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PROPAGATION AND BREEDING OF KAKROL (*MOMORDICA DIOICA* ROXB.)

Ph. D. THESIS

BY

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RAJSHAHI
AUGUST 1997

PLANT BREEDING LABORATORY
DEPARTMENT OF BOTANY
UNIVERSITY OF RAJSHAHI.
RAJSHAHI, BANGLADESH

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A THESIS

SUBMITTED TO THE UNIVERSITY OF RAJSHAHI, BANGLADESH IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY

IN

BOTANY

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BY

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DECLARATION

I hereby declare that the whole of the work now submitted as a thesis for the degree of Doctor of Philosophy in Botany of the University of Rajshahi, is the result of my own investigation.

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CERTIFICATE

I hereby certify that the work embodied in this thesis has not been submitted in substance for any degree, and has not been concurrently submitted in candidature for any degree.

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TO

MY PARENTS

ABSTRACT

Prime objective of this study was to establish an effecient method for mass production of propagules of diploid, triploid and tetraploid kakrol (*Momordica dioica* Roxb.). The induction of somaclonal variation and reproductive behavior of the kakrol were also studied for undertaking future breeding programme.

Kakrol cheifly propagates through tuberous root. However, production of tuberous root is low. In addition to this due to hard seed coat, seed germination in kakrol under normal condition, is very low. In this investigation, mass propagation was successfully done in kakrol through treating vine cuttings with 1.0 mgl^{-1} IBA for 30 min. Enhanced rate of seed germination was acheived by removing the seed coat prior to seed sowing.

Results of *in vitro* studies show that, true to type kakrol could also be raised through culture of different explants. Nodal and shoot tip explants of field grown plant rapidly induced multiple shoot *in vitro.* Among the different media formulations tested 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA in MS (Murashige and Skoog, 1962) salt was the best for the induction of multiple shoot from both nodal and shoot tip explant of tetraploid kakrol.

Morphological differentiation from juvenile tissues such as cotyledon, embryo and hypocotyl of germinating seeds of tetraploid kakrol in different culture media formulations was investigated. Morphogenic response of cotyledon markedly varied with the ontogenic stage of the explant as well as with growth regulator composition of the media formulations. Cotyledons collected 18-21 days after pollination induced direct multiple proliferation when cultured on MS medium supplemented with 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA + 0.1 mgl^{-1} GA_3.

Callus development occurred from cotyledon and hypocotyl explants in MS medium containing auxin alone or in combination with cytokinin. Growth, morphological nature and organogenic potentiality of the calli varied with explant type and growth regulator supplements. All BA with NAA combinations accentuated organic potentiality of the primary callus. Organic potentiality of cotyledoner callus was more than hypocotyl derived callus. Optimum shoot regeneration occurred from cotyledoner calli when subcultured in 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA. Whereas, 3.0 mgl^{-1} BA + 0.5 mgl^{-1} NAA was optimum for hypocotylar callus. Callus culture was maintained for a long time through subculturing in MS medium containing BA+ NAA or IAA + KIN combinations.

Rooting potentiality of the microcuttings varied with their sources of explants. Among the various media compositions tested MS with 1.0 mgl^{-1} IBA medium was the best for root induction.

Somaclonal variation in respect of fruit weight among the somaclones regenerated from induced callus was observed. Some of the somaclones had higher fruit weight than normal.

Bisexual flowers could be induced in tetraploid, triploid and diploid type of kakrol by treating shoots with $AgNO_3$. Most of the $AgNO_3$ treated vines produced continuous female flowers when sprayed with 100 and 200 ppm $AgNO_3$ at 5 days, 7 days or 10 days interval. $AgNO_3$ at 100 ppm on individual twig at 7 days interval produced highest number of normal female flowers in tetraploid type of kakrol.

The application of $AgNO_3$ to female plants developed bisexual flowers. Stamens in induced bisexual flowers developed from the base of style and anther reached just beneath the stigma. $AgNO_3$ at 300 ppm produced the highest number of bisexual flowers per vine in diploid type of kakrol whereas 400 and 500 ppm $AgNO_3$ produced highest number of bisexual flowers per vine in triploid and tetraploid type of kakrol respectively.

Morphological studies of the reproductive structures reveal that bract size and position on the peduncle of the bisexual flower were different from those of normal

male and female flowers. Most of the induced bisexual flowers were bigger than those of corresponding normal male and female flowers. Leaf area in male and female tetraploid plants were larger than corresponding diploid and triploid.

Number of viable pollen in induced bisexual flower was higher in tetraploid than other types of kakrol. The highest percentage of pollen grain germination was 85% recorded in tetraploid and diploid male flowers when 20% glucose solutions were used for 45 minutes.

The pollens of bisexual was as effective as that of male flowers on the normal female flowers. In diploid, percentage of success in crosses with pollens from diploid bisexual flowers was same as found in control. The pollen of induced bisexual flower was not effective on the same or other induced bisexual flowers. However, induced bisexual flower did not develop fruit under self or sib or even cross pollination with normal pollen. Fluorescent microscopic observations reveal that 76% of developing pollen tubes of ♀ tetraploid were able to enter the stigma but they failed to penetrate further due to tumor formation.

When pollen grains of normal tetraploid flower were used to pollinate different types of pistillate flowers, fruit setting was observed only in ♀ tetra ×♂ tetra and ♀ dip ×♂ tetra crosses. Fluorescence microscopy shows that, numerous pollen tubes in both cases were found to develop through the stigmatic papillae to perform fertilization.

Pollen grains colleeted from male and bisexual flowers of triploid failed to give successful crosses with any types of pistillate flowers. Following pollination both localized and diffused callose production was observed on the stigmatic papillae which eventually prevented pollen germination. On the other hand the stigmatic papillae from unpollinated pistils did not produce any kind of callose.

The fruit produced through the crossing of the stigmas of diploid or tetraploid with the pollens of induced bisexual flowers were greater in size than the fruits developed through other crosses. Seeds from induced ♀ pollen × ♀ dip or tetra crosses developed only female plants that could be used for large scale seed production.

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ABBREVIATIONS

BA	6-benzyladenine
cm	Centimeter (s)
°C	Celsius
2, 4-D	2, 4-dichlorophenoxyacetic acid
DW	Distilled water
DDW	Double distilled water
et al.,	Et alli = Other people
GA_3	Gibberellic acid
gl^{-1}	Gram per litre
h	Hour
IAA	Indole-3- acetic acid
IBA	Indole-3- butyric acid
Kg	Kilogram (s)
KIN	6-furfuryl amino purine
mg	Miligram
mgl^{-1}	Miligram per litre
MS	Murashige and Skoog (1962) medium
O. 1N	0.1 Normal
NAA	α-naphthalene acetic acid
pH	Negative logarithm of hydrogen ion (H^-) concentration
sp	Species
viz.	Namely
%	Percentage
min	minutes
Tetra	Tetraploid
Trip	Triploid
Dip	Diploid
♀	Female
♂	Male
⚥	Bisexual

Chapter - 1

INTRODUCTION

INTRODUCTION

The propagation of plants has been a fundamental occupation of human kind since civilization began. Plant propagation is the multiplication of plants by both sexual and asexual means. A study of plant propagation has three different aspects. First of all, successful propagation of plant requires a knowledge of mechanical, environmental and chemical manipulations, and technical skills that take a certain amount of practice and experience to master, such as how to bud or graft, how to make cuttings, or how to use tissue culture procedures. Second, successful propagation requires a knowledge of plant growth, development and morphology. Third aspect of successful plant propagation is a knowledge of the different kinds of plants and the various possible methods by which certain plants can be propagated. To a large extent, the method selected, must be related to the responses of the kinds of plants being propagated and to the situation at hand.

1.1. GENERAL ACCOUNT :

Kakrol (*Momordica dioica* Roxb.) is a popular vegetable in Bangladesh, India and neighboring countries. It has become an important and probably the most expensive summer vegetable in Bangladesh (Rashid, 1976). Kakrol belongs to the family Cucurbitaceae, is a herbaceous perennial climber. Its fruits are ovoid in shape with soft spines. Its arial parts die during winter and sprout from underground tuberous root with the onset of rains in March.

There are two cultivars one diploid and another tetraploid types of kakrol found in this region. The somatic chromosome number of diploid cultivars is 2n = 28, and tetraploid is 4n = 56. Fruits of tetraploids are larger than those of the diploid cultivars. Both cultivars are called *Momordica dioica* Roxb. (Mishra *et al.*, 1983).

Kakrol is dioecious, producing male and female flowers on separate plants. In absence of male plant the female does not normally bear fruits. Tetraploid cultivar is suitable for commercial cultivation. The plant growth is vigorous and it sprouts in spring from underground tubers. Diploid cultivars are not cultivated on large scale in Bangladesh. Fruits of diploid cultivars are available only in vegetable markets of Bihar in India (Mishra *et al.,* 1983). A few tribals have domesticated diploid cultivar in their backyards. In the diploid cultivar the flower opens during night (7 pm to 12 pm), whereas, in the tetraploid it opens in the morning (6 am). Leaf size, petiole length, peduncle length and the size of sepals and corolla are more in the tetraploid cultivar than in the diploid.

A cross between diploid and tetraploid produces a triploid which has characters intermediate between the two cultivars. They are sterile. Another species known as *Momordica cochinchinensis* Spreng is found in our country. It is wild one, which is a tetraploid, having chromosome number 4n = 56 (Hossain *et al.,* 1992). It is also found in Taiwan, Hong Kong, China and India. It is cultivated in Khoto Herbal Garden, Japan. It is probably native to Vietnam (Mishra *et al.,* 1983). It has very large fruits and flowers. The leaves of this species are deeply lobed and have umbilicate glands in lamina base, whereas those of *M. dioica* do not have such gland. A new species, *M. littorea* is found in coastal regions of southern Somalia and Kenya. It is a dioecious climber with succulent 3-foliolate leaves and conspicuously bracteate flowers (Thulin, 1991).

1.1.1. BOTANY :

Kakrol is a cucurbitaceous summer vegetable of Indo-Malayan origin (Rashid, 1976; Singh, 1990). Both tetraploid and diploid have been cultivated in India, Bangladesh and neighboring countries for a long time. Its wild types are found in the Himalayas, Chittagong Hilltrack, hills of Rajmahal, Hazaribagh, Rajgir in Bihar, hill regions of Pune, Rajastan and Srilanka, The genus *Momordica* is native to tropical regions of Asia, Africa and South America (Shesadri, 1986).

Momordica L. is a genus of the old world tropics belonging to the Tribe Cucurbitaceae. The genus *Momordica* L. with about 40 species of annual or perennial climbing herbs in tropical Africa and Asia may be monoecious or dioecious. Three species of this genus have been reported in Bengal (Prain, 1963 reprint), *Momordica dioica* Roxb., *M. cochinchinensis* Spreng and *M. charantia* ex Willd.

The tuberous roots of tetraploid, triploid and diploid types of kakrol are various sizes and shapes viz., tuberous type with both ends tapering, round, elliptical; and sometimes very irregular in shape having smaller to larger in size. Some of them are of moniliform in nature developing irregularly throughout the whole length of the root. Almost all parts of a tuberous root may give rise to new shoots although these new shoots are mostly concentrated to the basal part of the root. These characters are more or less similar in all the plant types studied.

External morphology of arial parts of different types of kakrol are described under following heads:

Descri-ption	Tetraploid	Triploid	Diploid
Stem	Solid climber with 5 sharply angular ridges. It possesses twisting habit with spirally arranged leaves and extra-axillary tendrils. Unlike many other Cucurbits the tendril is unbranched. The young tendril is slender but subsequently coil at the middle region like a spring, after clasping a support to shorter its length. As a result the twig is pulled either upwards or forwards on the support. These characters are similar in all the plant types except slight variation in tendril length. The length and diameter of the internodes are also slightly variable in different plant types.	Solid, climber with 5 angular ridges, tendril extra axillary and unbranched slender.	Solid, soft, hairy, tendril unbranched.

Description	Tetraploid	Triploid	Diploid
Leaf	Deep green, cordate shape with prominent palmately reticulate vain and midrib, 10.2 ± 0.35 cm long and 11.6 ± 0.36 cm wide. The spirally arranged simple leaves produced at the nodes have dentate margin.	Light green, cordate shape with prominent palmately reticulate vine and midrib, 9.6 ± 0.4 cm Long and 10.48 ± 0. 46 cm wide.	Green, palmately lobed, spirally arranged, 8.5 ± 0.1 cm long and 9.5 ± 0.3 cm breadth.
Flower	Axillary, the aestivation of petals is imbricate (male and female) and colour is creamy with black round spot at the base of the upper three petals, corolla rotate, hair is present; Patals 5 parted nearly to base. Calyx blackish with brown tip and pointed shape like as candle flame. The aestivation of calyx is imbricate in male flowers and valvate in female flowers. Prominent sessile bract of male flower is much longer than female flowers, peduncle and pedicel present, slender like. The solitary flower of male plant produced at each node. It has a large bract in the bud stage and the flower is covered by the bract. In the female plant the flower is not produced at each node as that of the male plant and is not enclosed by bract.	Axillary, the aestivation of whitish yellow coloured petals of both male and female is imbricate having attractive black spots at the base of the innermost three petals. The solitary flower of male plant produced at each node. As the male flowers covered by the bract the sepals are not green but appear yellowish initially and become grayish subsequently: peduncle and pedicel present, slender like.	Yellow, flowers axillary, about 4 cm in diameter, bract present, calyx brown in colour, 5 corolla.
Female	The ovary is epigynous, short (length above 1.5 cm); short style, 2 bilobate stigma 3.	The epigynous ovary short (length above 1.3 cm). short style, 2 bilobate stigma 3. the ovary is elliptical in appearance.	ovary short with short style. 2 bilobate stigma 3.
Male	Stamens 5 synandrous (2)+(2)+1, outer colour black with brown thin binigs, pollen colour yellow or redish.	Stamens 5, synandrous (2) + (2) + 1, outer colour blackish with brown thin binigs, pollen colour yellow or redish.	Five stamens are synandrous (2) + (2) + 1 brown colour, pollen whitish.

Descri-ption	Tetraploid	Triploid	Diploid
Fruit	Fruit is pepo ovoid shape with medium thick dense conical spines: pedicel long. The body of the fruit extends almost up to the base of persistent calyx. In tapered-end type the fruit develops a neck near the base of persistent calyx. The elongated-round-end type is almost cylindrical in appearance while the elongated-tapered-end type is elliptical thus varying in weight although both types possess almost similar length and diameter. The appearance of yellowish colour of tuberculate spines on the fruit indicates its maturity.	—	Green ovoid in shape, peduncle and pedicel slender like and short. The appearance of yellowish colour of tuberculate spines on the fruit indicates its maturity.
Seed	Seeds are blackish with rough surface; margin irregular; aril present.	—	The seed is blackish and brown, round surface, ovoid in shape, margin regular, aril present.

Flowering in kakrol occurs on nodes 8th to 26th on the primary stem. In some plants the first flower appears on the primary branch, on node 1-13 on the secondary branches and on node 1 onwards on the tertiary branches. Begining with the emergence of the stems from the soil the opening of the first female flowers of the different plant requires an average of 53 days (range 32-60 days). The male plant produces the first flower on the main stem on 75 days after emergence from the soil (Hussain and Rashid, 1974). So the female flower buds develop quicker than the male ones. Fruit setting requires transfer of pollen from the male to female flowers. The male flower bud of tetraploid cultivar takes 22-28 days from visible initiation of full flowering, whereas the female bud takes 19-22 days. Anthesis in female flower occurs at 5.3 am and in the male at 6.30 am. The anther dehiscence commences at 10.45 pm and completes at 12.30 pm with peak at 11 pm. The stigma remains receptive 12 h

before anthesis to 12 h after anthesis. The flowers of the diploid plant open at 7.30 pm, with slight variations from season to season (Mishra *et al.*, 1983; Hussain and Rashid, 1974). Anthers are found to dehiscence at the time of opening of the flowers and the release of pollen grains continues for several hours. The pollen grains are sticky and redish in colour. Normally pollen grains stored at room temperature remain viable for 12 hours.

The fruit set in all the cultivars is unsatisfactory. Pollination in the cucurbits is usually accomplished by insects, however, artificial hand pollination has been found to increase fruit setting. Average fruit set in the tetraploid is 22.89 percent by natural pollination during the mid August. The fruit setting in the diploid is still worse. It flowers at night when natural pollinating agents are fewer (Mishra *et al.*, 1983). Fruits reach harvesting within 15-20 days after fruit set. The edible fruits are harvested when tender and slightly green. If harvesting is delayed, fruit colour becomes yellow and then red. An average vine yield is 42-60 fruits. Yield per hectare is 4-15 ton (Fakir *et al.*, 1992). For effective pollination, using a small camel-hair brush pollens taken from male flowers are placed on the stigma of female flowers. In this was the yield can be increased four times.

1.1.2. AGRONOMY :

Kakrol is grown luxuriantly in hot humid summer with minimum care. Usually kakrol is found throughout India from the Himalayas (1,500 m) to Srilanka. It is found on hedges in warm humid places (Mishra *et al.*, 1983). Soil conditions and summer climates of this region are suitable for kakrol cultivation.

Kakrol can be propagated by seed, tubers, and vine cutting. It is propagated less commonly by seeds. However, seed propagation is not common for commercial cultivation in kakrol. Fresh seeds don't germinate, but remain dormant for about 9 months. The seeded vine usually may not bear fruit in the first year of its life. The plant propagated mainly by tuberous roots. The underground roots are not easily available, because the owner does not want to part with them. Also these roots are not easily detectable in the off season. This limits its large scale cultivation.

1.1.3. IMPORTANCE :

The fruit of kakrol contains a very high amount of vitamin C (Bhuiya *et al.*, 1977). These vegetables are ideal for patient of diabetes and gout. Unlike bitter gourd (*M. charantia*) it is not better in taste. Flowers during the wet and cold seasons produces a fruit which when green mid tender, is eaten in curries by the natives. It may be cooked in different methods; fried and cooked with or without meat or fish and thus gives high palatability. The young twig and leaves of this crop are also used as vegetable (Fakir *et al.*, 1992). The tuberous roots of the female plant are also consumed in belgaum as an expectorant and externally in ague cases as an absorbent. The root of the male creeper is used in ulcers, especially those caused by snake bites. The unripe fruit is used as a vegetable and given as a delicacy to patients recovering from fever.

Fruit has good nutritional value. In Bangladesh, there are three cultivars of kakrol named as Kathali, Narikeli and Boll (Hossain *et al.*, 1987). These have been analysed for edible protein and dry matter contains of the fruits at three stages of harvest (Fakir *et al.*, 1992). The comparative nutritional values of these three varieties at different harvesting stages are given below :

Stage of harvesting	Cultivar / variety	% Crude protein	% Fat	% Ash	% Dry matter
Fully-green	Kathali	1.275	4.25	6.820	15.110 a
	Narikeli	1.240	3.85	7.255	14.510 b
	Boll	1.28	4.25	6.695	15.022 a
Half-yellow	Kathali	1.323	5.765	6.68	15.75 a
half-green	Narikeli	1.258	4.275	7.06	14.61 b
	Boll	1.380	4.013	6.53	15.89 a
Fully-	Kathali	1.373	4.133	6.680	16.153 a
yellow	Narikeli	1.543	4.288	7.055	15.135 b
	Boll	1.880	4.510	6.525	16.333 a

* The values with same letters in each column are not statistically different.

Recently, kakrol has become a major vegetable in Bangladesh. Because of its high export potential and demand in international market, it is primarily exported to

United Kingdom and middle East countries. It is available in May to October, the lean period for vegetables also acts in favour of its high demand. However, the crop is not grown on large scale because of the want of sufficient planting material and lack of appropriate cultivation technique (Hossain, 1974).

1.2. PAST RESEARCH :

Improvement of kakrol has not been attempted, perhaps because of its dioecious nature and its vegetative mode of propagation, or because it has, until recently, been a minor vegetable with a poor yield.

In normal cultivation practices tuberous roots are cut into pieces and planted during warm climate (March / April). Sprouts grow very rapidly to form a 3-4 m branched vine at maturity. Male and female plants are grown together at a ratio of 1 male: 15 female (Rashid, 1976; Vijay, 1978). Flowers start to appear 1-2 months after planting and continue until the start of senescence of the vines (Hussain and Rashid, 1974). Fruits are harvested 15-20 days after anthesis of the respective female flowers, before the fruits turn yellow and the seeds become hard. Even in its native habitat, the vines die in October, as temperatures fall, but the plants perenate via the non growing tuberous roots in the following year. Tetraploid plants are characterised by larger leaves, flowers and fruits and longer peduncles than the diploids (Siddique and Rahman, 1987). Its (*M.dioica* Roxb.) wild relative *Momordica cochinchinensis* is perennial has attributes like drought and cold tolerance (Islam *et al.*, 1992).

There are some evidences of variation in flower and fruit morphotypes among the plants grown in native areas. In addition, there may be a scope to transfer useful characteristics such as, from its close relatives, e.g. *M. charantia* L., *M. balsamina* L., *M. subangulata* Blume, *M. tuberosa* Roxb. (Rashid, 1976; Vijay *et al.*, 1977; Singh, 1978, 1990).

Morphological features of the indigenous types of kakrol are not well described (Rashid, 1976). Floral biology of *M. dioica* has been reported by Hussain and Rashid

(1974) and of other species (*Momordica cochinchinensis*) by Vijay *et al.* (1977). A general morphology of *M. dioica* and of other Cucurbits have been studied by Hossain (1974) and Hoque (1971) respectively.

Tarik and Reza (1992) collected twenty six female and seven male germplasms of *M. dioica* Roxb. from the extensive kakrol growing areas. One female germplasm of wild species was also collected from Jamalpur. The 26 female cultivated germplasms collected were classified into 16 genotypes. Seven male germplasms were different from one another.

Seed germination of kakrol is difficult due to hard seed coat. One hundred percent germination from decoated seeds was reported by Ali *et al.*, (1991) in *M. dioica.* Mishra and Sahu (1983) reported that fresh seeds do not germinate but remain dormant for nine months. Hartmann and Kester (1968) reported scarification or gibberellin treatment of seeds can improve seed germination.

Induction of bisexual and staminate flowers on pistillate plant using $AgNO_3$ is well established in cucumber and tomato (Beyer, 1976); in pickling cucumber (Kalloo and Franken, 1978); in *Momordica charantia* (Kabir *et el.*, 1989) and in *Cucumis sativa* (More and Munger, 1986). Induction of bisexual flowers on pistillate *M. dioica* has been reported (Ali *et al.*, 1991). Self pollination of breeding lines of dioecious plants has been made possible by changes in flower sex using stimulation or inhibition of ethylene action (McMurry and Miller, 1968; Iwahori *et al.*, 1970; Beyer, 1976; Kalloo and Franken, 1978; More and Munger, 1986; More and Seshadri, 1988).

Germination of pollen on the stigma was observed within 8 h after pollination (Hussain and Rashid, 1984) with sucrose and glucose solution, a concentration of 15% gave the highest germination of pollen grain (38.2 & 33.3 respectively) and longest pollen tube lengths (85.7 and 53.44 μm, respectively). A 3 ppm boric acid solution produce the highest germination percentage (33.1%) and among the grown

plants, 21 were female and 25 were male i.e., there was a 1:1 ratio (Ali *et al.*, 1991) which show the low yield. The cause of vine senescence with a fall in temperature and whether there is dormancy of the tuberous roots are questions that have not yet been addressed..

Until recent past kakrol had been considered as a minor vegetable. However, its popularity in internal and for export market has been increased sharply during last decade. As a result commercial cultivation of kakrol has also been expanded. Obviously the growers are now demanding information on improved varieties and production technology.

The multiplication of kakrol through conventional method is very slow. Tissue culture techniques were explored with kakrol, with the intention of developing the information needed to multiply the plants on a large scale. Through this method 10-11 thousands saplings or clones can be raised by *in vitro* culture of different organs (viz. cotyledon, hypocotyle, shoot tip, node and internode) within 4-5 months from a single explant.

Dioecy is a serious hindrance, to generation advancement in plant breeding. Induction of bisexual is an important aspect and may open the way for generation advanced breeding in kakrol. As kakrol is unisexual, selfing of female plant is not possible. Through induction of bisexuality female × female crosses is possible. As a result segregation of characters in the cross progeny, release of potential variability and development of inbreed line are also possible. Genotypes with superior characters may be obtained through somaclonal variation. Production of any female plants on the large scale may be possible by self seeds.

Thus it appears that idea about the pollen-pistil interactions following self and cross-pollinations among the different types of kakrol is important for effective breeding programme. A clear understanding regarding these interactions certainly help to obtain fertile seeds out of various pollinations. The fate of the different

pollinations is not understood unless the post-pollination interactions among the various types of kakrol are known. The major steps of the pollen-pistil interaction are considered to be pollen adhesion, hydration and germination, followed by pollen tube penetration, tube growth through the stigma, style, ovary and finally fertilization. Longest pollen tube length was 58.5 μm (Dubey and Gaur, 1989). Pollen storage at 0% relative humidity resulted in a loss of viability after 84 h but viability was maintained for up to 45 days at 3°C (Dubey and Gaur, 1989).

Hybridization between a tetraploid female and diploid male produced only male flowers while in the following season it also produced many female flowers, sex expression had changed from dioecious to monoecious (Jha and Roy, 1989).

1.3. RATIONALE AND OBJECTIVES :

During past few decades the *in vitro* technique of plant tissue culture has been developed as a new and powerful tool as a possible way of propagation as well as increasing genetic variability for plant improvement. The ever-increasing pressure of an expanding world population on limited food, fibre and industrial crop productivity will require a continued plant breeding effort. Plant breeding is a complex activity and it is therefore unlikely that any one technological advance will be solely responsible for more efficient plant improvement, within this century knowledge of genetics has had a major impact on plant breeding. Subsequently, cytogenetic techniques, polyploidy, mutagenesis, heterosis, quantitative genetics, better understanding of bio-chemical and physiological processes and plant pathology have all been integrated into what is now refferred to as conventional plant breeding. So, too will tissue culture methods become integrated into plant breeding procedures.

As a crop, kakrol has a number of problems, including low yield. Fruits become inedible at maturity owing to the presence of a large number of hard seeds. Production of tuberous root pieces per plant is low; 10-20 tuberous pieces are produced per year. Germination of the seeds is very difficult or impossible because of

the hard seed coat (Rashid, 1976). Moreover, it is impossible to predict the sex of seed produced plants. In this investigation comparative analysis has been carried out among the different stages of buds to find out the appearance of incompatible responses in these plants. In fluorescent microscopic study emphasis has been given to get a clear idea about various types of callose responses on the stigmatic papillae following self as well as cross-pollinations. The events like pollen germination and tube elongation following such pollinations have been investigated along with the extent of fertilization with the help of fluorescent microscopy.

The present study was, therefore, primarily directed at :

1. Development of information needed to multiply the plants on a large scale through seeds and cuttings to initiate a breeding programme in kakrol.

2. Stimulation of sex conversion with an aim for self-pollination of breeding lines of different dioecious plants of kakrol.

3. Development of information on cross compatibility among the different types of kakrol plants through the investigation of various phases of pollen-pistil interaction at pre and post fertilization stages with the help of fluorescent microscopic study.

4. Establishment of *in vitro* culture technique for multiplication through micropropagation and crop improvement through somaclonal variation. Formulation of optimum culture condition through the adjustment of different exogenous growth regulators and selections of suitable explant sources.

Chapter - 2

MATERIALS & METHODS

MATERIALS AND METHODS

2.1. MATERIALS :

Tetraploid, diploid and triploid types of kakrol (*Momordica dioica* Roxb.) collected from the root stocks maintained at the Fruit Research Center, Shampur, Rajshahi were used as experimental materials for this investigation.

2.2. METHODS :

2.2.1. SEED GERMINATION : Seeds were germinated on the seed bed.

(i) Preparation of seed beds : Seed beds were prepared throughly and fertilized with cowdung and chemical fertilizers. Bed size was 10m × 1m having 30 rows of 1m size. Proper irrigation was made as and when necessary.

(ii) Seed sowing : Almost 300 seeds of tetraploid type and 300 seeds of diploid type with seed coat (unpeeled), without seed coat (decoated) and scarified were sown on 20th April, 1995. The seed coats were scarified with the help of sharp scalpel. Twenty seeds were sown per row for each type. Observations were made on regular basis.

2.2.2. VEGETATIVE PROPAGATION :

(i) By tuberous roots : The tuberous roots of tetraploid, triploid and diploid male, female type were collected from the Fruit Research Centre, Shampur, Rajshahi. The tuberous roots were dugup from the soil in late October when the plants were about senescent. As a control treatment, they were treated with fungicide and kept under the soil for 35 days in an unheated house and then allowed to sprout. Within

two weeks almost all the tuberous roots sprouted and these were planted in the field on 15 February, 1995.

(ii) By vine cutting : Vines from originally tuberous root grown plants were cut into pieces with one or two leaves each and one axillary bud. The bottom part of the cuttings were about 2-3 cm long from the lower node and the upper part was 1-2 cm long from the upper node. Both the ends had a slant cut. The bottom ends of the cuttings were dipped into different concentrations of 3-indolebutyric acid (IBA) for 30 minutes and the cuttings were planted on soil in a perforated tray. The IBA hormone promoted rooting. The cuttings were kept covered with polythene bags for a week.

2.2.3. *IN VITRO* PROPAGATION

The methods involved for *in vitro* propagation are described under the following headings :

A. CULTURE MEDIA

MS (Murashige and Skoog, 1962) salt mixture was used in the preparation of culture media as follows :

The first step in the preparation of the culture media was the preparation of the stock solution. The various constituents of media were prepared into stock solutions for ready use during preparation of medium. As different media constituents were required in different concentrations, separate stock solutions of macronutrients, micronutrients, organic compounds, Fe-EDTA (Iron-stock), vitamins and amino-acids, growth regulators (auxin, cytokinin and gibberellin) etc. were prepared.

(i) Stock solution of macro-nutrients :

This stock solution was made upto 10 times the final strength of the medium in 1000 ml of distilled water. At first 10 times the weight of the salts present per litre of the medium was weighed accurately, dissolved once at a time and sequentially in 500 ml of distilled water and then made upto 1000 ml. The stock solution was stored in deep freeze.

(ii) Stock solution of micro-nutrients :

Stock solutions of micro-nutrients were made up to 10 times the final strength of the medium in 100 ml of distilled water as described for the stock solution of the macro-nutrients. This stock was filtered and stored in a refrigerator at 0°C.

(iii) Stock solution of organic components :

Each of the recommended ingredients of the organic components were made into stock solutions separately. Ten times of each of the required ingredients were taken in a volumetric flask and dissolved in 50 ml of distilled water. Then the final volume was made upto 100 ml by further addition of distilled water. These were poured seperately into plastic bottles and stored in deep freeze.

(iv) Stock solution of $FeSO_4$ and Na-EDTA :

It was made ten times the final strength of the medium in 100 ml of distilled water. Here, two constituents $FeSO_4$ and Na - EDTA, were dissolved separately in distilled water and was chelated for 24 hours at 58°C by placing it in an incubator. Then the two solutions were mixed and volume was made to 100 ml by adding distilled water. The pH of the solution was adjusted at 5.6 and after filtering it was stored at 4°C in refrigerator.

(v) Stock solution of vitamins and amino acids :

The following vitamins and amino acids were used in the present investigation:

Pyridoxin HCl (Vitamin B_6).

Thiamine HCl (Vitamin B_1).

Nicotinic acid (Vitamin B_3).

Glycine

Myoinositol (Inositol)

Ten times each of the above mentioned vitamins and amino acids were dissolved separately in distilled water. They were then mixed and the volume was made to 100 ml by additional distilled water. The stock solution was stored in a refrigerator at 0°C.

(vi) Stock solution of growth regulators :

In addition to the nutrients, it is generally necessary to add one or more growth regulators such as auxin, cytokinin or gibberellic acid to the media to support good growth of tissues and organs (Bhojwani and Razdan, 1983). Stock solutions of different phytohormones were prepared separately. Details of the methods of preparation of stock solution are given below.

Different plant growth regulators and their solvents.

Growth regulators	Amount taken (mgl^{-1})	Dissolving solvent (ml)	Final volume of the stock solution with DDW (ml).	Strength of the stock solution.
IAA	10	0.1N KOH 1ml	10	1
IBA	10	0.1N KOH 1ml	10	1
NAA	10	0.1N KOH 1ml	10	1
2,4-D	10	70% ethyl alcohol 0.5ml	10	1
BA	10	0.1 N HCl 0.5 ml	10	1
KIN	10	0.1 N HCl 0.5 ml	10	1
GA_3	10	0.1 N HCl 0.5 ml	10	1

To prepare stock solutions, 10 mg of any of the growth regulators was taken in a clear test tube and dissolved in required volume of appropriate solvent. Final volume of the solution was made up to 10 ml by adding DDW. Thus stock solutions of all growth regulators were prepared and stored at 4°C.

(vii) Sterilent solution :

$HgCl_2$ solution at various concentrations was used for surface sterilization of plant materials. To prepare 0.1% solution, 0.1 of $HgCl_2$ was dissolved in 100 ml DW. Freshly prepared $HgCl_2$ was always used. Generally $HgCl_2$ solution was prepared 1 hour before use.

B. PREPARATION OF CULTURE MEDIUM :

To prepare 1 litre of any of the above mentioned culture medium the following steps were involved :

(i) 30 g of sucrose for MS medium was dissolved in 500 ml of distilled water. In case of determining the effect of different concentrations of sucrose on shoot proliferation and elongation 20, 30, 40, 50 and 60 g of sucrose were used.

(ii) 100 ml of stock solution of macronutrients, 10 ml of stock solution of micronutrients, 10 ml of stock solution of Fe- EDTA and 10 ml of stock solution of vitamins were added to the above mentioned 500 ml of sucrose solution and mixed well.

(iii) Different concentrations of hormonal supplements as required was added single or in combination to the solution and thoroughly mixed. Since 1 ml of each of the hormonal stock solutions contained 1 mg solute, therefore, addition of 1 ml stock solution of any of the hormone to 1 litre medium resulted 1 mgl^{-1} concentration. Hormonal concentration was made different by varying the volume of the stock solution as per requirement.

(iv) Other supplements such as glutamine, tyrosine, caseinhydrolysate, malt extract, etc. if required, were added.

(v) Final volume of the medium was made 1 litre by adding DDW.

(vi) The pH of the medium was usually adjusted to 5.0-6.0, before autoclaving. In the present findings pH of the medium was adjusted to 5.8 by a pH meter with the help of 0.1 N NaOH or 0.1 N HCl whichever was necessary.

(vii) The culture nutrient media were gelled with agar. In the present experiments, 6 g of Carolina Biological Supply Co. agar was added for one litre of medium. Then, the whole mixture was gently heated until the agar was melted completely making the turbid solution clear. Care was taken so as not to be boiled the solution while melting agar. At the time of gentle heating, continuous stirring procedure was applied to the solution till complete dissolution of agar.

(viii) The prepared melted medium was dispensed into culture vessels like test tubes or conical flasks while medium still hot. The culture vessels were plugged with non-absorbent cotton plugs or heavy duty aluminium foil and marked with the help of a glass marker to indicate specific hormonal supplement.

(ix) The culture vessels containing medium were then autoclaved at 1.1 kg cm^2 pressure and at the temperature of 121°C for 20 minutes to ensure sterilization. In case of test tubes, the medium was allowed to cool vertically or as slants after sterilization.

As GA_3 is degraded at higher temperature and by autoclaving, this hormone was filter sterilized and added to cool autoclaved medium using microfilter of pore size 0.2 μm.

C. COLLECTION, SURFACE STERILIZATION AND PREPARATION OF EXPLANT :

Different explants used in this investigation, their sources and process of preparation are summarized below :

Explant types, sources and methods of surface sterilization.

Plant materials	Source of plant materials	Method of washing	Surface sterilization with $HgCl_2$	
			Conc.	Duration (min)
Embryo, cotyledon	Seeds of ripe and various stages of developing fruits	Washed in 1% Savlon +4 drops Tween 80 for 10 mins followed by 4 rinses with DW.	0.1	12
Hypocotyl	Aseptically grown seedling	-	-	-
Leaf, nodal and internodal segments	*In vitro* regenerated shoots or seedlings	-	-	-
Shoot tips and nodal explants	Field grown mature plants	Washed in running tap water for ½ to 1h, washed again in DW	0.1	4-8

Embryo and Cotyledon : For culture of embryo and cotyledons, the seeds were collected from mature green fruits (*Momordica dioica* Roxb.) and washed with a few drops of tween 80 and 1% savlon (v/v, cetrimide) for ten minutes with constant shaking. Then the seeds were washed 4 to 5 times with DW and were taken into running Laminar-air-flow cabinet and transferred to 250 ml sterilized conical flask. Surface sterilization was done with 0.1% $HgCl_2$ for 12 minutes. They were then washed at least 6 times with sterile distilled water. The sterilized seeds were taken into a sterilized petridish and seed coat was removed very carefully. After removing seed coat from the seeds, embryo and cotyledon were isolated carefully. The cotyledons either in intact condition or cut into pieces and incubated in the culture vessels containing media. The embryos were also incubated in the culture media. For future use, the fruits were stored in refrigerator at 4°C.

Hypocotyl explant : For this purpose, seeds from the ripe fruits were collected and sterilized as mentioned earlier. The sterilized seeds were incubated (16h light / 8h dark) in culture vessel containing MS medium, 3.0% sucrose, 0.6% agar having pH 5.8. Hypocotyl explants (0.7 - 1.0 cm in length) dissected from aseptically grown seedlings were used as inoculum.

Shoot tips and nodal explants : Healthy, disease free tender twigs were collected from mature field grown plants. The shoots, after trimming off larger leaves, were brought to laboratory and washed thoroughly in running tap water for 1 h. The shoots were then cut into pieces each containing 8-10 nodes and excess parts were removed. The pieces were treated with 1% Savlon + 2 drops of Tween 80 as wetting agent for 10 mins and rewashed thrice with DW. The materials were then sorted as shoot tips and nodes and transferred separately. Surface sterilization was carried out by gentle shaking in $HgCl_2$ solution for 4-8 mins depending on hardness of the materials. The shoot apices were trimmed to (1.0 -0.5 cm) terminal parts of lightly furled leaves 2-3 visible nodes and used as shoot tip explants. The remaining segments were further cut into pieces each containing 1-2 nodes were treated as nodal explants. Prepared explants were placed single in 25×150 mm culture tubes containing 20 ml of different growth regulators supplemented agar gelled media. For the first week of culture, at least one additional change of media was given depending on the degree of phenolic exudation and media staining. Subsequently, the cultures were transferred to the fresh medium after every 2-3 weeks and proliferating shoot clumps were maintained in 25×150 mm tubes.

Leaves, shoot tips and nodal segments: *In vitro* regenerated shoots (from both juvenile and mature tissue) were aseptically taken out from culture vessel and leaves excluding petiole and stem segments (shoot tips and nodes) were dissected. These explants were cultured singly in 25×150 mm culture tubes or 250 ml flasks containing culture media.

D. INOCULATION TECHNIQUE :

Before starting inoculation, the floor of laminar-air-flow cabinet was cleaned with 70% ethanol after put it on for 20 minutes. All the inoculations and aseptic manipulations were carried out in front of the running laminar-air-flow cabinet. All the dissecting instruments like forceps, scalpels, needles, surgical blades etc. were covered with aluminum foil and autoclaved by steam autoclaving procedures. Then these were brought to the laminar-air-flow cabinet. Before every use, the above instruments were dipped into absolute alcohol and flamed over a spirit lamp. Other requirements like petridishes, distilled water etc. were sterilized by steam sterilization procedure. Before starting inoculation, hands were also washed thoroughly by soap and made sterile by spraying 70% ethanol. For surgical operations, sufficient care was taken as usual to obtain possible contamination free condition.

Sterilized materials were collected in a petridish by a sterilized forceps and incised at right angles by a cold sterile scalpel at required size of explant depending on the nature of experiment and explant. During incision, efficient care was taken to avoid injury to the explants.

Prepared explants were carefully inoculated in culture vessels containing sterilized agar gelled medium. The plugs of the culture vessels were removed inside laminar-air-flow cabinet in presence of spirit lamp flame. Then the inoculation procedure was applied. Inoculation of explants was made singly or in groups of two to four explants per culture vessels depending on the diameter of culture vessels and nature of experiment. In case of callus induction two to three explants were used in the same culture vessels. During inoculation, special care was taken that the explant must touch the medium equally and not dip into the medium.

After inoculation, the culture vessels were sealed by parafilm 'M' and labelled by glass marker with inoculation date. Then the culture vessels were ready for incubation.

E. INCUBATION :

The inoculated culture vessels were incubated in a growth chamber containing special culture environment. The vessels were put on the shelves of a cupboard in the growth chamber. Unless mentioned specially, all cultures were grown in the growth chamber illuminated by 40 watts while fluorescent tubes fitted at a distance of 30-40 cm from the culture shelves. The cultures were maintained at 27±2°C under the warm fluorescent light intensity varied from 2000-3000 Lux. The photoperiod maintained generally 16 hours light and 8 hours dark. The vessels were checked daily to note the response. In some cases of callus induction, the vessels were incubated on dark shelves and with the appearance of callus, the vessels were transferred to another shelves under low intensity fluorescent light at the same temperature. During the growth of shoots, always 16 hours photoperiod was maintained.

F. MAINTENANCE OF CULTURES :

Different processes were followed for maintaining the proliferating cultures through subcultures and are discussed under separate heads.

Adventitious bud proliferating cultures : The explants when cultured on suitable medium were induced to proliferate adventitious buds within 4-5 weeks. At this stage, the proliferating cultures were subcultured again in the same initial medium in order to increase budding frequency. After another 4 week incubation the proliferating cultures were transferred to different media for bud elongation. The elongated shoots were excised from the proliferated cultures and transferred individually to the rooting media. Some of the shoots after isolating leaves, were cut into pieces and cultured individually or in groups for further adventitious and axillary regeneration. The stock cultures after excising usable shoots were transferred to the initial medium and then to the shoot elongation medium for further adventitious regeneration. The process was repeated for several times in order to establish continuous production of shoots.

Callus culture : The calli derived from cotyledon and hypocotyl explants were cut into pieces of 0.5 to 1 cm in diameter and subcultured on to media with different concentrations and types of growth regulators and additives for observing morphogenic potentialities regarding shoot differentiation or further callus proliferation.

Shoot regenerating cultures from mature tissues : Shoot tips and nodal explants from mature field grown plants induced precocious axillary buds after culturing onto suitable media within 4-5 weeks. These buds had very slow growth and showed frequent premature leaf and bud breaking. In order to rejuvinate explants with growing buds were subcultured repeatedly. The axillary shoots thus proliferated showed normal growth, were cut into pieces, each containing 1-2 nodes. These segments were recultured on to fresh medium, within 4-5 weeks of subculture each segment developed 3-5 shoots which were again subdivided and subcultured repeatedly. This process was continued for regenerating sufficient number of shoots. For conducting rooting experiments, a steady production of shoot cuttings was maintained by reculturing the stock cultures after each bach of cuttings were harvested.

Microcutting preparation and their rooting : The usable shoots were collected aseptically from proliferating cultures of different types of explants at different stages of subculture. Until transferred to the rooting media the shoots were kept in a sterilized glass beaker lined with moistened filter paper to avoid desiccation. The cuttings were prepared from these shoots by snaping off the basal leaves and cultured individually in 25×150 mm tubes containing different strength of basal salt composition and different auxin concentrations. The cultures were incubated under different physical factors.

G. PREPARATION OF MICROCLONES UNDER *IN VIVO* ENVIRONMENT :

Rooted plants were taken out from the culture tubes and washed carefully under running tap water for complete removal of remains of the medium. Polythene

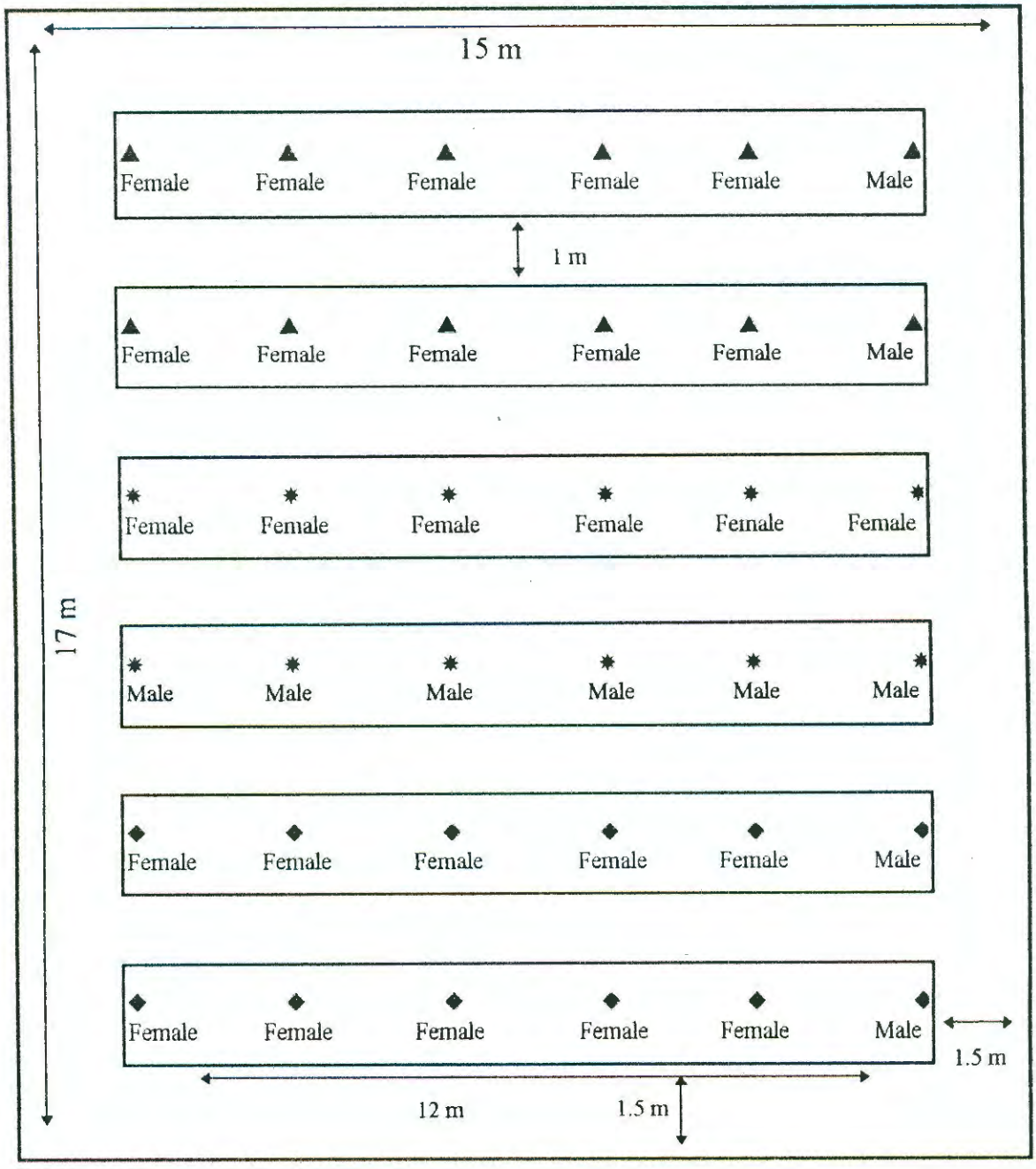
bags (9×15 cm) were kept ready filled with garden soil, organic manure and sand in the proportion of 2:1:1 respectively. The soil in the polythene bags was moistured uniformly and treated with agroson (250 mgl^{-1}, w/v) two days before transplantation. The plants were then transplanted into bags containing soil (one in each bag) with special care and sealed with the help of a piece of thread for providing high humidity to the plants. The bags containing transplanted plants were kept in the culture room under artificial illumination. On the 5/6th day after transplantation the polythene bags near proximity were perforated to allow ventilation to the plants. The polythene bags were finally reopened on the 10th day. After 15 days the transplanted plantlets were subjected to sunlight periodically and progressively. This practice facilitated the transplants for gradual acclimatization in *ex vitro* stress environment. After 20 days, the plants were transferred to the garden soil.

2.2.4. FIELD SELECTION FOR BREEDING EXPERIMENTS :

The experiment was conducted at Fruit Research Centre, Shampur, Rajshahi. The selected field was sandyloam and was free from water logging.

Field design : The experiment was laid out in 6 blocks, with 6 plants in each block. The 36 plants of which 12 for tetraploid, 12 for triploid and 12 for diploid were randomly assignment in these 6 blocks. The block size was 12×1.5 m having 1m foot path in each block and 1.5 m foot path all round the blocks.

FIELD DESIGN



1 cm = 1m

▲ = Tetraploid ; ✷ = Triploid ; ◆ = Diploid

Field preparation : The experimental field was ploughed deeply (200 cm) four times and weeds were removed. The field was pulverized and cowdung was mixed thoroughly with repeated ploughing. The field was leveled properly by laddering.

Transplantation of plant materials : The plant materials (tuberous roots) were transplanted in the field on 28th February, 1995 at the Fruit Research Center, Shampur, Rajshahi. Basins were prepared 30 to 45 cm diameter and 30 cm depth, 2 kg compost, 2 kg cowdung, 0.5 kg mustard oil cake, 80 g organic fertilizer containing equal parts of N P and K and 3 g furadan were used per basin. After plantation irrigation was made on regular basis by hand. The shoots were allowed to trail over bamboo trailers approximately 80 cm above soil surface.

Optimum cultural practices were made during the crop production which included weeding, fungicide spray, insecticide spray, use of organic fertilizer, irrigation etc.

2.2.5. PREPARATION OF $AgNO_3$ SOLUTION :

One g $AgNO_3$ was weighted and dissolved in 1000 ml distilled. The solution thus prepared was 1000 ppm of $AgNO_3$ and stored at 4°C as a stock solution.

From this 1000 ppm stock solution following six different concentrations were made and used for induction of flowering.

Prepared ppm	Amount of water (ml)	Amount of 1000 ppm $AgNO_3$ (ml)
600	40	60
500	50	50
400	60	40
300	70	30
200	80	20
100	90	10

$AgNO_3$ solution was applied following two methods (a) spraying directly on the twigs and (b) by dipping the tips of the twigs into solution.

a) Spray with $AgNO_3$ solution : The solution was sprayed by a sprayer on twigs of female plants of tetraploid, diploid and triploid types of kakrol. The spraying operation was done from the tip down to 7th leaf of the twig.

b) Immersion in $AgNO_3$ solution : The twigs of three types of female plants were drawn and immersed in $AgNO_3$ solution for 10, 20, 30, 40, 50 and 60 seconds.

2.2.6. POLLINATION METHOD :

Pollination was made with stigma of freshly opened flowers. Kakrol is a dioecious plant and the flowers are unisexual. As a result self pollination does not occur. The flowers of tetraploid type open at 5.30 to 6.30 am and those for diploid type open at 7.30 to 10.00 pm, where as, those for triploid (♀ dip ×♂ tetra) open at 3 am to 4.30 am. The stigma of female flowers of all these three types of kakrol remain receptive for 12 h before anthesis and 12 h after anthesis. For crossing, bagging of both male and female flowers were done in the afternoon a day before anthesis and pollination was carried out on the following morning. In case of bisexual flowers emasculation was made a day before anthesis to prevent self-pollination. Male flowers were collected in sterilized petridish and pollen grains were dusted on the stigma of mature female flowers. The pollinated flowers were kept covered with polythene bags for conjunctive two days after pollination. Proper labelling was made and labels were kept till fruits were harvested.

2.2.7. CROSSING SCHEDULE :

The description of pistillate flowers, pollen source and mode of pollination in different crossings are summarized below :

Pistillate flower	Pollen source	Mode of pollination
♀ tetra		Cross (control)
♀ (♂ tetra)		Cross
♀ trip	♂ tetra	Cross
♀ (♂ trip)		Cross
♀ dip		Cross
♀ (♂ dip)		Cross
♀ tetra		Sib (same plant)
♀ tetra		Sib (different plant)
♀ (♂ tetra)		Sib (same flower)
♀ trip		Cross
♀ (♂ trip)	♂ (♂ tetra)	Cross
♀ dip		Cross
♀ (♂ dip)		Cross
♀ tetra		Cross (control)
♀ (♂ tetra)		Cross
♀ trip	♂ dip	Cross
♀ (♂ trip)		Cross
♀ dip		Cross
♀ (♂ dip)		Cross
♀ tetra		Cross
♀ (♂ tetra)		Cross
♀ trip		Cross
♀ (♂ trip)	♂ (♂ dip)	Cross
♀ dip		Sib (same plant)
♀ dip		Sib (different plant)
♀ (♂ dip)		Sib (same flower)

Pistillate flower	Pollen source	Mode of pollination
♀ tetra		Cross
♀ (♂ tetra)		Cross
♀ trip	♂ trip	Cross
♀ (♂ trip)		Cross
♀ dip		Cross
♀ (♂ dip)		Cross
♀ tetra		Cross
♀ (♂ tetra)		Cross
♀ trip		Sib (same plant)
♀ trip		Sib (different plant)
♀ (♂ trip)	♂ (♂ trip)	Sib (same flower)
♀ dip		Cross
♀ (♂ dip)		Cross

tetra = Tetraploid, trip = Triploid, dip = Diploid.

2.2.8. COLLECTION OF POLLINATED PISTILS :

Self and cross-pollinated pistils were collected 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 12 h, 16 h, 18 h, 20 h and 24 h after pollination in vials containing acetoalcohol solution (1:3 v/v).

2.2.9. DETERMINATION OF POLLEN GERMINATION AND POLLEN TUBE GROWTH

To determine pollen germination and pollen tube growth, the collected pistils were washed for three times with distilled water to make them free from fixative (acetoalcohol solution). Then these were kept in 1N NaOH and incubated at 60°C for 30 minutes for softening. After cooling the materials were washed for three times with distilled water to make them free from sodium hydroxide. For staining, the materials were kept in 0.1% (W/V) decolourized aniline blue solution for 20 minutes. Aniline blue solution was prepared by dissolving aniline blue (BDH) in 0.I M tri-potassium orthophosphate. The solution was allowed to stand for one week for decolorization.

Following staining, materials were mounted in a 50% (v/v) aqueous solution of glycerol prior to examination under a Nikon (Optiphot) microscope fitted with epifluorescence UV illumination system having filter combination of UV-2A and V-2A. For each occation callose reaction, pollen germination, tube growth and number of fertilized ovules were recorded following the methods of Kho and Baer (1968).

2.2.10. POLLEN GERMINATION IN LABORATORY :

Pollen grains were collected from different types of kakrol. Separate solutions of sucrose and glucose (15%, 20%, 25% and 30%) were employed. Boric acid solutions dissolved in DW at 3, 4, 5 and 6 ppm were also used seperately as germination media.

2.2.11. DATA COLLECTION :

In this investigation following data were recorded :

1. **Seed germination :** Numbers of seeds germinated were counted at 10 days interval up to 60 days and expressed in percentage.

$$\text{Percentage of seed germination} = \frac{\text{No. of seeds germinated}}{\text{Total no. of seeds sown}}$$

2. **Survival of vine cuttings :** The survival of vine cuttings was recorded on the basis of rooting in percentage.

$$\text{Percentage of success} = \frac{\text{No. of cuttings survived}}{\text{Total no. of cuttings planted}} \times 100$$

3. ***In vitro* growth response :** Different growth parameters were considered to study morphogenic responses of various explants under different culture conditions. The parameters considered are :

   i. Frequency of callus proliferating explants : Numbers of explants induced callus were expressed as % and data were recorded 4 weeks after culture.

   ii. Frequency of shoot formation (adventitious / axillary) : Numbers of explants induced axillary / adventitious buds were recorded 5 weeks after culture and expressed as % of axillary / adventitious shoot regenerating explants.

   iii. Percentage of explants induced roots : This parameter was used for rooting experiment and data on percentage of shoot produced roots were recorded 4 weeks after culture.

   iv. Number of shoots (adventitious / axillary) per explant : Data on this parameter were recorded 4-8 weeks after culture.

   v. Number of roots per shoot : Number of roots per shoot was recorded 5 weeks after culture.

vi. Shoot length : Shoot height was measured in cm usually after 5 weeks of culture. The length of the longest shoot was considered if more than one shoots were present in a culture.

vii. Root length : Length of the longest root was measured in cm while the plants were transplanted.

viii. Fresh weight of callus : Each culture vessel containing proper amount of culture media was weighed carefully with an electronic balance before placing explants in it. Each culture vessel was weighed again after placing explant (single explant / culture vessel) and weight of the explant was found out as follows :

Wt. of explant = (Wt. of culture vessel + explant) – Wt. of culture vessel.

After 4 weeks of culture each explant with proliferating callus was taken out and weighed. Entire process was operated in front of running laminar-air-flow cabinet and sterilized petridishes were used to avoid contamination of the calli. Fresh weight of callus was found out as follows :

Fresh weight of callus = Wt. of explant with callus - Wt. of explant.

Weight of explants and calli were taken in gram. Degree of callus development from various explants was scored by the indices listed below :

Index	Description of callus formation
–	No callus formation
+	Callus tissue just formed on parts of explant
++	Callus tissue less than 0.5 cm in diameter.
+++	Callus tissue more than 0.5 cm in diameter.

4. **Sex convertion and continuous flowering :** Observation on sex conversion was made until the normal sex of the plant would be identified. Three different types of flowers were obtained after treatment with $AgNO_3$: flowers with large

bracts, flowers with full developed anther (bisexual) and flowers with aberrant anthers. The continuous flower opening sequence of these induced and uninduced (normal types) flowers was also recorded.

i. Normal Bisexual flower : Numbers of induced female flowers per twig that developed complete anthers were recorded and termed as normal bisexual.

ii. Abnormal bisexual : Female flowers with aberrant anthers were considered as abnormal bisexual. Numbers of such flowers per twig were counted and recorded.

iii. Normal female flower : Flowers that developed large bracts like those of male due to $AgNO_3$ treatment but did not develop any anthers in them were considered as normal female. Numbers of such flowers per treated twig were counted and recorded.

iv. Flowers with bracts : Numbers of flowers per twig that developed large bracts due to $AgNO_3$ treatment were counted and recorded. These included bisexual, abnormal bisexual and normal female flowers developed in twigs treated with $AgNO_3$.

5. **Floral morphology :** Following flower characters of normal and induced flowers were recorded.

Bract length : The length from the base to the tip of the bract was measured in cm.

Bract breadth : Breadth was measured at the middle of the bract in cm.

Calyx length : Calyx length was measured from base to apex in cm.

Calyx breadth : Calyx breadth was measured in cm at the widest point.

Pedicel length : For female and bisexual flowers the distance from bract to the base of the ovary was measured in cm. For male flowers pedicel length was measured from bract to the calyx in cm.

Ovary length : The ovary was cut lengthwise and the length of the ovary was measured in cm from base to the apex.

Ovary diameter : Ovary diameter was measured in cm by slide calipar.

Petal length : Petal length was measured in cm from base to apex.

Petal breadth : Petal breadth was measured in cm at the widest point.

Petal area : Petal area was measured by area meter.

6. **Leaf area :** Leaf area of different types of kakrol was measured by green leaf area meter.

7. **Pollen characters :** Following pollen characters were recorded.

i. Pollen viability : Viability of pollens was determined through acetocarmine staining. The pollens that took stain were considered as viable pollens and the pollens that did not take stain were considered as non-viable pollens. Numbers of viable and non-viable pollens were counted and recorded.

ii. Percentage of pollen germination : Sucrose, glucose and boric acid solutions were used for pollen germination. Numbers of germinated pollens were counted at different duration and recorded in percentage.

$$\% \text{ of germination} = \frac{\text{No of germinated pollens}}{\text{Total number of pollens}} \times 100$$

iii. Observation of callose responses on the stigmatic papillae : To examine callose responses stigmatic papillae from 10 randomly selected pollinated flowers were observed under fluorescence microscope. The various types of callose reactions produced by the stigmatic papillae were recorded.

iv. Observation of pollen germination and tube penetration : Total numbers of germinated and non-germinated pollen grains were scored from 10 pollinated pistils following pollination. A pollen grain was recorded as germinated if the tube tip emerged and extended at least half the diameter of

the pollen grain. A tube was recorded as penetrated the stigmatic papillae if the tip passed through the cuticle of a stigmatic papilla and observed at the base of the papilla.

v. Observation of pollen tube within style and ovary : Pollinated pistils were observed under fluorescence microscope to monitor the growth of the pollen tube within the style. To study the effective pollinations the number of ovules fertilized by the pollen tubes were scored and recorded.

8. **Fruit characters :** Following fruit characters were considered and recorded:

Fruit weight : Mature fruits were harvested from different pollinated flowers and weight was taken in gram.

Fruit volume : After harvesting of mature fruits the volume of the fruit was taken.

Seeds per fruit : Total numbers of seeds in a fruit were counted and recorded.

Seed weight of fruit : Weight of total sun dried seeds collected from a fruit were taken in gram and recorded.

2.2.12. DATA ANALYSIS :

For evaluating and interpreting the results, collected data were analysed following biometrical techniques developed by Mather (1949) and Allard (1960) based on the mathematical models of Fisher *et. al.* (1932). The methods of analysis are described below :

Mean and standard error of mean : The mean of a particular parameter from different replications was calculated by taking arithmetic mean using the following formula :

$$\bar{Y} = \frac{1}{n}\sum_{i=1}^{n} Yi$$

Where, $\bar{Y}$ = Arithmetic mean

n = Number of observation

$\sum Yi$ = Summation of variable.

Standard errors of mean (S.E.) were calculated as follows :

S.E. or $\delta\bar{X} = \sqrt{S^2 / n}$

Where, $\bar{X}$ = Sample mean

S^2 = Sample variance

n = Sample size.

Chapter - 3

RESULTS

RESULTS

A number of experiments on propagation and breeding of kakrol (*Momordica dioica* Roxb.) were conducted in this investigation. Observations made on different experiments considering various aspects are discussed under different heads according to the nature of objectives and type of experiments conducted.

3.1. EFFECT OF SEED COAT ON SEED GERMINATION :

Germination of seeds in kakrol usually require longer time as compared to other cucurbits. This experiment was conducted on seed bed. Seeds of tetraploid and diploid types of kakrol were shown either with entact seed coat (unpeeled) or without seed coat (decoated seed) and another type was scarified seed. Results obtained are shown in **Table 1**.

On average, germination percentage was higher in diploid type of kakrol than that of tetraploid type. It was 59% in diploid and 53% in tetraploid when the seeds were germinated with intact seed coat. On the contrary, the percentage of germination was higher in decoated seeds and it was 80% in diploid type and 73% in tetraploid type. It is therefore, evident from the result that the germination percentage was always higher in decoated seeds than with coated and scarified seeds.

For decoated seeds complete germination occurred within 20 days from the date of seeding. It was observed that 35% and 38% of the total seeds for tetraploid and

diploid type respectively germinated in 10 days and the rest of the seeds germinated within 20 days of seeding.

In case of unpeeled seeds, the rate of germination was slow and continued up to 60 days. Within 10 days only 4% of the tetraploid type and 6% of the diploid type of seeds germinated. Within 20 days, 14% and 16% of seeds of tetraploid and diploid germinated respectively. These results indicate that coated seeds required more time for germination than decoated and scarified seeds. The seed germination percentage of scarified seed was higher than the coated seed.

Table 1 : Effect of seed coat on seed germination in tetraploid and diploid kakrol.

Types of kakrol	Number of seeds	Number of seeds germinated						
		After 10 days	After 20 days	After 30 days	After 40 days	After 50 days	After 60 days	% of germination
Tetraploid *	100	4	14	16	10	6	3	53
Diploid *	100	6	16	17	11	6	3	59
Tetraploid **	100	35	38	-	-	-	-	73
Diploid **	100	38	42	-	-	-	-	80
Tetraploid ***	100	8	21	19	10	4	2	64
Diploid ***	100	10	23	20	10	3	1	68

* With seed coat ; ** Without seed coat ; *** Scarified seed.

3.2 VEGETATIVE PROPAGATION :

Vegetative or asexual propagation is used to produce a plant identical to source (mother) plant and it possible to induce roots and/or shoots on stems or roots. Vegetative propagation from these parts is possible because living cells contain genetic information in their nuclei necessary to reproduce the entire plant. Vine cuttings were used for vegetative propagation and the results are presented below.

3.2.1 BY VINE CUTTING :

Vegetative propagation through vine cutting was tested in this experiment. Vines from originally tuberous root-grown plants were cut into pieces with one or two leaves each and one axilliary bud. The bottom part of the cuttings was about 2-3 cm long from the lower node and the upper part was 1-2 cm long from the upper node. Both the ends had a slant cut. Normally the vine cuttings could not produce roots, therefore, these were treated with an auxin. The bottom ends of the cuttings were dipped in various concentrations of 3-indolbutyric acid (IBA) such as 0.5, 1.0 and 2.0 mgl^{-1} for 30 minutes and the cuttings were planted on soil in a perforated tray. Data were recorded after 21 days of treatment and are shown in **Table 2**. The IBA hormone promoted rooting (**Plate I : Fig. J, L, M, N**) on vine cuttings. The planted cuttings were kept in a lighted place, but away from direct sunlight. The cuttings were covered with polythene bag for a week. The plants were irrigated lightly after planting. To prevent wilting of leaves spraying of water was done from a sprayer.

The root initiation was observed within 6 to 8 days after plantation and day by day the roots developed. On average highest number of roots per cutting (5.42) was recorded in 1.0 mgl^{-1} IBA for diploid female kakrol. The maximum root length was also (3.85) obtained at the same treatment for diploid male kakrol.

Table 2 : Effect of IBA on vine cutting. Each value is an average of five replications. (Data recorded after 21 days of treatment).

Type	IBA concentration used for 30 min.	Male		Female		Mean	
		Root no.	Root length (cm)	Root no.	Root length (cm)	Root no.	Root length (cm)
Tetra	0	–	–	–	–	–	–
	0.5	2.88	2.76	2.77	2.92	**2.82**	**2.84**
	1.0	4.21	3.65	4.53	3.38	**4.37**	**3.51**
	2.0	4.43	3.58	4.58	3.26	**4.5**	**3.42**
Mean		**3.84**	**3.33**	**3.96**	**3.18**		
Trip	0	–	–	–	–	–	–
	0.5	3.92	2.88	3.2	3.3	**3.56**	**3.09**
	1.0	4.42	3.76	4.56	3.58	**4.49**	**3.67**
	2.0	4.55	3.82	4.62	3.36	**4.58**	**3.59**
Mean		**4.29**	**3.48**	**4.12**	**3.41**		
Dip	0	–	–	–	–	–	–
	0.5	4.21	3.36	4.25	3.42	**4.23**	**3.39**
	1.0	5.36	3.85	5.42	3.78	**5.39**	**3.81**
	2.0	5.28	3.52	5.32	3.32	**5.3**	**3.42**
Mean		**4.95**	**3.57**	**4.99**	**3.5**		

Tetra = Tetraploid ; Trip = Triploid ; Dip = Diploid.

PLATE I

Plate IA. Different types of female twigs.
1. Diploid, 2. Triploid, 3. Tetraploid.

Plate IB. Different types of male twigs.
1. Diploid, 2. Triploid, 3. Tetraploid.

Plate IC. Different types of fruits.
1. Tetraploid, 2. Triploid, 3. Diploid.

Plate ID. Corolla of tetraploid, 1. Female, 2. Male.

Plate IE. Flowers of triploid, 1. Male, 2. Female.

Plate IF. Flowers of diploid, 1. Bisexual, 2. Male, 3. Female.

Plate IG. Tuberous roots of tetraploid female kakrol.

Plate IH. Tuberous roots of triploid female kakrol.

Plate II. Tuberous roots of diploid female kakrol.

Plate IJ. Adventitious root formation in different types of kakrol.

Plate IK. Growing plants from treated (1.0 mgl^{-1} IBA for 30 min) cuttings of 1. triploid, 2. tetraploid and 3. diploid female kakrol after 15 days of plantation.

Plate IL. Adventitious root formation on 8th cutting of tetraploid, 1. male, 2. female kakrol after treating with 1.0 mgl^{-1} IBA for 30 min. (Photograph was taken after 20 days of treatment).

Plate IM. Adventitious root formation on 7th cutting of triploid, 1. male, 2. female kakrol after treating with 1.0 mgl^{-1} IBA for 30 min. (Photograph was taken after 21 days of treatment).

Plate IN. Adventitious root formation diploid 1. female 2. male kakrol after treating with 1.0 mgl^{-1} IBA for 30 min. (Photograph was taken after 21 days of treatment).

PLATE - I

3.3 *IN VITRO* CULTURE :

Different experiments were set up to cover some of the aspects of *in vitro* response of *Momordica dioica* Roxb. Embryos and cotyledon from developing seeds; hypocotyl from *in vitro* grown seedlings, and shoot tip and nodal explants from *in vitro* and field grown shoots were used. The investigation had two objectives : (i) induction of shoot regeneration directly from the explants and (ii) induction of callus and subsequent plant regeneration from the induced callus.

3.3.1. DIRECT SHOOT FORMATION :

Cotyledon, embryo, shoot tip and nodal segments were used as explants in this experiment for direct shoot regeneration. The results are described in respect of type of explants and growth regulators under following separate heads.

3.3.1.1 From Cotyledon Explants :

Results concerning with various experiments involving different cultural aspects for finding out optimum media formulation and culture condition for adventitious bud regeneration from cotyledon explants are described below :

Effect of cytokinin :

For cotyledon culture seeds from developing green fruits were washed with a drop of tween 80 and washed thoroughly. After surface sterilization with 0.1% $HgCl_2$ the explants were cultured in MS medium supplemented with different concentrations of BA or KIN. The results of the experiment are given in **Table 3**. Pattern of morphogenic differentiation of the cultured explants varied with the concentrations of BA or KIN present in the culture media. First visible changes after transferring of explants in the culture media occurred by enlargement in size. The explants were white in colour before transferring to the media but became green within 7-9 days of culture through chlorophyll synthesis. During resuming new growth most of the explants in all treatments induced to develop trace of callus at the cut surface of the explants.

In case of BA most of the media formulations induced the explants to produce adventitious buds except at 0.1, 0.2 and 0.5 mgl^{-1} BA. Initially adventitious buds were visible as tiny nodule like structures (**Plate II : Fig. A**) within 14-16 days of culture. These nodule like protrusions subsequently developed into adventitious buds (**Plate II : Fig. B**) within 24-28 days of culture. In most cases bud proliferation occurred throughout the entire surface of explant. In a few cases adventitious bud regeneration occurred only from cut surface of the explant. There was no visible difference between dorsal and ventral surface of the explant regarding adventitious bud regeneration.

Degree of adventitious shoot regeneration was markedly influenced by amount of BA present in the culture media. The maximum 60% of adventitious bud regenerating explants was recorded at 4.0 mgl^{-1} BA. Greater number 14.55 and 12.45 shoots per explant were recorded respectively at 4.0 mgl^{-1} and 3.0 mgl^{-1} BA. The lowest number of shoots per explant was recorded in media with low concentration of BA (1.0 mgl^{-1}). In a few cases adventitious shoots were found to develop from callus formed at the cut surface.

Lower frequency of adventitious bud regenerating explants was recorded when KIN was used alone and the explants formed very small amount of callus at their cut surface when cultured with KIN alone. KIN alone showed very less effect on degree of shoot proliferation (**Table 3**). The highest mean number of shoots per explant was 6.85 in medium with 4.0 mgl^{-1} KIN and the lowest number was 2.25 in medium with 1.5 mgl^{-1} KIN. KIN concentrations at 0.1, 0.2, 0.5 and 1.0 mgl^{-1} failed to induce the explants to develop any adventitious buds.

Table 3 : Effect of different concentrations of BA or KIN in MS medium for direct shoot formation from cotyledon explant after 4 weeks of culture. Each value is an average of two replications and each replication consisted of 10 - 15 explants.

Growth regulators mgl^{-1}	Degree of callus formation	% of bud forming explant	Mean no of shoots per explant
BA			
0.1	-	-	-
0.2	-	-	-
0.5	-	-	-
1.0	-	10	2.15
1.5	-	20	5.22
2.0	+	35	7.48
2.5	+	45	10.25
3.0	++	55	12.45
4.0	++	60	14.55
5.0	++	50	10.35
KIN			
0.1	-	-	-
0.2	-	-	-
0.5	-	-	-
1.0	-	-	-
1.5	+	5	2.25
2.0	+	5	2.35
2.5	+	5	2.45
3.0	+	10	4.68
4.0	+	20	6.85
5.0	+	20	6.25

Effect of Cytokinin with Auxin

Differential morphogenic responses of cotyledon explants in MS medium supplemented with different concentration and combination of cytokinin and auxin were observed and the results of this experiment are shown in **Table 4**. Pattern of morphogenic differentiation of the cultured explants varied with concentration and combination of cytokinin and auxin present in the culture media.

When the explants were cultured on MS medium supplemented with different concentrations of BA (0.5, 1.0, 2.0, 3.0 and 4.0 mgl^{-1}) along with different NAA concentrations (0.1, 0.2, 0.5 and 1.0 mgl^{-1}), showed variable response towards callus formation, development of adventitious buds and number of shoots per explant. The result reveals that adventitious bud initiation was more frequent in all BA + NAA formulation, except when 0.5 BA + 0.1 NAA and 0.5 BA + 0.2 NAA were used. The explants were found to be more prone to callus as well as adventitious bud proliferation when the culture media had higher concentration of BA and lower concentration of NAA. The highest 75% of bud forming explants was recorded in media having 2.0 mgl^{-1} BA with 0.2 mgl^{-1} NAA. The maximum mean number of shoots per explant (16.55) was also recorded in this medium (**Plate II : Fig. E**). The lowest 10% of explants induced adventitious buds in medium with 0.5 mgl^{-1} BA and 1.0 mgl^{-1} NAA. These adventitious buds began to appear as tiny nodule shaped structures within 14-21 days of culture and subsequently developed into adventitious buds.

In case of KIN + IAA, the highest 70% of bud forming explants was recorded in media having 1.0 mgl^{-1} KIN with 2.0 mgl^{-1} IAA and the maximum mean number of shoots per explant was 15.65 in the same formulation. Media containing 0.1, 0.2 and 0.5 mgl^{-1} KIN with 0.5 mgl^{-1} IAA failed to induce any adventitious buds on the cultured explants.

Table 4 : Effect of different concentrations and combinations of BA and NAA in MS medium for direct shoot formation from cotyledon explant after 4 weeks of culture. Each value is an average of two replications and each replication consisted of 10 - 15 explants.

Growth regulators mgl^{-1}		Degree of callus formation	% of bud forming explant	Mean no.of shoots per explant
Cytokinin	Auxin			
BA +	**NAA**			
	0.1	+	-	-
	0.2	+	-	-
0.5	0.5	++	5	2.25
	1.0	++	10	2.55
	0.1	+	15	2.60
1.0	0.2	++	20	3.26
	0.5	++	30	4.56
	1.0	++	40	6.88
	0.1	+	60	10.85
2.0	0.2	+++	75	16.55
	0.5	+++	65	14.35
	1.0	++	60	12.28
	0.1	++	55	12.55
3.0	0.2	+++	60	14.45
	0.5	+++	65	13.36
	1.0	++	55	12.20
	0.1	++	55	10.35
4.0	0.2	+++	60	11.26
	0.5	+++	50	10.38
	1.0	++	45	8.45

Table 4 : (continued)

Table 4 : (continued)

Growth regulators mgl^{-1}		Degree of callus formation	% of bud forming explant	Mean no. of shoots per explant
Cytokinin	Auxin			
KIN +	**IAA**			
0.1		-	-	-
0.2	0.5	-	-	-
0.5		-	-	-
1.0		-	5	2.38
0.1		+	10	3.62
0.2	1.0	+	20	4.25
0.5		+	30	6.26
1.0		+	40	8.35
0.1		+	50	9.85
0.2	2.0	+	60	10.63
0.5		+	60	12.55
1.0		++	70	15.65
0.1		+	60	14.62
0.2	3.0	+	60	14.50
0.5		+	65	15.25
1.0		++	65	15.35
0.1		+	60	10.85
0.2	4.0	+	65	12.38
0.5		++	60	12.26
1.0		++	60	10.25

Effect of Cytokinin, Auxin and Gibberellic Acid :

In this experiment BA, NAA and GA_3 were used in different concentrations and combinations in MS medium to see their effect in the production of adventitious buds from cotyledon explants. The results obtained are presented in **Table 5**. Results reveal that adventitious bud initiation was more frequent in all BA + NAA + GA_3 formulations.

It was observed that all BA + NAA + GA_3 combinations produced callus from cut surface of the explants. In case of callus initiation the explants began to swell up and subsequently led to callus formation within 2-3 weeks of culture.

The explants were found to be more prone to develop adventitious buds when they were cultured in media with high concentration of BA and low concentration of NAA and GA_3. Greater percentages of adventitious bud regenerating explants were recorded at BA + NAA + GA_3 formulations. The highest 80% of bud forming explants was recorded at 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA + 0.1 mgl^{-1} GA_3 followed by 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA + 0.2 mgl^{-1} GA_3 and 3.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA + 0.1 mgl^{-1} GA_3. The media with 0.5 mgl^{-1} BA + 0.1 mgl^{-1} NAA + 0.01 mgl^{-1} GA_3 and 0.5 mgl^{-1} BA + 0.1 mgl^{-1} NAA + 0.1 mgl^{-1} GA_3 produced the lowest (1%) of bud formation.

The combination BA + NAA + GA_3 was found to be better than either BA alone or BA + NAA combination. Maximum 19.33 shoots per explant was recorded in media with 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA + 0.1 mgl^{-1} GA_3 followed by 3.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA + 0.1 mgl^{-1} GA_3. Shoot proliferation was the lowest (2.35) in medium containing 0.5 mgl^{-1} BA + 0.1 mgl^{-1} NAA + 0.01 mgl^{-1} GA_3. The adventitious buds began to appear as tiny nodule shaped structures within 21-28 days of culture [**Plate II : Fig. F (arrows)**] . These tiny nodule shaped structures subsequently developed into adventitious buds (**Plate II : Fig. C**).

Effect of different parts of cotyledon : In order to assess the regenerability of different parts of cotyledon, the explants were dissected into basal, middle and apical parts. These different parts were cultured individually in MS medium supplemented with different concentrations or combinations of auxin, cytokinin and gibberellic acid. Morphogenic potentialities of the explants were found to vary among different parts and full cotyledons produced maximum adventitious bud regenerating explants (60 - 80%) where cotyledons were attached with the embryoaxes. Full cotyledon explants also showed higher mean number of shoots per explant.

Among the different forms of explants cultured with different growth regulators formulations, the full cotyledon produced the highest frequency of bud regenerating explants (80%) and the maximum number of shoots per explant (19.33), where cotyledons were attached with embryo in media with 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA + 0.1 mgl^{-1} GA_3 (**Plate II : Fig. H**).

Table 5 : Effect of different concentrations and combinations of BA, NAA and GA_3 in MS medium for direct shoot formation from cotyledon explant after 4 weeks of culture. Each value is an average of two replications and each replication consisted of 10 - 15 explants.

Growth regulators mgl^{-1}			Degree of callus formation	% of bud forming explant	Mean no.of shoots per explant
Cytokinin	Auxin	Gibberellic acid			
BA +	**NAA** +	**GA_3**			
		0.01	+	10	2.35
0.5	0.1	0.1	+	10	3.65
		0.2	+	15	5.37
		0.5	+	20	6.72
		0.01	+	20	9.32
1.0	0.2	0.1	+	30	12.25
		0.2	+	40	15.36
		0.5	+	50	14.52
		0.01	++	70	17.54
2.0	0.2	0.1	++	80	19.33
		0.2	++	75	18.25
		0.5	+	70	16.15
		0.01	++	70	16.56
3.0	0.2	0.1	++	75	18.35
		0.2	++	70	15.20
		0.5	+	60	14.58
		0.01	++	60	16.36
4.0	0.2	0.1	+++	60	17.75
		0.2	+++	55	16.25
		0.5	++	50	12.38

PLATE II

Plate IIA. Callus initiation from cotyledon on MS+0.2 mgl^{-1} NAA after 15 days of inoculation.

Plate IIB. Callus induction and development of shoot bud from cotyledon on MS+2.0 mgl^{-1} BA+0.2 mgl^{-1} NAA after 21 days of inoculation.

Plate IIC. Callus induction and development of shoot bud from cotyledon on MS+3.0 mgl^{-1} BA+0.2 mgl^{-1} NAA after 20 days of inoculation.

Plate IID. Direct shoot regeneration from cotyledon explant on 3.0 mgl^{-1} BA+0.5 mgl^{-1} NAA after 22 days of inoculation.

Plate IIE. Shoot multiplication from callus and developing adventitious bud on MS+2.0 mgl^{-1} BA+0.2 mgl^{-1} NAA+0.1 mgl^{-1} GA_3 after 28 days of inoculation.

Plate IIF. Direct shoot formation from cotyledon with embryo explant on MS+2.0 mgl^{-1} BA+0.2 mgl^{-1} NAA after 21 days of inoculation.

Plate IIG. Shoot multiplication from *in vitro* grown shoot tip culture on 2.0 mgl^{-1} IAA+1.0 mgl^{-1} KIN after 21 days of inoculation.

Plate IIH. Shoot multiplication from *in vitro* grown shoot tip culture on 2.0 mgl^{-1} BA+0.2 mgl^{-1} NAA after 21 days of inoculation.

Plate III. Shoot formation from *in vitro* grown nodal explant on 2.0 mgl^{-1} BA+0.2 mgl^{-1} NAA after 21 days of inoculation.

Plate IIJ. Shoot formation from *in vitro* grown nodal explant on 2.0 mgl^{-1} IAA+1.0 mgl^{-1} KIN after 28 days of inoculation.

Plate IIK. Root induction on 1. *In vitro* grown explant 2. Field grown explant after 21 days of culture on MS+1.0 mgl^{-1} IBA.

PLATE - II

3.3.1.2 From Shoot Tip and Nodal Explants :

A number of experiments were conducted to establish a standard protocol for plant regeneration from *in vitro* grown shoot cultures. Results on these experiments are discussed below according to the physical and chemical aspects of the experiments.

Effect of growth regulators for better shoot proliferation from *in vitro* grown shoot tip and nodal explants :

Shoot tip and nodal explants were excised from *in vitro* grown explants that grew in MS medium supplemented with 2.0 mgl^{-1} IAA + 1.0 mgl^{-1} KIN and 2.0 mgl^{-1} BA+0.2 mgl^{-1} NAA and were cultured on agar gelled MS medium supplemented with different concentrations and combinations of auxin and cytokinin (**Table 6**). Shoot proliferation was noticed in all 44 media formulations and all the cultures resumed new growth.

First growth responses in both type of explants were evident by the precocious development of bud primordia from leaf axils. Within 1-2 weeks of subculture newly formed buds grew considerably accompanied with unfurling of new leaves. In most of the cases, the explants showed more than one shoot buds from their each axil. Multiple shoot bud proliferation was more frequent in those explants which were cultured with 3.0 mgl^{-1} BA+0.2 mgl^{-1} NAA. Growth regulator formulations showed pronounced effect on frequency of explants resuming new growth. Media containing BA 3.0 mgl^{-1} alone or with low concentration of NAA (0.2 - 0.5 mgl^{-1}) showed the best performance in inducing new growth of the explant. However, BA more than 3.0 mgl^{-1} with or without auxin suppressed the explants to proliferate new buds. The media containing 3.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA induced 85% shoot tips and 80% nodal explants to develop highest number of new buds. The maximum shoot length was recorded 10.16 cm after 35 days of culture in media having 2.0 mgl^{-1} IAA + 1.0 mgl^{-1} KIN and shoot proliferating percentage was second highest from both explants in this media composition. Similarly, new growth of explant was also suppressed by IAA alone. Over all performance in respect of resuming new growth of shoot tip explants were found to be better than nodal explants.

Table 6 : Effect of BA and IAA either singly or in combination with NAA or KIN in MS medium for better direct shoot proliferation from *in vitro* grown shoot tip and nodal explants. Each value is an average of two replications and each replication consisted of 10 - 15 explants. (Data recorded after 35 days of culture).

Growth regulators in preculture mgl^{-1}	Growth regulators in sub-culture mgl^{-1}	% of explant resumed new growth			
		Shoot tip		Nodal explant	
		% of shoot form-ation	Mean length of the longest shoot	% of shoot form-ation	Mean length of the longest shoot
2 IAA + 1 KIN	**BA**				
	0.1	10	3.67	10	3.53
	0.2	20	3.89	20	3.82
	0.5	30	4.10	30	4.09
	1.0	35	4.15	35	4.12
	1.5	40	4.28	45	5.05
	2.0	55	6.12	50	5.62
	2.5	65	7.26	60	6.28
	3.0	70	7.65	65	6.86
	4.0	65	6.68	60	6.36
	5.0	50	5.39	50	5.03
2 BA + 0.2 NAA	**IAA**				
	0.1	05	4.15	05	4.10
	0.2	15	4.59	15	4.28
	0.5	20	5.68	20	5.16
	1.0	35	7.10	30	5.62
	1.5	40	8.12	35	6.29
	2.0	50	8.36	45	7.46
	2.5	45	7.28	40	7.17
	3.0	40	7.14	35	6.38
	4.0	40	6.89	35	6.25
	5.0	35	5.62	30	5.22

Table 6 : (continued)

Table 6 : (continued)

Growth regulators in preculture mgl^{-1}	Growth regulators in sub-culture mgl^{-1}	% of explant resumed new growth			
		Shoot tip		Nodal explant	
		% of shoot form-ation	Mean length of the longest shoot	% of shoot form-ation	Mean length of the longest shoot
2 IAA + 1 KIN	**BA + NAA**				
	0.2	35	5.32	30	5.25
	1.0 + 0.5	40	6.21	35	6.13
	1.0	45	7.25	40	7.14
	0.2	70	9.15	65	7.28
	2.0 + 0.5	75	9.51	70	8.53
	1.0	75	9.62	70	8.68
	0.2	85	10.06	80	9.62
	3.0 + 0.5	80	10.02	75	9.26
	1.0	75	9.13	70	8.48
	0.2	75	9.10	70	8.38
	4.0 + 0.5	70	8.38	65	7.88
	1.0	65	7.87	60	6.92
2BA +0.2 NAA	**IAA + KIN**				
	0.2	40	5.64	40	5.48
	1.0 + 0.5	50	6.26	50	6.22
	1.0	60	6.78	55	6.58
	0.2	70	8.95	65	8.35
	2.0 + 0.5	75	9.89	70	9.26
	1.0	80	10.16	75	9.85
	0.2	75	9.98	70	9.36
	3.0 + 0.5	75	9.86	70	9.14
	1.0	70	8.62	65	8.05
	0.2	70	9.38	70	8.03
	4.0 + 0.5	65	8.58	65	7.62
	1.0	60	7.28	60	6.86

3.3.2. INDIRECT REGENERATION VIA CALLUS INDUCTION :

Cotyledon from seeds (21 days after anthesis) of developing green fruits and hypocotyls from 5-8 day-old aseptically grown seedlings of tetraploid type kakrol were used as explants for inducing callus development and subsequent plant regeneration. A number of experiments were conducted to find out optimum culture media for callus induction and subsequent plant regeneration. Results obtained from these experiments are discussed below according to the nature of experiments.

3.3.2.1. Plant Regeneration From Cotyledon Derived Callus :

Cotyledon explants were cultured in MS medium supplemented with different concentrations and combinations of NAA, 2, 4-D, BA and KIN. Cultures were maintained under 16 h light condition. Results obtained on morphogenic response of the cultured explants are shown in **Table 7**.

Morphogenic responses of the explants were found to vary with hormonal formulations present in the culture media. Callus proliferation was noticed in all 91 media formulations tested. There was a wide range of variation regarding morphological nature and degree of callus proliferation among them. First visible changes of the cultured explant occurred through lateral swelling. In most of the cases, callus proliferation began at the cut surfaces of the explant. But localized callus proliferation (callus proliferation other than cut surface) was also noticed at the end of 3 weeks of culture. Colour and texture of callus was also found to vary with hormonal supplements. Texture of calli was hard, spongy and friable and colour was green, white, greenish white, greenish yellow and brownish white.

Cent precent of the explants induced to form callus when cultured in media supplemented with 4.0 - 5.0 mgl^{-1} NAA. Similarly, 70-95% of callus forming explants was recorded in media having NAA ranges from 1.0 - 3.0 mgl^{-1}. Lower callusing frequency was found at low concentration of this auxin. Optimum callus growth was observed when the explants were cultured with 4.0 or 5.0 mgl^{-1} NAA

(fresh weight 1.65 - 1.75 g). Calli proliferated with NAA ranges from 3.0 - 5.0 mgl^{-1} were spongy, greenish-white and composed of rapidly growing large vacuolated cells. This type of calli did not show organogenesis even after 4–5 weeks of culture. Fresh weight of callus was the lowest 0.75g at 0.2 mgl^{-1} NAA. Calli proliferated at lower concentration of NAA were hard, greenish and showed organogenic potentialities. Calli proliferated within 0.2 - 2.0 mgl^{-1} NAA developed shoot buds although the frequency was low. Within 0.2 - 5.0 mgl^{-1} NAA, the calli did not show any root formation.

Explants cultured with 8 different concentrations of 2, 4-D were found more responsive to callusing. Callusing frequency was 90 - 100% which recorded at 2, 4-D ranging from 1.5 - 5.0 mgl^{-1}. Maximum degree of callus proliferation was also recorded in these ranges of 2, 4-D. The highest 1.80g fresh weight of callus was recorded at 5.0 mgl^{-1} 2, 4-D. The explants at lower concentration of this auxin showed lower degree of callus proliferation. The calli proliferated in 2, 4-D employed media failed to show any organogenesis. Calli proliferated in 2, 4-D were spongy to hard and brownish–white.

Degree of callusing was less but percentage of shooting was more when NAA was used with BA and 65-85% callusing was recorded in these treatments. Callusing frequency was high when high amount of NAA was supplied. The lowest frequency of callusing was observed at 0.5 + 0.2 mgl^{-1} BA and NAA. Degree of callus growth was also higher in those media which contained higher proportion of NAA. Among the 25 BA-NAA combinations, fresh weight of callus was maximum of 1.56 and 1.42g recorded at 4.0 + 2.0 and 4.0 + 1.5 mgl^{-1} respectively. Calli grew in these media were spongy, green and greenish yellow. Calli proliferated in media having lower concentrations of NAA and BA were soft, green to greenish yellow and composed of small compact cells. A few percentage of these calli showed root and maximum showed shoot bud differentiation. Two types of organogenic differentiation were observed, all BA + NAA combinations, calli showing only shoots and calli showing

both shoots and roots (**Plate III : Fig. C**). These shoot buds initially grew as globular embryoid like structures (**Plate II : Fig. B**). These globular embryo like protrutions eventually grew to shoot buds during later period. The maximum number of shoots per callus was recorded 15.5 in media having 2.0 + 0.2 mgl^{-1} BA and NAA. The second highest number of shoot per callus was recorded 14.6 in media having 2.0 mgl^{-1} BA with 0.5 mgl^{-1} NAA. The highest rooting percentage was recorded 10 in media having 3.0 + 0.2 mgl^{-1} and 4.0 + 0.2 mgl^{-1} BA with NAA.

KIN and 2, 4-D also showed promotive effect on callus induction from cotyledon explants and more than 60% of the explants developed callus in all 25 media formulations. However, the degree of callus proliferation was found to vary with media composition. In general, callus proliferation was higher when the media contained higher proportion of 2, 4-D. Among 25 KIN -2, 4-D formulations, fresh weight of callus was maximum (2.02 g) recorded in media with 4.0 mgl^{-1} KIN + 2.0 mgl^{-1} 2, 4-D at the end of 4 weeks of culture. Calli developed in KIN -2, 4-D fortified media were hard white to greenish white and brownish in colour. These calli did not show any organogenesis.

Callus proliferation and shoot formation were observed in media with KIN and NAA formulations. Degree of callus proliferation was higher but shoot regeneration was lower in these media compositions. Root initiation was not observed in any KIN-NAA formulations. The highest percentage of shoot forming callus was recorded 25 in media having 2.0 + 0.2 mgl^{-1} KIN with NAA and the maximum number of shoot per callus was recorded 6.2 in media having 0.5 + 0.2 mgl^{-1} KIN with NAA. Calli proliferated in media having lower proportion of KIN and NAA were hard green and showed organogenic potentiality. The calli proliferated in comparatively higher concentration of NAA were hard greenish–white or yellowish–green in colour.

Table 7 : Effect of NAA and 2, 4-D either alone or in combination with BA or KIN on callus initiation and callus growth from cotyledon explant after 4 weeks of culture. (12-15 explants were cultured per treatment).

Growth regulators mgl^{-1}	% of callus forming explants	Fresh wt. of callus (gm)	% of organic callus		Mean no. of shoot/callus
			Root	Shoot	
NAA					
0.2	45	0.75	-	25	7.5
0.5	55	0.85	-	20	8.5
1.0	70	0.90	-	15	6.0
1.5	90	1.25	-	15	3.0
2.0	95	1.35	-	10	2.5
3.0	95	1.40	-	-	-
4.0	100	1.65	-	-	-
5.0	100	1.75	-	-	-
2, 4-D					
0.2	50	0.85	-	-	-
0.5	60	0.95	-	-	-
1.0	75	1.35	-	-	-
1.5	90	1.45	-	-	-
2.0	95	1.55	-	-	-
3.0	100	1.65	-	-	-
4.0	100	1.75	-	-	-
5.0	100	1.80	-	-	-
BA + NAA					
0.2	35	0.60	-	25	8.2
0.5	40	0.70	-	25	8.5
0.5 + 1.0	45	0.75	-	30	9.1
1.5	55	0.86	-	30	9.5
2.0	70	0.92	-	35	9.2
0.2	35	0.65	-	30	10.5
0.5	45	0.72	-	30	10.2
1.0 + 1.0	55	0.76	-	35	9.7
1.5	65	0.88	-	35	8.5
2.0	75	0.96	-	40	8.2

Table 7 : (continued)

Table 7 : (continued)

Growth regulators mgl^{-1}	% of callus forming explants	Fresh wt. of callus (gm)	% of organic callus		Mean no. of shoot/callus
			Root	Shoot	
BA + NAA					
0.2	50	0.72	5	40	15.5 **
0.5	55	0.86	5	45	14.6
2.0 + 1.0	65	0.92	-	45	12.5
1.5	70	1.25	-	50	11.6
2.0	75	1.36	-	55	10.3
0.2	60	0.85	10	45	12.5
0.5	65	0.92	5	40	11.6
3.0 + 1.0	70	0.98	-	35	10.2
1.5	80	1.12	-	35	8.5
2.0	80	1.35	-	30	7.1
0.2	60	0.89	10	50	11.2
0.5	65	0.99	5	45	9.5
4.0 + 1.0	70	1.26	-	40	9.2
1.5	80	1.42	-	40	8.3
2.0	85	1.56	-	35	6.5
KIN + 2, 4-D					
0.2	65	0.62	-	-	-
0.5	75	0.73	-	-	-
0.5 + 1.0	85	0.86	-	-	-
1.5	90	1.24	-	-	-
2.0	95	1.48	-	-	-
0.2	70	0.68	-	-	-
0.5	75	0.82	-	-	-
1.0 + 1.0	80	0.97	-	-	-
1.5	90	1.35	-	-	-
2.0	100	1.66	-	-	-
0.2	100	1.66	-	-	-
0.5	70	0.98	-	-	-
2.0 + 1.0	75	1.13	-	-	-
1.5	85	1.48	-	-	-
2.0	100	1.92	-	-	-

Table 7 : (continued)

Table 7 : (continued)

Growth regulators mgl^{-1}	% of callus forming explants	Fresh wt. of callus (gm)	% of organic callus		Mean no. of shoot/callus
			Root	Shoot	
KIN + 2, 4-D					
0.2	65	0.75	-	-	-
0.5	75	0.89	-	-	-
3.0 + 1.0	80	1.18	-	-	-
1.5	80	1.45	-	-	-
2.0	95	1.85	-	-	-
0.2	70	0.92	-	-	-
0.5	75	1.23	-	-	-
4.0 + 1.0	85	1.38	-	-	-
1.5	90	1.86	-	-	-
2.0	100	2.02	-	-	-
KIN + NAA					
0.2	30	0.46	-	15	6.2
0.5	35	0.52	-	15	5.8
0.5 + 1.0	50	0.62	-	10	5.5
1.5	60	0.76	-	10	4.6
2.0	70	0.86	-	10	4.2
0.2	35	0.53	-	20	6.0
0.5	45	0.66	-	20	5.8
1.0 + 1.0	55	0.75	-	15	5.5
1.5	65	0.86	-	15	5.2
2.0	75	0.92	-	15	4.2
0.2	35	0.58	-	25	5.5
0.5	45	0.64	-	20	5.3
2.0 + 1.0	50	0.76	-	25	4.8
1.5	65	0.87	-	20	4.6
2.0	75	0.98	-	15	3.8
0.2	30	0.59	-	20	4.3
0.5	45	0.66	-	20	4.1
3.0 + 1.0	50	0.78	-	15	3.8
1.5	60	0.95	-	15	3.5
2.0	70	1.22	-	10	2.5
0.2	30	0.55	-	20	4.1
0.5	40	0.62	-	15	3.8
4.0 + 1.0	50	0.76	-	15	3.5
1.5	60	0.85	-	10	2.8
2.0	65	0.92	-	10	2.5

3.3.2.2. Plant Regeneration From Hypocotyl Derived Callus :

Morphogenic responses of hypocotyl explants were found to vary with growth regulator formulations present in the culture media (**Table 8**). Callus proliferation occurred in all 8 concentrations of NAA. Explants cultured in medium with 3.0, 4.0 and 5.0 mgl^{-1} NAA induced centpercent callusing. Similarly, 50-95 callus forming explant was recorded at NAA ranges from 0.2 - 2.0 mgl^{-1}. Callusing frequency and shooting percentage were high (%) at 0.2 mgl^{-1} NAA.

Degree of callus proliferation was found to vary with the variation in concentration of NAA. Highest fresh weight of callus was recorded 1.68 g at 5.0 mgl^{-1} NAA and the lowest was 0.72g recorded at 0.2 mgl^{-1} NAA. Usually, callus proliferation began to form at the cut surface of the explants. Calli proliferated in media with higher concentrations of NAA were spongy, white to yellowish-white. Whereas, calli were hard and green to greenish white when cultured in media having 0.2 to 2.0 mgl^{-1} NAA. These calli showed shoot differentiation and highest 30 percent of shoot forming calli was recorded in media having 0.2 mgl^{-1} NAA. Maximum number of shoots per callus was recorded 7.2 at 0.2 mgl^{-1} NAA. The media containing 3.0, 4.0 and 5.0 mgl^{-1} NAA did not show any organogenic differentiation.

All 2, 4-D supplemented media produced the best percentage of callus. However, the degree of callus proliferation varied considerably with the concentration of 2, 4-D. Among the 8 different formulations of 2, 4-D, 3.0, 4.0 and 5.0 mgl^{-1} were found to be most effective for callus induction. The highest fresh weight of callus was recorded 1.78g at 5.0 mgl^{-1} 2, 4-D and the lowest fresh weight of callus (0.82 g) was observed at 0.2 mgl^{-1} 2, 4-D. Calli proliferated within 2.0 - 5.0 mgl^{-1} 2, 4-D were hard and yellowish white. Whereas, proliferated calli were hard and green at 0.2 mgl^{-1}. No organogenic differentiation was observed from the calli cultured in 2, 4-D supplemented media.

Supplementation of BA and NAA together in basal media showed promotive effect on callusing (**Table 8**). Callus proliferation from most of the explants was recorded in all 25 BA - NA supplemented media. But frequency of callusing varied with proportion of BA and NAA. The media having higher proportion of BA than NAA induced more than 80% of the explants of callusing (**Plate III : Fig. A**). Degree of callus proliferation and morphological nature of callus were also found to vary with BA - NAA proportion. In most of the treatments, maximum callus growth occurred when the media was supplemented with 2.0 - 4.0 mgl^{-1} BA with low concentration of NAA. Calli proliferated in media having 2.0 - 4.0 mgl^{-1} BA with 0.2 - 0.5 mgl^{-1} NAA were friable, whitish or greenish and showed organogenic differentiation. Root and shoot proliferation from calli was observed within 4 weeks in media with 2.0 - 4.0 mgl^{-1} BA with 0.2 - 0.5 mgl^{-1} NAA (**Plate III : Fig. B**). The maximum number of shoots per callus was recorded 11.6 in media having 4.0 + 0.2 mgl^{-1} BA and NAA. The highest fresh weight of callus in BA - NAA combination was 1.42 g in media having 4.0 + 2.0 mgl^{-1} BA and NAA at the end of 4 weeks of culture.

Effect of KIN with 2, 4-D on callus initiation was also pronounced even more than 2, 4-D alone. In most of the treatments more than 65% of the explants produced calli. But the degree of callus proliferation was found to vary with media composition. In general, callus proliferation was higher when the media contained higher proportion of 2, 4-D. Among 25 KIN - 2, 4-D formulations, fresh weight of callus was maximum (1.73g) recorded in media with 4.0 + 2.0 mgl^{-1} KIN and 2, 4-D, at the end of 4 weeks of culture. Calli developed in KIN - 2, 4-D fortified media were hard, white to greenish white and brownish in colour. In these calli, organogenic differentiation was not observed in any of the KIN - 2, 4-D treatments.

From hypocotyl explants, callus proliferation was also observed at all KIN - NAA formulations. Degree of callus proliferations was also higher but shoot

regeneration was lower in these media formulations. Root initiation was not observed in any of the KIN - NAA formulations. The highest percentage (30%) of shoot forming callus was recorded in media having 2.0+0.2 mgl^{-1} KIN and NAA and the maximum number or shoots per callus was recorded 6.3 in media having 1.0+2.0 mgl^{-1} KIN with NAA. Calli proliferated in media having lower proportion of KIN and NAA were hard and greenish white and showed organogenic potentiality. The calli proliferated in comparatively higher concentration of NAA were hard, greenish white or yellowish green in colour.

Table 8 : Effect of NAA and 2, 4-D either alone or in combination with BA or KIN on initiation and growth of callus from hypocotyl explant. In each treatment 12-15 explants were cultured.

Growth regulators	% of callus forming explants	Fresh wt. of callus	% of organogenic callus		Mean no. of shoot per callus
			Root	Shoot	
NAA					
0.2	50	0.72	-	30	7.2
0.5	60	0.81	-	25	7.0
1.0	75	0.89	-	20	6.5
1.5	95	1.18	-	20	4.5
2.0	95	1.42	-	15	3.2
3.0	100	1.58	-	-	-
4.0	100	1.62	-	-	-
5.0	100	1.68	-	-	-
2, 4-D					
0.2	60	0.82	-	-	-
0.5	65	1.12	-	-	-
1.0	80	1.32	-	-	-
1.5	95	1.38	-	-	-
2.0	100	1.58	-	-	-
3.0	100	1.61	-	-	-
4.0	100	1.72	-	-	-
5.0	100	1.78	-	-	-
BA + NAA					
0.2	40	0.58	-	20	7.8
0.5	45	0.71	-	25	7.6
0.5 + 1.0	45	0.76	-	30	8.3
1.5	55	0.85	-	35	8.6
2.0	75	0.90	-	35	8.5
0.2	40	0.62	-	25	9.2
0.5	45	0.74	-	30	9.5
1.0 + 1.0	55	0.78	-	30	10.2
1.5	65	0.82	-	35	8.8
2.0	80	0.92	-	40	8.6
0.2	55	0.68	-	35	9.5
0.5	60	0.82	-	35	8.9
2.0 + 1.0	65	0.91	-	45	8.5
1.5	70	1.12	-	45	8.6
2.0	75	1.18	-	50	7.5

Table 8 : (continued)

Table 8 : (continued)

Growth regulators	% of callus forming explants	Fresh wt. of callus	% of organogenic callus		Mean no. of shoot per callus
			Root	Shoot	
BA + NAA					
0.2	60	0.81	5	50	12.2
0.5	65	0.88	5	45	12.4
3.0 + 1.0	75	0.92	-	45	11.5
1.5	85	1.09	-	40	11.2
2.0	90	1.25	-	40	10.3
0.2	65	0.85	5	50	11.6
0.5	70	0.89	-	45	10.8
4.0 + 1.0	75	1.16	-	40	10.2
1.5	80	1.32	-	40	8.3
2.0	85	1.42	-	35	8.1
KIN + 2, 4-D					
0.2	65	0.57	-	-	-
0.5	80	0.68	-	-	-
0.5 + 1.0	90	0.82	-	-	-
1.5	95	1.14	-	-	-
2.0	100	1.32	-	-	-
0.2	70	0.62	-	-	-
0.5	75	0.78	-	-	-
1.0 + 1.0	85	0.88	-	-	-
1.5	90	1.25	-	-	-
2.0	100	1.48	-	-	-
0.2	70	0.86	-	-	-
0.5	75	0.95	-	-	-
2.0 + 1.0	85	1.08	-	-	-
1.5	90	1.42	-	-	-
2.0	100	1.56	-	-	-
0.2	65	0.72	-	-	-
0.5	75	0.83	-	-	-
3.0 + 1.0	85	0.98	-	-	-
1.5	85	1.26	-	-	-
2.0	95	1.46	-	-	-

Table 8 : (continued)

Table 8 : (continued)

Growth regulators	% of callus forming explants	Fresh wt. of callus	% of organogenic callus		Mean no. of shoot per callus
			Root	Shoot	
KIN+2, 4-D					
0.2	75	0.84	-	-	-
0.5	80	1.07	-	-	-
4.0 + 1.0	95	1.26	-	-	-
1.5	95	1.54	-	-	-
2.0	100	1.73	-	-	-
KIN + NAA					
0.2	35	0.38	-	15	5.6
0.5	40	0.48	-	15	5.3
0.5 + 1.0	50	0.58	-	15	5.1
1.5	65	0.72	-	10	4.8
2.0	75	0.82	-	10	4.0
0.2	35	0.46	-	20	6.3
0.5	50	0.57	-	25	5.8
1.0 + 1.0	60	0.69	-	20	5.3
1.5	70	0.80	-	15	4.2
2.0	75	0.89	-	10	3.8
0.2	40	0.52	-	30	5.8
0.5	45	0.62	-	20	5.2
2.0 + 1.0	60	0.68	-	15	4.6
1.5	65	0.79	-	10	4.2
2.0	75	0.89	-	10	3.8
0.2	40	0.49	-	20	5.2
0.5	45	0.64	-	20	4.6
3.0 + 1.0	50	0.74	-	15	4.2
1.5	65	0.87	-	15	3.9
2.0	75	1.02	-	15	3.3
0.2	35	0.46	-	15	4.5
0.5	45	0.56	-	15	4.2
4.0 + 1.0	55	0.68	-	10	3.8
1.5	65	0.74	-	10	3.2
2.0	70	0.86	-	10	3.1

3.3.2.3. Effect of Recurrent Sub-culture on Callus Growth and Shoot Formation :

Undifferentiated callus proliferated from cotyledon and hypocotyl explants in previous experiments were recurrently sub-cultured onto MS medium employed with 2.0 mgl^{-1} BA, 1.0 mgl^{-1} KIN, 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA and 2.0 mgl^{-1} IAA + 1.0 mgl^{-1} KIN. The calli were cut into 5-6 mm size pieces and used as explants of each of the subsequent subculture. Subculture was done at 4 weeks interval. Mean number of shoots per callus upto 6th subculture was recorded at the end of 4 week culture and data recorded are shown in **Table 9**.

Mean number of shoots per callus upto 6th subcultures were found to vary with hormonal supplement, calli subcultured with BA-NAA showed optimum growth upto 5th subculture and then decreased in 6th subculture. Similarly, the callus subcultured in media having 2.0 mgl^{-1} IAA + 1.0 mgl^{-1} KIN media produced less number of shoots per callus in the 6th subculture. Loss of callus growth and shoot regeneration during recurrent subculture was also observed in 1.0 mgl^{-1} KIN supplemented media. Callus subcultured onto media fortified with 2.0 mgl^{-1} BA showed more or less same degree of shoot regeneration upto 6th subculture without any loss of vigour (**Plate III : Fig. D**). Mean number of shoot regeneration and morphological nature of the callus of two different explants were found more or less same.

Table 9 : Effect of BA and KIN either singly or in combination with NAA or IAA in MS medium on shoot regeneration from cotyledon and hypocotyl derived callus during recurrent subculture.

Subculture	Source of callus regenerating explant	Growth regulators and mean number of shoot per callus.			
		2 BA	1 KIN	2 BA + 0.2 NAA	2 IAA+1 KIN
	Cotyledon	8.56	3.65	12.36	10.42
SC_1	Hypocotyl	7.62	2.86	10.48	8.67
	Cotyledon	9.86	3.82	14.62	11.52
SC_2	Hypocotyl	8.25	3.05	12.38	10.25
	Cotyledon	10.22	4.03	18.32	14.38
SC_3	Hypocotyl	9.36	3.80	16.54	13.25
	Cotyledon	10.15	3.89	17.63	14.25
SC_4	Hypocotyl	9.28	3.76	14.28	13.22
	Cotyledon	9.85	3.76	17.25	14.03
SC_5	Hypocotyl	8.38	3.25	14.09	12.32
	Cotyledon	8.62	3.25	15.38	10.35
SC_6	Hypocotyl	8.12	3.12	12.89	8.48

3.3.3. ROOTING OF MICROCUTTINGS :

Proliferating shoots of different types of explants took 3-4 weeks from the time of shoot formation to attain suitable size for rooting (3 cm or more). Different experiments were conducted for rooting and the results of these experiments are discussed below :

Effect of different concentrations of IBA for root induction :

Individual shoots from *in vitro* grown shoot clumps were excised and after trimming off basal leaves they were transferred to rooting media. Rooting media composed of MS basal salt having 0.6% agar, 3% sucrose and fortified with different concentrations of IBA viz., 0.1, 0.2, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 5.0 mgl^{-1}. All these media composition showed good response of root induction. A large number of lateral roots developed from the main roots when hormonal concentrations were increased and at the same time main root initiation decreased but number of lateral roots increased. Data were recorded as mean number of roots per explant and mean length of the longest root per explant in cm after 14, 21 and 28 days of inoculation and are tabulated in **Table 10**.

The highest number of roots per explant was 15.5 after 14 days, 18.6 after 21 days and 22.4 after 28 days of inoculation respectively in MS media having 5.0 mgl^{-1} IBA but this IBA concentration showed the lowest root length. The lowest number of roots per explant was 2.4 after 14 days and 3.3 after 21 days of inoculation respectively in media having 0.1 mgl^{-1} IBA.

The highest length of the longest root was 4.6 cm after 14 days and 6.8 cm after 21 days of culture in media having 1.0 mgl^{-1} IBA (**Plate III : Fig. E**) but 8.95 cm after 28 days of culture in media having 1.0 mgl^{-1} IBA. The lowest length of the longest root was 2.8 cm after 14 days, 4.0 cm after 21 days and 5.3 cm. after 28 days of culture in media having 0.1 mgl^{-1} IBA.

Table 10 : Effect of different concentrations of IBA in MS basal medium on root induction. (At least 15 shoots were used per-treatment).

Media composition mgl^{-1}	Morphogenic response					
	Mean number of roots per explant after days			Mean length of the longest root after days (in cm)		
	14	21	28	14	21	28
IBA						
0.1	2.4	3.3	7.6	2.8	4.0	5.3
0.2	5.6	7.2	12.3	3.8	5.9	6.8
0.5	6.8	9.5	13.6	4.3	5.0	7.2
1.0	9.2	10.4	15.5	4.6	6.8	8.95
1.5	10.2	12.4	16.2	4.4	6.6	8.62
2.0	12.1	13.6	17.5	4.2	6.4	7.85
2.5	12.5	13.9	17.8	3.9	5.9	7.12
3.0	12.8	14.5	18.2	3.8	5.6	6.86
4.0	14.2	15.2	19.3	3.2	5.2	5.8
5.0	15.5	18.6	22.4	2.9	4.6	5.4

3.4. PERFORMANCE OF REGENERATED PLANTLETS UNDER *IN VIVO* CONDITION :

3.4.1. INITIAL ESTABLISHMENT :

When the plantlets developed few leaves and few roots on the rooting medium, they were taken out from the culture vessels, washed thoroughly under running tap water to remove the debris of agar with care not to damage the roots and transferred to a pot (10 cm×9 cm) filled with 2:1 garden soil and compost. The potted plants (one plantlets per pot) were then put into large polythene bags (25 cm×15 cm). To maintain high humidity, open portion of the large bag was made air tight and kept them in growth chamber under artificial illumination. Within 5-7 days, the potted plants began to form new leaves and resumed new growth. Within 7-9 days the covering bags were finally removed.

However, the potted plants were brought out from the growth chamber and kept under full sun light for 2-3 hours only. Potted plants were successfully acclimated with natural condition through gradual increasing of duration under sun light and they eventually became suitable for final plantation in field. It was found that more than 85% of the planted microcuttings survived during initial establishment. In general, plantlets with active growth of primary roots (1-2 cm) showed greater survival and faster initial growth as compared to the plantlets having longer and branched root system (3-5 cm) at the time of transplantation.

3.4.2. FIELD PERFORMANCE :

Most of the transplanted plantlets showed orthotopic mode of development upto 25-30 days. Transplants started primary branching only after that period. In some of the cases the transplants started primary branching within 40-45 days after transplantation. Survival percentage of the plants was also higher in the field under natural environment. About 88% of the potted plants survived in the field successfully.

However, plants transplanted during warm humid condition showed necrotic lesson on their leaves and shoot tips. This type of necrosis generally did not affect the survival of the plantlets. Somaclonal variation among the transplants was also studied.

3.4.3. COMPARISON OF FRUIT CHARACTERS OF CALLUS GROWING PLANTS AND NORMAL PLANTS :

Different fruit characters viz. fruit weight, fruit volume, seed number and seed weight per fruit of callus derived tetraploid plants were compared with normal tetraploid plants. Results are shown in **Table 11**.

In case of callus derived tetraploid plants, fruit weight was 110.85 ± 2.14 and the fruit volume was 120.46 ± 3.24. The seed number of per fruit was 16.76 ± 1.29 and the seed weight per fruit was 4.38 ± 0.52. On the other hand, in normal tetraploid plants, the fruit weight was 90.63 ± 1.56 and the fruit volume was 99.66 ± 2.85. The seed number of normal fruit was 22.5 ± 1.35 and the seed weight per fruit was 5.62 ± 0.38. In both cases pollen source was same. It appears from the results, the fruits of callus growing plant were always greater in size than those from normal growing plants (**Plate III : Fig. H, I**).

Table 11 : Different fruit characters of callus growing tetraploid plants to compare with normal tetraploid plants. (Results presented are the mean values of 10 replicates with the standard error of the mean).

Plant type	Fruit weight (g)	Fruit volume (c c)	Seed number	Seed weight
Callus growing tetraploid plant	110.85 ± 2.14	120.46 ± 3.24	16.76 ± 1.29	4.38 ± 0.52
Normal tetraploid plant	90.63 ± 1.56	99.66 ± 2.85	22.5 ± 1.35	5.62 ± 0.38

PLATE III

Plate IIIA. Callus initiation from hypocotyl explants on MS + 3.0 mgl^{-1} BA +0.2 mgl^{-1} NAA after 15 days of inoculation.

Plate IIIB. Callus induction and development of shoot bud from hypocotyl on 4.0 mgl^{-1} BA+0.2 mgl^{-1} NAA after 21 days of inoculation.

Plate IIIC. Callus induction and development of shoot bud after 21 days of subculture from hypocotyl on 2.0 mgl^{-1} IAA+1.0 mgl^{-1} KIN.

Plate IIID. Shoot multiplication from *in vitro* grown hypocotylar shoot on 2.0 mgl^{-1} BA+0.2 mgl^{-1} NAA after 28 days of culture.

Plate IIIE. Root induction from *in vitro* grown nodal explant on MS+1.0 mgl^{-1} IBA after 21 days of subculture.

Plate IIIF. Root induction after 15(1) and 21 (2) days of culture on MS+1.0 mgl^{-1} IBA.

Plate IIIG. Normal fruits of 1, 2 tetraploid and 3,4 diploid type of kakrol.

Plate IIIH. Tetraploid fruit of callus growing plant.

Plate IIII. Different size of tetraploid fruits (from callus derived plant).

Plate IIIJ. Fruiting after transplantation of *in vitro* grown plants.

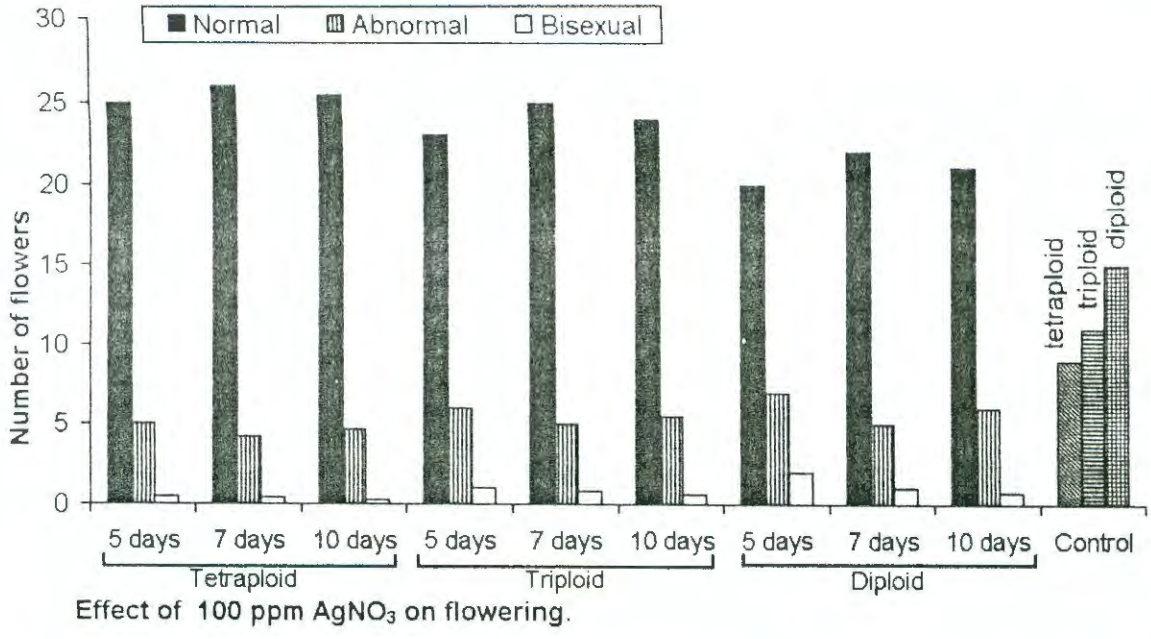
PLATE - III

3.5. INDUCTION OF FLOWERING AND BISEXUALITY :

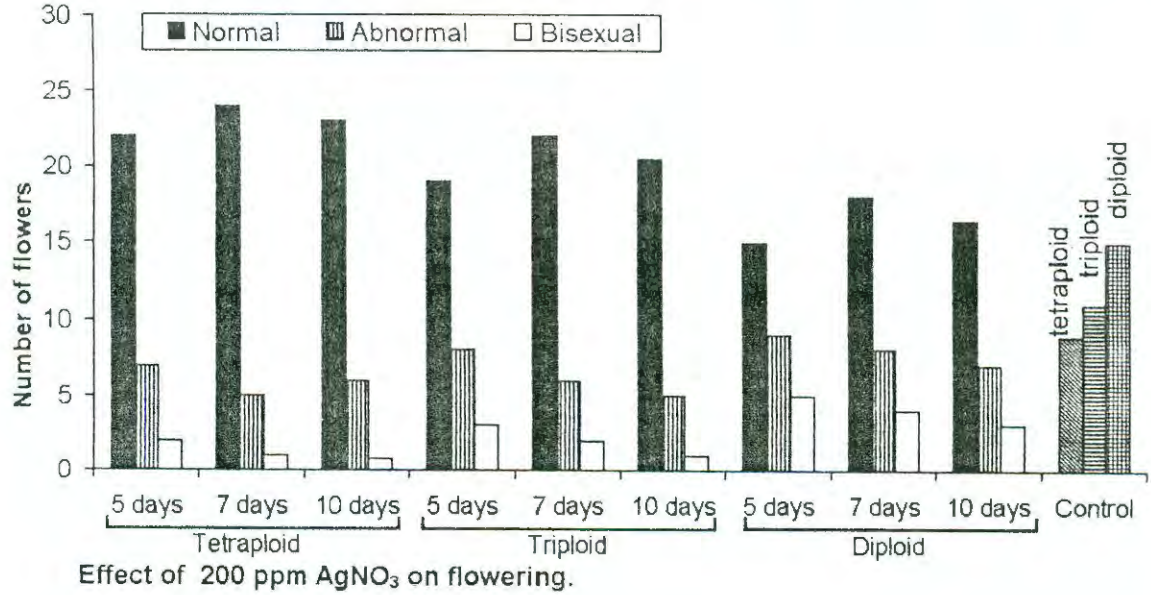
3.5.1. EFFECT OF $AGNO_3$ ON FLOWERING :

Opening of flowers depends on the node on which the flower bud has developed. It maintains a regular sequence of opening of flower from 1st node onword. It means that 1st bud opens first and maintains this sequence all through. Flowers opening sequence in a vine treated with individual strength of $AgNO_3$ from 100 and 200 ppm and vines where no $AgNO_3$ treated were studied at 5 days, 7 days and 10 days interval for individual twig. Results are shown in **Graph 1**.

The vines of the different types of kakrol induced to develope flower continuously (**Plate IV : Fig. B, F2**) when 100 and 200 ppm $AgNO_3$ were sprayed at 5, 7 and 10 days interval (**Graph 1**). However, flowering in untreated vines was discontinuious and sparse (**Plate IV : Fig. A**). Most of the flowers induced by $AgNO_3$ were normal female with large bract. A few of the induced flowers were abnormal bisexual with illdeveloped anther. The flowers developed from untreated vine were normal female without any bract. Out of 30 nodes the 26 nodes developed flowers when 100 ppm $AgNO_3$ was sprayed at 7 days interval for tetraploid type.



Effect of 100 ppm $AgNO_3$ on flowering.



Effect of 200 ppm $AgNO_3$ on flowering.

Graph 1 : Effect of 100 and 200 ppm $AgNO_3$ on flowering in different types of kakrol. $AgNO_3$ was sprayed at 5 days, 7 days and 10 days interval (30 nodes from 5 vines were used for each concentration).

3.5.2. EFFECT OF $AGNO_3$ ON SEX CONVERSION :

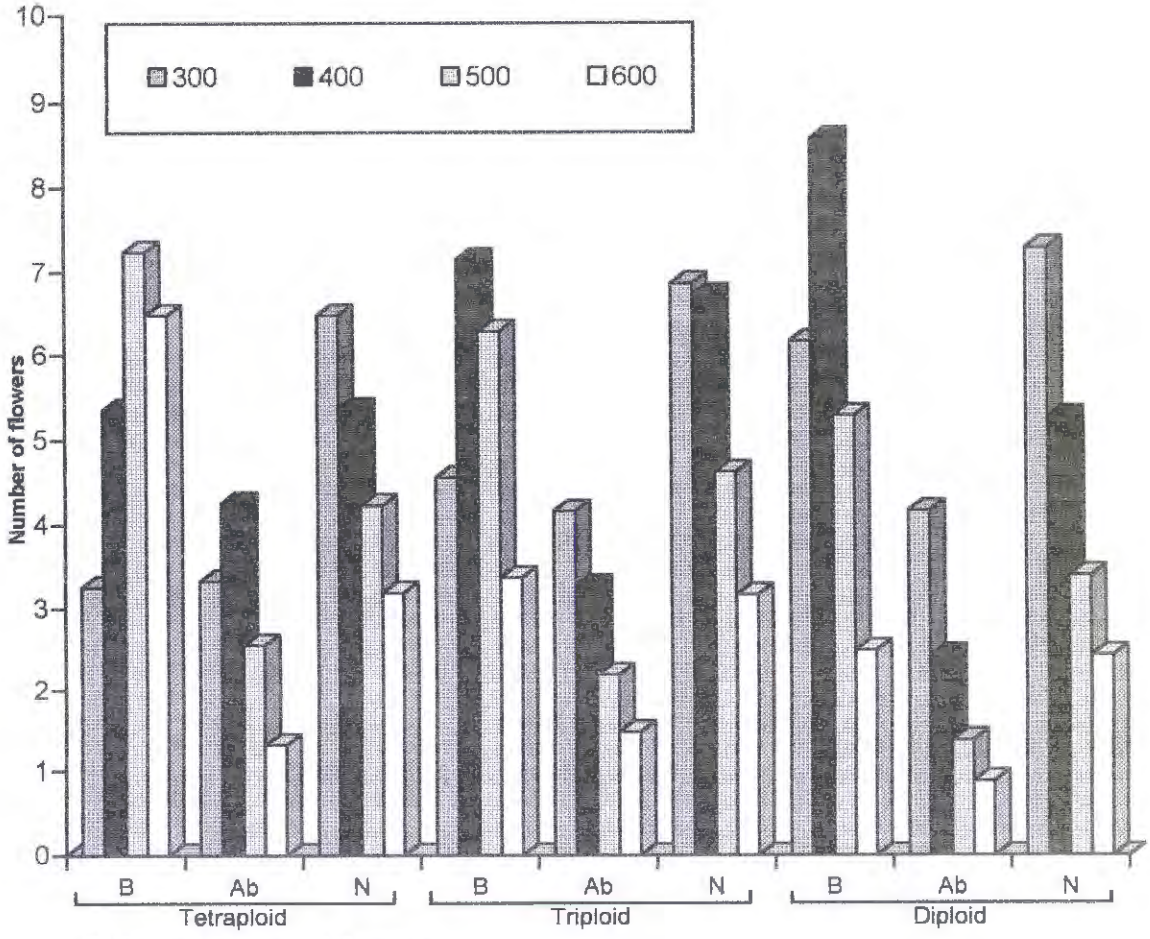
Bisexual flowers were induced from normal female flowers through the application of $AgNO_3$. $AgNO_3$ at 300, 400, 500 and 600 ppm were sprayed on to the shoot of female twigs. Spraying was done until the solution ran off the shoots. Spraying was done on shoots down to 10 leaves from the tip. Observations were made until the normal sex of the flower (female or bisexual) would be identified and 10 twigs were used per treatment. $AgNO_3$ spraying was done on tetraploid, triploid and diploid type of female kakrol plants. Results obtained from the treatment of $AgNO_3$ solution at 4 different strength are presented in graphical forms (**Graph 2**).

$AgNO_3$ application on female vines tip leads to development of three types of flowers viz. normal female, abnormal bisexual and normal bisexual. All the flowers treated with $AgNO_3$ produced large bracts. Few of these flowers developed weak and abnormal anther and some others developed complete anther (**Plate IV : Fig. J1**).

All the strength of $AgNO_3$ tested induced to develop bisexual flowers but the numbers of flowers per twig were different in different treatments. The highest mean number of unisexual flower converted to bisexual flower per tetraploid female twig was 7.24 recorded in vines where 500 ppm $AgNO_3$ was sprayed. For triploid female, the highest mean number of unisexual flower converted to bisexual flower per twig was 7.15 recorded when 400 ppm $AgNO_3$ was sprayed. Whereas, the highest number of bisexual flowers was 8.56 recorded from diploid female with same treatment of $AgNO_3$.

Mean numbers of flowers converted to abnormal bisexual flowers due to different strength of $AgNO_3$ are shown in **Graph 2**. Highest number of female flowers per twig converted to abnormal bisexual flowers was 4.26 when 400 ppm $AgNO_3$ was sprayed on tetraploid female vines. On the other hand, in triploid and diploid the highest numbers of abnormal bisexual flowers were recorded 4.16 and 4.38 respectively when 300 ppm $AgNO_3$ solution was sprayed.

When higher strength of $AgNO_3$ was sprayed the length of internodes reduced considerably in all types of kakrol (**Plate IV : Fig. E, D**).



Different types of flowers.

B = Bisexual ; Ab = Abnormal bisexual ; N = Normal

Graph 2 : Effect of 300, 400, 500 and 600 ppm $AgNO_3$ on sex conversion in different types of kakrol.

PLATE IV

Plate IVA. Nontreated twig of tetraploid type of kakrol.

Plate IVB. Continuous flowering of treated (100 ppm $AgNO_3$) twig of tetraploid type of kakrol.

Plate IVC. Continuous fruiting of treated (200 ppm $AgNO_3$) twig of tetraploid type of kakrol.

Plate IVD. Continuous flowering of treated (600 ppm $AgNO_3$) twig of tetraploid type of kakrol.

Plate IVE. Continuous flowering of treated (300 ppm) twig of 1. tetraploid, 2. triploid, 3. diploid type of kakrol.

Plate IVF. 1. Nontreated and 2. treated (100 ppm $AgNO_3$) twig of diploid type of kakrol

Plate IVG. Ovary of treated (1. tetraploid, 2. triploid, 3. diploid) with 300 ppm $AgNO_3$ and nontreated (4. tetraploid, 5. triploid, 6. diploid) flowers of different types of kakrol.

Plate IVH. Different types of normal male and induced bisexual flowers; 1. Bisexual tetra, 2. Normal male tetra 3. Bisexual trip, 4. Normal male trip, 5. Bisexual dip and 6. Normal male dip.

Plate IVI. Three types of induced bisexual flowers; 1. Tetraploid, 2. Triploid and 3. Diploid.

Plate IVJ. Induced bisexual of kakrol tetraploid showing both 1. stamen and 2. stigma.

Plate IVK. 1. Induced bisexual of tetraploid type.

2. Induced female flower of tetraploid type with large bract.

3. Induced bisexual of triploid type.

4. Induced female flower of triploid type with large bract.

PLATE - IV

3.6. MORPHOLOGICAL STUDIES :

Comparative studies on morphological features such as reproductive organs and leaf area were done among diploid, triploid and tetraploid kakrols. Results are described under different heads.

3.6.1. REPRODUCTIVE ORGANS :

Reproductive characters such as pedicel, bract, calyx, corolla, androecium and gynoecium of three types of kakrol are shown in **Plate V and Table 12**. The results are described as follows :

Pedicel : The length of pedicel was 7.69 ± 0.232 cm in tetraploid type of female kakrol. The bisexual flowers developed from tetraploid female had two distinct types of pedicel. In one type the pedicel was the shortest. The flower with short pedicel had larger bract. The pedicel in some tetraploid bisexual flowers was the longest (9.46 ± 0.335 cm) however, bract of this flowers were smaller in size.

The pedicel of the female flower of triploid kakrol was 5.41 ± 0.125 cm in length which was almost double in size than the pedicel of normal female flowers of diploid type. The pedicel of the bisexual flowers developed from female triploid type was smaller than those of corresponding normal one. The pedicel of male flowers of three types of kakrol were generally smaller than those of the corresponding female flowers.

Bract : The bracts of normal tetraploid female flowers was smaller (0.268 ± 0.024 cm.) than the bracts of normal male flowers (2.81 ± 0.023 cm.in length). Whereas, the bracts of some of the induced bisexual flowers of tetraploid was the largest (3.625 ± 0.037 cm in length and 4.02 ± 0.08 in breadth) among all the types studied. (**Plate V : Fig. C**).

In general the bract of normal female diploid and triploid types were smaller than the corresponding male types. Bract size also increased with the induction of bisexuality in diploid and triploid types.

Calyx : Like bracts, calyx of male flowers of three types of kakrol in general was bigger in size than corresponding female flowers (**Table 12**).

Among the three types of kakrol, tetraploid had the largest calyx. Calyx size also increased with the induction of bisexuality in all types of kakrol (**Plate V : Fig. B8**)

Petal : Like other reproductive organs, the petals of tetraploid male flowers was larger in size than those of triploid and diploid male flowers. Petals of female flowers in general were smaller than the corresponding male. However, like other reproductive organs petals size was not significantly increased with the induction of bisexuality.

Ovary : The ovary of the tetraploids was the largest followed by triploid and diploid (**Table 12**). The size of the ovary in all types of kakrol increased with the induction of bisexuality with treatments of $AgNO_3$.

3.6.2. LEAF :

Leaf area in tetraploids was the largest followed by triploids and diploids (**Table 13**). Leaf size in the male and female plants of tetraploids and triploids was more or less same. However the leaves of diploid female were nearly double in size than those of corresponding males.

Table 12 : Reproductive characters in different types of kakrol.

Plant types	Pedicel length (cm)		Bract		Calyx		Ovary		Petal	
	Bract to ovary distance	Bract to calyx distance	Length (cm)	Breadth (cm)	Length (cm)	Breadth (cm)	Length (cm)	Diameter	Length (cm)	Breadth (cm)
♀ tetra	7.69 ± 0.232	-	0.268 ± 0.024	0.336 ± 0.024	1.382 ± 0.015	0.323 ± 0.033	1.690 ± 0.027	0.791 ± 0.016	5.213 ± 0.263	4.011 ± 0.343
♂ tetra	-	1.215 ± 0.021	2.81 ± 0.023	3.820 ± 0.018	1.574 ± 0.014	0.635 ± 0.004	-	-	6.361 ± 0.128	3.686 ± 0.217
*♀ tetra	9.46 ± 0.335	-	3.634 ± 0.036	4.021 ± 0.084	1.598 ± 0.051	0.532 ± 0.021	2.456 ± 0.078	1.301 ± 0.012	6.581 ± 0.054	4.585 ± 0.026
♀ dip	2.11 ± 0.071	-	0.242 ± 0.014	0.241 ± 0.004	0.850 ± 0.003	0.219 ± 0.001	1.062 ± 0.029	0.643 ± 0.121	2.335 ± 0.056	1.176 ± 0.012
♂ dip	-	0.56 ± 0.210	0.886 ± 0.023	1.620 ± 0.058	0.883 ± 0.031	0.213 ± 0.005	-	-	2.305 ± 0.036	1.187 ± 0.031
*♀ dip	1.43 ± 0.212	-	1.430 ± 0.016	2.590 ± 0.017	0.956 ± 0.012	0.243 ± 0.012	1.568 ± 0.051	1.171 ± 0.028	2.660 ± 0.058	1.314 ± 0.232
♀ trip	5.41 ± 0.125	-	0.245 ± 0.023	0.279 ± 0.013	0.989 ± 0.052	0.293 ± 0.004	1.411 ± 0.031	0.660 ± 0.032	4.441 ± 0.215	2.812 ± 0.211
♂ trip	-	0.831 ± 0.012	1.860 ± 0.089	2.860 ± 0.007	1.321 ± 0.021	0.503 ± 0.082	-	-	5.126 ± 0.232	3.460 ± 0.051
*♀ trip	3.25 ± 0.215	-	1.983 ± 0.102	3.041 ± 0.013	1.341 ± 0.076	0.584 ± 0.043	1.971 ± 0.105	0.676 ± 0.213	5.774 ± 0.023	3.048 ± 0.124

Figures presented are the mean values of 10 replicates with standard error of mean.

* Bisexuality was induced by dipping shoot tips in 300-500 ppm $AgNO_3$ for 30 second.

Table 13 : Comparative leaf area study in different types of kakrol.

Node number from tip	Type	Leaf area cm^2	
		Range	Mean
8-20	Tetraploid ♂	3.9 - 58.2	34.05 ± 3.429
8-20	Tetraploid ♀	3.0 - 73.4	35.66 ± 4.630
8-20	Triploid ♂	4.8 - 48.2	30.63 ± 2.514
8-20	Triploid ♀	3.5 - 70.7	33.11 ± 3.751
8-20	Diploid ♂	2.4 - 25.9	13.53 ± 1.463
8-20	Diploid ♀	4.1 - 50.7	24.24 ± 3.164

3.7. POLLEN :

Different pollen characters viz. pollen viability, pollen germination were studied.

3.7.1. POLLEN VIABILITY :

Pollen grains from male and induced bisexual flowers developed from female tetraploid, diploid and triploid kakrols were collected and their viability was determined by acetocarmine staining method. Pollen grains those took full stain and became flacid to round shape were considered as viable pollens and those did not were considered as non-viable pollens. Single anther was squashed in 1% acetocarmin and pollens were counted from 5 focuses. The average of total number of viable and non viable pollen grains from five counts were calculated and expressed as percentage. The results of pollen viability are given in **Table 14**.

The highest percentages (94-96%) of viable pollen grains per flower were recorded in tetraploid male and induced bisexual at the time of opening and after 24 hours of opening. The mean differences between these two counts was non-significant. Dropping of pollen grains was found to be more in diploid type of kakrol as compared to tetraploid type. Highest percentages (92-96%) of non-viable pollens were recorded in normal male and induced bisexual flowers of triploid type.

Table 14 : Number of viable and non-viable pollen grains in different types of kakrol.

Type		Time	Total number of pollens counted	% of viable pollen	% of non-viable pollen
♂	Tetra	M	356	95.22	4.77
		FM	348	94.25	5.74
♀	Tetra	M	340	96.17	3.82
		FM	336	95.23	4.76
♂	Dip	M	372	59.13	40.86
		FM	366	55.46	44.53
♀	Dip	M	376	65.95	34.04
		FM	362	64.08	35.91
♂	Trip	M	336	4.76	95.23
		FM	328	3.65	96.34
♀	Trip	M	362	7.18	92.81
		FM	358	5.86	94.13

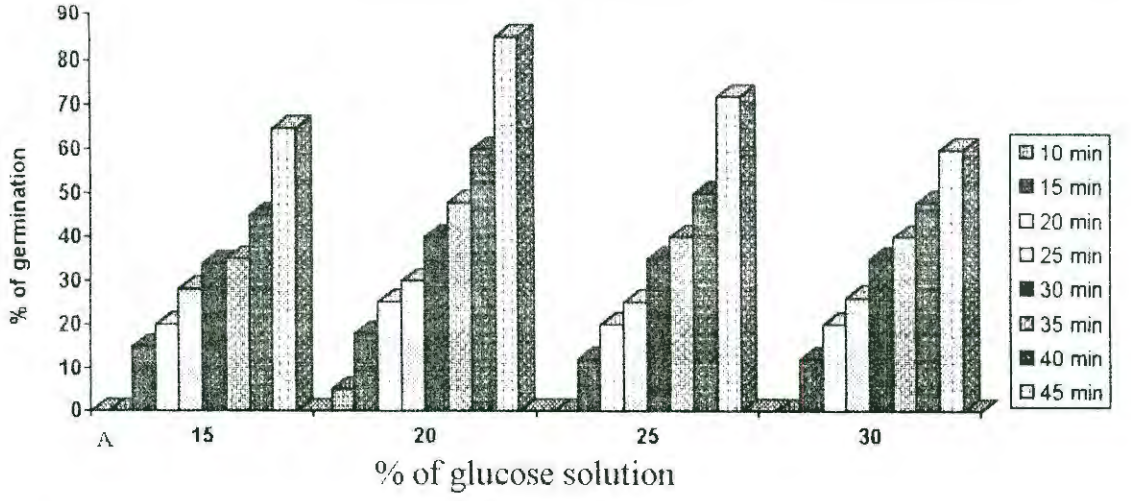
M = Morning ; FM = Following day morning.

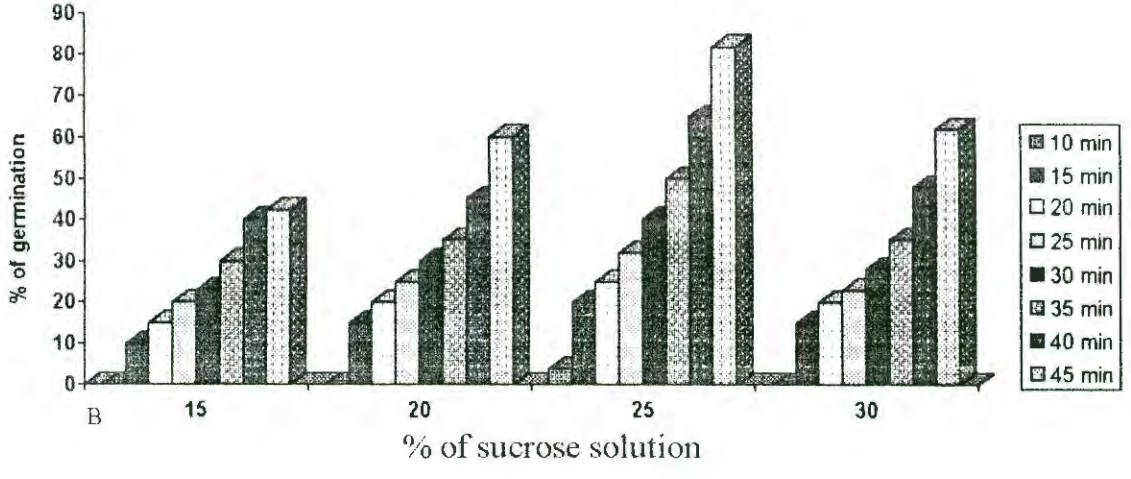
3.7.2. POLLEN GERMINATION ABILITY :

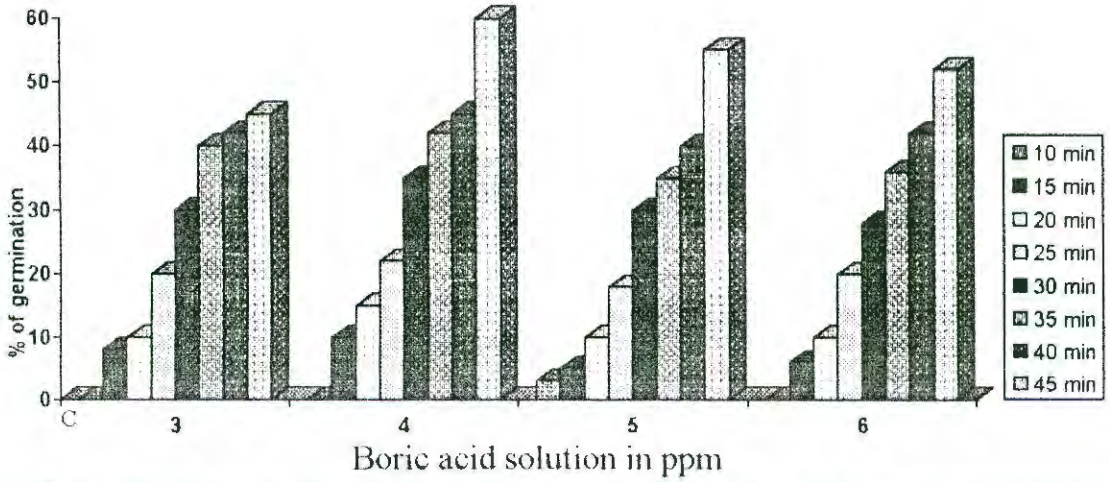
Different concentrations of glucose, sucrose and boric acid solutions were used for pollen germination. Pollen grains from normal male flowers and induced bisexual flowers of tetraploid and diploid types were collected and mixed them separately in various concentrations of glucose, sucrose and boric acid solutions and the percentage of pollen germination were recorded at different time intervals. Results obtained are shown in **Graph 3** for tetraploid male, **Graph 4** for tetraploid bisexual, **Graph 5** for diploid male and **Graph 6** for diploid bisexual.

The highest percentage of pollen grain germination was 85% recorded for tetra ♂ and dip ♂ when 20% glucose solution was used for 45 minutes (**Graph 3A and Graph 5A**). In case of bisexual flowers the highest 65% germinating pollen grains was recorded for tetraploid and diploid types when 20% glucose solution was used as germination medium for 45 minutes. (**Graph 4A and Graph 6A**).

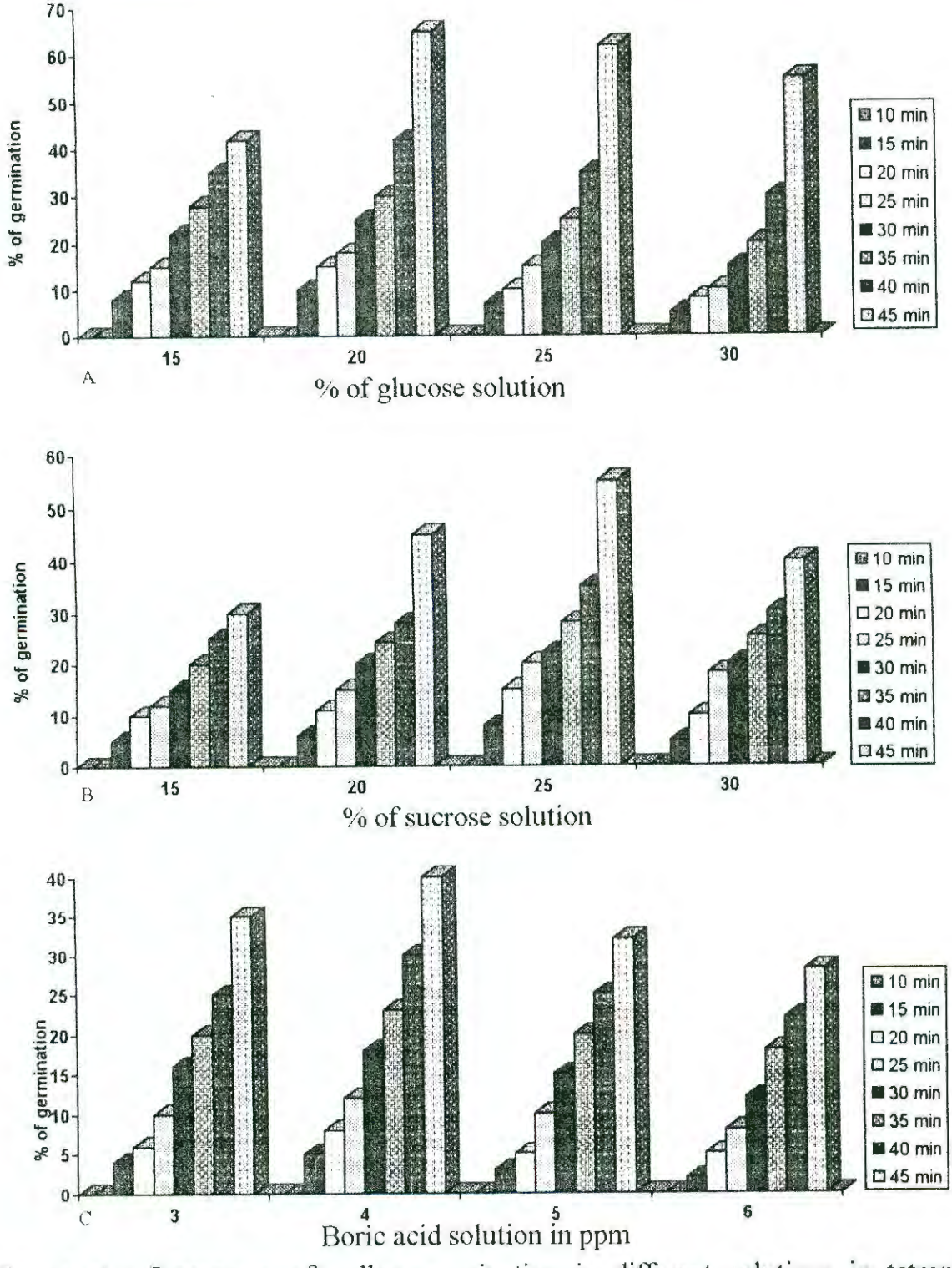
Boric acid solutions were not found suitable for the induction of pollen germination (**Graph 4C**).



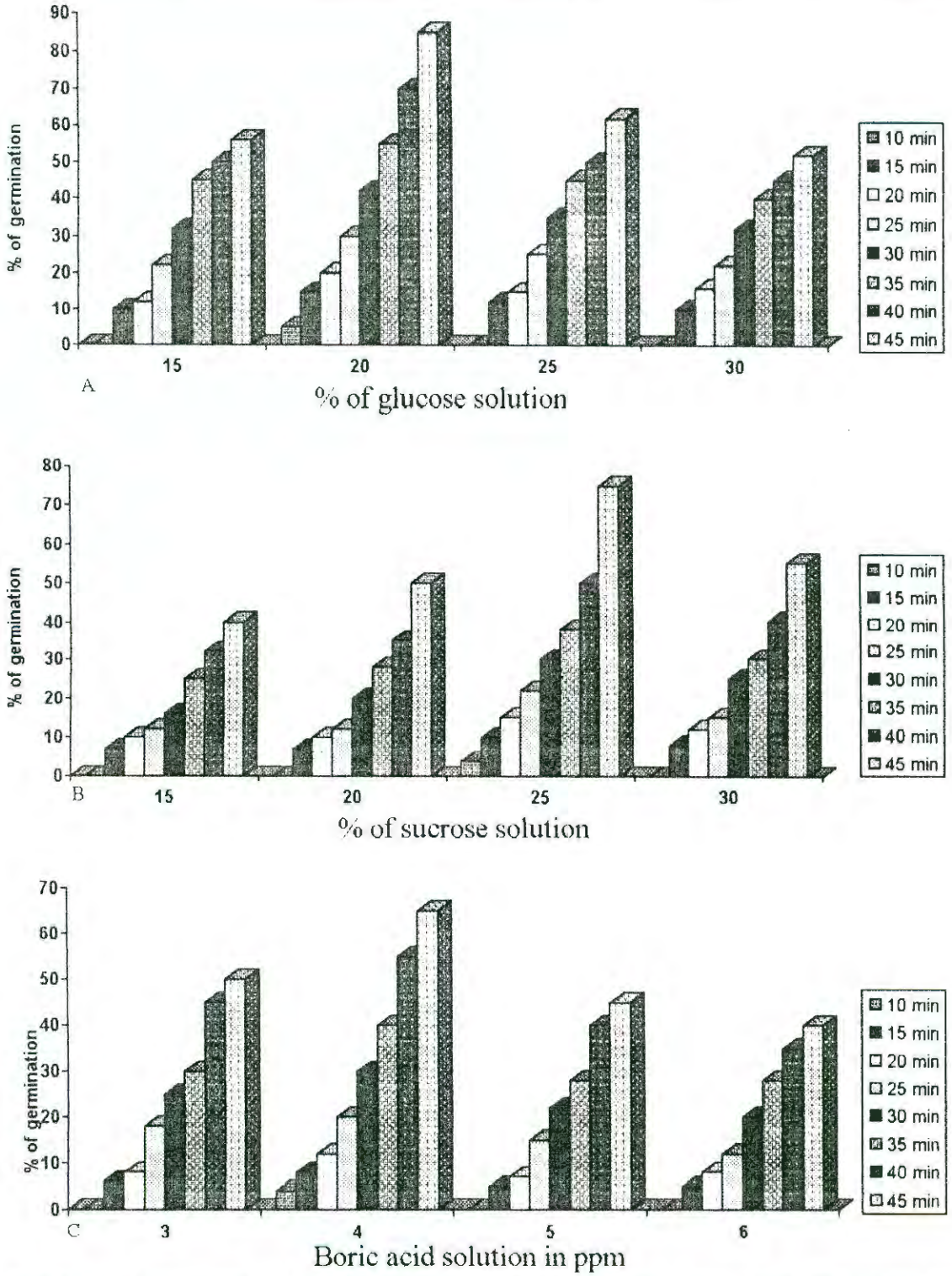




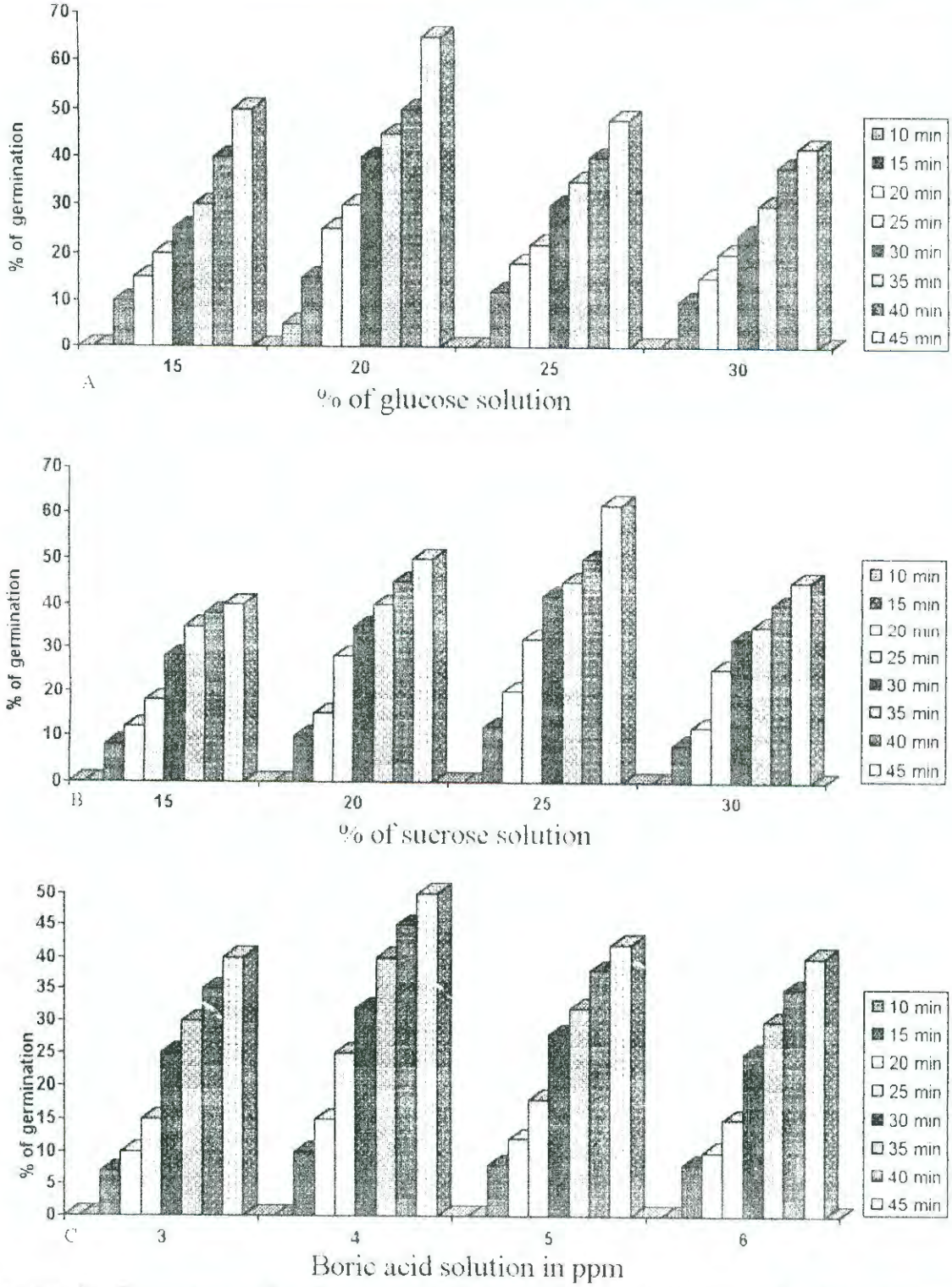
Graph 3 : Percentage of pollen germination in different solutions in tetraploid male kakrol.



Graph 4 : Percentage of pollen germination in different solutions in tetraploid bisexual type of kakrol .



Graph 5 : Percentage of pollen germination in different solutions in diploid male kakrol .



Graph 6 : Percentage of pollen germination in different solutions in diploid bisexual kakrol.

3.8. POLLEN–STIGMA INTERACTION WITHIN AND BETWEEN DIPLOID, TRIPLOID AND TETRAPLOID KAKROL :

In order to determine the pollen-stigma interaction, the stigma of diploid, teriploid and tetraploid plants of kakrol were pollinated with the corresponding diploid, triploid and tetraploid flower. Similarly, the stigma of three types of plants were reciprocally pollinated within themselves. The detail of crossing programme and their corresponding results of post pollination interactions are shown in **Table 15**. Sources of pollens are shown in **Plate IV : Fig. H**.

Post pollination pollen-stigma interactions was studied under fluorescent microscope using following parameters : pollen germination, tube penetration, tube elongation, callose responses and event of fertilization.

Pollen grains started to germination on the stigmatic papillae after 15 minutes of pollination and most of the pollens were found to germinate within 30 minutes. Pollen germination time was found more or less same for all the self and cross combination.

When pollen grains of normal tetraploid ♂ flowers were used to pollinate different types of pistillate flowers (**Table 15**) success was obtained between ♂ tetra × ♀ tetra and ♂ tetra × ♀ dip only. The percentages of fruit setting were 84 and 86 respectively, for these two cross combinations.

Fluorescent microscopic study reveals that 97% and 95% of pollens germinated on the stigmatic surface for normal tetra ♀ × normal tetra ♂ and normal dip ♀ × normal tetra ♂ cross-combinations, respectively. In these cross-combinations pollen tubes continued to grow through stigmatic papillae. Most of the pollen tubes of germinating pollen grains were found advancing towards the ovary (**Plate VI : Fig. A**). The development of pollen tube within the stylar tissue was monitored by the conspicous callose wall of the developing pollen tubes (**Plate VI : Fig. B**).

Fruit setting was not recorded when the pistil of induced ♀ tetraploid flower was pollinated with the pollen of normal diploid, triploid and tetraploid♂. Moreover, induced tetra ♀ flowers did not develop any fruit under self or sib pollinations. Similar results were also recorded in case of stigmas of induced diploid or triploid ♀ flowers pollinated under any cross combination. Pollen germination on the stigmatic surface of the induced flowers of tetraploid and diploid was normal. More than 70% pollen grains germinated normally within 30 minutes of pollination. In most of the cases the pollen grains began to develope tubes (**Plate V : Fig. G**). The growth of the tubes at initial stage was also normal (**Plate V : Fig. G**). At the later stage the growth of the pollen tubes slowed down and ultimately restricted within upper and mid region of the stylar tissue (**Plate V : Fig. H**). Instead of normal growth, the tip of the pollen tubes started to diform and vertually develop tumor (**Plate V : Fig. H**). Prominant callose tissue formation was also noticed around these tumers (**Plate V : Fig. N [arrows]**).

Bisexuality was also induced in female flowers of triploid (**Plate IV : Fig. I2**). Pollen grains collected from male and bisexual flowers (**Plate V : Fig. J**) of triploid failed to develop fruit when they were crossed with any types of pistillate flowers listed in **Table 15**. Most of the pollen grains of ♀ triploid flower failed to germinate on the stigmatic surface (**Plate VI : Fig. E**). Pollen germination was noticed in a few of the cases but the germinating pollen grains failed to develop tubes. Both localized and diffused callose production was observed on the stigmatic papillae (**Plate VI : Fig. F**) in these cross-combinations.

Table 15 : Results of pollination using pollens from different sources and post pollination interactions in different types of kakrol.

Pistillate flower	Pollen source	Mode of pollination	No. of flowers crossed	Pollen grain germination percentage	No. of fruits set	Success percentage (%)
♀ tetra		Cross (control)	100	97.23	84	84.00
♀ (♂ tetra)		Cross	100	96.24	00	00
♀ trip		Cross	70	5.36	00	00
♀ (♂ trip)	♂ tetra	Cross	70	00	00	00
♀ dip		Cross	100	95.58	86	86.00
♀ (♂ dip)		Cross	75	78.35	00	00
♀ tetra		(same plant)	85	88.52	56	65.88
♀ tetra		(different plant)	85	87.48	67	78.82
♀ (♂ tetra)		(same flower)	75	75.35	00	00
♀ trip	♂ (♂ tetra)	Cross	60	00	00	00
♀ (♂ trip)		Cross	60	00	00	00
♀ dip		Cross	95	92.36	82	86.31
♀ (♂ dip)		Cross	50	79.13	00	00
♀ tetra		Cross (control)	80	55.24	00	00
♀ (♂ tetra)		Cross	50	46.16	00	00
♀ trip		Cross	50	00	00	00
♀ (♂ trip)	♂ dip	Cross	50	00	00	00
♀ dip		Cross	80	94.45	63	78.75
♀ (♂ dip)		Cross	50	92.36	00	00
♀ tetra		Cross	50	72.35	00	00
♀ (♂ tetra)		Cross	50	52.46	00	00
♀ trip		Cross	50	00	00	00
♀ (♂ trip)	♂ (♂ dip)	Cross	50	00	00	00
♀ dip		(same plant)	100	88.35	88	88.00
♀ dip		(different plant)	95	83.24	83	87.36
♀ (♂ dip)		(same flower)	50	76.28	00	00

Table 15 : (continued)

Table 15 : (continued)

Pistillate flower	Pollen source	Mode of pollination	No. of flowers crossed	Pollen grain germination percentage	No. of fruits set	Success percentage (%)
♀ tetra		Cross	75	00	00	00
♀ (♂ tetra)		Cross	75	00	00	00
♀ trip		Cross	80	00	00	00
♀ (♂ trip)	♂ trip	Cross	75	00	00	00
♀ dip		Cross	80	00	00	00
♀ (♂ dip)		Cross	75	00	00	00
♀ tetra		Cross	75	00	00	00
♀ (♂ tetra)		Cross	50	00	00	00
♀ trip		(same plant)	75	00	00	00
♀ trip	♂ (♂ trip)	(different plant)	75	00	00	00
♀ (♂ trip)		(same flower)	50	00	00	00
♀ dip		Cross	50	00	00	00
♀ (♂ dip)		Cross	50	00	00	00

tetra = tetraploid ; trip = triploid ; dip = diploid.

PLATE V

Plate VA. Different types of flower bud; 3. bisexual tetra, 4. bisexual trip, 5. bisexual dip, 6. normal male tetra, 7. normal male trip, 8. normal male dip.

Plate VB. Different types of calyx showing ; 3. diploid female, 4. triploid female, 5. tetraploid female, 6. diploid induced bisexual, 7. triploid induced bisexual, 8. tetraploid induced bisexual.

Plate VC. Different types of induced bract; 4. diploid bisexual, 5. triploid bisexual, 6. tetraploid bisexual.

Plate VD. Different types of induced bisexual flower; 4. tetraploid, 5. triploid, 6. diploid.

Plate VE. Leaves of male plant ; 4. diploid, 5. triploid, 6. tetraploid.

Plate VF. Leaves of female plant; 4. diploid, 5. triploid, 6. tetraploid,

Plate VG-N. Fluorescent micrographs showing the post pollination development.

Plate VG. A portion of the self-pollinated pistils of tetraploid [♀ tetra (♂ tetra) × tetra ♂ (♀ tetra)] kakrol showing development of pollen tubes. (magnification 4×10X, after 24 h of pollination).

Plate VH. Large number of pollen tubes growing into the style, but the tip of pollen tubes formed tumor (arrows). (magnificant 4×10X, after 24 h of pollination).

Plate VI. Unfertilized ovule of tetraploid ♀ type.

Plate VJ. A portion of the self-pollinated pistils of triploid (trip ♀ × trip ♀) kakrol, 100% pollen grains failed to germinate on the stigmatic surface (magnification 4 × 10 X, after 24 h of pollination).

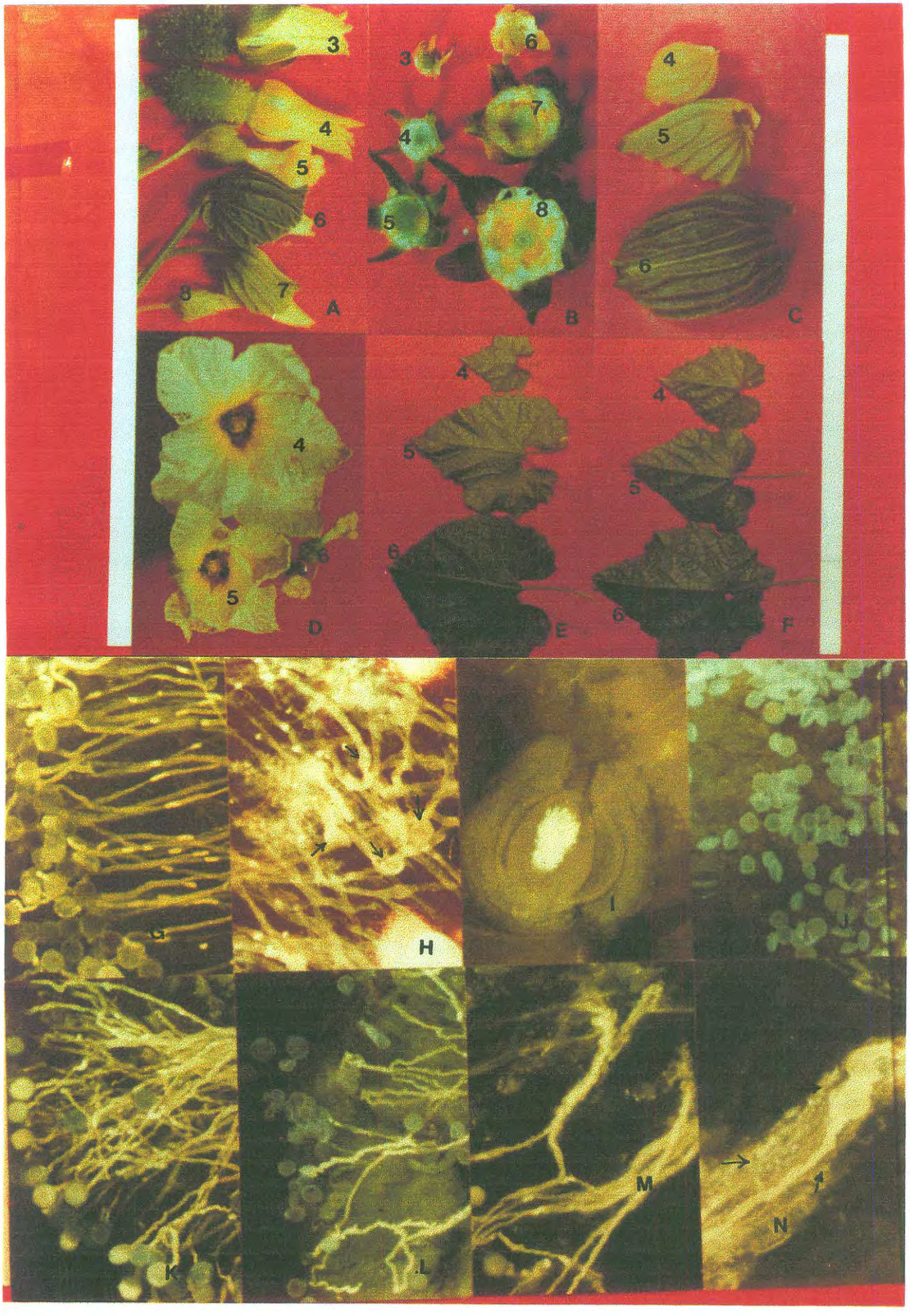
Plate VK. A portion of the pistil of diploid (dip ♀ × tetra ♂) kakrol showing development of pollen tubes (magnification 4×10X, after 8 h of pollination).

Plate VL. A portion of the self-pollinated pistil of diploid (dip ♀ × dip ♀) showing development of pollen tubes (magnification 4×10X, after 16 h of pollination).

Plate VM. Same as Fig. VL, but the tip of pollen tubes formed tumer like structure (magnification 4×10X, after 20 h of pollination).

Plate VN. Same as Fig. VM, but the tip of pollen tubes formed tumer. Deposition of localized callose (arrows) at the end of the stylar region, (magnification 4×10X, after 20 h of pollination).

PLATE - V



3.9. EFFECT OF POLLEN SOURCES ON FRUIT CHARACTERS :

The fruits of successful crosses between and within tetraploid and diploid types were characterized using four parameters, like fruit weight, fruit volume, seed number and seed weight. Results obtained are shown in **Table 16**.

In these cases, three types of pistillate flowers were noticed, one was normal female without bract, second type was normal female with large bract and third type was abnormal bisexual with large bract.

Fruit weights of the crosses tetra N♀ × tetra ♂, tetra W ♀ × tetra ♂, tetra A ♀ × tetra ♂ were 99.81 ± 2.15, 113.2 ± 1.84, 115.75 ± 2.07, respectively. The fruit volumes of these crosses were 119.2 ± 3.08, 121.4 ± 3.36 and 123.3 ± 3.35, respectively. The seed number per fruit and seed weight per fruit of these three types of crosses are shown in **Table 16**. The fruit weight and fruit volume per fruit were high in crosses tetra N ♀ × tetra ♀ **(Plate VI : Fig. J1)**, tetra W ♀ × tetra ♀, tetra A ♀ × tetra ♀ **(Plate VI : Fig. K)**.

Fruit weight, fruit volume, seed number and seed weight per fruit of dip N ♀ × dip ♂, dip W ♀ × dip ♂, dip A ♀ × dip ♂ cross were 15.4 ± 0.87, 14.45 ± 0.73, 15.45 ± 0.88 (weight); 15.74 ± 0.86, 15.91 ± 0.85, 16.89 ± 0.89 (volume); 15.3 ± 1.04, 15.7 ± 0.85, 16.6 ± 1.49 (seed no.) and 1.39 ± 0.053, 1.47 ± 0.067, 1.49 ± 0.073 (seed weight) respectively. These characters were almost same as those obtained in dip N ♀ × tetra ♂ crosses.

The fruit weight of dip N♀ × dip ♂ cross was 15.43 ± 0.87 g. The fruit weight as obtained from dip N♀ × dip ♂ was significantly greater **(Plate VI : Fig. L2)** than those obtained from dip N♀ × dip ♀ cross **(Plate VI : Fig. L1)**. The seed number and seed weight per fruit of the cross dip N ♀ × dip ♀ were 15.6 ± 1.38 and 1.41 ± 0.054, respectively. Seed number and seed weight per fruit of the cross dip N♀ × dip ♂ were almost same as those of dip N ♀ × dip ♀ .

Table 16 : Fruit characteristics of different crosses made within and between different types of kakrol.

Pistillate flowers	Pollen source	Fruit weight		Fruit volume		Seed number		Seed weight	
		Range	Mean	Range	Mean	Range	Mean	Range	Mean
Tetra N ♀	Tetra ♂	83.42 -107.65	99.81 ± 2.15	103-135	119.2 ± 3.08	16-31	22.6 ± 1.43	3.72-6.2	5.37 ± 0.25
Tetra W ♀	Tetra ♂	105.04 -124.17	113.2 ± 1.84	105-136	121.4 ± 3.36	17-31	23.6 ± 1.33	4.38-7.5	5.89 ± 0.33
Tetra A ♂♀	Tetra ♂	107.03 -126.28	115.75 ± 2.07	105-136	123.3 ± 3.35	18-33	24.5 ± 1.32	4.63-8.19	6.08 ± 0.35
Dip N ♀	Dip ♂	11.21 -20.97	15.43 ± 0.87	12-21.2	15.74 ± 0.86	10-19	15.3 ± 1.04	1.11-1.78	1.39 ± 0.053
Dip W ♀	Dip ♂	11.98 -22.13	14.45 ± 0.73	12.1-21.8	15.91 ± 0.85	11-20	15.7 ± 0.85	1.23-1.88	1.47 ± 0.067
Dip A ♂♀	Dip ♂	11.23 -20.99	15.45 ± 0.88	13.6-21.4	16.89 ± 0.89	11-26	16.6 ± 1.49	1.25-1.89	1.49 ± 0.073
Tetra N ♀	Tetra ♂♀	85.36 -10972	101.71 ± 2.18	104-134	120.4 ± 3.29	16-32	22.8 ± 1.44	3.76-6.8	5.42 ± 0.27
Tetra W ♀	Tetra ♂♀	108.61 -127.22	117.35 ± 2.08	105-138	122.3 ± 3.34	17-32	23.45 ± 1.35	4.43-7.1	5.91 ± 0.34
Tetra A ♂♀	Tetra ♂♀	109.03 -128.45	119.64 ± 2.12	106-139	124.2 ± 3.38	18-34	24.6 ± 1.31	4.64-8.21	6.27 ± 0.36
Dip N ♀	Dip ♂♀	11.28 -21.83	16.46 ± 0.86	12-22.3	16.19 ± 0.87	10-20	15.6 ± 1.38	1.12-1.8	1.41 ± 0.054
Dip W ♀	Dip ♂♀	12.85 -23.42	16.39 ± 0.84	12.3-22.4	16.75 ± 0.86	11-20	15.8 ± 0.96	1.24-1.86	1.46 ± 0.068
Dip A ♂♀	Dip ♂♀	12.92 -23.38	16.28 ± 0.87	12.7-23.5	16.82 ± 0.88	11-24	16.4 ± 1.38	1.26-1.88	1.51 ± 0.078

Figures presented are the mean values of 10 replicates with standard error of mean.

N = Normal female without bract, W = Normal female with bract, A = Abnormal bisexual.

PLATE VI

Plate VIA-H. Fluorescent micrographs showing some of the post pollination responses:

Plate VIA. Pollen tube development within the stigmatic papillae where no specific callose reactions occurred. (magnification 4×10X, cross tetra ♀ × tetra ♂, after 8 h of pollination).

Plate VIB. Large number of pollen tubes growing into the style following (tetra ♀ × tetra ♂) cross pollination in tetraploid kakrol (magnification 4 × 10X, after 12 h of pollination).

Plate VIC. Same as Fig. VIB. with developing pollen tubes entering into the ovules.

Plate VID. Same as Fig. VIC, pollen tubes entering into the ovules following cross pollination.

Plate VIE. A portion of the pistils of triploid (trip ♀ × tetra ♂) kakrol showing 100% pollen grains failed to germinate on the stigmatic surface (magnification 4 × 10X, after 24 h of pollination).

Plate VIF. Same as Fig. VIE, in higher magnification (10 × 10X), but formation of bright callose on entire stigmatic papillae (arrows).

Plate VIG. A portion of the pistils of triploid (trip ♀ × tetra ♂) kakrol showing single pollen germination on the stigmatic surface (magnification 4 × 10X, after 16 h of pollination).

Plate VIH. Inner portion of triploid fruit, showing single seed set (arrows).

Plate VI I. Field of tetraploid female type of kakrol.

Plate VIJ. Fruits of different types, 1. tetraploid (tetra W ♀ × tetra ♀, same plant) 2. triploid (trip ♀× tetra ♂), 3. diploid (dip W ♀ × tetra ♂).

Plate VIK. Fruits developed from selfing of tetra A ♀ × tetra ♀ (same plant), fruit is larger than those developed from pollination with normal male.

Plate VIL. Fruits of diploid type; 1. dip ♀× dip ♂, 2. dip W ♀ × dip ♀ (different. plant).

Plate VIM. Field of diploid female type of kakrol.

Plate VIN. Field of triploid female type of kakrol.

Plate VIO. Fruits of triploid type; 1. trip ♀ × dip ♂, 2. trip W ♀ × tetra ♂.

Plate VIP. Inner portion of triploid fruits (trip W ♀ × tetra ♂).

PLATE - VI

Chapter - 4

DISCUSSION

DISCUSSION

Kakrol (*Momordica dioica*) is a dioecious cucurbitaceous vegetable crop. Seed grown population is highly heterozygous due to unavoidable cross pollination. Moreover, seed germination is difficult because of prolong dormant period and hard seed coat. The results of the present investigation reveals that removal of seed coat prior to seed sowing increased germination rate remarkably. Similar result was also reported by Ali *et al.*, (1991). Mishra and Sahu (1983) reported that fresh seeds before germination remain dormant for nine months. Commercial propagation of kakrol through seed is not feasible because seed propagated plants are highly heterozygous and male female sex expression ratio is 1:1 (Ali *et al.*, 1991).

Vegetative propagation has several advantages over seed propagation specially for dioecious and cross pollinated plant. Kakrol could be propagated with tuberous root. However, low tuberous root production hinders mass propagation of kakrol. Present study reveals that mass propagation of kakrol could be done through vine cutting. Treatment of vine cutting for 30 min with low concentration IBA induced to develop roots in cent percent plants that might be used for large scale propagation.

In vitro culture is a proved alternative technique over traditional breeding for mass propagation as well as genetic improvement of a crop through the induction and manipulation of somaclonal variation. A tissue culture cycle involves the establishment of a more or less differentiated cell or tissue culture under defined culture conditions, proliferation for a number of cell generations and the subsequent regeneration of plants (Scowcroft and Larkin, 1982).

for callus induction and successive plant regeneration through organogenesis or embryogenesis (Konar and Oberoi, 1965; Arnold and Eriksson, 1978; Ohyama and Oka, 1980; Hammerschlag *et al.*, 1985; Espinasse *et al.*, 1989; Miller and Chandra, 1990; Dong and Jia, 1991). Nevertheless, mature tissues are less responsive when compared with juvenile tissues (Sommer and Caldras, 1981; Lazzeri *et al.*, 1985).

In the present study a number of experiments were conducted on *in vitro* response of *M. dioica* Roxb. using different types of explants such as cotyledon, hypocotyl, shoot tip and nodal explant. Throughout the investigation MS (Murashige and Skoog, 1962) medium was used with growth regulators NAA, IAA, IBA, 2, 4-D as auxin, BA, KIN as cytokinin and GA_3 in different combinations and concentrations.

In case of field grown explants, surface sterilization was made before inoculation because of the presence of loose contaminations on the explant surface. Due to the toxic nature of $HgCl_2$, problems of tissue killing occurred and it was overcome by using lower concentrations of $HgCl_2$ for some long duration of time. On the other hand, when *in vitro* grown explants were used as explants they showed more potentiality to response.

The use of cotyledon explant has several advantages (Gogala and Camloh, 1988). Microbial contamination of such explant has never been a serious problem. Nevertheless, cotyledons have been shown to possess high morphogenic potential (Fagekas *et al.*, 1986; Rao *et al.*, 1981; Singh *et al.*, 1981; Zee and Hui, 1977). Tissue culture methodology for plant regeneration from cotyledon explant is well established for herbaceous, ornamentals, fruits or vegetable crops specially for those belong to family cucurbitaceae and cruciferae.

Present investigation demonstrated successful plant regeneration directly or through callus phase from cotyledon explant of *M. dioica* Roxb. The formation of adventitious shoots from cotyledon of *M. dioica* was independent to the presence of embryonic axis which indicates that the full potential for plant regeneration was

present within the cotyledon itself. Reports on plant regeneration from cotyledon explant of apple (Kouider *et al.,* 1985) and of water melon (Dong and Jia, 1991) confirm the results of present study. Nevertheless, plant regeneration from cotyledon needed proper combination and concentration of growth regulator supplement. Among the different concentrations and combinations of growth regulators used in the present study, higher concentration of BA (2.0 - 4.0 mgl^{-1}) in combination with low concentration of NAA (0.2 - 0.5 mgl^{-1}) gave better result in direct adventitious shoot regeneration. Organogenesis and plant formation in presence of BA with NAA was also reported from cotyledon explant of *Brassica* sp. (Jain *et al.,* 1988; George and Rao, 1980). Although BA alone showed less promotive effect but found to be better than KIN alone. Among the different concentrations of BA, 4.0 mgl^{-1} gave better result. Shoot proliferation suppressed with the decrease of BA concentration. Superior effect of BA on adventitious bud proliferation from cotyledon explant has been reported by Kouider *et al.,* (1985) for apple; Kim *et al.,* (1988); Niedz *et al.,* (1989); Dirks and Van Buggenum (1989) for water melon. But contrary to these Dong and Jia (1991) reported that higher concentration of BA (7.0 mgl^{-1}) gave the best result in shoot regeneration from cotyledon of watermelon. The medium BA 2.0 mgl^{-1} + 0.2 mgl^{-1} NAA + 0.1 mgl^{-1} GA_3 was found to be the best formulation for the highest degree of shoot regeneration (80% with 19.33 shoots) from cotyledon of *M. dioica*. Promotive effect of GA_3 in combination with BA on shoot regeneration from cotyledon of melon was also reported by Niedz *et al.,* (1989) and Kathal *et al.,* (1986).

BA alone or BA-NAA combinations had little effect on callus proliferation from cotyledon explant of *M. dioica.* However, this hormonal supplement induced the explants to form trace of callus at their cut surface. In a few cases, adventitious buds developed from the callus. In most of the cases shoots proliferated directly from the surface of the explant. Similar results have been reported by Ahad (1992) from cotyledon of watermelon. Adventitious buds initiated as tube like protrusions also

reported by Srivastava *et al.*, (1989); Dong and Jia (1991) from cotyledon of water melon.

It was found in the present investigation that KIN in combination with IAA promoted callusing. KIN with higher concentration of IAA also supported callus growth but lower than corresponding BA with NAA. IAA and KIN although at lower frequency but stimulated adventitious shoot regeneration from the callus. Arya *et al.*, (1981) reported adventitious shoot regeneration of *A. marmelos* from hypocotyl derived callus in KIN supplemented medium.

It was noticed in the present study that physiological state of the cotyledon explants influenced their shoot regeneration potentiality. Young cotyledons (8-10 days old after pollination) did not exhibit shoot regeneration other than callus proliferation. Optimum age for the highest degree of shoot regeneration of cotyledon explant of *M. dioica* was found to be 18-21 days old after pollination. Shoot regeneration capacity decreased with the increase of age of cotyledon. A similar observation also was made with cotyledon of *Prunus persica* (Mante *et al.*, 1989); peach embryogenic calli derived from immature embryo (Hammerschlag *et al.*, 1985); apple cotyledon (Kouider *et al.*, 1985) and melon cotyledon (Neidz *et al.*, 1989). The cotyledon decreased their regeneration capacity after maturity which may be explained in terms of shoot factors being translocated or repressed (Kouider *et al.*, 1985; Ting, 1982).

Multiple shooting is very important in relation with rapid mass propagation of cucurbits species. Shoot tip and nodal explants were excised from *in vitro* grown shoot cultures and cultured on MS media supplemented with different concentrations and combinations of auxin, cytokinin and gibberellic acid. The media containing 3.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA gave the optimum result for developing highest number of new buds.

Roy and Datta (1985) described that multiple shoots were obtained from shoot tip explants of mature trees of *Albizia procera* on a defined MS medium

supplemented with 2.0 mgl^{-1} BA. Das and Mitra (1990) reported multiplication of axillary shoot bud from nodal segment and copiced shoots in MMS/MS media with BA and NAA in *Eucalyptus tereticornis* Smith.

Reports of Arya *et al.*, (1981) have proven the applicability of hypocotyl segments as explant in case of *M. dioica* tissue culture. Many of the problems of inducing callus from plant tissues may be overcome by using parts of freshly germinated seedlings, ensuring that tissue fragments composed of callus with high growth potential (Yeoman and Forche, 1980). Hence in the present experiment to induce callus and subsequent plant regeneration, only young parts such as cotyledon and hypocotyl segments from *in vitro* grown seedlings were selected as explants.

In the present work, different concentrations of auxins and cytokinins were used either alone or in combinations. It was found that callus proliferation strictly depended on exogenous growth regulator supplementation. In absence of exogenous growth regulator, the explants failed to induce callus. Callus proliferation was observed from both types of explants in all concentrations of auxins (NAA and 2, 4-D). Induction and growth of callus for both cotyledon and hypocotyl explants were linearly correlated with the concentration of auxins tested. Between two auxins, 2, 4-D had more promotive effect on callus growth through increasing fresh weight of callus. Many workers observed 2, 4-D as the best auxin for callus induction as common in monocot and even in dicot (Gonzalez *et al.*, 1985; Wang *et al.*, 1987; Chee, 1990).

Morphological nature of proliferated calli varied with type and concentration of auxins. Calli proliferated from both explants in low concentration of NAA (0.5-2.5 mgl^{-1}) were hard and green. Nodular structures appeared on the callus surface during the course of development. Primary callus of both explants proliferated in 2, 4-D did not show any organogenesis. Calli of 2, 4-D supplemented media were spongy to hard and yellowish white for both type of explants. The auxin 2, 4-D has been widely used in induction of embryogenic callus in monocot specially in Graminae (Vasil *et al.*, 1984; Ho and Vasil, 1983). In case of dicot 2, 4-D also promoted callus growth but suppressed organogenesis or embryogenesis (Lang and Kohlenbach, 1975). However,

Miller and Chandra (1990) reported callogenesis in strawberry in presence of low concentration of 2, 4-D (2 μ-10 μm).

Among the different combinations of KIN and 2, 4-D, the media with 4.0 mgl^{-1} KIN + 2.0 mgl^{-1} 2, 4-D were proved to be the optimum hormonal supplement for induction and growth of callus from cotyledon and hypocotyl explants. On the other than callus proliferated in these hormonal supplemented media did not induce any root-shoot differentiation from primary callus. Gamborg *et al.*, (1976) stated that 2, 4-D is a powerful suppressant of organogenesis and should not be used in experiments involving root-shoot differentiation which supports the present experimental findings.

In the present study, BA-NAA in MS medium not only accentuated callus growth from both explants bud also induced organogenesis and rhizogenesis from primary callus. Callus growth and its further dedifferentiation was found to be controlled by BA - NAA ratios. Among different BA-NAA combinations 3.0 mgl^{-1} BA and 2.0 mgl^{-1} NAA found to be the best for induction and growth of callus from hypocotyl explant and 4.0 mgl^{-1} BA and 2.0 mgl^{-1} NAA combination found to be the best for induction and growth of callus from cotyledon explant. Shoot and root induction was observed in the media with low concentration of NAA along with higher concentration of BA. Combinations of BA and NAA have successfully been employed to induce plant regeneration from hypocotyl explant in a wide range of species (Mroginski and Kartha, 1981, Rubluo *et al.*, 1984; Kameya and Widholm, 1986). Using BA - NAA or BA-auxin, shoot regeneration from cotyledon explant has also been achieved in a wide range of plant species (Bornman, 1983; Dong and Jia, 1991; Mante *et al.*, 1989; Kim *et al.*, 1988).

Different concentrations of auxins and cytokinins were used either alone or in combinations and BA-NAA proved to be the best for callus induction and also induced shoot differentiation from primary callus obtained from both (cotyledon and hypocotyl) the explants.

Long term maintenance of callus culture has many fold applications. The variation of a desirable trait observed with serial subcultures of callus may be exploited for crop improvement (Heinz and Mee, 1971) or may give rise to material rich in phytoproducts (Ellis, 1982).

In the present investigation efforts were made to maintain callus through serial subcultures at 4 week interval each. Calli initiated from cotyledon and hypocotyl could be maintained for long time. Among the different growth regulators tested, 2.0 mgl^{-1} BA + 0.2 mgl^{-1} NAA and 2.0 mgl^{-1} IAA + 1.0 mgl^{-1} KIN were proved to be suitable for long term maintenance of callus cultures. Calli maintained in media with these growth regulators did not decline their growth. Yamamoto *et al.*, (1982) reported consistency of pigment formation upto 24 subcultures. Embryogenic callus of Shamouti orange gradually declined its growth after 18th subculture (Button and Kochba, 1977). The differences observed in the present experiment might be due to growth regulators used.

In general, production of good quality, usable shoots was dependent on number of subcultures done. For all sources of explants, number of usable shoots and their quality rating increased gradually upto 5th subculture and then declined. Economou and Read (1986) mentioned that microcutting production of three clones of deciduous azala increased and then declined over a reculture period of five passages.

Before transplanting from the artificial heterotrophic environment of the test tube to an autotrophic free-living existence and onto their ultimate location, shoots regenerated from different explants need root initiation and their healthy growth. So, experiments were conducted with basal MS medium supplemented with different types of auxins. Auxins were tried singly or in combination with other auxins in various concentrations. Among different auxins, IBA was found to be the best for root induction and maximum mean number of roots after 35 days of culture was recorded as 22.5 per explant.

Most plants require the presence of auxin for efficient root regeneration. Herbaceous plants require lower concentrations of auxins for efficient rooting. In the present findings, MS + 1.0 mgl^{-1} IBA was proved most efficient for rooting. Efficiency of IBA in root induction was also observed in chickpea (Hoque *et al.*, 1984) and grape (Chakravorty, 1986). The present findings also showed similarities

to Roy and De (1986, 1990) in *Calotropis gigantea*; Agrawal *et al.*, (1989) in *Prunus* sp., as they obtained roots only with IBA. After sufficient development of roots, plantlets of *M. dioica* were successfully transplanted in pots and finally established to the field condition.

An aspect of plant tissue culture technology which at present is being strongly recommended for crop improvement is somaclonal variation. Somaclonal variation induced *in vitro* is a widespread phenomenon irrespective of the mode of reproduction sexual or vegetative and ploidy status of the species. Somaclonal variation refers to heritable changes which accumulate in the callus (differentiated growth) from a somatic explant and expresses in the progeny of *in vitro* regenerants obtained from the callus. Even though relative data from deliberately designed experiments is not yet available, indications of possible advantages of somaclonals viz-a-viz induced mutagenesis are; (i) the frequency of variation seems to be far greater than the yield of induced mutations; (ii) the changes are very suitable and may not involve drastic alteration in the genetic background. It is, therefore, possible to accumulate somaclonally induced advantages in already improved cultivar; (iii) somaclonal variation occurs for traits of both nuclear and cytoplasmic origin. The variation of cytoplasmic genes obtained by this method is a distinct advantage; and (iv) in wide crosses, somaclonals provide a mechanism of gene introgression. Immature embryos of the wide cross can be callused and plants with the introgressed desired gene (or gene complex) are selected among the regenerants or their progenies.

Already, somaclonal variation has been processed into commercial cultivars of sugarcane in Hawii and in India. The characteristics improved by this procedure include yield and disease resistance.

In vegetable crops somaclonal variation has been confirmed in tomato (Evans and Sharp 1983) and lettuce (Engler and Grogan, 1984). About 13 different nuclear mutations have been recovered among the progeneis of only 230 regenerants of tomato (Evans and Sharp, 1983).

This technology can be utilized for the improvement of tetraploid kakrol. Different characters viz. fruit quality and yield would be the best parameters of somaclonal variant plants, which could be compared with normal plants. The results of the present investigation reveals a marked somaclonal variation in respect of fruit weight noticed among the callus derived plants.

Induction of bisexual and staminate flowers on pistillate plants using $AgNO_3$ is well established in cucumber and tomato (Beyer, 1976); in pickling cucumber (Kalloo and Franken, 1978); in *M. charantia* (Kabir *et al.*, 1989) and in *Cucumis sativa* (More and Munger, 1986). Induction of bisexual flowers on pistillate *Momordica dioica* have been reported (Ali *et al.*, 1991). In the present study $AgNO_3$ was applied to induce bisexual flowers in tetraploid, triploid and diploid type of kakrol.

Most of the $AgNO_3$ treated vines produced flowers continuously from 5th upto 14th nodes, unlike the untreated control vines where flower formation was sparse. Similar effects of $AgNO_3$ on kakrol have been reported previously (Ali *et al.*, 1991).

Effectiveness of $AgNO_3$ on the induction of bisexual flowers on female plants could be identified before anthesis by observing the clavate shape of bisexual flower bud in contrast to the oblong female bud (Ali *et al.*, 1991). Present study indicated that 10-20% of the clavate shape flower buds did not produce normal anthers. An irregular position of the bract on the peduncle was another identifying feature of induction (Ali *et al.*, 1991) but it was not always true as found in this study. Higher ppm of $AgNO_3$ induced bisexuality in pistillate plant without bracts on the peduncle. A large ovary and calyx were also a diagnostic feature of the induced bisexual flower buds. Ali *et al.*, (1991) reported similar large ovary and calyx in induced bisexuals.

In other Cucurbitaceae (McMurray and Miller, 1968; Iwahori *et al.*, 1970) ethephon application at an early seedling stage converted the sex. In *M. dioica* $AgNO_3$ treatment on twig with very small visible buds was found to be very effective. The fact that changes of sex of female kakrol flowers with Ag+ suggesting that an ethephon treatment would work under proper application conditions.

The application of $AgNO_3$ to female plants produced bisexual flowers. Each of the stamen developed from the base of the style and anther reached just beneath the stigma. From the base of style three rudimentary structures appeared which developed into androecium. Among the different concentrations of $AgNO_3$ treated, 300 ppm produced the highest number of bisexual flowers per vine in diploid kakrol whereas 400 and 500 ppm $AgNO_3$ produced highest bisexual flowers per vine in triploid and tetraploid type of kakrol respectively. Ali *et al.*, (1991) reported that 400 ppm of $AgNO_3$ produced highest number of bisexual flowers per vine.

The size of the reproductive organs of kakrol depends on the degree of ploidy. In general all reproductive organs were bigger in size in tetraploids than triploids and diploids. Increasing ploidy level enhances the vigour and size of the organs has been reported in other plants (Zeilinga and Schouten, 1968). Moreover, the reproductive organs of the bisexual flower induced from the tetraploid, triploid and diploid female plants showed more vigour than the corresponding normal types. Tetraploid, triploid and diploid female plants induced bisexual flower upon the application of $AgNO_3$. Application of $AgNO_3$ may bring some sorts of physiological change in the growing shoots of kakrol which eventually influence in the increase of vigour of the reproductive organs.

Pollen viability of normal male and induced bisexual in tetraploid, triploid and diploid was different. Bisexual tetraploids produced more viable pollens than triploids and diploids. $AgNO_3$ used to induce bisexual flowers might have unknown physiological effect which eventually influenced pollen viability.

For pollen germination, different concentrations of glucose, sucrose and boric acid solutions were used at different time intervals and the highest percentage of pollen grain germination was 85% recorded in tetraploid and diploid male flowers when 20% glucose solutions were used for 45 minutes. The lowest percentage of pollen grain germination was 28% recorded in tetraploid induced bisexual flowers when these pollen grains were germinated in 6 ppm boric acid solution for 45 minutes.

Several investigators have noted upsets of varying degrees in seed setting, seed development and in germination capacity following intercrossing between diploids and their corresponding autotetraploids (Griffiths *et al.*, 1971). In some instances the triploid embryos abort and none or very few triploid individuals are developed e.g. in rye (Hakansson and Ellerstrom, 1950; Muntzing, 1951) and in maize (Cooper, 1951; Cavanagh and Alexander, 1963). In other cases the triploids may be fully vigorous but sterile, e.g. beet, where triploids are freely formed.

The pollens of induced bisexuals were as effective as that of normal flowers. In diploids and tetraploids the percentage of success in crossing with pollens from bisexual flowers was as high as that of control. The pollen of the bisexual flower was not effective on the same flower or other bisexual flowers. Moreover, when these bisexual flowers pollinated with the pollens of normal male flowers, no fruit development was observed. Microscopic observations revealed 76% pollens germinated normally but after a certain growth of pollen tube tumor developed at the tip that formed barrier for tube penetration into the ovule. As a result, the pollen tubes failed to elongate and the ovary remained unfertilized.

The stigma of normal tetraploid ♀ received pollens only from normal ♂ tetra and induced tetra ♂. In contrast Muntzing (1951) reported that in the styles of tetraploid rye, the pollen tubes of haploid pollen grains grow faster than the pollen tubes of 2n pollen grains (Hagberg and Ellerstrom, 1959). Randolph (1935, 1941) found pollen of diploid maize to be more effective than that of tetraploids in bringing about fertilization in both diploid and tetraploid maize under conditions of open pollination. However, normal diploid ♀ accepted pollen from normal dip, tetra ♂ as well as from induced bisexuals. In both cases, numerous pollen tubes were found to develop from the germinated pollen grains and elongated through the stigmatic papillae. The study reveal that numerous developing pollen tubes were able to enter the stigma. This study further indicated that maximum number of ovules received

pollen tubes to perform fertilization. The stigmas of triploids did not accept pollens from any source.

The pollen grains started to germinate on the stigmatic papillae after 15 minutes from the time of pollination and the phase of germination completed within 30 minuets. Pollens from all sources except from triploid germinated uniformly in case of all the pollinated stigmas studied. Cent percent pollen grains of triploid type failed to germinate on the stigmatic surface. In most cases both localized and diffused callose production was observed on the stigmatic papillae which might prevent pollen germination. However, stigmatic papillae from unpollinated pistils did not produce any callose.

In exceptional cases, cross between ♂ tetra and ♀ trip a few germinating pollen grains produced tubes to grow within the stigmatic papillar cells. Careful examination showed that on an average only one or two ovules per ovary received pollen tubes and set one or two seeds. Jha and Roy (1989) reported that crosses between ♀ tetraploid and ♂ diploid produced only male flowering plants. They also reported that in the following season one branch of the hybrid produced hermophrodite flowers but no fruit setting occurred.

The fruits of successful crosses between and within tetraploid and diploid types were characterised using four parameters like fruit weight, fruit volume, seed number and seed weight. The fruits produced through crossing of the flowers of same plant with pollen from bisexual flowers were greater in size in both tetraploid and diploid type of kakrol, and the seed number and seed weight were more or less same as compared with fruits of normal crosses. The fruit weight and fruit volume per fruit were high in crosses like tetra N♀ × tetra ♂, tetra W ♀ × tetra ♂ and tetra A ♂ × tetra ♂.

The induction of bisexual flowers on female plants opens the way for generation advancement by sib or self pollination (More and Seshadri, 1988). The

cause of failure in crosses of intra and inter bisexual flowers was determined through post-pollination interactions study using flurescent microscope. However, the transfer of characters from both male and female plants to another genotype could be achieved by using the pollens from both of the sex types. The difficulty caused by dioecy in transferring characteristics from one plant to another and the advancement of generations could be overcome by induction of bisexual flowers. Large scale production of only female plants, might also be possible by using the self or sib seeds developed from bisexual flowers of female plants.

Chapter - 5

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APPENDICES

APPENDICES

MS medium (Murashige and Skoog. 1962) :

Components	(Concentrations mgl^{-1})
Macro-nutrients :	
KNO_3	1900.00
NH_4NO_3	1650.00
KH_2PO_4	170.00
$CaCl_2.\ 2H_2O$	440.00
$MgSO_4.\ 7H_2O$	370.00
Micro-nutrients :	
$FeSO_4.\ 7H_2O$	27.80
$Na_2.$ EDTA	37.30
$MnSO_4.4H_20$	22.30
H_3BO_3	6.20
$ZnSO_4.4H_2O$	8.60
KI	0.83
$Na_2M_OO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Organic-nutrients:	
Glycine	2.00
Nicotinic acid	0.50
Pyridoxine-HCl	0.50
Thiamine -HCl	0.10
Inositol	100.00
Sucrose	30000.00

pH adjusted to 5.8 before autoclaving.