CHAPTER - 3

MATERIALS AND METHODS

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3:1 Collection of seeds and live plants

During the course of present work, seeds and live plants of various species of the genus Amaranthus L. were collected from different places of the state of Orissa, mostly from coastal plains and some from tribal belts. Seeds and live plants were collected from kitchen gardens, vegetable farms, open fields and way sides. Seeds of different cultivated varieties of various vegetable species and some ornamental types were collected from local seed suppliers. As some species are highly polymorphic, care was taken to collect the seeds of all such types varying in colour and inflorescence pattern etc.

Since few species are found in Orissa, attempt was made to collect seeds of various species from different parts of India and abroad such as National Botanical Research Institute, Lucknow, India; Botanical Garden, Sibpore, India; Botanical Garden, Barlin-Dahlem, West Germany; Botanical Garden, Copenhagen, Denmark; Botanical Garden, Stockholm, Sweden, Royal Botanical Garden, Kew, England; University Botanical Garden, Romania and Botanical Garden, Nepal. Seeds were sown in the experimental fields to raise the plants of various species for initial identification and fixing flower buds for cytological work. Every year plants were raised and seeds were collected from them and stored for the preservation of germ plasm. Representative plant specimens from which fixings were taken for chromosomal analysis were preserved in the Herbarium of Post-Graduate Department of Botany, Utkal University as voucher specimen.

3:2. Cytological technique

Chromosome study was carried out through out the year at different times in different species according to their flowering but in most of the cases during winter seasons. Meiotic and mitotic

analysis were done from the flower buds and root tips respectively. For meiotic analysis, the flower buds were fixed in acetic alcohol (1:3) at any time from 9 a.m. to 1 p.m. . After 24 hours, the flower buds were transferred to 70 % alcohol and stored in refrigerator for squashing in future. Anthers were squashed as far as possible within 72 hours of fixation to avoid staining of cytoplasm and chromosome stickyness. For the study of meiosis, simple aceto-carmine squash technique was followed using 1 % aceto carmine.

For mitotic analysis, the seeds were soaked in distilled water for 24 hours and then spread over moistened filter paper in petridishes and were kept in dark at 27°C-31°C for germination. The root tips at 0.2 mm - 0.5 mm length were taken and pre-treated with 0.2 % colchicine for 3 hours at 10± 2°C. Then the root tips were transferred to acetic alcohol (1:3) between 10 a.m. and 1 p.m. to get maximum cells in metakinesis. After 24 hours, the root tips were transferred from the fixative to 70 % alcohol and were stored in refrigerator. Root tips were squashed within 72 hours of fixing. Before squashing the root tips were hydrolysed in IN hydrochloric acid for five minutes with slight warming. Hydrolysed root tips were taken in few drops of 2 % aceto-orcein in a watch glass and warmed for few minutes and then allowed to cool. Finally deeply stained portion of the root tips were smeared in fresh 2 % aceto-orcein. The prepared slids were temporarily sealed in parafin wax and observed under the microscope. Suitable cells were photographed before making the slide permanent.

For making squash preparations permanent, the following schedules were found suitable.

A. Method using alcohol - xylol mixture

a) Slides and coverslips were separated in a mixture of glacial acetic acid : absolute alcohol : xylol : : 1:1:1

b) Slides and coverslips were passed through the following gradations

	Duration
I. Absolute alcohol:xylol :: 1:3	10 min.
II. Absolute alcohol : xylol :: 1:9	10 min.
III.Xylol	a dip

c) Mounted in Canda balsam.

B. Method using tertiary-butyl alcohol

a) Slides and coverslips were separated in tertiary-butyl alcohol.

b) Mounted in Euparol.

3:3. Colchicine treatment

Colchicine has been proved to be the most suitable chemical for inducing polyploidy in plants. Various methods of application of the chemical have been outlined. However during present investigation, the seedling treatment method was employed. Treatment of colchicine at the seedling stage has been found to be very successful and was tried in three grain species viz. A.hypochondriacus L., A.caudatus L. and A.hybridus L.

Seeds of these species were sown in earthenware pots. After the seedlings reached 4-leaf stage (two cotyledonary leaves and two foliage leaves), they were thinned out by removing some of the seedlings. After 3-4 days, when the seedlings were sturdy, colchicine treatments were given. Small round cotton plugs soaked in aqueous solution of colchicine were placed on the shoot apices. Two different concentrations , 0.2 % and o.5 % of colchicine were used for the treatment. The solution was dropped on cotton plugs through glass jet at regular intervals. The treatment was given continuously for six hours. After the stipulated duration of treatment, the cotton

plugs were removed. A control set was maintained for each species for comparison. When the seedlings were one week old , they were transplanted to the experimental plots. Treated plants of each species and their respective control sets were maintained in separate plots. Morphology and cytology of the treated plants were studied individually.

3:4. Hybridization technique

Hybridization was attempted between A.spinosus and A.dubius . The flowers being very small and aggregated in spikes, normal procedure of emasculation and pollination of individual flowers were not possible. As spikes contain male, female and hermaphrodite flowers, the following method was adopted.

Both the species were planted in close alternating rows. Just when the terminal spikes started emerging, the terminal portion of the plant bearing spikes of two species were tied together and the whole bunch was covered with paper bag. After maturity, seeds were collected from the spikes of both the parents separately to establish reciprocal crosses. Seeds were dried under sun and kept in close container. Though the chances of selfing was not eliminated, there were more hybrid plants raised from the seeds collected from each of the parental plants used for the cross than that found in nature.

Four hundred seeds of each parental line were sown in germinating pots. Number of seedlings were counted and when the seedlings were one week old, they were transplanted in separate experimental plots. When the plants were with fully matured spikes, number of parental types and number of F_1 hybrids from each parental line were established with the help of morphological parameters. Cytological tests of F_1 progeny plants were carried out to ascertain F_1 hybrids. Detailed morphological and cytological studies of such hybrid plants were made.

Since F_1 progeny were triploid and sterile, the young vegetative buds of the triploid hybrids were treated with colchicine to raise F_2 hexaploids. Small round cotton plugs soaked in aqueous solution of 0.5 % colchicine were placed on the apices of young vegetative buds. The solution was dropped on cotton plugs through glass jet at regular intervals. The treatment was continuously carried out for six hours after which the cotton plugs were removed and the treated vegetative buds were marked by labelling. Lateral branches growing from these vegetative buds produced viable seeds.

F_2 progeny were raised from these seeds and chromosomal analysis of different morphological types were carried out which were followed through F_3 and F_4 generations.

3:5. Mutagen treatment

Mutation was induced in A.hypochondriacus L. with the help of chemical and physical mutagens. For chemical mutagen treatment, seeds collected from pure line plants were soaked in distilled water for 2 hours. Pre-soaked seeds were treated with 30 ml. freshly prepared aqueous solution of ethyl methane sulphonate (EMS) and diethyl sulphate (DES) for six hours at room temperature (26°C). Different concentrations of EMS such as 0.1%, 0.2% and 0.4% and those of DES such as 0.012%, 0.025 % and 0.05 % were used. For DES treatment, freshly prepared solutions were being replenished every half an hour due to the short half life of hydrolysis of the chemical. After the treatment the seeds were thoroughly washed under running water for 30 minutes. For studying the effects of mutagen on germination and root and shoot growth, some of the seeds were maintained in petridishes while the other treated seeds were sown on earthenware pots. Percentage of seed gemination was recorded after one week of treatment. Percentage of seedling survival was recorded

at 4-leaf stage which were then transferred to the field. Plant heights were measured at 15 days and at maturity. Pollen sterility was determined by staining the matured pollen grains in a mixture of aceto-carmine and glycerine (1:1). At maturity the mutants were selfed and were harvested separately on individual spike basis. The morphology and the cytology of the mutant plants were studied at M_1 M_2 and M_3 generations.

For physical mutagen treatment, various doses (10 kr, 20 kr, 40 kr and 60 kr) of Gamma rays from Co^{60} source at Bhaba Atomic Research Centre, Trombay and Genetics department, Osmania University, Hyderabad, were applied to the dry seeds. Seven hundred seeds were being taken for each treatment. Half of the seeds from each treatment were maintained in petridishes to study the perecentage of germination, seedling survival, root and shoot length etc. and the other half were sown in earthenware pots. Rest of the methods were followed as in the case of chemical mutagen treatment.

3:6. Chromosome analysis

Both mitotic and meiotic sstudies were carried for detailed chromosomal analysis. Mitotic studies were carried out in different species of the genus in order to ascertain the chromosome number. Since the somatic chromosomes were very small in all the species worked out, karyotypic analysis could not be done.

Meiotic study was carried out in normal diploids, induced tetraploids at C_o,C_1 and C_2 generations, already existing induced tetraploid from C^5 to C^{10} generations, F_1 triploids and induced hexaploids at F_2, F_3 and F_4 generations. Meiotic analaysis was mainly confined to the observations at diakinesis, metaphase-I and anaphase-I in order to study the pairing behaviour, chromosome associations and abnormalities in chromosome separation.

3:7. Tissue and Protoplast culture

3:7.1 General tissue culture technique

In vitro study in A.hybridus L. was attempted through callus formation and regeneration from different explants according to the following methods.

Surface sterilization and inoculation

Before inoculating the seeds in germinating media, they were surface sterilized by treating the seeds in the following steps.

a)	0.1 % Mercuric chloride	10 minutes
b)	Calcium hypochlorite and Teepol (a mild detergent solution) mixture (7 cc. of calcium hypochlorite was added to 5 cc. of Teepol and final volume was made to 100 cc.)	10 minutes
c)	Thoroughly washed with sterile distilled water	6 minutes

Then the seeds were inoculated on sterile nutrient media without any growth hormone in 15 cm. petridishes. The petridishes containing seeds were kept in dark at a temperature of 29± 2°C. Explants like first leaf, hypocotyl and root (approximately 1 cm length) taken from 7-10 days old seedlings were transferred to culture flasks containing desired agar-gelled sterile media. The glasswares and the media were sterilized in autoclave following usual procedure. All operations during inoculation including surface sterilization of seeds were carried out inside a laminar airflow cabinet.

Incubation and maintenance of the culture.

All the cultures were incubated in racks inside the culture room with controlled condition of light and temperature. The

cultures were exposed to continuous illumination (1000 lux. cool white flourescent light, Phillips 40 WT) at 25 ± 2°C maintained inside the culture room.

Preparation of culture media

Different basic media viz. Murashige and Skoog (1962) and Uchimiya and Murashige (1974) were tried during the experiment. These two media were found to be suitable and followed. According to the need of different experiments, the formulation of Murashige and Skoog (MS) and Uchimiya and Murashige (UM) were suitably modified as detailed below. For convenience and to avoid weighing individual ingredients each time, concentrated stock solutions of selected components such as macronutrients , micronutrients, vitamins etc. of the media were prepared and stored frozen in a deep freezer. However, the stock solutions were prepared from time to time at an interval of 15 days to avoid contamination.

A. MS basic medium

Component	Concentration (mg/l)
$CaCl_2.2H_2O$	440
NH_4NO_3	1650
KNO_3	1950
KI	0.83
$CoCl_2.6H_2O$	0.025
KH_2PO_4	170
H_3BO_3	6.2
$Na_2MoO_4.2H_2O$	0.25
$MgSO_4.7H_2O$	370
$MnSO_4.4H_2O$	22.3
$CuSO_4.5H_2O$	0.025
$ZnSO_4.7H_2O$	8.6
$FeSO_4.7H_2O$	27.85
Na_2 EDTA	37.25

Component	Concentration (mg/l)
Glycine	2.0
Inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Sucrose	30000

MS based medium which was suitably modified with varied concentrations of NAA and BAP (Power and Chapman, 1985) was used and designated as MSP_1 = (MS + NAA - 2.0 mg/l and BAP - 0.5 mg/l).

B. UM basic medium

For preparation of UM basic medium , following additions to MS basic medium are made. (Uchimiya ,H. and Murashige, T., 1974) which was found suitable for certain types of cultures.

Component	Concentration (mg/l)
2,4-D	2.0
Kinetin	0.25
Thiamine	9.9
Pyridoxine	9.5
Nicotinic acid	4.5
Casein hydrolysate	2000

At the time of media preparations, the stock solutions were brought to room temperature and were mixed proportionately. Then 100 mgs of inositol (if present in the respective media) and 30 gms of sucrose were added to it. The final volume was made upto 1000 ml. and pH of the medium was adjusted to 5.8 by adding 0.1N NaOH or 0.1N Hcl. Then the solution was gelled with 8 gms of Difco-Bacto agar. Besides the basal components, some other additives like charcoal powder, PVP or

growth substances like IAA , NAA, 2,4-D, Kn and BAP were also included in the media whenever necessary. Since the concentration of these substances varied in different treatments, they have been mentioned at appropriate places.

3:7.2 Isolation, fusion and culture of protoplast

Since hybridization in most of the species of Amaranthus L. is difficult, it was planned to standardize methods for protoplast fusion to raise hybrids through somatic hybridization. For this purpose, A.hybridus L. and A.hypochondriacus L. were tried and the following procedure was followed.

While A.hypochondriacus L. was grown in the green house for the harvest of mesophyll protoplast directly from the leaf, explants from A.hybridus L. were cultured for raising callus from which protoplast could be derived through suspension culture for the purpose of isolation, fusion and culture of protoplast. The following materials were used.

A. Cell and Protoplast washing solution

This was composed of :

KH_2Po_4	27.2 mg/1
KNO_3	101.0 mg/1
$Cacl_2.2H_2O$	1480.0 mg/1
$MgSO_4.7H_2O$	246.0 mg/1
KI	0.16 mg/1
Mannitol	130 gm/1

The pH of this solution (CPW 13M) was adjusted at 5.8

B. Enzyme Mixture

(a) For leaf mesophyll protoplasts :

This was prepared by dissolving

Cellulase (R 10) - 2 gm

Pectolyase (Y 23) - 0.1 gm

in 100 ml of CPW 13 M solution to which 2 ml. of antibiotic of the following composition was added.

Ampicillin	400 mg/l
Gentamycin	10 mg/l
Tetracycline	10 mg/l

pH was adjusted at 5.8 and filter sterilized.

(b)For cell suspension protoplasts

This was prepared by dissolving

Rhozyme	2 gm
Meicelase	2 gm
Macerozyme R_{10}	0.03 gm

in 100 ml. of CPW 13 M solution. pH was adjusted at 5.8 and filter sterilized.

C. Fusion solution

(a) PEG -

Polyethyline glycol MW 6000	30 gm
Sucrose	4 gm
$CaCl_2.2H_2O$	0.147 gm

dissolved in 100 ml of sterile distilled water and autoclaved.

(b) High pH/Ca^{++}

Glycine	0.375 gm
$CaCl_2.2H_2O$	0.74 gm
Mannitol	9.0 gm

dissoslved in 100 ml of sterile distilled water, pH adjusted to 10.4 and filter sterilized.

D.Media used for Protoplast culture

Of various media tried, B5 (Gamborg et al., 1968) was found to be suitable for culture of Amaranthus L. protoplast. The B5 medium is of the following composition.

	mg/l
KNO_3	2500.0
$CaCl_2.2H_2O$	150.0
$MgSO_4.7H_2O$	250.0
$(NH_4)_2SO_4$	134.0
$NaH_2PO_4.H_2O$	150.0
KI	0.75
H_3BO_3	3.0
$MnSO_4$	10.0
$ZnSO_4.7H_2O$	2.0
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4,5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
FeNa EDTA	40.0
Inositol	100.0
Pyridoxine HCl	1.0
Thiamine HCl	10.0
Nicotinic acid	1.0
Kinetin	0.1
2,4-D	0.3
Sucrose	2000.0
pH 5.5	

Isolation of Protoplasts

Fresh leaves from five week old plant growing in green house were collected, washed and surface sterilized. These were peeled off their lowewr epidermis and were floated in petridishes containing CPW13M solution for 1 hr, then these were transferred to petridishes containing enzyme mixture (Ba) and maintained at 27°C for 16 hours in dark. After incubation, the leaf pieces were agitated in the enzyme solution and were squeezed out with the help of the pasteur pipette. The enzyme mixture containing the protoplasts were centrifused (100 g, 5 minutes). The supernatant was removed and the pellete was resuspended in CPW13M and centrifused. Again the supernatant was removed and the pellete was resuspended in CPW solution containing 21 % (W/V) sucrose and by centrifugation (120 g, 10 minutes), viable protoplasts were collected at the surface and were resuspended in CPW9m medium and the density of the protoplast was counted with haemocytometer.

The cells from the suspension lines collected by sieving were incucbated in the enzyme mixture (Bb) in light for 16 h on a rotary shaker. After incubation , protoplasts together with the enzyme solution were passed through a 64 nylon sieve. Protoplasts were centrifuged (100 g, 5 min.) and the enzyme supernatant was replaced by CPW21S medium. Following centrifugation (100g, 10 min.), viable protoplasts were collected at the surface , washed in CPW13M medium and resuspended in CPW9M medium and density was counted.

Fusion and culture of Protoplasts

Leaf mesophyll protoplasts at a density of 2×10^5 / ml and cell suspension protoplasts at a density of 8×10^5 /ml in

CPW9M solution were mixed. This is a departure from the usual 1:1 mixture of two types of protoplasts at a lower density. Aliquots (0.3 ml) of the protoplast mixture were slowly dropped in 5 cm. petridishes and protoplasts were allowed to settle down to the bottom of the dish for 12-15 minutes. Three drops of PEG (MW 6000) fusion solution (25 % W/V) were placed, each on the two sides of the settled protoplasts in such a way that the drops gradually coalesced with the protoplasts over a period of 15-20 minutes. Then the PEG solution was gradually replaced by high pH Ca^{++} fusion solution which in it's turn was replaced by CPW9M solution containing $CaCl_2.2H_2O$. Each washing with this solution was for 10 minutes and 3-4 washings were given before the protoplasts were flooded with 4 ml. of the liquid culture medium and the petridishes were maintained in continuous light in culture room at a temperature of 25± 1°C.

3.8. Photography

Coloured photographs of live materials were taken by the help of Practica Camera with 35 mm VR100 Kodak colour negative films. Similarly black and white photographs of live materials were taken with 35 mm ORWO NP22 (125 ASA) negative films.

Large number of photomicrographs were taken from the well stained PMC and root tip cells clearly showing the morphology of chromosomes of normal diploid, tetraploid and hybrid plants for the purpose of illustration. Exakta Camera with photomicrographic attachment was used and black and white ORWO MA8 as well as ORWO NP15 (25ASA) negative films were found to be most suitable. The negatives were processed in contrast developer (Agfa formula-D19). Enlarged prints were prepared with Agfa hard and normal

glossy photographic papers. All the chromosome photographs were produced at a magnification of X 2500 unless otherwise stated. Coloured prints were processed at colour process laboratory.

Abbreviations

BA/BAP	benzyladenine/benxylaminopurine
C_0	Colchicine treated generation
C_1	First generation after colchicine treatment and so on.
C°	degree centigrade
ml	milli litre
Cm	Centimeter
mm	millimeter
2,4-D	2,4-dichlorophenoxy acetic acid
EDTA	ethylene diamine tetra acetate
gm	gram
IAA	indole acetic acid
Kn	Kinetin
L	litre
M_1	First Mutant generation
M_2	Second mutant geneeration and so on.
u	micrometer
NAA	napthaline acetic acid
PVP	Polyvinyl Pyrrolidone
PMC	Pollen mother cell
Var	Variety
CV	Cultivar