1. Introduction:

Melon (Cucumis melo L.)

1.1 Melons of the world and India

*Cucumis melo* L. (2n=2x=24) is an important horticultural crop across wide areas of the world. Their use is extremely diverse, depending on the type of fruit (Akashi *et al.* 2002). Africa has been generally regarded as the centre of origin of *C. melo*, while India has been considered as an important centre of diversification. Strong viewpoints and arguments on African versus Indian origin are moot in the light of continental drift, South Eastern Africa and peninsular India were likely continuous or contiguous. The species *C. melo* is a polymorphic taxon encompassing a large number of botanical and horticultural varieties or groups. Melon is divided into two subspecies, *C. melo* ssp. *agrestis* and *C. melo* ssp. *melo*, differentiated by the pubescence on the hypanthium (Jeffrey 1990). Furthermore, the former has been subdivided into *conomon*, *makuwa*, *chinensis*, *acidulus* and *momordica* groups, the later into ten groups: *cantaloupe*, *reticulatus*, *adana*, *chandalak*, *ameri*, *inodorus*, *flexuosus*, *chate*, *tibish*, *dudaim* and *morren* (Pitrat 2008). The increasing number of varieties and morphological similarities among melons has necessitated the use of precise system for their identification and characterization. There are several local varieties of melon grown in different regions of India. Melons of India have large variability for fruit shape, size, skin characters, flesh colour, keeping quality and reaction towards insect pest and disease incidence. The non-dessert or culinary forms of *C. melo* is a distinct group distributed and adapted well essentially under humid tropics of Southern India (Fergany *et al.* 2011; Seshadri and More 1996). Great morphological variation exits in fruit characteristics such as size, shape, colour and texture, taste and composition, and *C. melo* is therefore considered the most diverse
species of the genus *Cucumis* (Kirkbride 1993; Whitekar and Davis 1962; Jeffrey 1980; Bates and Robinson 1995).

Culinary melon (*Cucumis melo* L. var. *acidulus*; 2n = 2x = 24) commonly called “vellari” is being cultivated in Kerala, Andhra Pradesh, Tamil Nadu and Karnataka states of India. This is a popular vegetables crop in humid tropic regions of South India, with a variety of common names viz., vellari, melon, pickling melon, preserving melon, culinary melon etc. A modest gene bank of the culinary melon has been established by N. P. S. Dhillon in the Department of Vegetable Crops, Punjab Agricultural University, Ludhiana, through explorations in Tamil Nadu and Kerala.

Snapmelon [*Cucumis melo* L. var. *momordica* (Roxb.) Duthie et Fuller; 2n = 2x = 24] is native to India, where it was commonly known as ‘phut’ or ‘phoont’ which means to split. Immature fruits are cooked or pickled; the low sugared mature fruits are eaten raw. Ripe fruits invariably crack. Snapmelon is cultivated in many parts of India and in the two Japanese Islands (Fujishita 2004), where it was used as food during the two world wars. Snapmelon germplasm has been found to be a very good source of disease (Cucumber mosaic virus, Zucchini yellow mosaic virus, Powdery mildew and Fusarium wilt) and insect resistance (*Aphis gossypii* and leaf miner) (Fergany et al. 2011).

Oriental pickling melon [*Cucumis melo* var. *conomon* Thunberg; (2n = 2x = 24)] is considered to be the most ancient form of melon domesticated in China (Jeffrey 1980; Walters 1989) and is cited several times in a book written between 1,000 and 500 years B.C.E. (Keng 1974). It is also held that it had originated from wild melon (var. *agrestis*) in China (Walters 1989). It is cultivated in Asia particularly in India, China, Japan, Korea and Southeast Asia. Munger and Robinson (1991) include two types of fruits, one used as a vegetable (non-sweet and eaten raw or pickled) and one
that can have high sugar content. Only the first one (oriental pickling melon) has
survived in the conomon group of (Robinson and Decker-Walters 1997). This variety
is characterised by dark green foliage, andromonoecious, elongated fruit, smooth thin
white or light green skin, white firm flesh, not sweet, not aromatic, not climacteric,
small yellow seeds.

1.2 Genetic diversity

Biological diversity is a commonly recognized value in natural resource
management. This diversity is often represented as a hierarchy of discrete units such
as species, ecosystem and landscapes. Genetic diversity is a measure of the possible
choices of information provided by a gene, when all or nearly all the members of a
population have the same allele at that gene. If many variants exist for a gene
sequence, that population has high genetic diversity at that gene. Study of genetic
diversity is the process by which variations among individuals or populations are
analyzed by a specific method or a combination of methods. The data often involve
numerical measurements and in many cases, combinations of different types of
variables. Diverse data sets have been used by researchers to analyze genetic diversity
in crop plants; most important among such data sets are pedigree data (Van Hintum
and Haalman 1994), passport data, morphological data (Bar-Hen et al. 1995),
biochemical data obtained by the isoenzymes (Hamrick and Godt 1997), storage
proteins (Smith et al. 1987), and DNA based markers data that allows more reliable
differentiation of genotypes.

1.2.1 Morphological variability

Genetic diversity in plants has traditionally been established using
morphological and biochemical markers. Phenotypic characterization is the first step
in the description and classification of genetic resources (Smith et al. 1987). With
respect to diversity in characters among populations, cluster analysis has been used to identify morphological variability in different crop species (Balkaya et al. 2010; Decker and Willson 1986). The assessment and description of trait variation are important tasks in the start-up of programmes aimed at the selection of genotypes with high-yield performance and qualitative traits useful to markets. In addition, studies on genetic variation of genetic resources are necessary to avoid storage of redundant germplasm that contributes to increase in the cost of germplasm management (Kumar 1999; Ricciardi and Filippetti 2000). Therefore, development of both procedures for characterization of genetic diversity and reducing collection size to manageable and accessible levels (core size) are important issues in gene bank studies (Brown 1989; Frankel 1984; Marshall 1990). Bio-agronomic characterization carried out by means of appropriate statistical methods continues to be a useful tool for the initial description and classification of germplasm, since it enables plant breeders to identify and select valuable genetic resources for direct use by farmers or in breeding programmes.

Genetic diversity in crop plants may be analyzed at different levels: individual genotypes such as inbred lines or clones, populations, germplasm accessions and species. Sampling strategies in each of the above cases would vary, primarily because of the differences in nature of the genetic material. Genetic distance is “the extent of gene difference between populations or species that is measured by some numerical quantity”. Genetic distance or similarity between two genotypes, populations or individuals may be calculated by various statistical measures depending on the data set.
1.2.2 Genetic variability analysis

1.2.2.1 Multivariate analysis

With the increase in the sample sizes of breeding materials and germplasm accessions used in the crop improvement programs, methods to classify and order genetic variability are of considerable significance. The use of established multivariate statistical algorithms is an important strategy for classifying germplasm and ordering variability for large number of accessions, or analyzing genetic relationships among breeding materials. Multivariate analytical techniques, which simultaneously analyze multiple measurements on each individual under investigation, are widely used in analysis of genetic diversity irrespective of data set (morphological, biochemical or molecular marker data). Among these algorithms, cluster analysis, Principle Component Analysis (PCA), Principal Coordinate Analysis (PCoA), and Multi Dimensional Scaling (MDS) are presently the most commonly employed and appear particularly useful (Brown et al. 2000; Johns et al. 1997).

1.2.2.2 Cluster analysis

Cluster analysis refers to “a group of multivariate techniques whose primary purpose is to group individuals or objects based on the characteristics they possess, so that individuals with similar descriptions are mathematically gathered into the same cluster” (Hair et al. 1995). There are broadly two types of clustering methods (i) distance based methods in which pair-wise distance matrix is used as an input for analysis by a specific clustering algorithms, leading to graphical representation (such as tree or dendrogram) in which cluster may be visually defined and (ii) model based methods, in which observations from each cluster are drawn from some parametric model. Inferences about clusters and each member are performed using some statistical methods as maximum likelihood or Bayesian method (Johnson and Wichern
Distance based clustering methods can be categorized into two groups: hierarchical and non-hierarchical. Hierarchical methods are found to be most commonly used for genetic diversity study in crop species. Among hierarchical methods, UPGMA (Un-weighed Paired Group Method using Arithmetic averages; Panchen 1992) is most commonly adopted method. Data analysis of genetic relationship in crop species is an important component of crop improvement programme. Many software packages are available for analyzing genetic diversity. Each data set has its own strength and constraints and there is no single or simple strategy to address effectively various complex issues related to genetic diversity and genetic relatedness.

The melon germplasm of the humid tropics of Southern India has been collected and assessed by Fergany et al. (2011). The melon germplasm of Karnataka was assessed by Manohar and Murthy (2011). The Kerala collection was not included in their study. So, present study was carried out with an objective of evaluation of morphological characters for the estimation of phenotypic divergence in melons collected from Kerala state, India and the second part of present investigation was focused on the production of the variant lines in two cultivars, cv. Arunima and cv. Mudicode of *Cucumis melo* var. *conomon* developed by Kerala Agricultural University, Trissure, India through gynogenetic haploid production.

1.3 Induction of haploids in *Cucumis melo* var. *conomon*

1.3.1 Crop improvement

Plant breeding is focused on continuously increasing crop production to meet the needs of an ever growing world population, improving food quality to ensure a long and healthy life and address the problems of global warming and environment pollution, together with the challenges of developing novel sources of bio fuels.
Breeding programs typically require several years to develop a new variety. The process begins with a cross-pollination to combine desirable parental traits. The first generation of offspring, the F₁, are heterozygous but genetically uniform. Segregation occurs during reproduction by the F₁. Segregation is the separation of homozygous chromosomes and genes from different parents at meiosis, and produce genetic variability within the F₂ population. In conventional breeding programme, pure lines are obtained after several generations of selfing and still may not be 100% homozygous (Germana 2006).

Plant breeders use traditional techniques and biotechnology to create and use novel genetic variations, aimed at selecting new elite and suitable varieties, and with improved traits to satisfy both farmers and consumers in terms of productivity, agricultural and quality performance, adaptability to marginal lands and regional environments, biotic and abiotic stress tolerance. Recent advances in biotechnology helps to enhance the efficiency and shorten the time required to reach the fixed purposes in a breeding program, as well as to address economic and ecological goals. Among these, haploid (H) and doubled haploid (DH) production through gametic embryogenesis has long been recognized as a valuable tool to help plant improvement.

1.3.2 Role of variable germplasm and doubled haploid homozygous lines in crop improvement

1.3.2.1 Production of haploids and doubled haploids

Haploidization techniques facilitate the production of pure lines from heterozygous plants in a single generation and represent significant advantages for breeders and geneticists. Haploid plants are the sporophytes with a gametophytic chromosome number and doubled haploids (DH) are haploids that have undergone chromosome duplication (Germana 2011). Haploidization and dihaploidization has
increased the efficiency in plant breeding programs in species of Solanaceae, Cucurbitaceae, Cruciferae and Gramineae families and some other crops. The advantage of doubled haploids for breeders is that homozygosity can be achieved in the first generation, whereas in breeding systems such as pedigree or backcrossing, several generations are needed to obtain high levels of homozygosity.

Haploids produced from diploid species (2n=2x), known as monoploids, contain only one set of chromosomes in the sporophytic phase (2n=x). They are smaller and exhibit a lower plant vigour compared to donor plants and are sterile due to the inability of their chromosomes to pair during meiosis. In order to propagate them through seed and to include them in breeding programs, their fertility has to be restored with spontaneous or induced chromosome doubling. The obtained DHs are homozygous at all loci and can represent a new variety (self-pollinated crops) or parental inbred line for the production of hybrid varieties (cross-pollinated crops).

Haploids from polyploid species have more than one set of chromosomes and are polyhaploids; for example dihaploids (2n=2x) from tetraploid potato (Solanum tuberosum ssp. tuberosum, 2n=4x), trihaploids (2n=3x) from heksaploid kiwifruit (Actinidia deliciosa, 2n=6x) etc. Dihaploids and trihaploids are not homozygous like doubled haploids, because they contain more than one set of chromosomes. They cannot be used as true-breeding lines but they enable the breeding of polyploid species at the diploid level and crossings with related cultivated or wild diploid species carrying genes of interest.

Doubled haploids can be produced in vivo or in vitro. Haploid embryos are produced in vivo by parthenogenesis, pseudogamy, or chromosome elimination after wide crossing. The haploid embryo is rescued, cultured, and chromosome-doubling produces doubled haploids. The in vitro methods include gynogenesis (ovary and
flower culture) and androgenesis (anther and microspore culture). Androgenesis is preferred method. Another method of producing the haploids is wide crossing. In barley, haploids can be produced by wide crossing with the related species *Hordeum bulbosum*, fertilization is affected, but during the early stages of seed development the *H. bulbosum* chromosomes are eliminated leaving a haploid embryo.

1.3.3 Techniques of haploid production

1.3.3.1 Induction of maternal haploids

*In situ* induction of maternal haploids can be initiated by pollination with pollen of the same species (e.g. maize), pollination with irradiated pollen, pollination with pollen of a wild relative (e.g. barley, potato) or unrelated species (e.g. wheat). Pollination can be followed by fertilization of the egg cell and development of a hybrid embryo, in which paternal chromosome elimination occurs in early embryogenesis or fertilization of the egg cell does not occur, and the development of the haploid embryo is triggered by pollination of polar nuclei and the development of endosperm.

1.3.3.2 Pollination with pollen of the same species

Maternal haploid induction in maize (*Zea mays* L.) is a result of legitimate crossing within one species with selected inducing genotypes (line, single cross or population). It results in a majority of regular hybrid embryos and a smaller proportion of haploid maternal embryos with normal triploid endosperms. In contrast to other induction techniques, no *in vitro* culture is needed, since kernels containing haploid embryos display a normal germination rate and lead to viable haploid seedlings. Haploid embryos can be selected early in the breeding process, based on morphological and physiological markers.
Pollination with irradiated pollen is another possibility for inducing the formation of maternal haploids using intra-specific pollination. Embryo development is stimulated by pollen germination on the stigma and growth of the pollen tube within the style, although irradiated pollen is unable to fertilize the egg cell. It has been used successfully in several species (Table 1). For most plant species, *in vitro* embryo rescue is necessary to recover haploid plants. The collection of mature seeds has only been reported for kiwifruit (Pandey *et al.* 1990; Chalak and Legave 1997), onion (Dore and Marie 1993), mandarin (Froelicher *et al.* 2007) and species of the genus *Nicotiana* (Pandey and Phung 1982). Even for the aforementioned species, *in vitro* germination of seeds enhanced the recovery of haploid plants.

*In vitro* induction of maternal haploids, so-called gynogenesis, is another pathway to the production of haploid embryos exclusively from a female gametophyte. It can be achieved with the *in vitro* culture of various un-pollinated flower parts, such as ovules, placenta attached ovules, ovaries or whole flower buds. Although gynogenetic regenerants show higher genetic stability and a lower rate of albino plants compared to androgenetic ones, gynogenesis is used mainly in plants in which other induction techniques, such as androgenesis and the pollination methods above described, have failed. Gynogenic induction using un-pollinated flower parts has been successful in several species, such as onion, sugar beet, cucumber, squash, gerbera, sunflower, wheat, barley etc. The female gametophyte is usually immature at inoculation and, in contrast to androgenesis, its development continues during *in vitro* culture, leading to a mature embryo sac (Musial *et al.* 2005). Mature embryo sacs contain several haploid cells theoretically capable of forming haploid embryos, such as the egg cell, synergids, antipodal cells and non-fused polar nuclei. However, under optimal conditions, the egg cells in most gynogenetic responsive species undergo
sporophytic development (haploid parthenogenesis) (Bohanec 2009). They can develop into haploid plants directly, avoiding the risk of gametoclonal variation, or through an intermediate callus phase.

1.3.3.3 Induction of paternal haploids

Haploid plants develop from anther culture either directly or indirectly through a callus phase. Direct androgenesis mimics zygotic embryogenesis; however, neither a suspensor nor an endosperm is present. At the globular stage of development, most of the embryos are released from the pollen cell wall (exine). They continue to develop, and after 4 to 8 weeks, the cotyledons unfold and plantlets emerge from the anthers. Direct androgenesis is primarily found among members of the tobacco (Solanaceae) and mustard (Cruciferae) families. During indirect androgenesis, the early cell division pattern is similar to that found in the zygotic embryogenic and direct androgenic pathways. After the globular stage, irregular and asynchronous divisions occur and callus is formed. This callus must then undergo organogenesis for haploid plants to be recovered. The cereals are among the species that undergo indirect androgenesis. The early cell divisions that occur in cultured anthers have been studied (Reynolds 1990). Due to its high effectiveness and applicability in numerous plant species, it has outstanding potential for plant breeding and commercial exploitation of DH. It is well established for plant breeding, genetic studies and/or induced mutations of many plant species, including barley, wheat, maize, rice, triticale, rye, tobacco, rapeseed, other plants from *Brassica* and other genera. The method relies on the ability of microspores and immature pollen grains to convert their developmental pathway from gametophytic (leading to mature pollen grain) to sporophytic, resulting in cell division at a haploid level followed by formation of calluses or embryos.
Anther culture was the first discovered haploid inducing technique of which efficiency was sufficient for plant breeding purposes (Maluszynski et al. 2003). It is still widely used, although isolated microspore culture is an improved alternative. During isolation of microspores, the anther wall tissues are removed, thus preventing interference of maternal sporophytic tissue during pollen embryogenesis and regeneration from somatic tissue. Moreover, basic research of haploid embryogenesis can be performed directly at the cellular, physiological, biochemical and molecular levels. The application of suitable physiochemical factors promotes a stress response, which arrests the microspores or young pollen grains in their gametophytic pathway. Their development is triggered through embryogenesis by promoting cell divisions and the formation of multicellular structures contained by the exine wall. Finally, the embryo-like structures are released from the exine wall (Maraschin et al. 2005).

1.3.3.4 Techniques of conversion of haploids into doubled haploids (Chromosome doubling)

Following regeneration, haploid plants obtained from either anther or ovule culture may grow normally under in vitro conditions or can even be acclimatized to form vital mature plants. Such plants often express reduced vigour but in some crops such as onion, even haploid plants might grow vigorously. At the flowering stage, haploid plants form inflorescences with evident malformations. Due to the absence of one set of homologous chromosomes, meiosis cannot occur, so there is no seed set. Duplication of the chromosome complement is therefore necessary. Various methods have been applied over several decades and are still in development. The most frequently used application is treatment with anti-microtubule drugs, such as colchicine (originally extracted from autumn crocus Colchicum autumnale), which inhibits microtubule polymerization by binding to tubulin. Although colchicine is
highly toxic, used at a millimolar concentration and known to be more efficient in animal than in plant tissues, it is still the most widely used chromosome doubling agent. Other options are oryzalin, amiprophosmethyl (APM), trifluralin and pronamide, all of which are used as herbicides and are effective in micromolar concentrations. Colchicine application on anther culture medium, for instance, showed a significant increase in embryo formation and green plant regeneration in wheat (Islam 2010). More often, duplication treatments are applied after regeneration at either embryo, shoot or plantlet level. Similarly, treatments of gynogenically derived embryos with colchicine have also been found to be appropriate. The treatment of plants at later developmental stages has the advantage that only already tested haploid regenerants are treated either in vitro (for instance at the shoot culture stage) or in vivo following acclimatization.

1.3.3.5 Identification of haploids: ploidy level determination and homozygosity testing

Several direct and indirect approaches are available for determining the ploidy level of regenerated plants. Indirect approaches are based on comparisons between regenerated and donor plants in terms of plant morphology (plant height, leaf dimensions and flower morphology), plant vigour and fertility, number of chloroplasts and their size in stomatal guard cells. They are fairly unreliable and subject to environmental effects but do not require costly equipment. Direct methods for ploidy determination are more robust and reliable and include conventional cytological techniques, such as counting the chromosome number in root tip cells and measurement of DNA content using flow cytometry. The latter provides a rapid and simple option for large-scale ploidy determination as early as in the in vitro culturing phase. It also enables detection of mixoploid regenerants (having cells with different ploidy) and the determination of their proportion. Haploids of higher plants can be
distinguished from their diploid equivalents in many ways. They are smaller in appearance, partly because of their smaller cell size which is directly related to their ploidy level. Haploid status of the plant can be confirmed by various methods which include direct measurements of the chromosome number using conventional cytological techniques and measurements of the DNA content using flow cytometry (Bohanec et al. 2003), and indirect methods based on guard cell and plastid dimensions (Lee and Hecht 1975; Yuan et al. 2009). At a genetic level, although the detection of homozygosity in doubled haploids using isozyme-based techniques (Liu and Douches 1993) is still practised (Bouvier et al. 2002; Toppino et al. 2008), these have now largely been replaced by methods based on DNA markers (Chani et al. 2000; Eimert et al. 2003; Belicuas et al. 2007; Diao et al. 2009). Cytological studies of cucurbits have lagged behind other crop plants primarily because their chromosomes are relatively small and stain poorly (Wang et al. 2007). Alternatively, more simple, reliable, rapid and cost effective methods are available. Measurements widely used for the identification of ploidy levels are pollen grain diameter and stomatal length. The number of plastids in guard cells is also often used as a selection criterion in ploidy identification (Kurtar et al. 2002, 2009).

_Cucumis melo_ var. _conomon_ is considered to be originated from wild melon (var. _agrestis_) in China (Walters 1989). It is cultivated in India, China, Japan, Korea and Southeast Asia. _Cucumis melo_ var. _conomon_ is popularly known as ‘kani vellari’ in Kerala state, India. Kerala Agricultural University has developed three kani vellari cultivars namely Arunima (accession CAKAU-01), Mudicode (accession CMKAU-02) and Saubhagya (accession CSKAU-03). All these three cultivars are high yielding. So, an attempt is made for the production of haploids in _Cucumis melo_ var. _conomon_ accession CAKAU-01 and _Cucumis melo_ var. _conomon_ accession CMKAU-
So, the present work was carried out with the following objectives,

1. To study the influence of various growth regulators individually and in combinations on induction of haploids by means of ovary slice and ovule culture.

2. To test the effect of various doses of γ-irradiation on induction of gynogenesis in melon cultivars.
Table 1. *In vitro* haploid production via parthenogenesis in some plants

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Barley</td>
<td>Powell <em>et al.</em> 1983</td>
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<tr>
<td>Blackberry</td>
<td>Naess <em>et al.</em> 1998</td>
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<tr>
<td>Kiwifruit</td>
<td>Pandey <em>et al.</em> 1990; Chalak and Legave 1997; Musial and Przywara 1998, 1999</td>
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<tr>
<td>Mandarin</td>
<td>Froelicher <em>et al.</em> 2007; Aleza <em>et al.</em> 2009</td>
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<tr>
<td>Onion</td>
<td>Dore and Marie 1993</td>
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<tr>
<td>Pear</td>
<td>Bouvier <em>et al.</em> 1993</td>
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<tr>
<td>Petunia</td>
<td>Raquin 1985</td>
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<tr>
<td>Rose</td>
<td>Meynet <em>et al.</em> 1994</td>
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<tr>
<td>Squash</td>
<td>Kurtar <em>et al.</em> 2002</td>
</tr>
<tr>
<td>Sunflower</td>
<td>Todorova <em>et al.</em> 1997</td>
</tr>
<tr>
<td>Sweet</td>
<td>Höfer and Grafe 2003</td>
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<tr>
<td>Watermelon</td>
<td>Sari <em>et al.</em> 1994</td>
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