MATERIALS AND METHODS
3. MATERIALS AND METHODS

3.1 Plant material

Seeds of *Cucumis melo* L. var. *acidulus* and *Cucumis melo* L. var. *momordica* were obtained from National Bureau of Plant Genetic Resources (NBPG), New Delhi, India. Plants were grown in the experimental garden of P.G. Department of Studies in Botany, Karnataka University, Dharwad. Plants were watered every alternate day. During their vegetative growth, they were supplied with urea and during the flowering phase with N:P:K (17:17:17) once in a week. Plants were protected with insecticides (Spark, Bayer Crop Science Ltd., Mumbai) and fungicides (Bavistin, BASF India Ltd., Mumbai) regularly throughout the growing season.

*Cucumis melo* var. *acidulus* (Fig. 2 A) and *C. melo* var. *momordica* (Fig. 10 A) are monoecious. Flowers are yellow, male flowers (Fig. 2 B and Fig. 10 B) are produced in axils generally in groups and female flowers (Fig. 2 C and Fig. 10 C) are solitary. Ovary is tricarpellary syncarpus inferior. They are sensitive to frost, hence require plenty of sunshine and do well in loamy soils. The fruits of variety *acidulus* and *momordica* are shown in Fig. 2 D and Fig. 10 D. The fruits of variety *momordica* split when they ripe as shown in the Fig. 10 D.

3.2 Chemicals and Glasswares

Analytical grade mineral salts, vitamins (myo-inositol, nicotinic acid, pyridoxine hydrochloride, thiamine hydrochloride, biotin etc.), amino acids, sucrose, agar, mercuric chloride, colchicine, growth regulators such as IAA, IBA, NAA, 2,4-D, TDZ, BAP and KN were procured from Hi-media Laboratories, Mumbai, India. Nylon filters were procured from RFCL Ltd, Mumbai. Glasswares were procured from Borosil Glass Works Ltd. (Mumbai, India) and Riviera (Mumbai, India) which were autoclaved before use.
3.3 Media preparation

Media were prepared using standard composition of MS (Murashige and Skoog, 1962) and E20A (Sauton and Dumas de Vaulx, 1987) media with the composition shown in the Table 2 solidified with 0.8% agar with a pH of 5.8 adjusted using 1N or 0.1N hydrochloric acid or sodium hydroxide as per the requirement. The hot medium (15 ml) was dispensed into culture tubes (150 mm x 25 mm) or jam bottles (50 ml into 250 ml bottle). Culture tubes, which were plugged with non-absorbent cotton plugs, and bottles were sterilized by autoclaving at 121°C under 1.06 Kg/cm² pressure for 15 mins. Thermolabile compounds such as IAA, TDZ etc. were filter sterilized using nylon filters with pore size of 0.2/0.45 µM and added into the medium before dispensing into the culture vessels.

Culture tubes and bottles containing autoclaved media, sterile distil water and sterilized instruments were kept in laminar airflow chamber under UV light for one hour prior to inoculation. Forceps, scalpel, needles were dipped in absolute alcohol and flamed over spirit lamp and cooled before use at regular intervals.

3.4 In vitro culture of ovules and ovary slices for the induction of gynogenesis in Cucumis melo cultivars

3.4.1 Collection of explants and surface sterilization

The unopened female flowers on the day of anthesis were collected early morning from healthy plants in Erlenmeyer flask. These flowers were washed in tap water for 3 minutes along with Bavistin - a fungicide. The fungicide is removed after thorough washing in running tap water. Then the flowers were taken inside the laminar air flow cabinet and were treated with 70% ethanol for 30 seconds followed by 0.1% mercuric chloride for 8 minutes and finally rinsed 4–5 times with sterile distilled water. For ovule culture, the ovules were dissected aseptically using stainless steel needles under stereo zoom microscope (Carl Zeiss) as the ovules were microscopic and transferred to the test
tubes using sterile camel hair brush immediately. Each test tube was inoculated with 5 undamaged ovules. For ovary culture, the outer surface of the ovary was shaved and longitudinal sections were made so as to separate the carpels individually and placed aseptically on the culture medium. Generally 1 cm long ovary slices were prepared for culture and from one ovary, 6 such slices were prepared for inoculation.

Ovules and ovary slices were inoculated onto the MS medium supplemented with cytokinins such as TDZ, KN and BAP and auxins such as 2,4-D, NAA, IAA and IBA at the concentration 0.25, 0.5, 1.0, 2.0, 5.0 & 10.0 µM individually along with 3% sucrose for induction of gynogenesis.

To test the effect of cytokinins in combinations with auxins at various concentrations, the ovules and ovary slices were cultured on MS medium containing 3% sucrose and supplemented with following combinations of auxins and cytokinins for induction of gynogenesis:

Ovule cultures of *Cucumis melo* var. *acidulus*

i. TDZ (0.25/0.5 µM) + 2,4-D/NAA (2.0, 5.0 & 10.0 µM)

Ovary cultures of *Cucumis melo* var. *acidulus*

i. BAP (0.88 µM) + NAA (0.27 µM) (Ficcadenti et al., 1999)

Ovule cultures of *Cucumis melo* var. *momordica*

i. TDZ (1.0/2.0 µM) + 2,4-D (5.0 & 10.0 µM)/NAA (2.0, 5.0 & 10.0 µM)

Ovary cultures of *Cucumis melo* var. *momordica*

i. BAP (0.88 µM) + NAA (0.27 µM)

3.5 Effect of gamma irradiation dose on fruit setting and seed development.

To test the effect of gamma irradiation dose to induce parthenogenesis, male flower buds were collected in Petri dishes one day before anthesis and the petals were removed. Then they were gamma irradiated at 150, 200, 250, 300 and 350 Gray (Gy) using cobalt-60 source (M.C.M Phoenix, Germany) with an output of 6.4683 Gy/min at
the Karnataka Institute of Medical Sciences, Hubballi and then stored overnight at room temperature as per the procedure of Kumar et al., (2003). Meanwhile the female flower buds were covered with bags made up of muslin cloth to prevent any chances of pollination. The following day, pollen were collected from three irradiated male flowers with a camel hair brush and dusted on the stigmatic surface of the female flowers and covered again with muslin cloth bags. Pollination were done early morning (before 8.00 am) to prevent the drying of the stigmatic surface and to facilitate rapid pollen germination.

Successful pollination results in fruit development. During development, the fruits and plants were protected with pesticides (Spark, Bayer Crop Science Ltd., Mumbai) and fungicides (Bavistin, BASF India Ltd., Mumbai). Well developed fruits were harvested after 3–4 weeks.

3.6 Embryo rescue technique

3.6.1 Surface sterilization and embryo culture

The fruits were washed with running tap water followed by Tween-20 for 5 min. Then, they were sprayed with 70% alcohol. Further surface sterilization was carried out inside the laminar air flow cabinet in aseptic condition with 0.1% HgCl₂ for 15 min followed by rinsing with sterile distilled water 4–5 times.

The sterilized fruits were dissected; each seed was cut open and checked for the developmental stages of embryo. The embryos thus obtained were cultured on E20A medium (Sauton and Dumas de Vaulx, 1987) supplemented with 2% sucrose and 0.06 µM IAA.

All cultures (ovule or ovary for in vitro gynogenesis and extracted embryo developed from the parthenogenetic fruits) were incubated at 26 ± 2°C for 16-h photoperiod of 40 µmol m⁻² s⁻¹ light provided by cool white fluorescent tubes (Philips, Kolkata, India).
3.6.2 Shoot multiplication

After 15 days, few of the cultured embryos germinated into plantlets. After six weeks of culture, plants attain a length of 15 – 20 cms with well developed root system. They were multiplied in vitro by nodal cutting on the modified E20A medium supplemented with 3% sucrose and 1 µM IAA. For nodal culture, 2 cm of node without leaves were cut and placed on the medium. New clones of plants would be developing in 6 weeks of culture.

3.6.3 Acclimatization of plantlets

Well developed plantlets (15–20 cm in height) after 6 weeks of culture were taken out of the culture vessel and washed thoroughly in sterile distilled water and transferred to plastic cups containing a mixture of autoclaved vermiculite, sand and soil in the ratio 1:1:1 and reared in a plant growth chamber (Sanyo, Osaka, Japan) at 25°C, 60% relative humidity, with a 16-h photoperiod (40 µmol m² s⁻¹) provided by 40-W fluorescent lamps. Alternatively a simple method of acclimatization using plastic transparent bags to cover the plants was adopted. After hardening for 3-4 weeks the plantlets were transferred to pots containing sand, soil and farmyard manure in the ratio 1:1:1 and maintained in the green house.

3.7 Ploidy analysis by flow cytometry

Ploidy analysis of regenerated plants was carried out using PA-I ploidy analyzer (Partec, Munster, Germany). Samples were prepared according to the instructions of the manufacturer. A piece of leaf approximately 0.5 cm² in size from five regenerates was finely chopped with a sharp razor blade in Petri dish (5 cm) in 400 µl of extraction buffer (Partec Cystain UV precise P). Following 2 min of lysis, the sample was filtered through a 50 µM Nylon mesh. The filtered nuclei were stained for 1 min with 1.6 ml of staining buffer (Partec Cystain UV precise P). The processing was done on ice and the samples were maintained on ice till analysis. The gain of the instrument was calibrated with trout
erythrocytes to give a CV of less than 1.0. The ploidy of the samples was determined in comparison with the peak positions of control plant. The control plant was analyzed at the beginning of the day, after each 10 samples and also at the end of the day to check for the variation in reading due to instrument drift, if any. In each sample 5,000-10,000 nuclei were counted and a minimum of six measurements were made for each plant.

3.8 Ploidy analysis by root tip cytology

To determine the ploidy level of regenerated plants, the root tips from healthy, acclimatized plants were treated with 0.1% colchicine for three hours and then the pretreated root tips were fixed in ethanol:glacial acetic acid (3:1) for 24 hours. Then, they were washed in running tap water and placed in a watch glass containing nine drops of 2% acetoorcein and one drop of 1N HCl and heated gently. After cooling, individual root tip was placed in a drop of fresh stain on a glass slide and covered with a cover glass. Slide was warmed gently and the material was squashed. The slides were observed under Carl Zeiss Axio Imager-M2 inverted microscope and photographed under oil-immersion.

3.9 Ploidy analysis by stomatal indices and guard cell chloroplast numbers

Stomatal dimensions, density and the number of chloroplasts in the guard cell can also be used for the determination of the ploidy level. Fully expanded fifth or sixth leaf from the apex was taken from the haploid and the control plant. Two samples of epidermal cells were obtained from lower surface of each leaf by epidermal peelings and randomly 8 stomata were measured. Chloroplasts present in the stomatal guard cells were counted on each sample after staining with aqueous 1% silver nitrate. The length and diameter of the stomata was measured using inbuilt micrometry software in Carl Zeiss Axio Imager-M2 inverted microscope.
3.10 Induction of doubled haploids (2n) from haploid (n) plants of *Cucumis melo* cultivars

3.10.1 *In vitro* colchicine application

Colchicine application was based on the methods described by Solmaz et al., (2011). Fifty regenerated plants were treated with colchicine solution prepared in sterile distilled water at doses of 250 mg/l and 500 mg/l for induction of double haploids (2n). Colchicine solution was sterilized by filtering with sterile nylon syringe filter with a pore size of 0.2 µM. Plantlets were kept in colchicine solution for 4h in culture jars. After rinsing 4 times with sterile distilled water, old leaves were stripped off and plantlets were cut into pieces which have minimum of one node. Later on they were blotted on sterile filter paper and were placed into test tubes containing E20A medium. The treated micro-cuttings were placed in growth chamber with 25°C temperature and 16/8 h light/dark period conditions.

3.10.2 *In vivo* colchicine application

Application of *in vivo* colchicine solution was begun when the main stem of the plants were reached about 30-40 cm. The application of colchicine was done by “via-the-root” method as described by Linde-Laursen (1973). In *via*-the-root method, 50 acclimatized plants of about 30 – 40 cm were allowed to absorb 250 mg/l or 500 mg/l colchicine solution for 4 h. Thereafter the roots were rinsed and the plants were transferred to pots. Haploid and dihaploid plants were identified by root tip cytology.

3.11 Histological studies to assess the embryo development

Starting from the day of anthesis, before and after pollination with irradiated pollen at regular intervals, the fruits from *Cucumis melo* var. *momordica* were harvested and fixed in FAA (formalin, glacial acetic acid and ethanol) for 24 hours at room temperature, dehydrated through a graded ethanol-butyl alcohol series and embedded in paraffin wax (Fowke and Rennie, 1996). The tissues were sectioned at a thickness of 8 µM and stained.
either with 0.5% toluidine blue or with 1% safranine solution and examined under Carl Zeiss Axio Imager-M2 inverted microscope.

3.12 Data collection and analysis

In all the ovule culture experiments, 60 ovules were cultured (with 12 replicated test tubes containing 5 ovules each) and for ovary culture, ovary slices were cultured (one slice each in test tube with 12 replicates) in each treatment and the experiments were repeated thrice. The cultures were observed periodically and changes, if any, were recorded at weekly intervals. The number of responding ovules/ovary slices and their percentage were recorded. The results were statistically analysed by analysis of variance (ANOVA) and mean values were separated according to Duncan’s multiple range test at $p=0.05$ level.