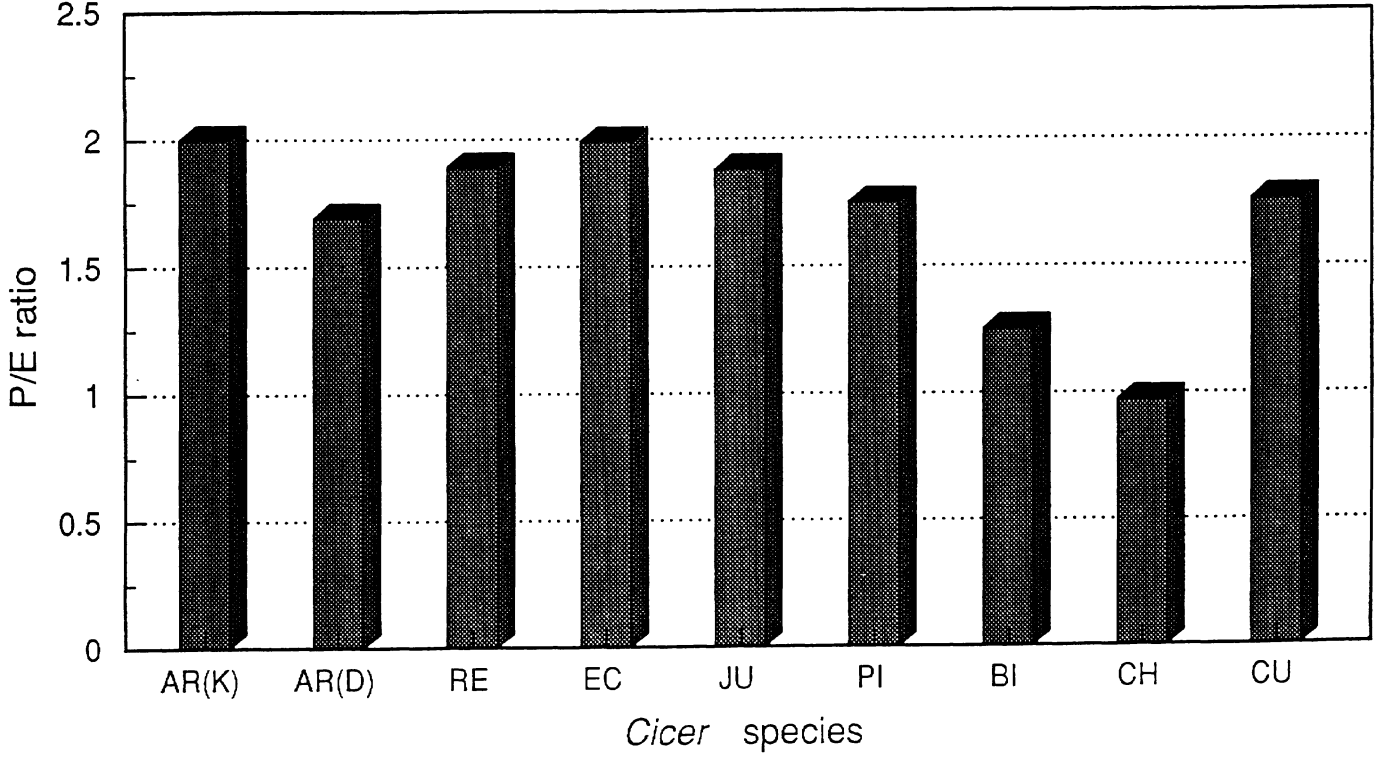
Scanning electron microscopy of the pollen grains in genus *Cicer* revealed very clearly that there was not much difference in the pollen of different species. Combining the present data with that obtained by various other workers (Vishnu-Mitre and Sharma, 1962; Gapochka , 1974; Clarke and Kupicha, 1976; Ahmad, 1988) for *Cicer* species, it can be concluded that the genus *Cicer* is stenopalynous since all the species studied have general similarity in pollen morphology, and the differences were not so prominent as to use it to study the species relationship in the genus *Cicer*. But some relationship can be worked out looking at the characters of individual pollen belonging to different species. There is a clear relationship between *C. echinospermum, C. judaicum, C. bijugum, C. pinnatifidum,* and *C. chorassanicum* as the values of P/E ratio and number of brochi/10 μm^2 in

these species seem to follow an order where *C. arietinum* and *C. echinospermum* showed the largest values of P/E ratio and *C. chorassanicum* has the smallest, whereas, *C. cuneatum* fell apart from this group (**Figure 7**), while *C. reticulatum* showed the largest value for number of brochi/10μm^2 of the pollen grain and *C. cuneatum* and *C. chorassanicum* showed the lowest values (**Figure 8**).

However, some differences in pollen morphology were evident, specially *C. chorassanicum* and *C. bijugum*, showing gross morphological differences as compared to other species. Pollen grains of these two species are slightly smaller than the other *Cicer* species. More prominently, these species show syncolporate colpi, which was not observed in other species. This character could be of some evolutionary value. Syncolporate colpi were observed previously in *C. chorassanicum* (Clarke and Kupicha, 1976; Ahmad, 1988), *C. bijugum* (Ahmad, 1988) and also in many perennial *Cicer* species (Gapochka, 1974; Clarke and Kupicha, 1976). This character may be associated with primitiveness of a species, and accordingly it is suggested that *C. chorassanicum* and *C. bijugum* are more primitive than other wild annual *Cicer* species and it seems they are more closer to perennial species, as it is known that annual species are evolved from the perennial species (Gupta and Bahl, 1983).

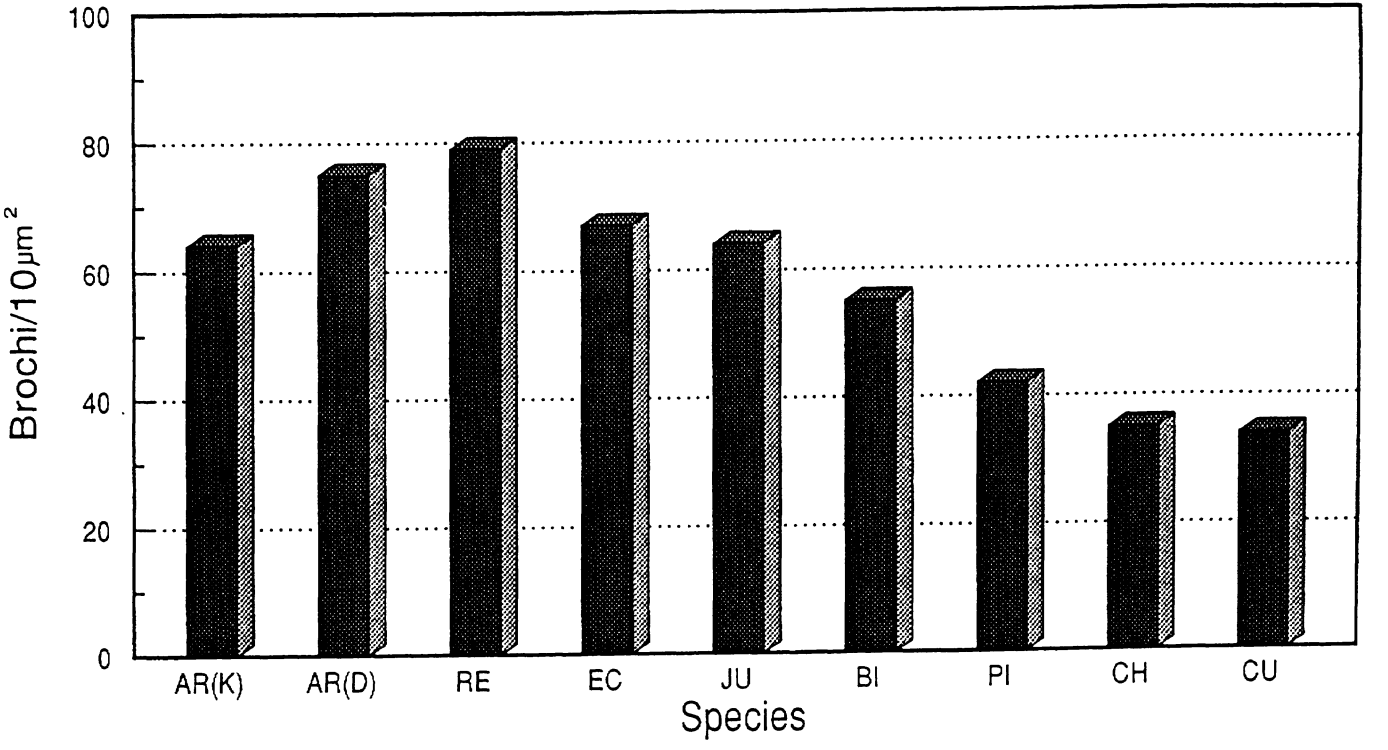
Pollen characters which are considered to indicate the primitiveness of a particular species are: small pollen size, long ectocolpi, ectocolpi without margo, lolongate endoapertures, reticulate pattern of exine, thin and uninterrupted murri, convex polar and equatorial sides, spheroidal pollen shape and small lumina (Punt, 1976). The characters listed above were more or less present in all the *Cicer* species studied, indicating that these *Cicer* species are primitive.

Figure 7: P/E ratios of the pollen grains of the *Cicer* species.



AR- *C. arietinum* RE- *C. reticulatum* EH- *C. echinospermum* BI- *C. bijugum* D -*desi*
JU- *C. judaicum* PI- *C. pinnatifidum* CH- *C. chorassanicum* CU- *C. cuneatum* K -*kabuli*

Figure 8: **No. of brochi/10μm^2 in pollen grains of *Cicer* species**



AR- *C. arietinum* RE- *C. reticulatum* EH- *C. echinospermum* BI- *C. bijugum* D *-desi*

JU- *C. judaicum* PI- *C. pinnatifidum* CH- *C. chorassanicum* CU- *C. cuneatum* K *-kabuli*

All the annual *Cicer* species studied here have lower P/E ratio, large lolongate endopori with thin margins, broad colpi bordered by rather wide unornamented bands and the reticulate ornamentation of mesocolpia extending to the poles, makes *Cicer* pollen distinctly different from the tribe Viceae. These pollen characters are in support of the exclusion of *Cicer* from the tribe Viceae and placement in its own monogeneric tribe Cicereae.

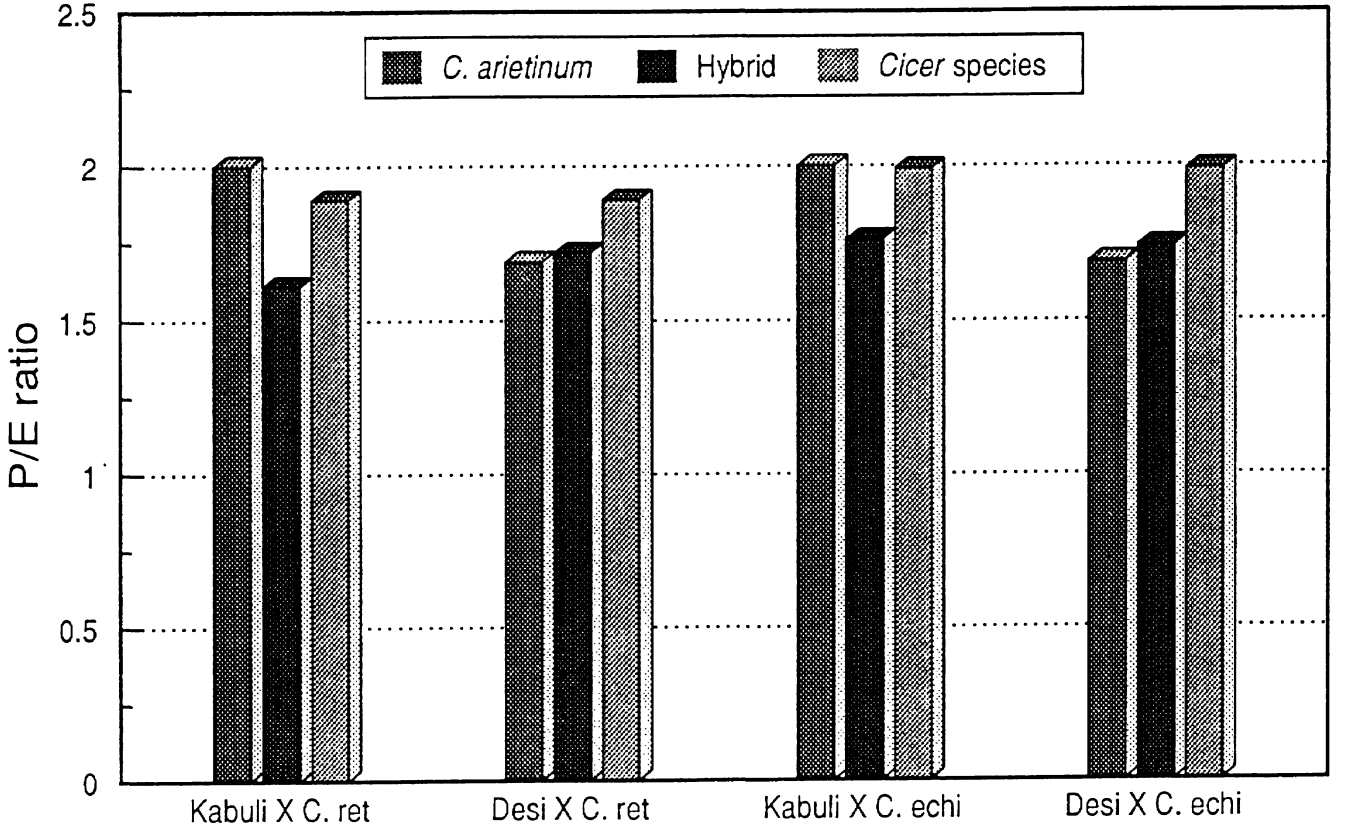
5.4.2 POLLEN MORPHOLOGY OF INTERSPECIFIC HYBRIDS

The hybrid pollen grains scanned did not resemble either of the parents, which confirmed them to be true hybrids. In the crosses involving *C. arietinum X C. echinospermum*, colpi are more deeply placed than those of the parents. Poles more pointed than the female parent (both *kabuli* and *desi*), resembling the wild parent. Slight protrusion of ectoaperture at the equator is observed in crosses involving *C. arietinum X C. reticulatum* a character present in *C. reticulatum*.

P/E ratios of the hybrids involving *C. arietinum* (*kabuli*) as one of the parents in crosses with both *C. reticulatum* and *C. echinospermum*, were always less than those of either of the parents. However, the value was less than the male wild parent and more than female *desi* chickpea parent (**Figure 9**). This can be explained by the fact that P/E ratio of the male wild parent was always less than the female *kabuli* chickpea parent. But when *desi* chickpea was used as female parent the P/E ratio of the hybrid was more than that of the female parent but less than male wild parent. This clearly shows that the greater value of P/E ratio is inherited from the male wild parent.

Hybrids always showed greater number of brochi per 10 μm^2 than the female parent and less than wild male parent, except in the cross *C. arietinum* (*desi*) X *C. echinospermum* where number of brochi/10 μm^2 were less in

Figure 9: P/E ratios of the pollen grains of the interspecific hybrids of genus *Cicer*.



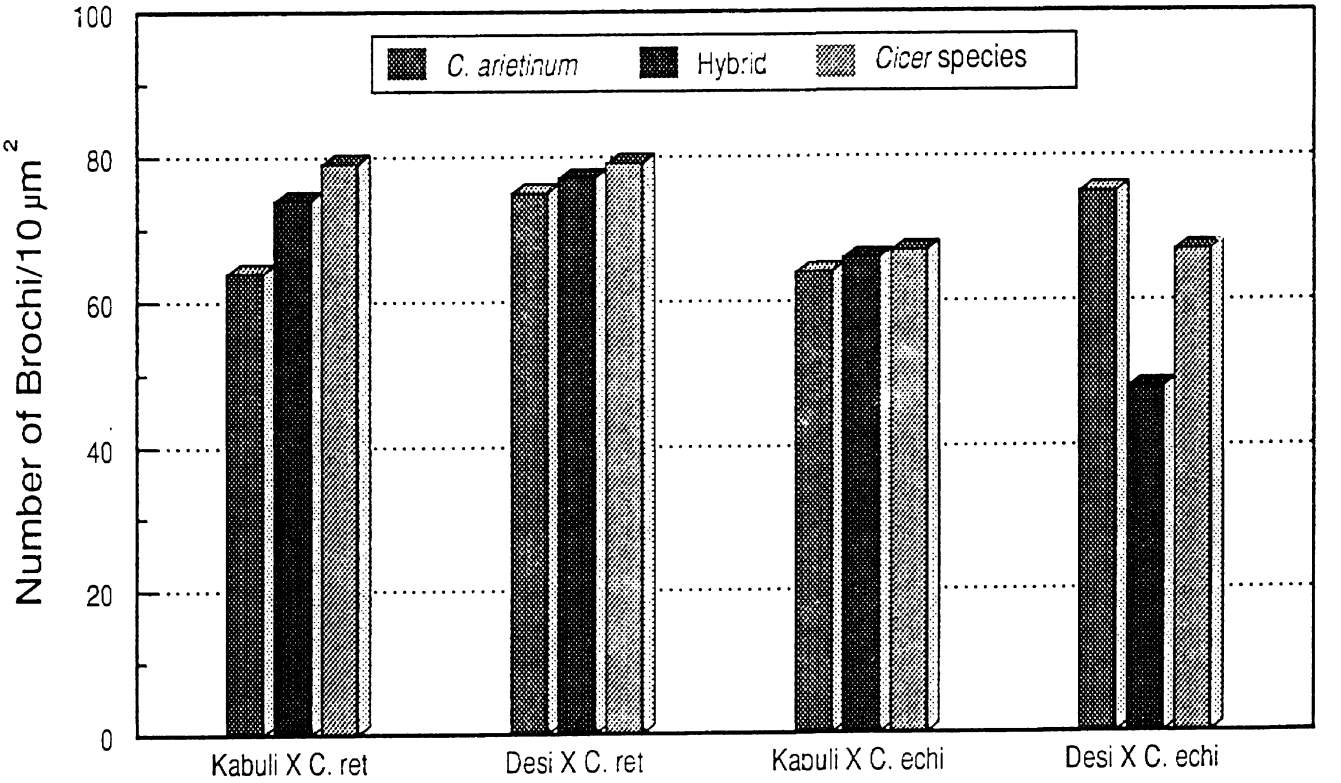
C. echinospermum than *desi* chickpea (**Figure 10**). This also explains that the character of greater number of brochi was inherited from the male wild parent.

5.5 SPECIES RELATIONSHIP BASED ON RESULTS OF INTERSPECIFIC HYBRIDIZATION

Chromosome pairing in the interspecific hybrids is a valuable tool to determine the species relationship (Kimber 1984). Genus *Cicer* being little amenable to the interspecific hybridization, only five interspecific hybrids were produced (*C. arietinum X C. reticulatum, C. reticulatum X C. arietinum, C. arietinum X C. echinospermum, C. arietinum X C. pinnatifidum* and *C. chorassanicum X C. pinnatifidum*) and only three could be characterized cytologically (*C. arietinum X C. reticulatum, C. reticulatum X C. arietinum* and *C. arietinum X C. echinospermum*) during the present investigation. Rest two could not survive up to maturity. The hybrids which were characterized cytologically were also studied earlier (Ladizinsky and Adler, 1976a,b; Ahmad, 1988) and the results of the cytological studies were more or less similar.

Certain conclusions can be drawn by pooling all information on interspecific hybridization in the genus *Cicer*. The cultivated chickpea, *C. arietinum*, has so far been hybridized only with two wild annual *Cicer* species, *C. reticulatum* and *C. echinospermum*, apart from the new hybrid produced during the present study with *C. pinnatifidum*, which could not survive up to flowering to carry out cytological studies. Looking to the results of the cytological studies with the two hybrids it becomes clear that *C. arietinum* is genetically closer to *C. reticulatum* than *C. echinospermum*, since it regularly forms eight bivalents with the former species (present study, Ladizinsky and Adler, 1976a; Ahmad, 1988), while it forms a quadrivalent at metaphase I and differ by a reciprocal translocation from the later species (present study,

Figure 10: Number of brochi/10μm^2 in the pollen grains of interspecific hybrids in genus *Cicer.*



Ladizinsky and Adler, 1976a). Closely related species differ genetically from one another with regard to one or a few genes, and thus a genetic barrier between the species can exist without cytological differences. However, there can be no unsurmountable cytological obstacles if no differences in genes between the species are present (Lamprecht, 1948). The hybridization between *C. arietinum* and *C. pinnatifidum* produced a hybrid plant which was albino and could not survive for more than 20 days in all genotypic combinations regardless of the route of its production, through *in vitro* embryo rescue, or through artificial pollination. This proved that *C. arietinum* is more distant to *C. pinnatifidum* than it is to *C. reticulatum* and *C. echinospermum*. Thus, the results obtained from the present study, and elsewhere, indicate closer phylogenetic affinity among *C. arietinum*, *C. reticulatum* and *C. echinospermum*, than *C. pinnatifidum*. *C. arietinum* is more distantly related to the remaining annual wild species. This agrees with the earlier studies where *C. yamashitae* was also included, and no interspecific hybrids were produced between *C. arietinum* and these species.

Phylogenetically the three species, *C. pinnatifidum, C. judaicum* and *C. bijugum* are quite close. Morphology of *C. pinnatifidum* and *C. judaicum*, indicate that they are very similar to each other, such that they have often been treated as variants of the same species (Mercy *et al.* 1974a), while *C. bijugum* is morphologically distinct. Ahmad (1988) was able to produce the hybrid between *C. pinnatifidum X C. judaicum*, while Ladizinsky and Adler (1976b) were able to produce all three hybrids, viz., *C. judaicum X C. pinnatifidum, C. pinnatifidum X C. bijugum* and *C. judaicum X C. bijugum*. Meiosis in hybrids involving these three species was characterized mainly by bivalent association and occasional univalent formation (Ladizinsky and Adler, 1976b). The hybrid *C. pinnatifidum X C. bijugum* produced fewer univalents

than the hybrid *C. judaicum X C. pinnatifidum*. Thus, data on chromosome pairing in these three hybrids indicate that *C. pinnatifidum* and *C. judaicum* are substantially different genetically and should be treated as two different species, not merely variants of the same species. Furthermore, *C. pinnatifidum* is cytogenetically closer to *C. bijugum* than it is to *C. judaicum*. On the other hand, the hybrid *C. judaicum X C. chorassanicum* which survived up to flowering, was characterized by many univalents, indicating little genetic homology between these two species (Ahmad, 1988). Furthermore, the hybrid *C. chorassanicum X C. pinnatifidum* (Present study, Ahmad 1988) produced albino F_1 plants. Thus *C. chorassanicum* is isolated genetically from three species, *C. pinnatifidum*, *C. judaicum* and *C. bijugum*.

Crossability groups recognized by Ladizinsky and Adler (1976b), in which seven annual *Cicer* species were placed, such that interspecific hybrids between the groups were not readily produced but could be produced within groups. Thus, *C. arietinum, C. reticulatum* and *C. echinospermum* formed group I, while *C. pinnatifidum, C. judaicum* and *C. bijugum* formed group II. The third group comprised of a single species, *C. cuneatum*. The results of present study and that of Ahmad (1988) supported Ladizinsky and Adler's group I and Group III. However, support to their group II is less definite, partially because of the fact that no crosses were successfully produced with *C. bijugum* in this study. The lack of any successful crosses with *C. bijugum* in this and earlier studies may relate to the fact that *C. bijugum* was rather difficult to grow up to flowering stage, delayed and fewer number of flowers were produced, excessive flower drop and problems in release of pollen grains from anthers were noticed. Furthermore, Ladizinsky and Adler (1976b) did not study *C. chorassanicum*. The hybrid *C. arietinum X C. pinnatifidum* which was produced in this study did make it clear that *C. pinnatifidum* is

closer to *C. arietinum* than rest of the species of group II are. This is also proved by the karyotypic asymmetry of the species where *C. pinnatifidum* finds its place in the 2B group along with *C. arietinum* (**Table 14**). The two crosses, *C. chorassanicum X C. pinnatifidum* (present study, Ahmad, 1988) and *C. judaicum X C. chorassanicum* (Ahmad, 1988) suggest that *C. chorassanicum* should be included in the group II. The presence of *C. cuneatum* by itself in group III is supported by the present and earlier studies (Ahmad, 1988). Although, Singh and Singh (1989) reported the production of the hybrid *C. arietinum X C. cuneatum* through embryo rescue but the hybrid was sterile. Another species, *C. yamashitae*, not included in present study, could not be successfully hybridized with any of the other annual *Cicer* species (Ahmad, 1988) and, therefore, could be placed in a separate group IV. Previously, based on interspecific crossability, Smirnoff *et al.* (1981) indicated that, it could be placed in group II, even though no crossability data were provided. Thus, as supported by Ahmad (1988), until data are available to disprove it, *C. yamashitae* should be placed in group IV by itself.

Harlan and de Wet (1971) recognized three different gene pools, according to the difficulty in interspecific hybridization and gene transfer between them. The primary gene pool (GP-1) consists of the cultivated species and the wild species that intercross easily with the cultivated species to produce fertile hybrids which have good chromosome pairing and normal genetic segregation. The secondary gene pool (GP-2) includes those biological species which, although crossable, produce hybrids with high sterility and poor chromosome pairing. Both interspecific crosses and gene transfer are possible and no special techniques, such as embryo rescue, are required. The tertiary gene pool (GP-3) includes species that can cross with the species under study,

but only with the assistance of special techniques such as embryo rescue, bridge hybridization, grafting and tissue culture.

On the basis of the above classification, and supported by cytological data, *C. arietinum* and *C. reticulatum* should be placed in GP-1 and *C. echinospermum* in GP-2. The remaining *Cicer* species, *C. judaicum, C. bijugum, C. chorassanicum, C. cuneatum* and *C. yamashitae* should be placed in GP-3, since hybrids between these species and *C. arietinum* could not be successfully produced in either the present study or in the previous studies, although, some hybrid plants were produced in the cross *C. arietinum X C. pinnatifidum* that did not survive for more than 20-25 days, and *C. pinnatifidum* would be considered as part of GP-3.

Being mainly an Asian crop, chickpea has been neglected by workers in Europe and America. As a result, in comparison to wheat, barley and pea, little information regarding the origin of cultivated chickpea and its wild progenitor is available (Gupta and Bahl, 1983). Other reasons for lack of study in this crop are the inadequacy of world collections of annual and perennial species of *Cicer* and the difficulty in making interspecific crosses due to the small size and cleistogamous nature of the flowers (Ahmad, 1988). Results obtained in present study and elsewhere (Ladizinsky and Adler, 1975, 1976a; Ahmad, 1988) supported the belief that *C. reticulatum* is the progenitor species of the cultivated chickpea, which is also evident from several lines of studies, viz., small morphological differences presumably controlled by few genes, non-shattering nature of the pods, the ease of crossability, normal meiosis in F_1 hybrids, and similarities in karyotype. In fact, these two species are genetically so close that it has been suggested that *C. reticulatum* is a subspecies of *C. arietinum* (Moreno and Cubero, 1978).

The first essential step in the evolution of annual legumes is, evolution to annual state from the perennial state. Thus, discovery of the wild annual ancestor of chickpea represents evolution only at the secondary level and more work will be needed to discover the ancestral perennial species at primary level. Nevertheless, until this is settled, *C. reticulatum* should be considered as the progenitor of cultivated chickpea. *Cicer reticulatum* and *C. echinospermum* are restricted to different parts of Turkey and occupy different ecological niches (Ladizinsky and Adler, 1976b). *Cicer reticulatum* is apparently endemic to south-east Turkey, which also is the central part of the traditional Fertile Crescent where wheat, barley, pea and probably also lentil were domesticated (Zohary and Hopf, 1988). It is very likely that chickpea was also domesticated there, although it has been much less common in the remains unearthed in archaeological excavations. Chickpea seed is characterized having protruding beak of various lengths and shapes which can easily break off or be destroyed by fire, thus becoming almost indistinguishable from carbonized pea.

The *desi* type chickpea is regarded as a primitive type, from which *kabuli* type chickpea evolved through mutation, and selection for large light colored seeds and white flower color (Moreno and Cubero, 1978). Thus, the suggested evolutionary sequence will be from *C. reticulatum,* the wild progenitor to *C. arietinum,* first the primitive *desi* type chickpea and then more recent *kabuli* type chickpea are evolved. Recently, on the basis of the recovery of *desi* type segregants in F_3 from *kabuli* chickpea X *C. reticulatum* cross, Jaiswal *et al.* (1984) presented evidence suggesting the possibility that *kabuli* types are of a more primitive origin and that the *desi* types evolved as a consequence of introgression of *C. reticulatum* into the *kabuli* type chickpea, and all the three :- *desi* chickpea, *kabuli* chickpea and *C. reticulatum*, may have originated independently from a wild progenitor.

The wild *Cicer* species are very poorly represented in the world germplasm (van der Maesen, 1987). It is very important not only to collect more accessions of these wild *Cicer* species, but also to successfully maintain and systematically evaluate them to get better information on the traits that could be used in chickpea improvement.

Successful interspecific hybridization is difficult to achieve in the genus *Cicer* (Ladizinsky and Adler, 1976b; Pundir and van der maesen, 1983; Ahmad, 1988). The present study also indicates the same, since only one unreported interspecific hybrid (*C. arietinum X C. pinnatifidum*) could be produced, which was albino and non-viable. *C. pinnatifidum* being placed just after *C. echinospermum* in the crossability group (Ladizinsky and Adler 1976b) showing such difficulty in crossing with cultivated chickpea, proves the difficulty other species would pose in crossing with cultivated chickpea.

It is clear from the present as well as earlier studies (Ahmad 1988) that there is a strong post-fertilization barrier to the interspecific hybridization in the genus *Cicer*. Several approaches to overcome early embryo abortion need to be investigated. The approach of applying plant growth regulators in order to stimulate or enhance embryo development, helped to get a new hybrid *C. arietinum X C. pinnatifidum* which otherwise was never obtained. This also helped in obtaining large number of seeds of other hybrids. *In vitro* embryo culture technique was also investigated in view of rescuing wide crosses, which were not possible to get under field conditions.

The embryos of other hybrids were very slow in growth therefore, by the time it was possible to excise them and culture on the media they had already started showing signs of abortion, and could not be rescued. Therefore, ovules of these hybrids were excised and cultured on the ovule culture media,

so as to get the hybrid embryo rescued. But this could not be achieved because of eventual browning and death of ovules, after initial slow growth. In the present study, it has been shown clearly that interspecific hybridization barrier(s) between *C. arietinum* and the wild annual *Cicer* species is of post-fertilization nature. The results of embryo rescue also proved that the embryo abortion in the wide crosses takes place so early that it could not be rescued through embryo culture. The hybrid ovules when cultured *in vitro* in liquid medium, died within a week of culture, suggesting, either the media was not appropriate for the ovule culture of interspecific hybrids in genus *Cicer* or a strong effect of surrounding maternal tissue on the developing hybrid embryo inside the ovule. Development of an appropriate ovule culture media for rescuing interspecific hybrids in genus *Cicer* and the method of embryo implantation should be used where the hybrid embryo is removed before it gets aborted and kept on the nurse endosperm of female parent should be undertaken. This will eliminate the effect of surrounding tissue to the growing embryo which might be because of the effect of the deleterious gene(s) present. The results of present study can be supported by the fact that during speciation and evolution, population differentiates morphologically, physiologically, and/or genetically to an extent that each one becomes a distinct entity warranting a unique taxonomic status. Reproductive isolation at some stage prevents the gene flow among them, and the taxa are then described as incompatible with each other.

In spite of normal pollen germination and pollen tube growth, fusion between the two gametes may not occur; in the event of a normal fertilization the resulting hybrid zygote may collapse any time before it develops into an embryo or a seedling. Such a phenomenon may be due to lethality [e.g., *Gossypium davidsonii* when used as parent in crosses with most *Gossypium*

taxa (Lee, 1981)], genic disharmony, inefficient endosperm, or the failure of the embryo. While attempting to introduce beneficial foreign genes across interspecific barriers in plants, some deleterious genes also find their way into the embryo sac. These latter genes apparently interfere with the growth of the embryo, endosperm and maternal tissue, leading to embryo lethality and collapse of seeds. In few cases hybrid seeds and seedlings were formed, which then developed into plants, but these were sterile due to meiotic irregularities. They did not produce gametes, and not formed fruits and seeds. Interspecific incompatibility is believed to be controlled by one gene or a group of genes and is often accompanied by zygotic and post-zygotic inviability (Sastri, 1984).

Species in secondary and tertiary gene pools, although difficult to exploit, are more likely to possess desirable traits missing in the primary gene pool. Thus, it is important to rescue the embryos involving these species so that hybrid plants can be raised to assess their potential for further crossing. Manipulations in embryo rescue technique such as implantation of growing hybrid embryos in a nurse tissue, culturing in the ovule culture medium and using induced tetraploids in the breeding program so as to nullify any effect of chromosome segment elimination, are some of the possibilities which should be explored. Only then the potential of wild *Cicer* species for genetic improvement of the cultivated chickpea will be realized.

SUMMARY AND CONCLUSIONS

CHAPTER VI

SUMMARY & CONCLUSIONS

The cultivated chickpea, *Cicer arietinum* L., and seven wild annual *Cicer* species, viz., *C. reticulatum* Lad., *C. echinospermum* Dav., *C. pinnatifidum* J. & S., *C. judaicum* Boiss., *C. bijugum* Rich., *C. chorassanicum* (Bge.) M. Pop., and *C. cuneatum* Rich. were studied to gain information to assist in gene transfer through interspecific hybridization. Studies on interspecific hybridization included investigation of pre-fertilization barrier(s) and cytogenetic study of interspecific hybrids. Species relationships among the annual *Cicer* species were investigated by karyotyping, electron microscopy of

pollen grains, pollen-pistil interaction studies and based on the results of interspecific hybridization. The hybrids were characterized morphologically, cytologically and by electron microscopy of pollen grains.

The following conclusions were drawn from the present study:

(1) The following interspecific hybrids were produced and characterized cytologically, palynologically, and morphologically :

C. arietinum X C. reticulatum, C. reticulatum X C. arietinum, C. arietinum X C. echinospermum.

The cross *C. chorassanicum X C. pinnatifidum*, which produced albino seedling which died a few days after germination as had been reported earlier.

(2) A new hybrid, *C. arietinum X C. pinnatifidum*, was produced for the first time through embryo rescue. The hybrid seedlings obtained were albino and did not survive.

(3) *C. arietinum, C. reticulatum, C. echinospermum* and *C. pinnatifidum* have similar karyotype with a higher level of asymmetry and larger chromosomes than that of the remaining 4 annual *Cicer* species studied.

(4) All eight annual *Cicer* species studied were diploid with a somatic chromosome number of 16.

(5) Meiosis was normal in all eight species and eight bivalents were regularly formed.

(6) *C. arietinum* chromosomes paired normally with those of *C. reticulatum*. In the present material, it is logical to assume that there is no cytological barrier to cross between the two species. The meiotic study in the reciprocal cross confirms the previous view that *C. arietinum* and *C. reticulatum* differ by a translocation and a paracentric inversion. The other probable cause for the reciprocal difference could be maternal cytoplasm.

(7) The hybrid *C. arietinum X C. echinospermum* was characterized cytologically by a quadrivalent, indicating that these two species differed by a reciprocal translocation.

(8) In the pollen-pistil interaction studies in both *in-vivo* and *in-vitro* conditions pollen tubes entered the ovules in all interspecific crosses between *C. arietinum* and the wild species including reciprocal crosses.

(9) Embryo was formed in crosses between *C. arietinum* and the wild species, but the growth was extremely slow in all cross combinations except in crosses with *C. reticulatum*). However, embryos aborted 6-15 days after pollination. Thus, the barrier(s) to interspecific hybridization occurred post-fertilization.

(10) Variation among species for pollen morphology was minimal and, this trait was not useful for studying species relationships. But the hybrids obtained during the study were characterized by differences in shape (P/E ratio) and number of brochi/10 μm^2.

(11) Karyotypic and cytogenetic studies indicate that *C. arietinum, C. reticulatum* and *C. echinospermum* are genetically related, *C. reticulatum* being the closest relative of *C. arietinum*.

The problem of albino plants observed in the crosses *C. arietinum X C. pinnatifidum* and *C. chorassanicum X C. pinnatifidum* could be because of the genetic differences or the elimination of chromosome segment from one of the parents. This may be overcome by using different accessions, or by inducing polyploidy in one or both parents in the crossing programs and then selecting green plants in the hybrid population.

It was concluded that the genetic variability present in *C. reticulatum* and *C. echinospermum* could be presently utilized with little or no difficulty for the genetic improvement of the cultivated chickpea. However, utilization of genetic variability in the remaining five wild species will have to await development of appropriate *in vitro* technology to overcome the strong post-fertilization barrier(s) to interspecific hybridization.

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VITAE

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