

HAND BOOK OF
STUDIES ON *IN VITRO* MICRO PROPAGATION AND PROTOPLAST ISOLATION OF
***CUCUMIS MELO* L. VAR. UTILISSIMUS**

Dr. Mandaloju Venkateshwarlu

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**Published By:
EJBPS
2017**

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PREFACE

The aim of this book is to provide detailed “Studies on *In Vitro* Micro propagation and Protoplast isolation of *Cucumis melo* Linn. Plant tissue culture (PTC) broadly refers to cultivation of plant cells, tissues, organs, and plantlets on artificial medium under aseptic and controlled environmental conditions. PTC is as much an art as a science. It is the art of growing experimental plants, selecting a suitable plant organ or tissue to initiate cultures, cleaning, sterilization and trimming it to a suitable size, and planting it on a culture medium in right orientation while maintaining complete asepsis. It also requires an experienced and vigilant eye to select healthy and normal tissues for subculture. PTC involves a scientific approach to systematically optimize physical (nature of the substrate, pH, light, temperature and humidity), chemical (composition of the culture medium, particularly nutrients and growth regulators), biological (source, physiological status and size of the explant), and environmental (gaseous environment inside the culture vial) parameters to achieve the desired growth rate, cellular metabolism, and differentiation. The most important contribution made through PTC is the demonstration of the unique capacity of plant cells to regenerate full plants, via organogenesis or embryogenesis, irrespective of their source (root, leaf, stem, floral parts, pollen, endosperm) and ploidy level (haploid, diploid, triploid). PTC is also the best technique to exploit the cellular totipotency of plant cells for numerous practical applications, and offers technologies for crop improvement (haploid and triploid production, in vitro fertilization, hybrid embryo rescue, variant selection), clonal propagation (Micropropagation), virus elimination (shoot tip culture), germplasm conservation, production of industrial phytochemicals, and regeneration of plants from genetically manipulated cells by recombinant DNA technology (genetic engineering) or cell fusion (somatic hybridization).

The book includes seven chapters profusely illustrated with half-tone pictures and self-explanatory diagrams. Most of the chapters include relevant media compositions and protocols that should be helpful in conducting laboratory exercises. For those who are interested in further details, Suggested Further Reading is given at the end of each chapter. We hope that the readers will find it useful. Suggestions for further

improvement of the book are most welcome. During the past two decades or so research in the area of plant biotechnology has become a closed door activity because many renowned scientists have moved from public research laboratories in universities and institutions to the private industry. Consequently, detailed information on many recent developments is not readily available.

I would like to thank many friends who provided illustrations from their works and those who have helped us in completing this mammoth task. The help of Dr. M. Surekha Head Department of Botany Kakatiya University and Dr. V. Krishna Reddy BOS Department of Botany Kakatiya University in preparing some of the illustrations is gratefully acknowledged.

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CHAPTER- I**INTRODUCTION**

Development of plant tissue culture is closely linked to improvement in techniques of protoplast, cell tissue and organ culture, followed by the success achieved in regenerating whole plant from cultured plant materials. This field of experimental botany has advanced rapidly over the years due to extensive investigation into problems related to basic and applied aspects of plants. Knowledge of tissue culture has greatly contributed to our understanding of factors responsible for growth and differentiation of plant cells. Plant tissue culture is presently of great interest to molecular biologists, biotechnologists. Plant breeders and industrialists. Tissue culture methods have been employed as an important aid to conventional methods of plant improvement. They have also been used as tool for the propagation of genetically manipulated superior clone and for *ex-situ* conservation of valuable germplasm. The progress in use of cell or tissue culture in producing pathogen free plants, synthetic seeds. Secondary metabolites, Somatic hybrids and cybrids and also provide basic requirement for genetic transformation.

Plant tissue culture also offers a meaningful tool for isolating induced as well as spontaneously occurring variant cell lines for the increase of genetic diversity in crops. The use of this technique for mutant selection has many advantages over the use of other available methods. Mutation breeding in conjunction with tissue culture offers economy in space, time, labour and expenditure; Selection of mutants compared to other methods is also rapid.

The recent rapid progress in biology is due to development of new ideas and new methods of investigation. Modern biology attempts to elucidate progress which takes place at cellular level. The modern *in vitro* culture of protoplasts, cell, tissue and organ under aseptic conditions may be termed as tissue culture. The plant tissue culture was exploited both for basic and applied aspects of plants research, encompassing haploidy, mutagenesis, Somatic embryogenesis, Somaclonal variation, selection of cell lines for antibiotics metabolic analogue resistance, protoplast fusion, genetic manipulation and molecular biology.

Development of science of tissue culture is historically linked to the discovery of cell and subsequent propounding of the cell theory. Further tissue culture could be attributed to the cell doctrine, which implicitly admitted that a cell is capable of autonomy and even demonstrated the potential for totipotency-ability to regenerate an entire organism from a single plant cell.

Hanning (1904) initiated a new line of investigation involving the culture of embryogenic tissue which later became an important applied area of investigation, using *in vitro* techniques. Most promising results were obtained in the year 1908 by Simon, who achieved success in the regeneration of bulky callus and buds and roots from popular stem segments. Therefore, Simon may be credited with having established callogenesis and to some extent even micropropagation.

The concept of cellular totipotency i.e. ability to regenerate an entire organism from a single cell and plant part (Explant) proposed by Haberlandt (1902) emerged as corollary of the cell theory proposed by Schleiden (1838) and Schwann (1839). According to this concept, the genetic information necessary for the development of the entire organism is contained in all living cell. Consequently when the cells are released from the developmental controls, they are able to divide and differentiate into specific organs and whole organisms.

From the time Haberlandt presented his paper in 1902 until about 1934 hardly any progress was made in the field of plant tissue culture. Plant scientists took advantage of totipotency concept and developed media and methods for tissue culture. The nutrient media used for most of the cultures were the formulations developed in the early 1930's by White (1934). The other media widely used for plant tissue culture studies were LS medium (Linsmaier and Skoog, 1965), Blaydes (Blaydes 1966) B5 medium (Gamborg *et al.*, 1968), SH medium (Schenk and Hilderbrandt 1972) and Nitsch and Nitsch medium (1969) etc.

The first complete chemical defined nutrient medium was developed by Murashige and Skoog (1962). A standard culture medium consists of a balanced mixture of macro and micro nutrients, vitamin and plant growth regulators which play an important role in cell metabolism and cell membrane synthesis (Cocking, 1978). Beside growth substance, auxins were proved to be essential for establishing

successful culture of plant tissue due to their effects on the nucleic acid and protein metabolism (Gautheret, 1959, Rao and Swamy, 1972). Cytokinins influence cell growth by promoting nucleic acid metabolism and the synthesis of specific proteins required for cell division (Letham, 1968). Cytokinins bind to a specific receptor site in the cell to bring out organogenesis. Physical environment viz. state of medium, light, temperature, humidity besides source and size of explants are Known to play an important role in *in vitro* organogenesis (Murashige, 1974).

In 1957, Skoog and Miller showed that the differentiation of roots and shoots in tobacco pith cultures was a function of the auxin and cytokinin ratio, and the organ differentiation could be regulated by changing the relative concentration of two substances in the medium. Steward *et al.*, (1958) from Cornell University USA reported roots, then shoots and ultimately whole plants from culture of carrot roots in cell suspension. The rise of whole plant from a free cultured cell was first shown in tobacco plant by Vasil and Hilderbrandt (1965). According to Cocking (1986) the present tissue culture revolution began in 1969 when the Rockfellow Foundation sponsored a small conference on crop improvement through plant cell and tissue culture at the Villa Sebellani on Lake Como, Italy. Tissue from almost any organ can prove to be totipotent under appropriate condition. The mode of regeneration depends upon the species, the organ used and level of growth regulatory substance in the medium. The ability of shoots, roots and flowers to rise adventitiously is being exploited in tissue culture (Flick *et al.*, 1983).

Carbohydrate is demonstrated to act as energy and carbon source and as an osmoticum in shoot formation in tobacco callus (Brown *et al.*, 1979). The type of sugar supplied to the medium has a distinct effect on shoot organogenesis from cultured explants. The commonly used sugars for *in vitro* culture of explants are sucrose, glucose, fructose and maltose. The type of sugar and the concentration present in the media plays an important role in morphogenesis. Among the different carbon sources used in the finger millet, glucose and sucrose were effectively high in the induction and development of somatic embryos, while other were partially or completely inhibitory (Eapen and George, 1990). *Medicago stiva* induce the highest embryo yield in the presence of maltose (Strickland *et al.*, 1987). Increasing and decreasing levels of sugar also influence the production of secondary metabolites

(Mantell and Smith, 1983; Nigara *et al.*, 1990). Sucrose and glucose proved to be a suitable source for induction of multiple shoots and callus from *Solanum melongena* and the sugar in the medium has limited osmotic role in shoot organogenesis, apart from being an energy source (Mukharjee *et al.*, 1991).

Another interesting area of research in tissue culture is clonal propagation through which plants are multiplied using axillary buds, nodal segments and shoot meristems as explants. For rapid clonal propagation of plants, in *in vitro* method normally dormant axillary buds are induced to grow into multiple shoots by a judicious use of hormones especially cytokinins. Shoot number increased logarithmically with each subculture to give greatly enhanced multiplication rates. As this method involves only organized meristems, the plants obtained show minimum variability and allow recovery of genetically stable and true-to-type progeny (Murashige, 1974; Hu and Wang, 1983). A large number of plant species have been successfully propagated by this method includes several crop plants, ornamentals, fruit crops, woody trees and medicinal plant species (Hu and Wang, 1983; Pierik, 1987; Mascarenhas and Muralidharan, 1989; Dhawan, 1993; Huettmann and Preece, 1993; Kathiravan and Ignacimuthu, 1999).

Meristem culture has been identified as an excellent technique for rapid multiplication and producing virus-free plants. This meristem culture has been exploited successfully in herbaceous and medicinal plants (Bhojwani 1980 b; Kartha *et al.*, 1981; Wang and Hu, 1982; Mehra-palta, 1982; Bhaskarn *et al.*, 1992). Clonal propagation aims at producing exact replicates (true-to-type) of the original plant selected for its desirable characters. Considerable progress has been made in recent years in clonal propagation of many plant species (Bhojwani, 1980; Wang and Hu, 1982; Yadav *et al.*, 1990; Malathy and Pai, 1998; Bais *et al.*, 2000). Thus, micropropagation has several advantages over traditional methods of propagation for instance higher multiplication rates, lower requirements of space, propagation throughout the year and greater degree of control over chemical and physical environmental factors. Micropropagation provides a means of germplasm storage for maintenance of disease-free stock (Wilkins and Dodds, 1983; Withers, 1989). Fairly a large number of plant species has been propagated by *in vitro* techniques (Murashige, 1974; Narayana Swamy, 1977; Vasil and Vasil, 1980). For micropropagation of

tomato, direct regeneration from different explants via callogenesis of the explants has been reported. When callus of most *L.esculentum* cultivars is repeatedly transferred on to the same medium, the ability to regenerate plant from such *calli* upon transfer to cytokinin containing media is very limited (Morgan and Cocking, 1982). Cell suspension culture studies were carried out in tomato by Meredith (1979) and Evans and Gamborg (1982).

Mass propagation has been attempted through the use of various techniques, including shoot tip culture (Novak and Maskova, 1979; Padlikhikh and Yarmishin, 1990; Fari *et al.*, 1992; Izadpanah and Khosh Khui, 1992), Somatic embryogenesis (Chen and Adachi, 1994; Gill *et al.*, 1995; Kaparakis and Alderson, 2002), direct organogenesis from intact explants (El-Farash *et al.*, 1993) or protoplast culture (Muhlbach, 1980; Sink *et al.*, 1986). In tomato, adventitious shoot regeneration can be achieved either directly (Dwivedi *et al.*, 1990) or indirectly through an intermediate callus phase (Bekhi and Lesley, 1980; Geetha *et al.*, 1998). Indeed both callus and shoots may be produced together (Bhatia, 2003).

Callus formation from explant tissue involves the development of progressively more random planes of cell division less frequent specialization of cells and loss of organized structure (Thorpe, 1980). Cell suspensions are initiated by transfer of callus pieces into conical flasks with liquid medium, which are then placed on a gyratory-shaker to provide aeration to the cell (Rashid, 1988). As new calls are formed, they are dispersed into the liquid medium and become clusters and aggregates. Cells in suspensions can exhibit much higher rates of cell division than do cells in callus culture. Thus cell suspensions offer advantages when rapid cell division or many cell generations are desired or when a more uniform treatment application is required such as during cell selection procedures. Muir (1953) succeeded in mechanically picking single cells from callus in suspension cultures. The success achieved thus for in single cell cloning created great enthusiasm for research on the prospects of raising whole plants from a single cell. An important breakthrough was achieved in 1965 when Vasil and Hildebrandt observed that colonies arising from cloning of isolated cells of the hybrid *Nicotiana glutnosa* and *Nicotiana tabacum* regenerated plantlets.

Direct regeneration of adventitious shoots on explant placed on media with a high cytokinin/auxin ratio has been studied by many others (Padmanadhan *et al.*, 1974; Kartha *et al.*, 1976). In general, the observations of Kartha that media with benzyladenine or zeatin at concentration of (1.0-5.0 mg/L) combined with indoleacetic acid (0.1-0.5 mg/L) are most effective for shoot regeneration has been confirmed. Shoot formation by *L. esculentum* genotypes is often accompanied by callus formation on the explant. However on *L. peruvianum* explants, shoots often appear without the simultaneous appearance of callus tissue. Also within *L. esculentum* genotypes, genetic differences for shoot forming capacity have been described (Ohki *et al.*, 1978; Tan *et al.*, 1987 b;). four major cytokinins viz. Zeatin, BAP, and, Kinetin can be used either separately or in combination with auxins for organogenesis in Tomato. Auxins proved to be essential for establishing successful culture of plant tissues due to their effects on nucleic acid and protein metabolism (Gautheret, 1959; Rao and Swamy, 1972). Cytokinins influence cell growth by promoting nucleic acid metabolism and synthesis of specific protein required for cell division (Letham, 1968) physical environment viz., state of medium, light, temperature, humidity besides sources and size of explants are Known to play an important role in *in vitro* organogenesis (Murashige, 1974).

Cucurbitaceae Family

Cucurbitaceae is a major family for economically important species, particularly those with edible fruit such as cucumber, melon, gourds, pumpkin, zucchini, luffa and squashes. The family comprises 90 genera, 700 species, primarily in tropical and subtropical regions with a few representatives in temperate and cooler climates.

The Cucurbitaceae are mostly prostrate or climbing, herbaceous annuals with characteristically coiled tendrils and five-angled stems. Some of the major edible species domesticated as food plants covered in this volume include *Citrullus lanatus* (watermelon), *Cucumis sativus* (cucumber), *Cucumis melo* (honey-dew melon, musk melon, cantaloupe), *Cucumis metuliferus* (African horned cucumber), *Cucurbita moschata* (pumpkin, butternut squash, squash), *Cucurbit pepo* (squash, pumpkin, zucchini), *Cucurbita ficifolia* (fig leaf gourd), *Momordica charantia* (bitter melon), spiny bitter gourd (*Momordica cochinchinensis*), *Sechium edule* (chayote), *Luffa acutangula*, *Luffa aegyptiaca* (luffa), *Lagenaria siceraria* (bottle gourd), *Benincasa*

hispida (wax gourd), *Trichosanthes cucumerina* (snake gourd), *Coccinia grandis* (ivy gourd) and *Siraitia grosvenorii* (Arhat fruit, Buddha fruit, Luo han guo). Of these, three are more well known for their medicinal value: *Momordica charantia* (bitter melon), *Momordica cochinchinensis* (gac) and *Siraitia grosvenorii* (luo han guo). Luo Han Guo is rich in mogrosides, a group of triterpene glycosides. Mogroside V, the major constituent in the fruit extract is more than 400 times as sweet as sucrose and is extremely low in calories. This fruit and mogroside V will be valuable as an advanced food ingredient and a natural sweetener substitute for sucrose in the near future, and might be valuable as a source of the chemopreventive agents in chemical carcinogenesis (Konoshima and Takasaki 2002). Gac fruit contains by far the highest content of β -carotene and lycopene of any known fruit or vegetable. The concentration of lycopene in the gac seed membrane has been reported to be about ten-times higher than that in known lycopene-rich fruit and vegetables. Gac also has other carotenoids like zeaxanthin and b - cryptoxanthin. Research has confirmed that the b-carotene (vitamin A) in gac fruit is highly bioavailable. Bitter melon contains bioactive compounds such as momordicins, cucubitacins, glycosides, terpenoid compounds and cytotoxic ribosome-inactivating proteins (RIPs). Together these compounds have shown various pharmacological and biological activities including antidiabetic, antiobesity, anticancer, antimicrobial, anti-HIV, antifeedant and antioviposition activities.

Origin/Distribution

The oriental, pickling melon (*C. melo* ssp. *Melo* var. *Utilissimus*) is considered to be the most ancient form of melon domesticated in China (Jeffrey 1980; Walter 1989). It is also held that it had originated from wild melon (var. *Utilissimus*) in China (Walters 1989). Oriental melon is cultivated in Asia – India, China, Japan, Korea and Southeast Asia. Molecular polymorphism studies suggested that *Cucumis melo* vars. *makuwa* and *conomon* are derived from small seed type melon in east India (Kato et al., 2002). Vars. *makuwa* and *conomon* are supposed to have originated in India, the secondary center of genetic diversity of cultivated melon, and were introduced into China from the west via the Silk Road. (Kitamura 1950).

Agroecology

There is a wide array of cool temperate and warm tropical varieties of cultivars of oriental pickling melon. Generally, the crop is frost intolerant, abhors a very wet climate and is found from near sea level to 1,300 m elevation. It is not suited for areas with heavy rain of more than 1,500 mm per year unless grown in protected glasshouses. It grows in full sun in the fields, farms, home gardens and glasshouses. It performs best in well aerated, well-drained fertile, water retentive, loamy soils rich in organic matter, with a pH range of 5.5–6.8. It is intolerant of heavy clays and waterlogged conditions. It is quite drought tolerant but needs adequate water for good growth and development. It can grow sprawling on the ground or be trained up trellises.

Edible Plant Parts and Uses

Both immature and mature fruit is eaten raw or cooked, pickled or made into sweets and juice. Katsura-uri Japanese pickling melon (*Cucumis melo* var. *Utilissimus*) or Katsura-uri is an heirloom vegetable in Kyoto, Japan (Nakamura *et. al.*, 2008a, b). It is harvest half-ripe and used primarily in traditional Japanese cuisine. The fully ripened fruit of Katsura-uri has rarely been used in Japanese eating habits, because half-ripe fruit is utilized for making pickles, but the fully ripened fruit is no longer valuable for pickles due to the flesh being too soft. Nakamura *et. al.*, (2008a, b) reported that fully ripe Katsura-uri has potential for use in non-pickling products as it has the unique properties of not being sweet and the possession of a strong melon-like odour and has demonstrated health benefits. In south East Asia, both immature and ripe fruits can be made into pickles, *acar*, chutney and sweets (candies, *dodol*). Ripe fruit is relished as a refreshing drink when blended and mixed with sugar or honey and ice as is popularly done with the Timun Suri cultivar during the Ramadhan fasting month in Indonesia. In Indonesia, fruits of timun Krai cultivar is used raw or often steamed in a special Surabaya dish called rujuk Cingur (a special mixed fruit salad with savory, spicy peanut sauce). Young fruits are eaten raw as lalab or serve as sayur in Indonesia. The fruit can be finely diced and used as a seasoning in salads and soups. The fruits are also used in curries and stews. In Thailand, mature fruit is eaten fresh; young fruit is occasionally harvested and eaten as vegetable like cucumber either raw or cooked in sour curry; ripe fruit i.e. consumed as Thai traditional dessert. The seed is edible raw or cooked and yields a rich nutty flavored oil.

Morphological Characters

The plant is a trailing, prostrate, or climbing, much-branched, annual, monoecious, herb with angular, hirsute stem having a diameter of 5.5– 8.5 mm. Tendrils simple, unbranched. Leaves are alternate, simple, sub orbicular to ovate, shallowly 5–7 lobed, finely dentate margin and deeply cordate base, dark green, 7–18 cm long by 7–15 cm wide, 8–15 cm across, both surfaces covered with villous hairs. Flowers and monoecious, yellowish on 5–3 cm long penduncle. Male flowers fasciculated 2–4 flowered, 26–44 mm across, calyx campanulate, sepals subulate, corolla campanulate, 5-parted, yellow, stamens 3. Perfect flowers solitary with a short sericeous (short, adpressed hairs) ovary inferior, 3–5 carpels. Fruit, polymorphous, ellipsoid, oval-oblong, pyriform, globose, elongate pepo 11–30 cm long, smooth, glabrescent, colour variable white, yellow, golden-yellow, yellowish- white, yellowish with white longitudinal stripes, green, green with dark green longitudinal stripes, edible insipid to mildly sweet flesh, white, orange, yellow or pink and numerous white, elliptic, small (<8 mm seeds), flattened seeds.

Nutritive/Medicinal Properties

The sucrose content of Huangjingua (*Cucumis melo* var. *makuwa* Makino) was found to increase with fruit maturity but the sucrose content remained unchanged in Yuegua (*Cucumis melo* var. *conomon* Makino) (Zhang and Li 2005). In Huangjingua, both sucrose and total sugar gradients were observed, rising from mesocarp adjacent to pedicel, middle part of mesocarp, and up to mesocarp adjacent to the umbilicus. In terms of sweetness index, fructose was found to be the major contributor to sugar accumulation in both varieties. Also, Huangjingua could be comparatively considered as a high-sucrose accumulator and Yuegua a minor-sucrose accumulator. 3-methylthiopropionic acid ethyl ester, an odour producing compound, isolated from fully ripened Katsura-uri Japanese pickling melon fruit (*Cucumis melo* var. *conomon*) was found to have antimutagenic and anticarcinogenic activities (Nakamura *et. al.*, 2008a, b). It was found to be an antimutagen in *Escherichia coli* mutagenicity assay. It also demonstrated in-vivo animal cancer prevention, and induced differentiation, a chemotherapeutic strategy, in an in-vitro human colon-cancer cell system. Katsura oriental pickling melon showed higher bio-antimutagenicity and yield in the n-hexane, chloroform and ethyl acetate fractions than other common and traditional vegetables tested (Nakamura *et. al.*, 1998). In more recent studies, Nakamura *et. al.*,

(2010) identified six fragrant ingredients in fully-ripened Katsura-uri (Japanese pickling melon, *Cucumis melo* var. *conomon*). Four of them were sulfur-containing compounds [methylthioacetic acid ethyl ester (MTAE), acetic acid 2-methylthio ethyl ester (AMTE), 3-methylthiopropionic acid ethyl ester (MTPE), and acetic acid 3-methylthio propyl ester (AMTP)]; and the others were benzyl acetate and eugenol. MTAE and AMTP were found to possess antimutagenic activity as determined by their ability to inhibit the UV-induced mutation in repair-proficient *E. coli* B/r WP2. MTAE and MTPE (esters with thiocarbonic acid and alkyl alcohol) induced the differentiation of human colon cancer cells (RCM-1 cells), but AMTE and AMTP (esters with carbonic acid and thioalkyl alcohol) did not. AMTE, MTPE, AMTP, and eugenol had higher oxygen radical absorbing capacity than the antioxidative vitamin C. The quantity of MTPE, AMTP and eugenol increased 49-fold, >1,175-fold and 11-fold, respectively, in the fully-ripened fruit as compared to the half-ripened fruit. Three trypsin inhibitors (CMCTI-I, II, and III) were isolated from oriental pickling melon (*Cucumis melo* var. *conomon* Makino) seeds (Nishino *et. al.*, 1992). All three inhibitors could inhibit lysyl endopeptidase and trypsin at the enzyme-inhibitor ratio of 1:1. In folkloric ethnomedicine, the fruit has been used as a cooling cleanser or moisturiser for the skin and also used for burns, scald and abrasions. The fruit is also stomachic. The seed is digestive, antitussive, febrifuge and anthelmintic. When used as a vermifuge, the powdered seeds are made into an emulsion and consumed. The root is emetic and diuretic and the flowers are also emetic and expectorant.

Other Uses

Melons like cantaloupes can be used as animal feed. Respectively. Makino (1928) and Kitamura (1950) divided var. *makuwa* into eight different forms. Based on the length of ovary hair, Pitrat *et. al.*, (2000) divided *C. melo* into two ssp. *agrestis* and *melo*, and vars. *Conomon*, *makuwa*, *chinensis*, *momordica* and *acidulus* were grouped under ssp. *Agrestis*. According to Stepansky *et. al.*, (1999) var. *conomon* is closely related to African and Asian vars. *agrestis*, *momordica* and *chito*, rather than to vars. *flexuosus* and *inodorus*. Rahayu and Hartana (2002) investigated variations on morphology, anatomy, and isozyme banding patterns of *Cucumis sativus* and *Cucumis melo* by employing 18 populations of cultivars from 17 regions in Java. On the basis of those features, cultivars of *Cucumis* populations in Java were grouped into 9 different cultivars. Three of them were timun wuku, timun saloyo, and timun jepang, which

belonged to *C. sativus*. The remaining cultivars, namely krai rundu, timun suri, krai kapasan, bhalungkak, blewah, and melon, belonged to *C. melo*. The grouping based on morphological and anatomical characters showed that timun saloyo was closely related to timun jepang compared to timun wuku. Timun suri was closely related to bhalungkak, while blewah was closely related to melon compared to other cultivars.

Tissue culture work in Cucurbits is very scanty, especially in *Cucumis melo* var *Utilissimus* with regard to micro propagation in vitro mutagenesis and isolation of Somocloners. But there are few reports in *Cucumis* and his related species put forth by the researchers. Shoot bud differentiation in long term callus cultures of *Memordia* and *Cucumis* was reported by Holder and Gadgil (1982), who induced shoot bud and embryoids from callus cultures of both the above mentioned genera. Regeneration from callus was obtained on MS medium supplemented with NAA and BAP or IAA and BAP; but the frequency of regeneration was low and the process of plantlet formation was slow in Cold cultures. Schroeder (1968) reported the formation of embryoids in 2 ½ years old. Cultures of pericarp tissue of the fruit of the genera *Cucumis Pepo* when the tissue was placed on MS medium containing IAA.

Jelaska (1974) reported the embryoid formation in *Cucumis melo* from by Hypocotyl segments. The presence of tetracyclic triteapenoids (Cucurbitacins) that are distributed in the plant members of the family Cucurbitaceae was noticed by Lavie and Glotter (1971). According to Guha and Sen (1975) the role of the Cucurbitacins on plant growth and differentiation is not known. The inhibition of embryoid initiation and suppression of further development of embryoids (which are already formed in callus cultures) in carrot cultures was due to the addition of Cucurbitacins and is reported by Shirotria and Rangaswamy(1980).

Morphogenesis in *Cucumis sativus*, *Momordica charantia* and *Luffa acutangula* by the effect of different combination of IBA, 2,4-D and coconut milk has been reported by Halder and Gadgil (1981), but could only obtain just green spot; while in *Momordica charantia* and *Luffa acutangula*, only a few roots were observed at higher concentrations of NAA, but not the aerial out growths. Malepszys *et. al.*, (1982) studied somatic embryogenesis in cucumber (*Cucumis sativus*) and established the culture conditions for cell suspensions (Malepszy and Solarek 1986).

Nadolska, *et al.*, (1987) put forth histological analysis of leaf explants of *C. sativus* when cultured on media with 2,4-D or 2,4,5-T. Growth and development of a few members of Cucarbitaceae *in vitro* from excised ovaries were reported by Nitsch (1951) while Noel *et.al.*, (1992) studied the somatic embryogenesis from cotyledons in *Cucurbita pepo*. Orezyk and Malepszy, (1985) reported the stabilizing effect of amino acid (Glycine) on leaf protoplasts of *C. sativus*. Orezyk *et al.*, (1988) gave the procedure to regenerate wild *C. sativus*. A study relating to regenerate through somatic embryogenesis and organogenesis on *C. sativus*, *C.harwictiri* and *C.metaliferus* was given by Punja *et al.*, (1990).

A technique for the production of de novo of multiple shoots was established by Gambley and Dodd, (1990) in *C. sativus* by the action of cytokining the medium with various concentrations of cytokinins i.e., BAP, Kinetin and IAA. By removing the axillary buds, it was possible to active a yield of 23 shoots per cotyledon. The yield increased to 50 shoots per cotyledon by cutting the basal region of cotyledon into small pieces prior to culturing.

Ziv and Gadasi (1986) established plant regeneration methods and brought about enhancement of embryogenesis in cucumber (*C. sativus*). Several factors were also tested to modify the developmental pattern of embryoids *in vitro* such as osmolarity, reduced carbon source, growth regulators and culture conditions (Amiratio 1977; Amirato 1983; Johanson *et al.*, 1982).

A procedure for the regeneration of whole plants from cucumber (*C. sativus*) through embryogenesis from cell suspension cultures was established by Chee and Tricoli (1988). They initiated embryogenic callus from the primary leaf explants of 14-17 day old plants. Sixty to seventy percent of the embryos were pre washed with activated charcoal, and yet, germinated into plantlets with normal morphology. According to Fariet *et al* (1987) activated charcoal can remove the metabolites that inhibit the normal morphogenesis in plant tissue cultures. Johanson *et al.*, (1982) observed in the anther cultures of *Anemone canadensis* that embryogenesis was enhanced by charcoal activity.

Organogenesis and embryogenesis in *Cucumis* by the effect of auxin concentrations was studied by Tabei *et al.*, (1991) explants such as cotyledons of mature seeds,

hypocotyls of seedling, leaves and petioles of young plantlets could produce adventitious shoots and somatic embryos Blackman and Reynolds (1982) reported plant regeneration through adventitious shoot formation in *Cucumis* plant regeneration via somatic embryogenesis was reported by Oridate and Oosawa (1986). Formation of adventitious shoots and somatic embryoids from callus cultures of *Cucumis* on hormone free medium was reported by Rajasekharan *et al.*, (1983).

A technique for *in vitro* plant regeneration in *Cucumis* was established by Trulson and Shahin (1986). Somatic embryogenesis was induced on a medium consisting of Murashege and skoog salts supplemented with 1.0mg/L 2,4-D, 1.0 mg/L NAA and 0.5 mg/L BAP. Embryos matured on the same medium devoid of 2,4-D, Embryo9s developed into normal plants when placed on hormone free MS medium. Kathal *et al.*, (1988) reported the regenerations of plants from explants of *Cucumis* genus on MS basal salts supplemented with BAP and 2,4-D. Attridge *et al.*, (1984) reported the photo control effect on hypocotyls elongation of *Cucumis sativus* grown under different light conditions. Plant regeneration procedure from callus cultures of *Cucumis* genus was put forth by Moreno *et al.*, (1985). Punja *et al.*, (1990) obtained optimal protoplast yields from cotyledons and leaves of *C. sativus* and regenerated on MS basal salts fortified with 2,4-D and BAP. *In vitro* plant regeneration from leaf explants of *C. melo* was reported by Dirks and Buggenum (1989) on MS basal salts with 1.0 mg/L BAP.

Wehner and Locky (1981) reported the formation of adventitious shoot and root formation *in vitro* from explants of *Cucumis sativus* when placed on MS basal medium along with 1.0 mg/L BAP and 1.0 mg/L NAA and 3% sucrose. Petapczykowa *et al.*, (1972) obtained shoots from callus cultures at stem explants of *Luffa cylindrical* and *Trichosanthes anguina* were made by Rangaswamy and Shivanna (1975).

There are very few reports in *Cucumis melo* protoplast derived calli or musk melon, the development of embryo like structures was strongly dependent on light intensity was reported by Colijn – Hooymans *et al.*, (1988 a,b). Somatic embryogenesis in *Cucumis melo* was reported by number of workers in number variants (Young, *et al.*, 1983; Moreno *et al.*, 1985b; Oridate and Oosawa 1986; Branchard *et al.*, 1988; Kageyama *et al.*, 1990; Punja *et al.*, 1990a; kageyama *et al.*, 1991; Tabei *et al.*, 1991;

Oridate *et al.*, 1992). Attempted *in vitro* plant regeneration studies in the genus *Cucumis*. Moreno *et al.*, (1985b) reported somatic embryogenesis and organogenesis from protoplast derived cultures of musk melon (*Cucumis melo* L).

This variety is very popular in Mahrastra is cultivated nearly throughout upper India especially in U.P and Punjab. This variety resembles cucumber and is used as a vegetable. The fruits are slender and elongated, the length varying from a few inches to about 3ft. they are pale or dark green in colour, smooth or ridged, with soft downy hairs covering the skin when tender.

Are smaller than those of the musk melon. A number of promising types have been evolved, among which Lm.4 is considered to be the best. A large number of varieties are known; some are sweet and some sour or bitter. This variety is cultivated both as a hot weather crop and as a rainy season crop. It grows on any kind of soil, but thrives best on well-manured rich lomy soils with abundant water supply.

The fruits are ready for harvest when the plants are 3-4 months old. The yield is 80-100 per acre. The tender fruits are eaten raw or with salt in salads. They are also pickled and cooked into curries. The fruits form an important article or food during the hot weather; they are nourishing and useful in quenching thirst. The seeds are small and edible, and are used in confectionery, a refreshing drink in prepared from ground kernels. The seeds are cooling, diuretic and are said to be beneficial in painful micturition and suppression of urine. The members of Cucurbitaceac family play a vital role in our agriculture economy as it forms an important diet after cereals and legumes. The primary aim of this study has been to gain some knowledge about there genotypic differences for callus initiation and high frequency plant regeneration from long term callus cultures of *Cucumis melo* var. Utilissimus.

In view of its used as vegetable, a protocol for *in vitro* propagation would be of considerable of disease free plants with other agronomically important traits of added will be of advantage of commercialization of the species. Hence, the present investigations have been carried out with the aim to achieve the following objectives.

- To establish the *in vitro* seed germination of *Cucumis melo* var. Utilissimus.
- To establish the protocol for callus induction from different explants viz. cotyledon, hypocotyls and leaf etc.

- To standardise the hormonal concentration for callus mediated regeneration.
- To achieve the direct regeneration in different explants by modulating the concentration and combination of auxins and cytokinins and also plantlet establishment.
- To develop the protocol for micro propagation using shoot tip and nodal cultures
- To optimize the protocol for *in vitro* rooting.
- Acclimatization and hardening of *in vitro* developed plantlets.
- Transfer of *in vitro* regenerated plants into field.

CHAPTER- II**TISSUE CULTURE TECHNIQUES****Glassware**

Glassware used in all the experiments was procured from —Borosill, India. Test tubes (25 mm x 150 mm), glass bottles (70 mm x 125 mm), petri dishes (85 mm x 15 mm), conical flasks (100, 250, 500 and 1000 ml capacity) and pipettes (1, 2, 5, 10 and 25 ml capacity) were used during the course of study.

Preparation of Glassware

Glassware used for all the experiments was cleaned by boiling in a saturated solution of Sodium bicarbonate for 1h followed by repeated washing in tap water. Thereafter, it was immersed in 30% nitric acid solution for 30 min followed by repeated washing in tap water. Ashed glassware was thereafter rinsed with distilled water and dried at room temperature (ambient temperature) or in an oven at 200°C. Test tubes and flasks were plugged with absorbent cotton (Seasons Healthcare Ltd, Andhra Pradesh, India). Pipettes and petridishes were wrapped in brown paper and then sterilized in autoclavable polypropylene bag. Autoclaving of the glassware and above items was done at 121°C, 15 lb psi for 1 h.

Plastic ware

Sterile disposable filter sterilization units and petridishes (35 mm, 55 mm and 85 mm diameter) were procured from —Laxbroll, India. Eppendorf tubes (1.5 ml and 2 ml capacity), microtips (0-200 ml and 200-1000 ml capacity) were also obtained from —Laxbroll and —Tarsons, India. Wide bore microtips (0-200 ml) were procured from —Sigmall, USA.

Chemicals

All chemicals used in the tissue culture study were of analytical grade and were obtained from —Qualigens, —S.D fine chemicals or —Hi-medial, India. The chemicals used in molecular biology study were obtained from —Sigma Chemical Co., USA. Growth regulators, vitamins, antibiotics (except Cefotaxime) and Phytigel were also obtained from —Sigma Chemical Co., USA. Cefotaxime was procured from Russel India Ltd. Bombay, India. Sucrose, glucose, gelling agent and agar-agar

were obtained from —Qualigens[®] and—Hi-Media[®]. Bacto-Agar for microbial work was obtained from —DIFCO[®] laboratories, USA.

Preparation of culture media

Double distilled water was used for preparation of the media used in the study. After addition of all macro- and micro-nutrients, vitamins, growth regulators and other necessary carbohydrate source like sucrose or glucose, the pH of the media was adjusted to 5.8 before autoclaving using 0.1N NaOH or HCl. Volume was made and gelling agent was added as per requirement. The medium was steamed to melt the gelling agent. Melted medium was then dispensed into test tubes, flasks and thereafter sterilized by autoclaving at 121°C at 15 lb psi for 20 min. Thermo labile growth regulators and antibiotics were filter sterilized through a millipore membrane (0.22mm or 0.45mm pore size). These were added to autoclaved medium before dispensing. Compositions of Murashige and Skoog's (MS) macro-, micro elements and vitamins used in the present study are in (Table -1).

Macronutrients

The macronutrients provide the six major elements- Nitrogen (N), Phosphorus (P), Potassium (K), Calcium (Ca), Magnesium (Mg), and Sulfur (S)-required for plant cell or tissue growth. The optimum concentration of each nutrient for achieving maximum growth rates varies considerably among species.

Culture media should contain at least 25-60 mM of inorganic nitrogen for adequate plant cell growth. Plant cells may grow on nitrates alone, but considerably better results are obtained when the medium contains both a nitrate and ammonium nitrogen source. Certain species require ammonium or another source of reduced nitrogen for cell growth to occur. Nitrates are usually supplied in the range of 25-20 mM; typical ammonium concentrations range between 2 and 20 mM. However, ammonium concentrations in excess of 8 mM may be deleterious to cell growth of certain species. Cells can grow on a culture medium containing ammonium as the sole nitrogen source if one or more of the TCA cycle acids (e.g., citrate, succinate, or malate) are also included in the culture medium at concentrations of approximately 10 mM. When nitrate and ammonium sources of nitrogen are utilized together in the culture medium, the ammonium ions will be utilized together in the culture medium, the ammonium ions will be utilized more rapidly and before the nitrate ions.

Potassium is required for cell growth of most plant species. Most media contain K, in the nitrate or chloride form, at concentrations of 20-30 mM. The optimum concentrations of P, Mg, S, and Ca range from 1-3 mM when all other requirements for cell growth are satisfied. Higher concentrations of these nutrients may be required if deficiencies in other nutrients exist.

Micronutrients

The essential micronutrients for plant cell and tissue growth include Iron (Fe), Manganese (Mn), Zinc (Zn), Boron (B), Copper (Cu), and Molybdenum (Mo). Chelated forms of iron and Zinc are commonly used in preparing culture media. Iron may be the most critical of all the micronutrients. Iron citrate and tartrate may be used in culture media, but these compounds are difficult to dissolve and frequently precipitate after media are prepared. Murashige and Skoog used an Ethylene Diamine Tetra Acetic acid (EDTA)-iron chelate to bypass this problem.

Cobalt (Co) and Iodine (I) may also be added to certain media, but strict cell growth requirements for these elements have not been established. Sodium (Na) and Chlorine (Cl) are also used in some media but are not essential for cell growth. Copper and Cobalt are normally added to culture media at concentrations of 0.1 μM , Fe and Mo at 1 μM , I at 5 μM , Zn at 5-30 μM , Mn at 20-90 μM , and B at 25-100 μM .

Carbon and Energy Source

The preferred carbohydrate in plant cell culture media is sucrose. Glucose and fructose may be substituted in some cases, glucose being as effective as sucrose and fructose being somewhat less effective. Other carbohydrates that have been tested include lactose, galactose, raffinose, maltose, and starch. Sucrose concentrations of culture media normally range between 2 and 3 percent. Use of autoclaved fructose can be detrimental to cell growth.

Carbohydrates must be supplied to the culture medium because few plant cell lines have been isolated that are fully autotrophic, e.g., capable of supplying their own carbohydrate needs by CO_2 assimilation during photosynthesis.

Vitamins

Normal plants synthesize the vitamins required for their growth and development. Vitamins are required by plants as catalysts in various metabolic processes. When plant cells and tissues are grown in vitro, some vitamins may become limiting factors for cell growth. The vitamins most frequently used in cell and tissue culture media include thiamin (B1), nicotinic acid, pyridoxine (B6), and myo-inositol. Thiamin is the one vitamin that is basically required by all cells for growth. Thiamin is normally used at concentrations ranging from 0.1 to 10.0 mg/liter. Nicotinic acid and pyridoxine are often added to culture media but are not essential for cell growth in many species. Nicotinic acid is normally used at concentrations of 0.1-5.0 mg/liter; pyridoxine is used at 0.1-10.0 mg/liter.

Myo-inositol is commonly included in many vitamin stock solutions. Although it is a carbohydrate not a vitamin, it has been shown to stimulate growth in certain cell cultures. Its presence in the culture medium is not essential, but in small quantities myo-inositol stimulates cell growth in most species. Myo-inositol is generally used in plant cell and tissue culture media at concentrations of 50-5000 mg/liter. Other vitamins such as biotin, folic acid, ascorbic acid, pantothonic acid, vitamin E (Tocopherol), riboflavin, and p-aminobenzoic acid have been included in some cell culture media. The requirement for these vitamins by plant cell cultures is generally negligible, and they are not considered growth-limiting factors. These vitamins are generally added to the culture medium only when the concentration of thiamin is below the desired level or when it is desirable to grow cells at very low population densities.

Amino Acids or Other Nitrogen Supplements

Although cultured cells are normally capable of synthesizing all of the required amino acids, the addition of certain amino acids or amino acid mixtures may be used to further stimulate cell growth. The use of amino acids is particularly important for establishing cell cultures and protoplast cultures. Amino acids provide plant cells with an immediately available source of nitrogen, which generally can be taken up by the cells more rapidly than inorganic nitrogen.

The most common sources of organic nitrogen used in culture media are amino acid mixtures (e.g., casein hydrolysate), L-glutamine, L-asparagine, and adenine. Casein

hydrolysate is generally used at concentrations between 0.05 and 0.1 percent. When amino acids are added alone, care must be taken, as they can be inhibitory to cell growth. Examples of amino acids included in culture media to enhance cell growth are glycine at 2 mg/liter, glutamine up to 8 mM, asparagine at 100 mg/liter, L-arginine and cysteine at 10 mg/liter, and L-tyrosine at 100 mg/liter. Tyrosine has been used to stimulate morphogenesis in cell cultures but should only be used in an agar medium. Supplementation of the culture medium with adenine sulfate can stimulate cell growth and greatly enhance shoot formation.

Undefined Organic Supplements

Addition of a wide variety of organic extracts to culture media often results in favorable tissue responses. Supplements that have been tested include protein hydrolysates, coconut milk, yeast extracts, malt extracts, ground banana, orange juice, and tomato juice. However, undefined organic supplements should only be used as a last resort, and only coconut milk and protein hydrolysates are used to any extent today. Protein (casein) hydrolysates are generally added to culture media at a concentration of 0.05-0.1%, while coconut milk is commonly used at 5-20% (v/v).

The addition of Activated Charcoal (AC) to culture media may have a beneficial effect. The effect of AC is generally attributed to one of three factors: absorption of inhibitory compounds, absorption of growth regulators from the culture medium, or darkening of the medium. The inhibition of growth in the presence of AC is generally attributed to the absorption of phytohormones to AC. 1-Napthaleneacetic acid (NAA), Kinetin, (Kn) 6-Benzylaminopurine (BA), Indole-3-acetic acid (IAA), and 6- γ - γ -dimethylallylaminopurine (2iP) all bind to AC, with the latter two growth regulators binding quite rapidly. The stimulation of cell growth by AC is generally attributed to its ability to bind to toxic phenolic compound charcoal is generally acid-washed prior to addition to the culture medium at a concentration of 0.5-3.0 percent.

Solidifying Agents or Support Systems

Agar is the most commonly used gelling agent for preparing semisolid and solid plant tissue culture media. Agar has several advantages over other gelling agents. First, when agar is mixed with water, it forms a gel that melts at approximately 60°-100°C and solidifies at approximately 45°C; thus, agar gels are stable at all feasible incubation temperatures. Additionally, agar gels do not react with media constituents

and are not digested by plant enzymes. The firmness of an agar gel is controlled by the concentration and brand of agar used in the culture medium and the pH of the medium. The agar concentrations commonly used in plant cell culture media range between 0.5 and 1.0%; these concentrations give a firm gel at the pH's typical of plant cell culture media.

Another gelling agent commonly used for commercial as well as research purposes is Gelrite. This product is synthetic and should be used at 1.25-2.5 g/liter, resulting in a clear gel which aids in detecting contamination.

Alternative methods of support have included use of perforated cellophane, filter paper bridges, filter paper wicks, polyurethane foam, and polyester fleece. Whether explants grow best on agar or on other supporting agents varies from one species of plant to the next.

Growth Regulators

Four broad classes of growth regulators are important in plant tissue culture; the auxins, cytokinins, gibberellins, and abscisic acid. Skoog and Miller were the first to report that the ration of auxin to cytokinin determined the type and extent of organogenesis in plant cell cultures. Both an auxin and cytokinin are usually added to culture media in order to obtain morphogenesis, although the ratio of hormones required for root and shoot induction is not universally the same. Considerable variability exists among genera, species, and even cultivars in the type and amount of auxin and cytokinin required for induction of morphogenesis.

The auxins commonly used in plant tissue culture media are 1H-indole-3-acetic acid (IAA), 1H-indole-3-butyric acid (IBA), (2,4-dichlorophenoxy) acetic acid (2,4-D), and 1-napthaleneacetic acid (NAA). The only naturally occurring auxin found in plant tissues is IAA. Other synthetic auxins that have been used in plant cell culture include 4-chlorophenoxyacetic acid or p-chlorophenoxyacetic acid (4-CPA, PCPA), (2,4,5-trichlorophenoxy)acetic acid (2,4,5-T), 3,6-dichloro-2-ethoxybenzoic acid (Dicamba), and 4-amino-3,5,6-trichloropicolinic acid (Picloram).

The various auxins differ in their physiological activity and in the extent to which they move through tissue, are bound to the cells, or metabolized. Naturally occurring

IAA has been shown to have less physiological activity than synthetic auxins. Based on stem curvature assays, 2,4-D has eight to twelve times the activity, 2,4,5-T has four times the activity, PCPA and Picloram are produced during culture. Active nutrients exist. have two to four times the activity, and NAA has two times the activity of IAA. Although 2,4-D, 2,4,5-T, PCPA, and Picloram have two to four times the activity, and NAA has two times the activity of IAA. Although 2,4-D, 2,4,5-T, PCPA, and Picloram are often used to induce rapid cell proliferation, exposure to high levels or prolonged exposure to these auxins, particularly 2,4-D, results in suppressed morphogenic activity. Auxins are generally included in a culture medium to stimulate callus production and cell growth, to initiate shoots, particularly roots, and to induce somatic embryogenesis and stimulate growth from shoot apices and shoot tip cultures.

The cytokinins commonly used in the culture media include 6-benzylaminopurine or 6-benzyladenine (BAP, BA), 6- γ - γ dimethylaminopurine (2iP), N-(2-furanylmethyl)-1H-purine-6-amine (kinetin), and 6-(4-hydroxy-3-methyl-trans-2-butenylamino) purine (zeatin). Zeatin and 2iP are considered to be naturally occurring cytokinins, while BA and kinetin are synthetically derived cytokinins. Adenine, another naturally occurring compound, has a base structure similar to that of the cytokinins and has shown cytokinin-like activity in some cases. Many plant tissues have an absolute requirement for a specific cytokinin for morphogenesis to occur, whereas some tissues are considered to be cytokinin independent, i.e., no cytokinin or a specific cytokinin may be required for organogenesis.

The cytokinins are generally added to a culture medium to stimulate cell division, to induce shoot formation and axillary shoot proliferation, and to inhibit root formation. The type of morphogenesis that occurs in a plant tissue culture largely depends upon the ratio and concentrations of auxins and cytokinins present in the medium. Root initiation of plantlets, embryogenesis, and callus initiation all generally occur when the ratio of auxin to cytokinin is high, whereas adventitious and axillary shoot proliferation occurs when the ratio is low. The concentrations of auxins and cytokinins are equally as important as their ratio.

Gibberellins (GA3) and abscisic acid (ABA) are two other growth regulators occasionally used in culture media. Plant tissue cultures can usually be induced to

grow without either GA3 or ABA, although, certain species may require these hormones for enhanced growth. Generally, GA3 is added to culture media to promote the growth of low-density cell cultures, to enhance callus growth, and to elongate dwarfed or stunted plantlets. Absciscic acid is generally added to culture media to either inhibit or stimulate callus growth (depending upon the species), to enhance, inhibit, or stimulate callus growth (depending upon the species), to enhance shoot or bud proliferation, and to inhibit latter stages of embryo development.

Stock Solutions

The use of stock solutions reduces the number of repetitive operations involved in media preparation and, hence, the chance of human or experimental error. Moreover direct weighing of media components (e.g., micronutrients and hormones) that are required only in milligram or microgram quantities in the final formulation cannot be performed with sufficient accuracy for tissue culture work. For these components, preparation of concentrated stock solutions and subsequent dilution into the final media is standard procedure. In addition, concentrated solutions of some materials are more stable and can be stored for longer periods than more dilute solutions.

To prepare a stock solution, weigh out the required amount of the compound and place it in a clean flask. It is common practice to make a stock solution 10x or 100x, depending upon the solubility of the compound. Once the chemical is in the flask, dissolved it in a small amount of water, ethyl alcohol, 1 N NaOH, or 1N HCl. Next, slowly add double-distilled water to the flask, while agitating. Continue this until the proper volume is reached. Label the flask with the name of the solution, preparation and expiration dates, and the name of the person who prepared the solution. Certain items, e.g., IAA, must be prepared and stored in amber bottles to prevent photodecomposition. The volumes of stock solutions prepared at various concentrations that must be used to achieve various final concentrations are presented in tabular form in the Plant Growth Regulator Section.

Macronutrients of Stock Solutions

Stock solutions of macronutrients can be prepared at 10 times the concentration of the final medium. A separate stock solution for calcium salts may be required to prevent precipitation. Stock solution of macronutrients can be stored safely for several weeks in a refrigerator at 2°-4°C.

Micronutrients of Stock Solutions

Micronutrient stock solutions are generally made up at 100 times their final strength. It is recommended that micronutrient stocks be stored in either a refrigerator or freezer until needed. Micronutrient stock solutions could be stored in a refrigerator for up to 1 year without appreciable deterioration. Iron stock solutions should be prepared and stored separately from other micronutrients in an amber storage bottle. Formulations for preparing stock solutions of iron are presented later.

Vitamins of Stock Solutions

Vitamins are prepared as 100X or 1000X stock solutions and stored in a freezer (-20°C) until used. Vitamin stock solutions should be made up each time media is prepared if a refrigerator or freezer is not available. Vitamin stock solutions can be stored safely in a refrigerator for 2-3 months but should be discarded after that time.

Growth Regulators of Stock Solutions

The auxins NAA and 2,4-D are considered to be stable and can be stored at 4°C for several months; IAA should be stored at -20°C. Auxin stock solutions are generally prepared at 100-1000 times the final desired concentrations. Solution of NAA and 2,4-D can be stored for several months in a refrigerator or indefinitely at -20°C. Generally IAA and 2,4-D are dissolved in a small volume of 95% ethyl alcohol or KOH and then brought to volume with double-distilled water; NAA can be dissolved in a small amount of 1 N NaOH or KOH, which also can be used to dissolve 2,4-D and IAA.

The cytokinins are considered to be stable and can be stored at -20°C. Cytokinin stock solutions are generally prepared at 100X to 1000X concentrations. Many of the cytokinins are difficult to dissolve, and a few drops of either 1 N HCL, 1 N NaOH, in KOH or DMSO, is required to bring them into solution.

Preparation of Medium: The chemicals (as per Table 1) were dissolved in distilled or dematerialized water, the stock solutions along with the carbohydrate source (mainly sucrose) were added, and the solution was made to volume after adding the plant growth regulators. The pH of the medium was adjusted between 5.6-5.8 by adding 0.1 NaOH or HCl, Finally 0.8% (w/v) Agar-agar powder is added to obtain semi-solid gel, a supporting material for growing tissue.

Storage of Stock Solutions

Storage conditions for most stock solutions have already been pointed out; however, some additional points can be made. For convenience, many labs prepare stock solutions and then divide them into aliquots sufficient to prepare from 1 to 10 liter of medium; these aliquots are stored in small vials or plastic bags in a freezer. This procedure removes the inconvenience of having to un-thaw a large volume of frozen stock each time medium is prepared. Some have found that heating in a microwave oven is a satisfactory and quick method of thawing concentrated medium procedure. In addition, concentrated medium.

Media sterilization

Nutrient medium, glass ware and all the required instruments (surgical Knife, Spatula, needles, forceps etc) were sterilized by autoclaving at 121°C and 15-psi pressure for 15-20 minutes.

The culture vessels and media were sterilized separately. When the media are dispensed into appropriate culture vessels under aseptic conditions after autoclaving, phytohormones (Auxins or Cytokinins) are added using filter sterilized method.

Aseptic techniques for sterile transfer cabinet

All the aseptic manipulations were carried out in a laminar air-flow cabinet. The cabinet was first sterilized by exposing the UV radiation for 60-90 minutes and after that the surface was sterilized with 70% alcohol. The laminar air-flow cabinet was switched on 30 minutes before use to avoid interruption or reversal of air flow. All the required instruments used for aseptic manipulations (surgical Knife, forceps, spatula etc) were flame sterilized with 95% alcohol before use and every time also at the time of transfer of explants.

Surface sterilization of seeds and explants

The seeds were first soaked for 24 hrs in sterile distilled water. The seeds and explants were surface sterilized with 0.1% (w/v) Mercuric chloride (HgCl_2) for 3 to 5 minutes or with 5% (w/v) Sodium hypochlorite (NaOCl) for 10-20 minutes. After appropriate sterilization, the sterilizing agent was decanted and the seeds or explants were thoroughly washed three times with sterile distilled water and dried on a sterile filter paper.

Inoculation

The instruments used for aseptic manipulations such as surgical Knife, forceps, spathula and scalpel were sterilized by dipping in 95% ethanol followed by flaming inside laminar air-flow cabinet on the flame of spirit lamp and cooling before use. During inoculation the culture tubes and flasks containing the solid nutrient medium were kept horizontally. This strongly reduces the number of contaminations. Using forceps and inoculating loops different explants were inoculated into culture tubes and the culture tube was immediately closed tightly, with non-absorbent cotton plug.

Incubation

The inoculation culture vessels were incubated at $25 \pm 2^{\circ}\text{C}$ which is more suitable for plant regeneration *in vitro* and a light period of 16 hours followed by 8 hours dark period. These cultures were maintained under a cool white fluorescent light of $40\text{-}60 \mu\text{mol m}^{-2}\text{s}^{-1}$ intensity.

Statistical Analysis

The following formula was used for statistical analysis

$$\text{Mean} = \frac{\sum x}{n}$$

$$\text{Variance } S^2 = \frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n}$$

$$\text{Standard Deviation (S.D)} = \sqrt{\text{variance}}$$

$$\text{Standard Error (S.E)} = \frac{\text{S.D.}}{\sqrt{n}}$$

Hardening of the plantlets

In vitro rooted shoots were carefully taken out of the test tubes and gently washed under tap water so as to remove the agar and medium sticking to it. The shoots were dipped in 1 % aqueous solution of bavistin, a systemic fungicide (BASF, India) for 10-15 min and then washed with tap water. Thereafter the treated shoots were transferred in 8 cm earthen pots containing a mixture of autoclaved soil and sand (1:1) or soil: sand: compost (1:1:1). The pots were covered with polypropylene bags and kept in green house. The plants were watered once in a week. The top corners of polypropylene bags were cut after two weeks to gradually expose the plants to the outside environment. After 3-4 weeks, the polypropylene bags were completely removed.

Table 1: Composition of Plant Tissue Culture media MS (Murashige and Skoog 1962).

	Constituents	Chemical Formula	Amount in Stock (Mg/L)	Amount in Medium(Mg/L)
1	(a) Macronutrients			
2	Ammonium nitrate	NH_4NO_3	33,000	1650
3	Potassium nitrate	KNO_3	38,000	1900
4	Calcium-chloride	$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	8,800	440
5	Magnesium sulphate	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	7,400	370
	Potassium dihydrogen phosphate	KH_2PO_4		170
	(b) Micronutrients			
1	Potassium Iodide	KI		0.83
2	Boric acid	H_3BO_3		6.20
3	Manganese sulphate	$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$		22.30
4	Zinc sulphate	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$		8.60
5	Sodium molybdate	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$		0.25
6	Copper sulphate	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$		0.025
7	Cobalt chloride	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$		0.025
8	Ferric sulphate	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$		27.8
	(C) Fe (Iron) -EDTA			
1	Ferrous Sulphate Ethylene diamine tetra acetic acid disodium salt (Dihydrate)	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$		37.8
	(c) Vitamins and organics			
1	Meso-Inositol	$\text{C}_6\text{H}_{12}\text{O}_6$		100
2	Glycine (Amino acetic acid)	$\text{C}_2\text{H}_5\text{NO}_2$		2.0
3	Thiamine-HCl (Vit.B1)	$\text{N}_{12}\text{H}_{17}\text{ClN}_4 \cdot 5\text{HCl}$		0.1
4	Pyridoxine-HCl (vit.B6)	$\text{C}_8\text{H}_{11}\text{NO}_3 \cdot \text{HCl}$		0.5
5	Nicotinic acid (Pyridine-3-carboxylic acid)	$\text{C}_6\text{H}_5\text{NO}_2$		0.5
	(d) Sucrose			3000.0
	pH			5.6 to 5.8

Plate-I





Germination of *Cucumis melo* seeds

a) *Cucumis melo* fruit view b) aseptically on M S (Basal) Medium after 6 weeks old seedlings.

CHAPTER- III**CALLUS INDUCTION**

Plant callus is a mass of undifferentiated cells derived from plant tissue (explants) for use in biological research and biotechnology. In plant biology, callus cells are those cells that cover a plant wound. To induce callus formation, plant tissues are surface sterilized and then plated onto *in vitro* tissue culture medium. Plant hormones, such as auxins, cytokinins, and gibberellins are supplemented into the medium to initiate callus formation or somatic embryogenesis. Callus tissue initiation has been described for a number of plant taxonomic divisions. Calli are defined as tissues constituted by differentiated cells, which develop in response to a chemical or physical lesion, under determinate hormonal conditions (Mantell *et.al.*, 1994). They can be obtained from a tissue fragment and have the ability to differentiate into tissues, organs and even embryos, being able to regenerate whole plants (Pierik, 1990).

Callus cultures consist of an undifferentiated, proliferating mass of cells usually arising on wounds of differentiated tissues and cells. These can be initiated either from the source tissue synthesizing the secondary compound of interest or from other tissues like embryo. Generally the plants which accumulate relatively high yields of specific secondary metabolites, give rise to tissue cultures producing high levels of secondary metabolites and vice versa. This is because the capacity for the biosynthesis of secondary metabolites is genetically determined (Lindsey and Yeoman, 1985). Cell division usually occurs in parenchymatous cells by dedifferentiation. During this process adult cells temporarily revert to juvenile state (Rejuvenation) and hence show intense growth and division activity. The degree of dedifferentiation is different in different cells derived from the same explant (Lindsey and Yeoman, 1985). Normally juvenile and hence physiologically the most active tissues give better callus formation. The exogenous plant growth regulator requirement (Type, concentration, auxin to cytokinin ratio) for callus formation depends upon the genotype and endogenous hormone content of the tissues (Pierik, 1987).

During callus formation there are two distinct phases namely, the —wound response and the —growth response. The former phase is characterized by a rapid increase in metabolic activity when the explant is cultured on the growth medium but does not

lead to callus formation. The latter phase results in cell division and is dependent upon an exogenous supply of auxin. The cell structure also undergoes changes such as increased numbers of mitochondria and poly ribosome, disappearance of storage reserves like starch etc. Thus both the wound response and the growth response are characterized by distinct periods of m-RNA activity and the levels of t-RNA and r-RNA are regulated independently (Lindsey and Yeoman, 1985).

Due to the presence of diffusion gradients of nutrients, oxygen and other factors, the growth of callus is not always uniform and rapid and hence has less possibility for scale-up. But the chemical gradients, cell-to-cell contact and small degrees of differentiation seem to favor secondary metabolite production of some classes (Lindsey and Yeoman, 1985). Callus induction requires an environment where some of the cells can divide and proliferate. The strongest induction factor is the growth/nutrient medium supplemented with plant growth regulators. With prolonged culture, some of the callus cells may undergo some degree of differentiation and may change the spectrum of secondary metabolite synthesis. For many years, callus cultures have been employed in the study of secondary metabolism *in vitro*. In many of the callus cultures, conditions for callus induction and growth (Growth Medium) are not conducive for secondary metabolite production and the tissues need to be transferred to a new medium with a different composition (Production Medium). Production methods tend to be contingent upon defining conditions that allow maximum product accumulation rate.

Callus cells are not necessarily genetically homogeneous because a callus is often made from structural tissue, not individual cells. Nevertheless, callus cells are often considered similar enough for standard scientific analysis to be performed as if on a single subject. For example, an experiment may have half a callus undergo a treatment as the experimental group, while the other half undergoes a similar but non-active treatment as the control group.

Plant calli can differentiate into a whole plant, a process called regeneration, through addition of plant hormones in culture medium. This ability is known as totipotency. Regeneration of a whole plant from a single cell allows researchers to recover whole plants that have a copy of the transgenic in every cell. Regeneration of a whole plant that has some genetically transformed cells and some untransformed cells are called a

chimera In general, chimeras are not useful for genetic research or agricultural applications. Genes can be inserted into callus cells using biolistic bombardment, also known as a gene gun, or *Agrobacterium tumefaciens*. Cells that receive the gene of interest can then be recovered into whole plants using a combination of plant hormones. The whole plants that are recovered can be used for experiment to determine gene function (s), or to enhance crop plant traits for modern agriculture.

Callus tissue is of particular use in micropropagation where it can be used to grow genetically identical copies of plants with desirable characteristics.

The morphogenic response of a tissue culture depends on the endogenous growth substances present at the time of culture. The callus formation from wounds is due to the effect of endogenous growth hormones was reported for the first time by Sinnot (1960). It is also dependent on the concentration of growth substances such as auxins alone or auxin-cytokinins present in the medium for inducing the callus. Callus is made of an amorphous aggregate of loose, parenchyma cells which proliferate from the mother cells (explant). When an explant is wounded a callus is usually formed at the cut ends of the explant. Gautheret (1939) and Nobocourt (1939) first time reported the plant tissue culture of callus, involving explants of cambial tissues isolated from carrot root and carrot respectively. The general growth characteristic of a callus involves a complete relationship between the plant material used to induce callus and the composition of the medium and the culture conditions (light , temperature, etc) during the incubation period (Murashige,1974). All the plant tissues containing living cells may be induced to form dedifferentiated mass of cells, callus, but tissues may vary in terms of their lag period before an active growth is induced, mostly all the multicellular plants are potential source for ignition of callus (Yeoman and Macleod 1977).

The application of plant tissue culture method is crop improvement depends upon the induction of viable callus cultures and capable maintenance *in vitro* (Vasil *et.al.*, 1979). Establishment of callus cultures from the explants can be divided in to three stages induction of cell division, continued proliferation and structural and physiological dedifferentiation.

Some calli are heavily liquefied and hard in texture where as other break easily in to small fragments and readily separated are termed as —Friable callus‡ Generally in tissue cultures two types of calli are recognized.

They are

1. Embryogenic callus which is smooth, compact and cream or yellowish in colour and consist of iso-diametric cells.
2. Non – Embryogenic callus that is generally rough crystalline yellow to brown in colour.

The nature of the callus tissue its texture, compactness, friable and coloration also depends on the genotype, age of the explant and even the season. The callus growth within a plant species is also dependent on various factors such as the original position of the explant within the plant and the growth conditions.

Callus is basically a more or less non-organized tumor tissue, which usually arises on wounds of differentiated tissues and organs. Thus, in principle it is a non-organized and little-differentiated tissue. The cells in callus are of a parenchymatous nature, when critically examined callus culture is not homogenous mass of cells because it is usually made up of two types of tissue differentiated and non-differentiated. Example, plant tissue is a differentiated tissue (roots, stem, leaves, flowers, etc) which is used as a starting material for callus induction. These explant tissues generally show distinct planes of cell division, cell proliferation and organization into specialized structures such as vascular systems. If there are only differentiated cells present in an isolated explant, then dedifferentiated must take place before cell division, can occur parenchyma cells present in the explant usually undergo this differentiation. If the explant already contains meristematic tissues when isolated, then this can divided immediately without dedifferentiation taking place. Dedifferentiation plays a very important role, enabling mature cells in an explant isolated from an adult plant to be redetermined. In this process, adult cells are temporarily able to revert from the adult to the juvenile state. The rejuvenated cells have a greater growth and division potential and under special circumstances are able to regenerate in to organs and or embryos.

The exogenous supply of growth regulators is frequently necessary in calogenesis. This necessity refers to the type, concentration, and relation auxin/cytokinin, genotype of the donor plant and the endogenous content of hormones. According to George and Sherrington (1984), the combination of auxins and cytokinins promote cellular differentiation and also organogenesis. Among the growth regulators used in callus induction, 2,4-D (2,4- dichlorophenoxy acetic acid), NAA (1-naphthaleneacetic acid), BAP (6-benzylaminopurine) and TDZ (thidiazuron) are the most important.

Callus formation take place under the influence of exogenously supplied growth regulators present in the nutrient medium. The type of growth regulator requirement and its concentration in the medium depends strongly on the genotype and endogenous hormone content of an explant.

These requirements can be put into three categories.

1. Auxin alone (Especially in monocotyledons)
2. Cytokinin alone
3. Both Auxin and Cytokinin.

Callus is somewhat an abnormal tissue which has the potentially to produce normal roots or shoots and embryoids, which develop into complete plant lets depending on the level of phyto-hormones i.e. Auxin and Cytokinins present in the nutrient medium. Induction of callus from an explant plays a great role in tissue culture technology for production and multiplication of somaclonal variants with agronomically important characters isolation of pharmaceutically and therapeutically important chemicals from medicinally important plants using cell suspension cultures and also for conservation of the species. In view of all these, an attempt has been made in the present investigations to establish the effective protocol by using various growth regulators in inducing the callus from different explants in *Cucumis melo*. L medicinal and commercial plant.

Methodology

Seeds of *Cucumis melo*. L were soaked in sterile distilled water for 24hrs. These were surface sterilized with 0.1% (w/v). Mercuric chloride (HgCl₂) solution for 3-5 minutes followed by three rinses with sterile distilled water. These are germinated aseptically

on MS basal medium (Murashige and Skoog 1962) solidified with 0.8%(w/v) Difco bacto agar at pH 5.8 + 1.0 in 250 ml Ehrlenmeyer flasks (50 ml/medium /flask).

For callus induction the explants viz. Cotyledon (0.6 -1.0 cm²) hypocotyl (0.5-0.8 cm long) and Leaf (0.6 -0.8 cm²) from 3-4 weeks old axenic seedling and leaf (0.8-1.0 cm²) explants from 6 weeks old seedlings were excised these explants were inoculated to MS medium supplemented with various concentrations of (1.0-0.6 mg/L) of auxins such as 2,4-D chlorophenoxy acetic acid (2,4-D) Indole 3-acetic acid (IAA) Naphthalene acetic acid (NAA). All media were adjusted to pH 5.8 before addition of 0.8 % agar and autoclaved at 121oC and 103 Kpa for 20min cultures in 25 X 150mm cultures tubes.

Data Analysis

At least 10 replicates were maintained for each treatment and data was recorded after 4 weeks of cultures. Each experiment was repeated at least twice with similar results and data presented are of one representative experiment. All the data were statically analyzed.

Results

Callus induction ability of different explants such as cotyledon, hypocotyls and leaf was investigated by using varying concentrations of different auxins individually callus proliferation was initiated at the cut surface of the explants studied and latter it covered the entire surface. The results are presented in Table (6-8) and shown in (Plates V to VII) the explants viz. cotyledon, hypocotyls and leaf cultured on MS medium supplemented with different concentrations of (1.0 to 6.0 mg/L) of auxins such as 2, 4-D, IAA and NAA individually exhibited initiation of callus after 15 days of inoculation while it took 15-17 days in cotyledon cultures.

Effect of 2, 4-D

On 2,4-D supplemented medium early induction of callus was observed from all the explants cultured and in all the concentrations of 2,4-D above 80% callusing response was recorded in leaf cultured in all the concentration of 2,4-D maximum percentage of response was observed in leaf followed by hypocotyls and cotyledon cultures at as the concentration of 2,4-D was increase above 2.0 mg/L there was a gradual decrease in the response in cotyledon hypocotyls and leaf cultures. High amount of callus was

induced on 2,4-D in all the explants at 2.0 mg/L. morphology of callus was also found to be varied at different levels of 2,4-D (Table-2) friable callus was induced at 2.0mg/L 2,4-D in cotyledon. Whereas white compact calli were induced in almost all the concentrations of 2,4-D (Plate II).

Effect of IAA

Callusing efficiency of cotyledon, hypocotyl and leaf explants cultured on MS medium containing various concentrations of IAA is presented in (Table 3). High percentage of response was observed at low level of Auxins used in all the explants. High amount of callus was induced at (2.0 mg/L) IAA in cotyledon culture compared to all other concentrations of Auxins. Lower levels of Auxins (1.0 mg/L) showed little response. High levels of Auxins (6.0 mg/L) showed little response too. The explants on the MS medium supplemented with IAA (2.0 mg/L) 80%, 68% and 79% cultures respectively responded with white friable white compact callus induced from cotyledon, hypocotyls (Plate V Fig b) and leaf explants (Plate III) the percentage of growth response was low in the presence of IAA (2.0 mg/L) when compared to 2,4-D (2.0 mg/L).

Effect of NAA

Effect of NAA on callusing ability of cotyledon, hypocotyls and leaf explants is shown in (Table 4). High percentage (80%) of response was observed at (3.0 mg/L) NAA in cotyledon culture. Whereas less percentage of responding cultures were noted in all the concentrations of NAA used in leaf explant culture. NAA at (4.0 mg/L) to induced shoot from the explants. Cotyledon and leaf explants were responded well at all levels of NAA. Very high amount of callus was produced from cotyledon and hypocotyl cultures on MS medium supplemented with (4.0 mg/L) and (2.0 mg/L) NAA. More amount of callus was induced to cotyledon explants. Callus with roots was produced on (2.0 mg/L) NAA (Plate IV).

Discussion

The results from this study have shown the induction of callus from different explants. In the present investigation, the efficiency of callusing of different explants viz. cotyledon, hypocotyls and leaf explants were studied. The application of plant tissue culture method in crop improvement depends upon the induction of viable callus cultures and capable of maintenance *in vitro* (Vasil *et al.*, 1979).

Growth and development of the tissues from different explants used depended upon the composition of media used (Murashige 1978). Majority of excised tissue required the addition of one or more growth factors to the medium in order to stimulate callus development (Yeoman and Macleod 1977).

In the present investigations the callus was induced from all the explants viz. Cotyledon, hypocotyls and leaf in all the concentrations of Auxins 2,4-D, IAA and NAA at high concentration of 2,4-D the morphogenic response was inhibited in cotyledon and leaf explants callusing efficiency was found to be higher in leaf explants followed by cotyledon and hypocotyls explants of *C. melo*. Their difference in callusing ability suggests the presence of different levels of endogenous hormones in the tissues that Auxins such as 2,4-D, IAA and NAA alone suspended shoot bud formation in all the explants studied but promote callusing. Among the Auxins tested NAA induced the highest yield of callus followed by IAA and 2,4-D, similarly Omer (1988) observed the same findings with NAA in *Rhazya stricta* medicinal plant, whereas Skoog and Miller (1957) and Kitamura (1988) recorded the higher callogenesis, with auxin, the auxin 2,4-D has been determined has plants callus inducing phytohormone in studies with mangolant species. *Capsicum* (Gunay and Rao 1978, Philips and Hubstenberger, 1985). *Cucumis sativas* (Rajasekharan *et al.*, 1983) where as in the percent invalidations 2,4-D induced less amount of callus proliferation compared to all other Auxins used in all the explants studied Preveen *et al.*, 2001) have studied the callusing ability of different explants in *Strychnos Potatorum* on various growth substances viz. IAA, NAA and 2,4-D they observed that the maximum callus growth on MS medium containing 2,4-D in contrast to our present findings.

Callus production from different explants showed variability in texture form and coloration. The difference is dependent up on the response of plant tissue to various growth promoting substances. Thus successful callus, induction depends up on various factors such as composition of the nutrient medium hormonal balance besides the type age and genotype of the explant (Murashige, 1976, Narayana Swamy, 1977).

Table 2: Morphogenetic response of Cotyledon Hypocotyl and Leaf explants of *C.melo* on MS medium with different concentrations of 2, 4-D.

S. No	Explants	2,4-D concentration (mg/L)	% of cultures Response	Morphogenic Response
1	Cotyledon	1.0	50	Brown compact herd callus
		2.0	70	Friable callus
		4.0	75	Compact callus
		6.0	60	White compact callus
2	Hypocotyl	1.0	50	Compact callus
		2.0	75	Brown herd callus
		4.0	80	Friable callus
		6.0	60	White friable Globular callus
3	Leaf	1.0	75	Compact callus
		2.0	80	Friable callus
		4.0	70	White friable
		6.0	60	

Table 3: Morphogenetic response of Cotyledon Hypocotyl and Leaf explants of *C.melo* on MS medium with different concentrations of IAA.

S. No	Explant	IAA concentration Mg/L	% of cultures Response	Morphogenic response
1	Cotyledon	1.2	75	Brown compact
		2.0	80	White friable
		4.0	60	White compact
		6.0	58	Brown compact
2	Hypocotyl	1.0	53	White compact
		2.0	68	Brown heed
		4.0	62	White friable callus
		6.0	56	White globular
3	Leaf	1.0	78	Globular
		2.0	79	white compact
		4.0	60	white friable
		6.0	55	white compact

Table 4: Morphogenetic response of Cotyledon Hypocotyl and Leaf explants of *C.melo* on MS medium with different concentrations of NAA.

S. No	Explants	NAA concentration mg/L	% of cultures Response	Morphogenic response
1	Cotyledon	1.0	50	Rooting
		2.0	75	Callus + Rooting
		4.0	80	Shoot Rooting
		6.0	65	Callus
2	Hypocotyl	1.0	70	Friable Callus
		2.0	80	Rooting
		4.0	60	Callus + Rooting
		6.0	55	Rooting
3	Leaf	1.0	42	Callus +Rooting
		2.0	56	Callus +Rooting
		4.0	60	Callus +Rooting
		6.0	40	Callus +Rooting

Plate-II



Callusing ability of cotyledon and hypocotyls explants o *Cucumis melo* after 6 weeks.

- Induction of Brown compact callus on MS + 4.0 mg/L 2, 4- D from cotyledon explants.
- Induction of white friable callus on 2.0 mg/L IAA from cotyledon explants.

Plate-III



Callusing ability of leaf and hypocotyls explants of *Cucumis melo* after 6 weeks.

- a) Induction of white friable callus on MS + 2.0 mg/L 2,4 D from Leaf explants.
- b) Induction of cream compact callus on 2.0 mg/L NAA from Hypocotyls explants.

Plate-IV



Callusing ability of Leaf explants of *Cucumis melo* after 6 weeks.

- a) Induction of cream friable callus on MS + 4.0 mg/L 2, 4-D.
- b) Induction of white compact callus on MS + 2.0 mg/L. IAA.

CHAPTER- IV**DIRECT REGENERATION**

Plant Regeneration/Organogenesis is an important aspect of the tissue culture. This phenomenon plays a great role in *in vitro* plant micropropagation and conservation of germplasm. The development of complete organs callus and the control of this process by phytohormones, represent a major area of interest in plant tissue culture. Regeneration for explant such as cotyledon, hypocotyls and leaves is possible through direct regeneration or via a callus phase (Male pszyi 1988, Gambley and Dodd, 1990). Morphogenesis can be obtained directly from cultured explants and indirectly from callus or suspension cultures (George and Sherington 1984).

The ability to regenerate whole plants from single cell, tissue or organ is known as totipotency, which is often retained even after a cell has undergone final differentiation. The totipotency of somatic cells has been exploited in vegetative propagation of plant species for quite a considerable time. Differentiation into shoots and roots from various explants i.e. root leaves and stem of different taxa was successful that led to establishment of new individuals.

Differentiation is genetically irreversible in animals, whereas in plants, highly mature and differentiated cells retain the ability to refer to a meristematic state as long as they have intact membrane system and a viable nucleus (redifferentiation). Various cells, tissues and organs from numerous plant species can be cultured successfully to regenerate into whole plants (Murashige, 1974, Tisserat *et.al.*, 1977, Narayana swamy 1977; Vasil and Vasil 1980; Kartha 1981). The degree of regeneration varies considerably from species to species (Tisserat, 1979).

Morphogenesis *in vitro* was first observed by (White 1939 b) who reported the formation of shoots from callus cultures of tobacco maintained in liquid medium and not on the surface of agar medium. This observation was confirmed and extended by Skoog. (1944). Who also proved the ability of auxins to stimulate root formation and inhibit shoot formation, Further studies by Skoog and Miller (1957) revealed that a balance between auxin and cytokinin is necessary from regeneration of roots and shoots. Since then several workers have reported their findings on plant regeneration (Durbin 1979; Gamborg *et. al.*, 1981; Evans and Bravo 1983).

Plant regeneration takes place by the formation of super axillary adventitious buds on the explant or *de nova* organization of shoot and root meristem in callus (Vasil *et.al.*, 1979). Plant regeneration from tissue culture may be achieved via Organogenesis or Embryogenesis (Ammirato 1983 a, Flik *et al.*, 1983) Organogenesis is the process by which a cell or group of cells differentiates to form organs, Organogenesis refers equally to the formation of roots or shoots but since recovery of plants is usually the objective the formation of shoots is of greater interest. Organogenesis is commonly induced by manipulation of exogenous phytohormones, levels and occur either directly from explant tissue or from Callus, often, what is referred as direct organogenesis / regeneration is shoot proliferation or micro propagation from preexisting meristems instead of *de novo* formation of meristem. Even in callus mediated organogenesis /regeneration organ -forming capacity limited to primary callus indicates the potential existence of meristems embedded in the original explants. However the degree of regeneration varies considerably from species to species (Tisser 1987).

In vitro Organogenesis/Regeneration can be obtained by 3 ways.

1. Through callus mediated regeneration.
2. Somatic embryogenesis.
3. Direct organogenesis /shoot bud Proliferation.

Regeneration based on the genotype of the donor tissue, source of explant and growth regulators such as auxin or cytokinin. Combination and also the environmental conditions during the incubation period. The influence of these factors in *in vitro* was reported in *Capsicum annum* (Venkataiah and Subhash 2001) *Lycopersicon esculentum* (Tan 1987) *Brassica* (Narasimhulu and Chopra, 1988, Khehra and Mathais, 1992).

The plant tissue culture methods also provide base for the improvement of crop. To induce somaclonal variations, *in vitro* mutations, herbicide tolerance, di-haploid induction, genetic transformation of economically important genes and development of somatic hybrids, efficient plant regeneration protocol is required. Such advance techniques in combination with conventional breeding give a momentum to the improvement of a crop.

The purpose of this study on *Cucumis melo* is to establish an efficient and reproducible method for regeneration and the development of mass variable population of plants in short period of time. To reach this aim it was necessary to identify.

- a) Potential explants and.
- b) The suitable nutrient media and additives that would elicit optimum responses.

Methodology

Plant material

Seeds of *Cucumis melo* were soaked for 24hrs in sterilized distilled water and surface sterilized. With 0.1% HgCl_2 for 2-3 minutes. Then they were washed three times with sterile distilled water for 5- minutes and germinated aseptically on MS basal medium.

Culture media and culture conditions

Different explants such as hypocotyls (0.5-0.7 cm long) and cotyledon (0.6-0.8 cm²) from 2 weeks old seedling and leaves (0.5-0.9 cm²) from 6 week old seedling were excised and cultured on MS medium containing 3% w/v sucrose with various concentrations of cytokinin (BAP, Kn) alone and also in combination with auxin (See Tables 5-6) the pH of the media was adjusted to 5.8 $\pm$ 1 with 1 N HCl or 1N NaOH solidified with 0.8% Difco-Bacto agar and autoclaved at 121°C at 15 psi for 15-20 minutes single explant was inoculated in each culture tube and incubated at 25 $\pm$ 2°C under white fluorescent light of 40-60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ intensity for 16 hrs light /8 hrs dark period.

Data Analysis

20 replicates were maintained for each treatment was repeated at least once with similar result; Data were recorded after 6 weeks of culture.

Results

Multiple shoot buds proliferation was observed within 15-20 days of culture from the cut ends of cotyledon hypocotyls and leaf explants. The data on *in vitro* regeneration was presented in (Table 5-6).

Cotyledon explant

The morphogenetic response of the hypocotyls explants cultured on MS medium supplemented with various plant growth regulators is presented in (Table 5-6).

Effect of BAP and Kn

Table 5 represents, direct regeneration of seedling cotyledon explants to various concentration of cytokinins such as BAP and Kn alone in BAP (1.0 -6.0 mg /L) and Kn (1.0-6.0 mg/L) was studied on direct multiple shoot bud induction. Direct adventitious shoot regeneration on MS medium containing various results. Highest responding cultures with maximum frequency of multiple shoot bud induction was observed at (2.0 mg/L) BAP (4-6 shoots/explant) followed by 4.0 and 6.0 mg/L BAP, produced (2-4 and 3-4 shoots/explants) with 70 and 60% cultures responding. The number of shoots was considerably reduced, when BAP concentration was increased (Table-5).

Kn was less responsive compared to BAP in inducing shoot buds from the explant (Table -9) with 1.0 mg/L Kn the cotyledon explant produced single shoot and 52% culture responded 2.0 mg/L Kn was more responsive in inducing maximum number of shoots (2-3 shoots/explant) with greater frequency (65%) Kn at 4.0 and 6.0 mg/L Produced (1-2 and single shoot) with 60 and 55% cultures responded.

Effect of IAA + BAP and IAA + Kn

When the auxin was taken in combination with IAA (0.5 mg/L) + BAP (1.0 -6.0 mg/L) and IAA 0.5 mg/L + Kn (1.0-6.0 mg/L) (Table 5) in combination produced shoots from the explants. At 0.5 mg/L IAA with 1.0 mg/L BAP 60% cultures responded with (3-4 shoots /explants) maximum number of shoots (5-6 shoots/explant) with greater frequency 85% were produced at (2.0 mg/L) BAP + IAA (0.5 mg/L) (Plate V) As the concentration of BAP was increase from 4.0 mg/L to 6.0 mg/L the number of shoots were considerably reduced (Table -5). When IAA was taken in combination with Kn.

IAA + Kn was less responsive compared to IAA + BAP in including shoot buds from the explants (Table -9) with (1.0 mg/L) Kn and (0.5 mg/L) IAA produced single shoot and 55 % cultures responded 2.0 mg/L Kn was more responsive in inducing maximum number of shoots (4-5 shoots) (Plate V) with greater frequency (59%) Kn

at 4.0 and 6.0 mg/L produced 1-2 and shingle shoots with 60% and 55% cultures responding.

Hypocotyl explant

Hypocotyl explants cultured on MS medium containing various concentration of cytokinins BAP /Kn alone and in combination of IAA (0.5 mg/L) showed the direct regeneration (Table 5 and 6.)

Effect of BAP and Kn

Hypocotyl explants were cultured on MS medium supplemented with various levels of BAP (1.0-6.0 mg/L) Maximum frequency of shoot buds induction (3-4 shoots/explants) was noted at 4.0 mg/L BAP compared to all other concentration tested shoot capacity was gradually decreased at high concentration of BAP (Table 5) Similarly hypocotyls explants were cultured on MS medium supplemented with various levels of Kn (1.0 -6.0 mg/L). Highest percentage (60%) of responding cultures were observed at (4.0 mg/L) followed by (6.0 mg/L) and gradually reduced as the level of Kn was increased. Maximum number of shoots regeneration (2-3 shoots/explant) was found at (4.0 mg/L) Kn. whereas the shoot bud induction was decreased at high level of Kn.

Effect of IAA + BAP and IAA + Kn

To find out the efficiency of auxin – cytokinin combination the hypocotyls explants were cultured on MS medium supplemented with IAA (0.5 mg/L) in combination with various concentration of BAP /Kn (1.0 -6.0 mg/L). Direct shoot bud proliferation was found in all the concentrations and combinations of phytohormones used.

Hypocotyls culture on MS medium containing (0.5 mg/L)IAA in combination with (1.0 - 6.0 mg/L) BAP showed maximum responding culture and more number of shoots /explants (4-5 shoots /explant) at 2.0mg/L BAP. Average number of shoots production has been gradually decreased at high concentration of BAP (Table -10). Hypocotyls explants were cultured on (0.5 mg/L) IAA in combination with various concentration (1.0, 2.0, 4.0 and 6.0 mg/L) of Kn showed (Single shoot, 3-4, 2-3 and single shoot) with 50, 53, 50 and 45% responded.

Leaf explants

Multiple shoots development was observed directly from leaf explant on MS medium fortified with different concentration of (1.0 -6.0 mg/L) BAP and Kn alone tested and similarly leaf explants were cultured on MS medium fortified with IAA (0.5 mg/L) in combination of cytokinins such as BAP/Kn (1.0-6.0 mg/L). These explants were enlarged 3-4 fold with in one week of culture initiation. The results are presented in (Table 9-10) and shown in (Plate VI).

Effect of BAP and Kn

Shoots development was observed directly from leaf explants on MS medium fortified with deferent concentrations (1.0 -6.0 mg/L) of BAP and Kn (1.0 -6.0 mg/L) tested for shoots from leaf culture at (1.0 mg/L) BAP produced the (Single shoot/explant) (Table 5) the maximum number of shoots observed at (2.0 mg/L) BAP (4-6 shoots /explants) with 100% cultures responded (Plate VI) at (4.0 and 6.0 mg/L) BAP Produced (3-4 and 3-2 shoots/explant) with 90 and 70% cultures were responded. Leaf culture on MS medium containing Kn (1.0-5.0 mg/L) showed maximum responding culture and more number of shoots/explant. (2-3 shoots) at (4.0 mg/L) Kn with 85% cultures were responded. Compared to all other concentration of Kn and BAP as well but at high concentration of Kn the response was decrease (Table-9).

Effect of IAA + BAP and IAA +Kn

Leaf explants cultured on MS medium supplemented with (0.5 mg/L) IAA and different concentrations of BAP/Kn (1.0-6.0 mg/L) (Table -6) IAA (0.5 mg/L) in combination with BAP (1.0-6.0 mg/L) showed the direct shoot regeneration. Highest percentage (85%) of responding cultures were observed at 2.0 mg/L BAP gradually reduces the level of BAP was increased. The number of multiple shoots was also decreased.(6-7shoots/explant) (Plate VI) was also found (2.0 mg/L) BAP where as the multiple shoot bud induction was decreased at high level of BAP.

Similarly leaf explants were cultured on MS medium fortified with IAA (0.5 mg/L) in combination of Kn (1.0 -5.0 mg/L) Maximum number of shoot bud induction (4-5 shoots/explants) was noted at (2.0 mg/L) Kn compared to all other concentrations tested. Shoot regeneration capacity was gradually decreased at high concentration of Kn as observed (Table 6).

Discussion

We were successful in regeneration plants from, cotyledon, hypocotyl and leaf culture on MS medium fortified with different concentration of cytokinin ie BAP /Kn individually and also in combination on with (0.5 mg/L) IAA. Maximum number of shoot buds were induced (2.0 mg/L) BAP in comparison to Kn as a role growth regulators with low levels of auxin (0.5 mg/L) were added to the medium containing BAP/Kn it was interesting to find that the shoot induction was enhanced in all the concentrations of cytokinin tested. However the shoot bud proliferation was found to be more on (0.5 mg/L) IAA in combination with BAP/Kn to probably IAA might have triggered the action of BAP/Kn in a proper way for inducing more number of shoots per explant but the combination of IAA + BAP induced higher number of plantlet regeneration among all hormonal combinations and concentrations used.

The development of complete organs from callus and the control of this process by phytohormones represent a major area of interest in plant tissue culture. Regeneration from explant such as cotyledon, hypocotyls and leaves is possible through direct regeneration or via a callus phase (Ziv and Gadasi, 1986; malepszy: 1988: Gambley and Dodd, 1990). Morphogenesis can be obtained directly from cultured explants and indirectly from callus or suspension culture (George and Sheringaton, 1984).

In the present studies the induction of shoot bud was effective with addition of BAP, but kinetin did not yield positive result. The finding is corroborative with other reports. Cytokinins alone induced shoot formation in leaf discs of *Solanum laciniatum* of the cytokinins used BAP proved to be most effective than Kn in inducing shoot formation of *Capsicum* (Phillips and Hubstenburger, 1985: Sripichitt *et al.*, 1987). According to Gunay and Rao (1978). Shoot bud formation was not observed in the presence of kinetin in red paper. The quantity of callus used for regeneration was 300 mg and less than that did not favour differentiation. Similar result were observed in Italian millet (Rao *et al.*, 1988). Calli of 180 days old have shown positive result after successive subcultures on MS medium fortified with the previously mentioned hormones.

According to Skoog (1944) a balance between auxin and cytokinin controls organogenesis. In the present study, lower level of auxin along with the cytokinin was effective in organogenesis. Skoog and Miller (1957) observed induction of root

formation from tobacco callus by a relative high ratio of auxin and cytokinin, while a low ratio of the same hormones favoured shoot formation. NAA alone had inhibitory effect on shoot bud formation, while in combination with BAP has shown stimulatory effect. NAA in combination with either kinetin or BAP has shown stimulatory effect on shoot bud formation in *Brassica* (Wong and Loh 1987). George and Rao (1980) reported shoot buds on medium containing NAA and BAP. Although IAA was not very effective in promoting growth, it can be a substitute for NAA in combination with BAP. Similar observations were made in *Brassica* (Narasimhulu and Chopra 1988).

L- Glutamic acid at concentration of 2.0-2.5 mg/L along with 0.5 mg/L BAP Proved to be the best combination for shoot formation in the four Genera concerned, few amino acids also have proved to be effective for the growth of excised embryos (Monnier 1978) A mixture of 20 amino acids were tested by sander and Burkholder (1948) ten of which have been earlier found to be beneficial for plant growth which include L-Glutamic acid Media containing the amino acids enhanced differentiation and growth in tissues of *Pteris* (Ksihirsagar and Mehtha, 1978) and *Torenia* (Tammoto and Harada, 1982).

Similarly Hoque *et al.*, (2005) have reported the high frequency of plant regeneration on MS medium containing (2.0 mg/L) BAP in combination with (0.5 mg/L) IAA from cotyledon derived callus in *Momordica dioica*. They have also found the maximum number of shoots per explants on BAP compared to Kn. The essentially to both auxin cytokinin combination for inducing shoot organogenesis has been reported in leaf culture of *Cicer arietum* (Arockia swamy *et al.*, 2000) of the cytokinin used BAP proved as most effective than Kn in inducing shoots, the same finding were recorded in *Capsicum* spp (Phillips and Hubstenberger, 1985).

Table 5: Effect of BAP and Kn of Cotyledon, Hypocotyl and leaf culture of *C.melo*.

Hormone Conc. Mg/L	Cotyledon		Hypocoty		Leaf	
	Frequency	Mean of Shoos/explants	Frequency	Mean of Shoos/explants	Frequency	Mean of Shoos/explants
BAP						
1.0	50	Callus	50	Single Shoot	80	Single Shoot
2.0	85	4-6	60	3.0 Shoot	100	4-6
4.0	70	2-4	75	3-4	90	3-5
6.0	60	3-4	60	Single Shoot	70	3-2
Kn						
1.0	52	Single Shoot	50	Single Shoot	95	Single Shoot
2.0	65	2-3	55	2 Shoot	90	2 Shoot
4.0	60	1-2	60	2-3 Shoot	85	2-3 Shoot
6.0	55	Single Shoot	45	Single Shoot	80	Single Shoot

Table 6s: Effect of IAA+BAP and IAA+Kn Cotyledon, Hypocotyl and leaf culture of *Cucumis.melo*.

Hormone Conc. Mg/L	Cotyledon		Hypocoty		Leaf	
	Frequency	Mean of Shoos/explants	Frequency	Mean of Shoos/explants	Frequency	Mean of Shoos/explants
IAA+BAP						
0.5 + 1.0	60	3-4	52	Callus	65	Single Shoot
0.5 + 2.0	85	5-6	73	4-5	87	6-7
0.5 + 4.0	55	4-5	70	3-2	56	5-6
0.5 + 6.0	50	2-3	65	Single Shoot	50	3-4
IAA+Kn						
0.5 + 1.0	55	Single Shoot	50	Callus	59	Single Shoot
0.5 + 2.0	59	4-5	53	3-4	60	5-6
0.5 + 4.0	52	1-2	50	2-3	56	4-3
0.5 + 6.0	50	Single Shoot	45	Single Shoot	52	2-30.5 +

Plate-V



Direct multiple shoot bud proliferation of cotyledon explants in *Cucumis melo* after 6 weeks of cultures.

- a) Multiple shoot buds induced on MS + 0.5 mg/L IAA + 2.0 mg/L BAP.
- b) Formation of multiple shoot buds on MS + 0.5 mg/L IAA + 2.0 mg/L Kn.

Plate-VI



Direct multiple shoot bud proliferation of Leaf explants in *Cucumis melo* after 6 weeks of culture.

- a) Multiple shoot buds induced on MS + 2.0 mg/L BAP.
- b) Formation of multiple shoot buds on MS + 0.5 mg/L IAA + 2.0 mg/L BAP.

CHAPTER- V**IN VITRO MICROPROPAGATION**

Regeneration of whole plant through tissue culture is popularly called micro propagation-It is also a type of vegetative propagation in vitro and is rapidly expanding technique‘ *in vitro* micro propagation is an extremely space saving propagation method (Boxus *et al.*, 1977) wang and Hu,1982) following the successfully rapid multiplication of orchids by shoot meristem culture (Morel,1965) there has been an increasing interest in recent year in application of tissue culture techniques as an alternative means of asexual propagation of economically important plant shoot tip and bud culture are preferred over meristem culture in micro propagation when viral elimination is not part of the objective use of larger explants desirable they are easier to dissect and have much higher survival and growth rates than smaller explants.

Following stages are involved in the method of micro propagation

- I. Stage involves establishment of tissue *in vitro*.
- II. Involves multiplication of shoots (often media are not changed between stage I and stage II).
- III. Stage III concerns root formation and conditioning of propagules prior to transfer to the green house. This stage requires high intensity and alternation of media for promotion of root formation.
- IV. Involves growth in pots followed by field trials.

In vitro Micropropagation techniques are now often preferred and has the following potential advantages.

1. Only a small amount of plant tissue is needed as the initial explant for regeneration of millions of Clonal plants in one year. In comparison. It would take years to propagate an equal number of plants by conventional methods.
2. Many plants species are highly resistant to conventional bulk propagation practices. *In vitro* Micropropagation provides a possible alternative method for these species.
3. The *in vitro* technique provides a method for speedy international exchange of plant materials.
4. The *in vitro* stocks can be quickly proliferated at any time of the year (Boxus *et al.*, 1977: Huang and Millikan, 1980).

5. As a useful safe guard against plant loss.
6. For rapid introduction of new interesting material in recurrent selection (Brussel chicory, Dose, 1984).
7. As an efficient and reliable system of selection of characters whose expression is affected by the environment. Such is the case of curd quality in cauliflower as demonstrated by (Crisp and Gray 1979).
8. As an economic and easy way of maintaining of genotypes after virus elimination of necessary (Cauliflower, Grout and Crisp, 1977).
9. Multiplication of disease free plants.
10. Finally it represents an interesting possibility for seed production by cloning the parents of the hybrids. This aspects has been developed for hybrid seed production of Broccoli (Anderson and Carstens, 1977).

There are three possible approaches have been followed to achieve Micro propagation.

1. Through callusing: The potentiality of plant cells to multiply in culture and their totipotent nature permit a very rapid multiplication of several plant types. The genetic instability of callus and their cells sometimes causes objection against use of callus culture. This *in vitro* method of vegetative propagation is seen in *citrus* (Button and Kochba 1977 Chaturvedi, 1979) *Coffee* (Monaco *et al.*, 1977) and plams (Tisserat, 1981).

2. Enhanced release of axillary buds: The merit of using axillary bud proliferation from meristem, shoot tip or bud and nodal cultures as a means of regeneration is that the incipient shoot has already been differentiated *in vitro*. Thus to establish a complete plant, only elongation and root differentiation are required *in vitro* organogenesis and embryogenesis on the other hand, must undergo developmental changes which usually involve the formation of callus with subsequent reorganization into plantlets. This has not been easy to achieve in most plants. The induction of axillary bud proliferation seems to be applicable in many cases, eg. Carnation (Roset and Bokelmann 1981) and soyabean (Evans, 1981: Kartha *et al.*, 1981b) where methods of organogenesis and embryogenesis fail.

Plants of Cucurbitaceae suffer from several diseases including the watermelon mosaic virus (Greber, 1978). Cucumber green mottle mosaic virus (Nijsden, 1984) and bittergourd also suffer from fruit rot disease which seriously limits the crop production. Pathogen free (virus free) plants are recoverable from clones that are systematically infected.

Seedlings obtained from seeds will be genetically variable and each one will represent a new combination of genes brought about by meiosis. By contrast in the vegetative cycle. The unique characteristics of any individual plant or plantlet are perpetuated by growth multiplication of cells in which genes are copied exactly, at each mitotic division. Each new plant (remet) termed a clone produced by this method may be considered as an extension of the somatic cells line of one (sexually produced or mutant) individual.

Moral (1952) first demonstrated that virus free plants can be recovered from infected plants through shoot tip cultures. Subsequently in extending the demonstration to the orchids, a novel method of Clonal propagation was revealed by (Moral 1965) method that quickly become standard among the tissue culturists.

There have been less number of publications showing shoot development from multiple adventitious buds in few Cucurbitaceae members like Cucumber (Rhonda *et al.*, 1990) and with low yield of shoots (Welmer and Locky 1981) Axillary buds from pumpkin were reported by (Jelaska 1974) A transformation system using leaf disc for direct shoot production has been used successfully in tobacco, petunia and tomato (Horseh *et al.*, 1985; Mc Cormick *et al.*, 1986).

In the present investigation a simple and reproducible procedure was devised to obtain multiple shoots, and regeneration of complete plantlets from shoot tip and nodal explants of *Cucumis melo*. Different plant growth regulators along with Coconut milk and amino acids were supplemented to MS medium and the impact of them were investigated.

A. Shoot tip culture

Shoot regeneration from shoot apex / shoot tip is direct, relatively simple and is not prone to Somaclonal variation and chromosomal abnormalities. This is an elegant

methodology of multiplying *in vitro* sterling from a single shoot with obvious potential when applied to crop plants (Quak, 1977).

Culture of shoot meristem, especially through enhanced branching, permits rapid Clonal propagation and a high degree of genetic uniformity of the progeny (Sunitha and Handique, 2000). The meristem technology has been wide spread practical application in producing virus free plants in *in vitro* in recent year (Bhaskaran *et al.*, 1992, Sudarshan *et al.*, 2000) Although mainly used for virus elimination meristem tip culture has also enabled plants to be freed from other pathogens including viroids, mycoplasmas, bacteria and fungi, also, meristem freeze preservation as a method of conservation of germplasm has made possible to utilize when needed (Haskins and Kartha, 1980). Successful generation of entire plants from frozen meristem of *Arachis hypogaea* and *Cicer aurietinum* has been reported (Bajaj 1979).

The latest technology for delivery of plant tissues is the biolistic gun which require regenerable tissues. So target tissues may be callus, suspension cells, leaves, meristem tips or any other regenerable explant. Recently, shoot meristem tips have also been used for direct delivery of desired genes in soybean cotton and sorghum (Gould *et al.*, 1991; Bhaskaran *et al.*, 1992) In view of the importance of mericlonal technology in phyto pathology and genetic engineering experiment were conducted to achieve direct multiple shoot regeneration from shoot apex in *Cucumis melo*.

Methodology

Young plants of *Cucumis. Melo* collected from outside and were Department of Botany Kakatiya University Warangal. Were collected from one year old plants shoot tips comprising the apical dome and two or three leaf primordial were excised. The explants were washed thoroughly under running tap water, treated with 70% alcohol for 1 minute and washed four times with sterile distilled water, these were surfaced sterilized with 0.1% HgCl₂ for 4-5 minutes and washed thrice for 4 minutes each time in sterilized distilled water shoot meristems ranged from 0.6 cm to 0.8cm in length were cultured on MS medium containing different hormonal concentrations and combinations (Table 7-8) (Plate VII).

Culture media and culture conditions

MS media containing 3.0% sucrose and supplemented with various concentrations and combinations of auxins (NAA) Coconut milk, Glutamic acid and Cytokinins viz. BAP (1.0-6.0 mg/L) and Kn (1.0-6.0 mg/L) in combination on with GTA (1.0 mg /L) + BAP (1.0 -6.0 mg/L) Coconut milk (10-25%) + BAP (0.5 mg/L) NAA (1.0 mg/L)+ BAP (1.0-6.0 mg/L) and GTA (1.0 mg/L) + Kn(1.0-6.0/L) Coconut milk (10 - 25%) Kn (0.5 mg/L) NAA (1.0 mg/L) + Kn (1.0 - 6.0 mg/L) were used. The initial pH of the culture media was adjusted to 5.8 before addition of 0.8 % (w/v) agar agar. The media was dispensed into culture tubes (25 X 150 mm) each containing 15 ml of the culture medium. Capable with non- absorbent cotton and was autoclaved at 121°C for 15 minutes. In each culture tube one shoot tip explant was implanted. The cultures were maintained under 16hrs light provided with white fluorescent tubes ($40 \mu \text{mol m}^{-2} \text{s}^{-1}$) at $25 \pm 2^\circ\text{C}$.

Results

Data on multiple shoot induction from shoot tips cultured on MS medium fortified with different concentration of BAP and Kn, in combination with GTA CM and NAA is presented in (Table 7-8) and shown in flow Chart (I and II.).

Effect of BAP + GTA

The shoot tip explants cultured on MS medium supplemented with different concentrations of BAP (1.0 -6.0 mg/L) in combination with GTA (1.0 mg/L) are presented in (Table-7) and showed in (Plate VII) Direct multiple shoot proliferation was observed in shoot tip cultures in the above the treatment after 4 weeks of shoot tip culture, developed multiple shoots number of shoots increased with increased levels of BAP (Table 7) of the various treatment tested on MS medium fortified with GTA (1.0 mg/L) + (4.0 mg/L) BAP resulted in maximum number of shoots (4.0 ± 0.4 shoots / explant) at 2.0 mg/L BAP + 1.0 mg/L GTA resulted in (3.2 ± 0.2 shoots / explant) the minimum number of shoots/ explant were observed at (1.0 mg/L) BAP + (1.0 mg/L) GTA. As the concentration of BAP increased upto 4.0 mg/L the multiple shoots number was also increase but as High concentration of BAP (6.0 mg/L) resulted the number of shoots were reduced.

Effect of BAP +CM

Both BAP and Coconut milk were added in the medium to observe the effect on shoot tip response in *in vitro* of the various treatments tested on MS medium BAP (0.5 mg/L) and CM (10-25 %) resulted in maximum number of shoots (6.0 ± 0.4 shoots /explant) (Plate II Fig. b). as the percentage of CM was increased. The number of shoots per explants was also produced more but at high percentage of CM (25%) resulted the number of shoots were reduced.

Effect of Kn+GTA

Shoot tip explants showed direct shoot bud differentiation and plantlet formation on MS medium supplemented with different concentration of Kn (1.0 to 6.0 mg/L) + GTA (1.0 mg/L) 63% cultures responded on (4.0 mg/L) Kn +(1.0 mg/L) GTA with a shoot bud frequency was (3.5 ± 0.3 shoots / explant). At (6.0 mg/L)Kn + (1.0 mg/L) GTA induced 50% cultures responded with (1.5 ± 0.4 shoots /explant) As the concentration of Kn was increased to (1.0 and 5.0 mg/L) the number of shoots /explants were considerable increased to (2.2 ± 0.3 and 3.0 ± 0.2 shoots /explant) respectively (Table-7).

Effect of Kn +CM

When Kn was taken in combination with Coconut milk shoot bud differentiation was observed at (0.5 mg/L) Kn + 20% Coconut milk induced (5.0 ± 0.3 shoots/explant) with 68% frequency. Kn (0.5 mg/L) in combination with Coconut milk 10 and 15 % showed less number of shoots (2.0 ± 0.4 shoots /explant) with 63% cultures responding. In shoot tip explants at (0.5 mg/L) Kn + (20%) CM Produced Maximum number of shoots/explant.

Discussion

The result of present investigation show that shoot tip explants from mature plants of *C. melo* could be induced to produce multiple shoots *in vitro* maximum number of shoots were induced on MS medium fortified with combination of BAP + GTA, BAP +CM and Kn +GTA, Kn +CM(Table 2 and 3)these results are also in agreement with those on *Tectona grandis* (Gupta *et al.*, 1980) *Abizzia lebbeck* (Gharyl and Maheshwari 1982) multiple shoot induction was also observed in *ziziphus manritiana* (Sudharshan *et al.*, 2000) and *Vavilla plantifolia* (Geetha *et al.*, 2000) shoot tips cultured on MS+ cytokinin alone as it was observed in the present studies.

Das and Mitra (1990) and Roy *et al.*, (1993) have also reported the requirement of both auxin and cytokines for induction of multiple shoots in *Eucalyptus tereticosvis* and Jack fruit respectively. Thus a combination of both auxin and cytokinin improved the efficiency of multiple shoots development although it depended on the combination and concentrations employed.

Zamen *et al.*, 1996) have studied the effect of different cytokininss viz.BAP,Kn, 2-ip and Zeatin on multiple shoot induction from shoot tip culture in mulberry. According to their observation, BAP and Kn were superior to 2ip and Zeatin. The superiority of BAP over other cytokinins for multiple shoot formation has been reported as it was observed in the present inverstigation in Mulberry by Hossian *et al.*, (1991).

Khanan *et al.*, (1998) have recorded the maximum number of shoots/explant culture (22.5) on MS medium containing 5.0 mg/L BAP in strawberry shoot tip culture. Gupta *et al.*, (1997) reported the multiple shoot bud induction from shoot apex cultured on MS medium containing BAP in Gasipium Nasir *et al.*, (1997) have studied the shoot meristem culture in 16 cultures of cotton using several media formation. They observed the best shoot developmentation MS media containing Kn alone compared to other media with NAA/IAA in combination with Kn, these results are to the present observation in *Cucumis melo* which cytokinin with L-Glutamicacid and Coconut milk showed increase number of shoots/explant .Sharma and Dhiman (1998) have also observed the similar results when they have cultured the shoot tips of F1.hybrid of *Paulo whia*.They found the highest multiple shoot induction on MS +BAP/Kn alone.

In the present investigation direct shoot bud proliferation/ regeneration was found from the shoot apices with out on intervening callus stage which is advantageous because explants could be taken from selected elite plants. The plants which are produced by direct organogenesis exhibit greater genetic stablility than those produced from callus. Thus the protocol developed for shoot and root development from, meristamatic shoot tips is quite simplest and needs less time and labour to regenerate large number of plantlets. Hence this regeneration system can be exploited for screening of large number of biolistic gun transformed meristematic shoot tips for deserible genes as a model system.

B. Nodal culture

The axillary / nodal bud induction is one of the most efficient method of micropropagation in plants since the emerging buds, especially from meristematic organs and tissues possess a great potential for vigorous development (Yadav *et al.*, 1990, Pattanaik *et al.*, 1995). Axillary buds have been found to be most suitable for clonal propagation in several species such as *Morus niger* (Yadav *et al.*, 1990) *Morus alba* (Pattanaik *et al.*, 1995) *Jasminum officinale* (Bhattacharya and Bhattacharya 1997), *Ixora singaporensis* (Malathy and Pai 1998) *Vanilla planifolia* (Geetha and Shetty 2000) *Ocimum sanctum* (Shahzad and Siddique, 2000), *Melia azedarach* (Raghuraman and Ramanjam 1998).

The present investigation describes a micro propagation technique using nodal bud/ Axillary bud culture as the source of direct production of multiple shoots in *Cucumis melo*.

Methodology**Plant material**

Nodal segments (1.0 cm -2.5 cm) of *Cucumis melo* bearing an axillary bud were collected from healthy young branches of 3 months old plants grown in the Research field, Department of Botany, Kakatiya University Warangal. The explants were washed under running tap water and followed by treating with 5% teepol for 5 minutes. These were washed thoroughly under running tap water and then surface sterilized with 0.1 % w/v Mercuric chloride (HgCl_2) for 3-4 minutes and later rinsed at least thrice with sterile distilled water. Sterilized nodal segments were dried on sterile filter paper before inoculation.

Culture media and culture conditions

The explants were inoculated on MS medium containing 30 gm/L Sucrose fortified with different concentrations and combinations of cytokinins (BAP, Kn) and L Glutamic acid, Coconut milk Table (9 and 10) and solidified with 0.8 % agar, all media were adjusted pH 5.8 before addition of 0.8% agar and autoclaved at 121°C under 15 psi for 15-20 minutes.

Culture tubes were maintained at 25±2°C with 16hrs photo period under white fluorescent light (40-60 mol m⁻² s⁻¹) for subculture of differentiating explants MS+

BAP /Kn and also in combination with L- Glutamic acid /Coconut milk media was used. The proliferated axillary shoots were transferred to rooting medium after 4 weeks of culture.

***In vitro* shoot multiplication and rooting**

Shoots developed from the nodal segments (axillary buds) were multiplied on the respective medium, the microshoots were transferred to MS Basal medium or ½ strength MS Basal medium containing IBA/IAA (0.1 -1.0mg/L) rooting was observed within 3-4 weeks.

Data analysis

At least 15 replicates were maintained for each treatment and data were recorded after 6 weeks of culture. Each experiment was repeated at least twice with similar results and data presented are of one representative experiment data were statistically analyzed.

Results

The axillary/ nodal bud cultures on the development of multiple shoots and roots are shown in (Table 9 and 10). The nodal buds of *Cucumis melo* on different hormonal combinations showed varied results. The axillary buds became active within weeks after inoculation and new shoots become distinct by the second and third week with leaves and internodes.

The explants survival from nodal segments of mature plant of *Cucumis melo* varied with season. According to the present observations, the explants were collected from field grown plants throughout the year to determine the ideal season for culture establishment. Explant collected in August to November period showed less time for sprouting and quick shoot bud proliferation and also recorded high percentage of explants establishment during this period.

The result on nodal bud culture of *Cucumis melo* culture on MS medium supplemented with Kn/BAP and GTA /CM are presented in Table (9-10) and shown in (Plates VII and VIII).

Effect of BAP + GTA

The nodal explants were cultured on MS medium with different concentrations of BAP (1.0 to 6.0) mg/L) and GTA (1.0 mg/L) at (1.0 mg/L) BAP + (1.0 mg/L) GTA induced 5.2 ± 0.4 shoots / explant with 50% culture response were recorded at (2.0 mg/L) BAP + (1.0 mg/L) GTA produced 6.2 ± 0.9 shoots/ explants with 62% cultures were responded. Maximum number of shoots were produced at (4.0mg /L) BAP + (1.0 mg/L) GTA, 6.6 ± 0.3 shoots /explant (Plate VIII) as the concentration of BAP was increased up to (1.0 mg/L to 4.0 mg/L) gradually the shoot bud proliferation was also found to increased and when BAP concentration was increased above 4.0 mg/L or 6.0 mg/L the rate of shoot bud multiplication and elongation was reduced.

Effect of BAP + CM

The medium containing CM (10% to 25%) more number of shoots were formed explants and also with longer shoots in the medium fortified with (0.5 mg/L) BAP + 20% CM induced maximum number of shoots (9.0 ± 0.3) shoots /explant were recorded (Plate VIII) (Table-9) as the percentage of Coconut milk was increased up to 10% gradually the shoot bud proliferation was also found to be increased.

Effect of Kn +GTA

Addition of L- Glutamic acid (1.0mg/L) to cytokinin, Kn (1.0 to 6.0 mg/L) tested maximum number of (6.0 ± 0.4 shoots / explant) were produced at (1.0 mg/L) GTA and (4.0 mg/L) Kn with high percentage of responding (75%) (Table 10) (Plate- VIII) cultures compared to all other combinations and concentrations tested. As the concentration of Kn was increased from (1.0 mg/L to 4.0 mg/L) gradually the shoot bud proliferation was also found to be increased and multiple shoots were reduced at (6.0 mg/L/Kn + (1.0 mg/L) GTA.

Effect of Kn + CM

The result on nodal bud culture of *Cucumis melo* on MS medium fortified with Kn (0.5 mg/L) and Coconut milk (10% to 25%). The medium containing (0.5 mg/L) Kn + 20% CM induced maximum number of shoots (7.5 ± 0.7 shoots /explant) (Table 10) (Plate VIII) and also showed high percentage (58%) of responding cultures. As the percentage of CM was increased up to 10% gradually the shoot bud proliferation was also found to be increased.

Discussion

Influence of explanting season on culture establishment was also noted in *Tridax Procumbense* (Sahoo and Chand 1988) as we have observed in *Cucumis melo* similarly this was sown in other medicinal herbs including *Ocimum* species (Ahuja *et al.*, 1982).

During the present investigations multiple shoots were induced on MS medium supplemented with various concentrations of cytokinins such as BAP/Kn alone and also in combination with GTA /CM. similarly Sudharshan *et al.*, (2000) have observed the multiple shoot bud induction from nodal segments of *Ziziphus mauritiana* on MS medium supplemented with BAP alone. It was also recorded the same results in *Vanilla Planifolia* on MS+BAP alone (Geetha and Shetty, 2000), when BAP and Kn Concentration was increased above (2.0) the rate of shoot multiplication and elongation was reduced in the present investigation. Similar results were obtained in *Canavalia nirosa* (kathiravan and Ignacimuthu 1999). *Vigna radiata* (Gulati and Jaiswal, 1994) and *Pisonia alba* (Jagadish Chandra *et al.*, 1999) shoot tip and nodal were found to be the best explants for multiple shoot formation (Sharma *et al.*, 1991; Hiraoke and Oyangi 1998). In accordance with this in the present study also the shoot tip and nodal explants were found to be suitable for multiple shoot regeneration in *Cucumis melo*.

Although the effect of BAP on *in vitro* regeneration of multiple shoot regeneration in *Ocimum sanctum* has been reported earlier (Pattnaik and Chand 1996). At high concentration of cytokinin lateral bud break was suppressed but callus proliferation improved as observed by Shahzad *et al.*, (2000) and also reported by Ahuja *et al.*, (1982) Pattanaik and chand (1996) and Sahoo and Chand (1998).

Direct regeneration of multiple shoots from nodal explants as observed on IBA + BAP /Kn supports the finding of Shahzad *et al.*, (2000) on *Ocimum sanctum* .Gulati and Jaiwal (1992, 1994) on *Vigna radiates* have also observed the maximum number of shoots on MS medium supplemented with auxin and cytokinin combination in nodal culture of *Decalepis hamiltonii*.

The same synergistic effect was also recorded in *Plumbago indica* inducing maximum number of shoots /nodal explants on MS +IAA (0.1 mg/L) + BAP (3.0 mg/L).

Similarly this stimulatory effect of a single supplement of cytokinin was reported earlier in other medicinal species including *Curcuma* spp and *Zingiber officinale* (Balachandran *et al.*, 1990) *Chlorophytum borinilianum* (Pattanaik and Chand 1996; Sahoo *et al.*, 1997) and *Tridax Procumbens* Sahoo and Chand 1998).

Thus direct multiple shoot production was observed from nodal segments of the species studied. This type of Clonal propagation has advantage, by producing true to type plants from a single individual in a relatively short time (Figure. 1).

Our result on Micro propagation using Shoot meristem / Nodal bud culture shows the considerable importance for large scale propagation of *Cucumis melo* an important vegetable plant.

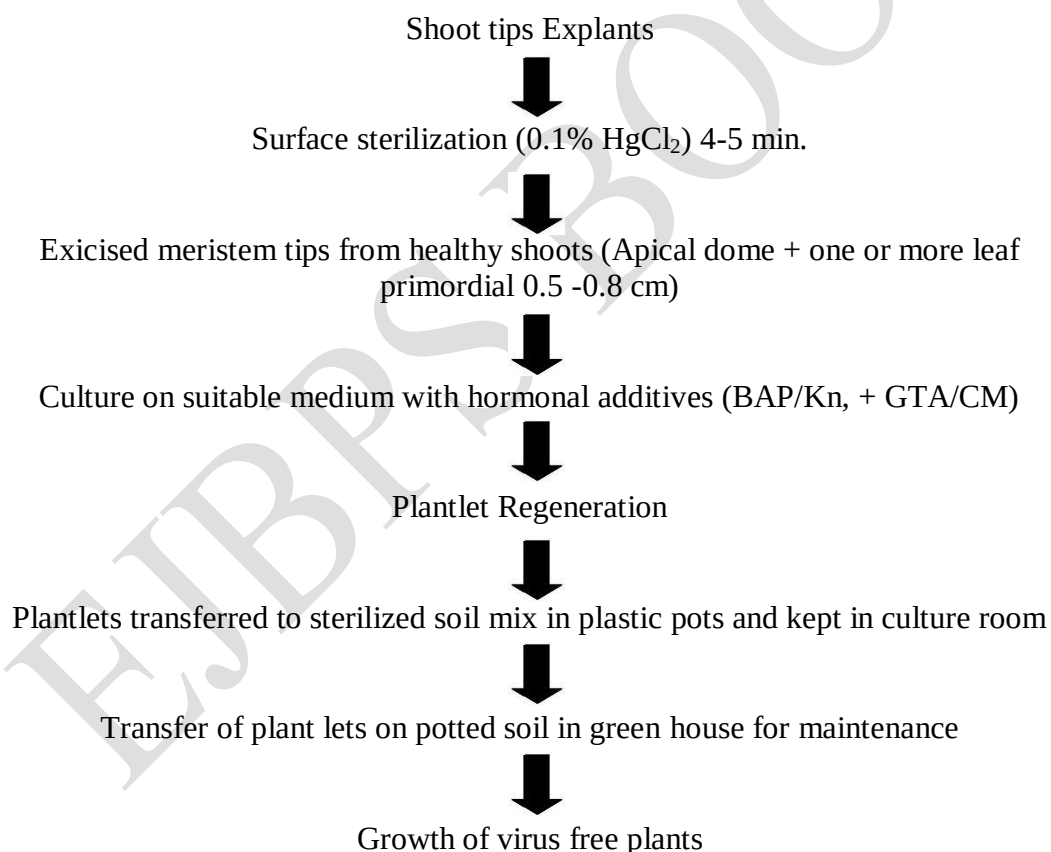


Figure I: Flow chart for Micro propagation through shoot meristem culture of *Cucumis melo*.

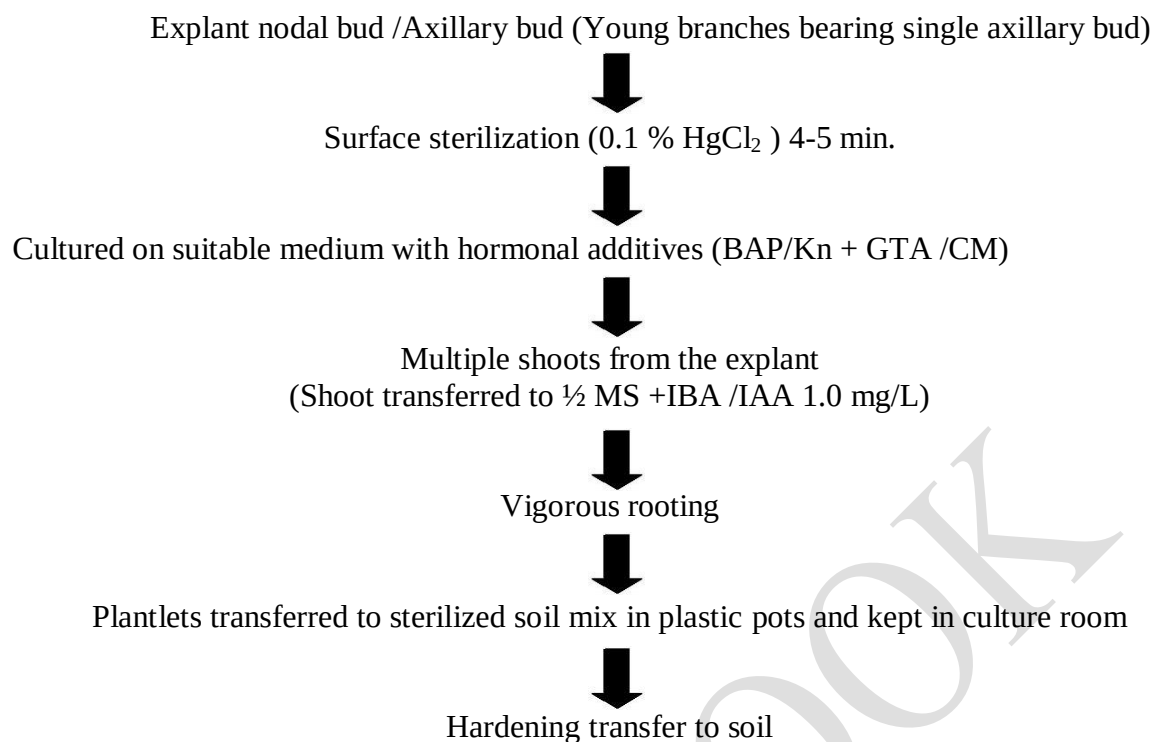


Figure II: Flow chart for Micro propagation through axillary bud/Nodal bud culture of *Cucumis melo*.

Table 7: Influence of L - Glutamic acid (GTA Coconut milk (CM), BAP in multiple shoot induction from shoot tip explant culture of *Cucumis melo*.

Treatment mg/L	% of cultures response	% of callus production	Average number of shoots /explant SE
BAP + GTA			
1.0 + 1.0	48	15	2.6 ± 0.2
2.0 + 1.0	55	12	3.2 ± 0.2
4.0 + 1.0	60	10	4.0 ± 0.4
6.0 + 1.0	53	08	1.8 ± 0.5
BAP + CM			
0.5 + 10%	57	10	3.0 ± 0.3
0.5 + 15%	50	07	4.5 ± 0.5
0.5 + 20%	58	05	6.0 ± 0.4
0.5 + 25%	53	10	2.0 ± 0.3

Table 8: Influence of L- Glutamic acid (GTA), Coconut milk (CM) Kn, on multiple shoot induction from nodal explant cultures of *Cucumis. Melo*.

Treatment mg/L	% of cultures response	% of Callus production	Average number of shoots/ explant S.E*
Kn + GA			
1.0 + 1.0	53	17	2.2 + 0.3
2.0 + 1.0	58	14	3.0 + 0.2
4.0 + 1.0	63	12	3.5 + 0.3
6.0 + 1.0	50	10	1.5 + 0.4
Kn + CM			
0.5 + 10%	58	12	2.0 + 0.4
0.5 + 15%	60	10	3.6 + 0.4
0.5 + 20%	68	10	5.0 + 0.3
0.5 + 25%	63	09	2.4 + 0.4

Table 9: Influence of L- Glutamic acid (GTA), Coconut milk (CM) BAP, on multiple shoot induction from nodal explant cultures of *Cucumis.melo*

Treatment mg/L	% of Cultures response	% of Callus production	Average number of shoots/ explant S.E*
BAP + GA			
1.0 + 1.0	50	15	5.2 + 0.4
2.0 + 1.0	62	14	6.2 + 0.9
4.0 + 1.0	56	13	6.6 + 0.3
6.0 + 1.0	54	10	2.4 + 0.4
BAP + CM			
0.5 + 10%	50	12	3.0 + 0.3
0.5 + 15%	48	08	4.0 + 0.4
0.5 + 20%	55	06	9.0 + 0.3
0.5 + 25%	40	10	2.0 + 0.4

Table 10: Influence of L- Glutamic acid (GTA), Coconut milk (CM) Kn and NAA on multiple shoot induction from nodal explant cultures of *Cucumis.melo*.

Treatment mg/L	% of cultures response	% of callus production	Average number of shoots/ explant S.E*
Kn+ GA			
1.0 + 1.0	53	17	4.2 + 0.4
2.0 + 1.0	60	14	5.3 + 0.5
4.0 + 1.0	75	16	6.0 + 0.4
6.0 + 1.0	50	18	2.5 + 0.4
Kn + CM			
0.5+ 10%	53	15	2.8 + 0.2
0.5+ 15%	47	10	3.5 + 0.4
0.5+ 20%	58	08	7.5 + 0.7
0.5+ 25%	43	12	3.2 + 0.6

Plate-VII



Multiple shoots formation from shoot tip explants of *Cucumis melo*.

- a) Induction of multiple shoots on MS + 1.0 mg/L GTA + 4.0 mg/L BAP.
- b) Multiple shoots developed on MS + 20% CM + 0.5 mg/L BAP.

Plate-VIII



Multiple shoots formation from nodal explants of *Cucumis melo*.

- a) Induction of multiple shoots on MS + 1.0 mg/L GTA + 4.0 mg/L BAP.
- b) Multiple shoots developed on MS + 20 % CM + 0.5 mg/L BAP.

Plate-IX



Multiple shoots formation from nodal explants of *Cucumis melo*.

- a) Induction of multiple shoots on MS + 1.0 mg /L GTA + 4.0 mg/L Kn.
- b) Multiple shots developed on MS + 20 % CM + 0.5 mg/L Kn.

CHAPTER- VI**PROTOPLAST ISOLATION**

Plant cells from which the cell wall has been removed are termed as protoplasts. Protoplasts are somewhat unique in plant cell culture in that they exist as separate cells without cytoplasmic continuity with neighboring cells. Treating tissues with a mixture of cell wall degrading enzymes Cellulase and Pectinase usually isolates Protoplasts; Osmotic stabilizers present in these solutions preserve the structures and viability of the protoplasts. The protoplasts regenerate a cell, undergo cell division and may grow into complete plants on suitable culture medium and environmental conditions.

The protoplast, also known as naked plant cell refers to all the components of plant cell excluding the cell wall, Hanstein introduced the term protoplast in 1880 to designate the living matter enclosed by plant cell membrane. The isolation of protoplasts from plant cells was first achieved by micro surgery on plasmolyzed cells by mechanical method (Klercker, 1892). Protoplast can be isolated from plant tissues (or) cultured cells by enzymatic digestion to remove the cell wall. Besides being useful for cell fusion studies, higher plant protoplasts can also take up. Through the naked plasma membrane, foreign DNA cell organelles, bacteria or virus particles these unique properties of protoplasts, combined with totipotent nature of plant cells, have opened up an entirely new area of fundamental and applied research in experimental biology and somatic cell genetics (Gleddie *et al.*, 1986).

Methods and procedure for protoplast isolation from plant tissues have long been known (Keller *et al.*, 1982) Recent advances in the isolation, culture and regeneration of plants from protoplasts of a wide diversity of species have been reported (Gleddie *et al.*, 1989).

Essential in the technique of genetic modification of plant cells through the protoplast system are

1. Isolation of protoplast
2. Culture of protoplasts to raise whole plant
3. Cell fusion
4. Introduction of foreign genetic material cell organelles into the protoplasts.

Source of material

The most convenient and populous source of plant protoplasts is the leaf because it allows the isolation of large number of relatively uniform cells without the necessity of killing the plants. Moreover, the mesophyll cells are loosely arranged, the enzymes have an easy access to the cell wall. When protoplasts are prepared from leaves, the age of the plant and the conditions under which it has grown may be critical. To achieve maximum control on the growth conditions of source plants several workers have used *in vitro* grown shoots (Binding, 1975; Schieder, 1978a; Butenko and Kuchko, 1980). In some species where it is difficult to isolate culturable protoplasts from leaf cells alternative source material of cultured cells have been used. The yield of protoplasts from cultured cells depends on the growth rate and growth phase of cells. Frequently sub cultured suspension cultures, and cells taken from the early log phase are almost suitable (Vasil and Vasil, 1979).

Enzyme treatment

The release of protoplasts is very much dependent on the nature and concentrations of enzymes used. The two enzymes regarded essential to isolate protoplasts from plant cells are cellulose & macerozyme. Driselase, having a number of zymolytic activities such as cellulase, pectinase laminarinase and zylanase has proved especially useful for isolating protoplasts from cultured cells. Increase in yield of mulberry protoplasts by treatment with chemical substances has been reported earlier (Ohnishi and Kiyama, 1987).

Osmoticum

A fundamental property of isolated protoplasts is their osmotic fragility and hence the need for suitable osmotic stabilizer to the enzyme solution, the protoplast washing medium and protoplast culture medium is necessary. A variety of solutes, ionic and non-ionic, have been tested for adjusting the osmotic pressure of the various solutions used in protoplast isolation and culture, but the most widely used osmotica are sorbitol and mannitol.

With advancement in plant tissue culture technology, plant regeneration systems from protoplasts have been developed in many mulberry species (Ohyama and Oka, 1975). Establishment of protoplast regeneration system using new technologies such as

protoplast fusion and gene transfer would contribute to the improvement of mulberry varieties.

Information on necessary conditions for in vitro culture and totipotency of cultured tissues, cells, and protoplasts is essential for somatic hybridization studies. Plant cells without cell walls are called protoplasts and they are appropriate receptors for transferring genes. In the *Solanum* genus, protoplast culture and plant regeneration procedures have been established for *S. tuberosum* (Shepard and Totten, 1977), *S. dulcamara* (Binding and Nehls, 1977), *S. nigrum* (Nehls 1978), *S. phureja* (Schumann *et al.*, 1980), *S. aviculare* (Gleddie *et al.*, 1985), and *S. tuberosum* (Wenzel *et al.*, 1979; Tavazza and Ancora, 1986). Optimization for large-scale isolation of viable protoplasts and subsequent in vitro plant regeneration are pre-requisites for producing plants with desired traits through somatic hybridization and genetic engineering techniques (Saxena *et al.*, 1987; Kaur *et al.*, 2006).

In the present study, attempts have been made to study protoplast isolation and purification using leaf explants of *Cucumis melo*.

MATERIAL AND METHODS

Isolation of protoplasts from mesophyll cells. Seeds of *Cucumis melo* were used in our study. The seeds were obtained from Research laboratory, Department of Botany, Kakatiya University, Warangal and the seeds were presoaked overnight and washed under tap water for 30 min to remove adherent particles. The seeds were immersed in 5% (v/v) Teepol for 10 min, rinsed with sterile double distilled water. This was followed by surface sterilization with 0.1% (m/v) HgCl_2 for 5 min and rinsed 5 times in sterile distilled water. The sterilized seeds were then placed on to basal Murashige and Skoog (1962) medium for germination.

The leaves of 2-3 cms in length and 1- 1.5 cm in width were excised from 6 weeks old seedlings. The leaves were cut into pieces smaller than 1mm and incubated in filter sterilized enzyme solution. The enzyme solution consisted of 20% cellulose —onuzuka R-10 and 1% macerozyme R 10 prepared in MS salts at pH 5.5 with 0.6 M mannitol and osmoticum. The sliced leaf pieces of all the cultivars were incubated in 10 ml of enzyme solution at 27°C and shaken at 40- 50 rpm for 4-5 hrs in dark.

Isolation of Protoplasts from callus cultures

Seeds of *Cucumis melo* were soaked for 24 hrs in sterilized water and surface sterilized with 0.1% HgCl₂ for 3 to 5 minutes. Then these were washed 3 times with sterile distilled water for 5 minutes and germinated aseptically on MS basal medium. The cotyledon (0.5 – 0.8 cm²) from 4- weeks old axenic – seedlings were excised and inoculated to MS medium supplemented with 2.0 mg/L 2,4-D. alone and gelled with 0.8% agar cultures were maintained at 25 ± 2°C under a 16 hrs. Photoperiod was provided by cool white fluorescent lamps.

15 – 20 days old. One – gram friable callus derived from cotyledon explants was gently broken into small pieces of callus. Incubation was carried out in 10ml of digestion solution containing 1% cellulose and 0.5% macerozyme with 0.6 m mannitol as osmoticum. Flasks were incubated at 27°C and shaken at 50 rpm for 3-4 hours in dark.

Purification of Mesophyll and callus derived Protoplasts.

The protoplasts were then purified by 60 µ m s steel mesh. The filtrate was collected in screw cap centrifuge tube and centrifuged at 50 g for 5 minutes. The supernatant was discarded and the pellet containing protoplasts was loaded on 20% sucrose solution for purification and centrifuged at 100g for 10 minutes to get a distinct protoplast band. The band was taken in a screw cap centrifuge tube and washed with 5ml of 0.6M mannitol by centrifuging at 50g for 5-7 minutes. The pellet was suspended in culture medium containing MS + 0.6 M mannitol + 2, 4-D and BAP at pH 5.7 and the protoplast yield was estimated using hemocytometer. Proto col is adopted for the purification of mesophyll derived protoplasts.

Results

A mixture of 2% cellulose and 1% macerozyme was suitable for isolation of viable protoplasts from mesophyll tissues of leaf cultures of *Cucumis melo* were investigated each enzyme was in effective by itself but when used in combination it resulted satisfactorily (Evans and Bravo 1983). For *in vitro* leaf explants above combination of macerozyme and cellulose gave an optimum yield.

In case of *Cucumis melo* cultivars, prolonged incubation period's i.e 10-12hrs were observed to be unfavorable shrinkage of protoplasts in these cultivars. Cell digestion

was taisy good when above mentioned conditions were applied for protoplast isolation. The number of protoplasts showed increase during shorter treatment time and reached a peak at 4-5 hours of incubation in dark. Beyond 5 hours of incubation the protoplast yield gradually decreased and further resulted in complete shrinkage of protoplasts at 10-12 hours of incubation from these result it was estimate that the adequate time for enzyme treatment to isolate maximum number of protoplasts from mesophyll cells of *Cucumis melo*.

Discussion

We were successful in protoplast isolation from callus culture and leaf mesophyll cells on the enzyme solution consisted of 2% cellulose —onuzuka R- 10 and 1% macerozyme R 10 prepared in MS salts at pH 5.5 with 0.6 M mannitol as osmoticum. Mesophyll cells and callus cells immersed in the enzyme mixture shaken at 40-50 rpm in dark for 10-12 hours resulted in better yield of protoplasts cell digestion was fairly good when above mentioned conditions were applied for protoplast isolation.

Several authors have reported that cell digestion and protoplasts yield in case of mulberry was fairly good during 12-13 hours incubation in dark (Tewary and Lakshmisita 1992).

Tavazza & Ancora (1986) found out that low concentrations of macerozyme avoided the toxic effects due to long exposures to this enzyme. Conversely high concentrations of the same enzyme yielded more protoplasts but there was more debris and non viable protoplasts. Protoplasts are fragile and sensitive to digesting enzymes. Plasmolysis prior to enzymatic digestion of source tissues in sugar alcohol solutions, such as sorbitol 0.5 M causes the cell to draw back from the cell wall and reduces cytoplasmic damage and spontaneous fusion of protoplasts from adjacent cells.

Protoplast yield and viability can be further enhanced by slicing source (preplasmolysed) tissues in a suitable osmotic solution (Power *et al.*, 2004). Also, the effect of the plasmolyzing solution (mannitol 13%) in the recovery of viable protoplasts was positive, which is similar to the results observed by Belarmino *et. al.*, (1994) working with sweet potato. It has been proposed that this solution reduces the osmotic shock during protoplast isolation by decreasing leakage of electrolytes

(Winkelmann *et. al.*, 2006). Most protoplasts have an optimum plating density related to their potential division. At this density (usually 104 -105 cel ml⁻¹) plated protoplasts produce cell colonies after 2-3 weeks of culture (Davey *et al.*, 2005).

In our experiments *Cucumis melo* yielded 105 -106 cel ml⁻¹ protoplasts with the isolation method proposed. With these densities the cell colonies arising from individual protoplasts tend to grow at a fairly early stage in culture. The nutritional components of the most commonly used culture media are not sufficient to induce divisions in protoplasts plated at low density. Generally high light intensity inhibits protoplasts growth when applied from the beginning of culture. It is better to initiate protoplasts culturing in darkness conditions.

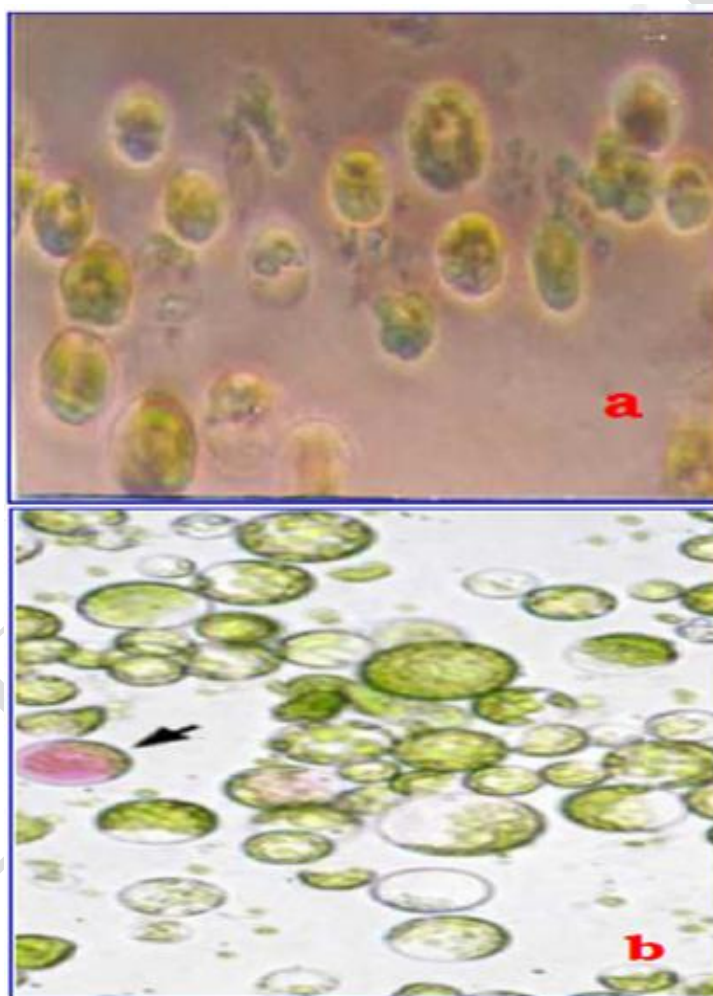
There are reports of better protoplasts growth when the cultures are kept in continuous darkness (Chawla, 2002). Growth and shoot initiation of potato protoplasts also depends on the composition of the culture media and genotype (Foulger & Jones, 1986). Protoplasts cultured on culture medium supplemented with 1% serum bovine albumin (Binding & Nehls, 1977) divided many times at a relatively high frequency leading to a high rate of colony formation in both species. The same pattern of cell division was observed by Fock *et. al.*, (2001) when they cultured protoplasts of *Cucumis melo*.

Protoplasts from *Cucumis melo* cultivar Desiree showed a higher division frequency when they cultured in alginate beads and culture medium. Likewise nutritional and physical factors affect colony formation from protoplasts, and dark conditions were suitable for culturing protoplasts and to induce colony development (Ehsanpour and Jones, 2001; Davey *et. al.*, 2005; Winkelmann *et. al.*, 2006). Low light conditions were beneficial for calli formation and regeneration (not shown). This result was similar to that mentioned by Shepard & Totten (1977) working with potato, who found out that high light intensities decreased the callus ability to form shoot buds. Shoots arose from non-embryogenic calli. It has been reported that shoot formation from calli derived from protoplasts is genotype dependent (Thomas, 1981).

However our findings agree with the results of who have examined a similar effect of enzyme treatment time for shorter duration (10- 12 hours) on protoplast isolation from seedling cotyledons of *Cucumis melo*.

While in the case of callus, cellulose 1% and macerozyme 0.5% yielded maximum number of protoplast. The use of callus as source for protoplast isolation may overcome the difficulty encountered with the production of leaf material in some recalcitrant *Cucumis melo* cultivars. Callus developed on 2.0 mg/L 2,4-D was used for protoplast isolation. A part from sub cultured callus the use of cell suspension culture is also avoided here to isolate protoplasts; this is because the suspension culture may accumulate changes in ploidy and aberrations due to soma clonal variations (Larkin and Scowcroft 1981).

Plate-X



Protoplast Isolation in Leaf explants of *Cucumis melo*.

Fig. a: Fresh and viable mesophyll derived Protoplast.

Fig. b: Clear view on Fresh and viable mesophyll derived Protoplast.

CHAPTER- VII**SUMMARY AND CONCLUSION**

Plant cell and culture technology could serve as a supplement to the conventional methods of plant breeding for production of elite plantlets *in vitro*. This is an important propagation technology to agriculture. During the last decade significant progress has been made in the propagation of plants with novel feature through tissue culture technology. To meet the ever growing commercial requirements. The realization of *in vitro* multiplication of large number of clonal plant with the improved characters has been gaining significance.

Cucumis melo Linn contains species which are useful as vegetable and medicine many *Cucumis* varieties have been exploited through *in vitro*. During the last ten years lot of research work has been gaining much importance in improvement of *Cucumis* varieties *in vitro*.

Considering the importance of the Genus *Cucumis melo* an attempt has been made to achieve the following objectives using the tissue culture technology for improvement of the species.

- To establish the protocol for callus induction from different explants viz. cotyledon, hypocotyls and leaf etc.
- To standardize the hormonal concentrations for callus mediated regeneration.
- To achieve the direct regeneration in different explants by modulating the concentrations and combinations of auxins and cytokinins and also plantlet establishment.
- Explants from 30-day-old seedlings raised in glass bottles produced a higher number of multiple shoots (8.0 shoots/explant) than those grown in glass tubes and cultured on the same shoot induction medium.
- Rooting of *in vitro* shoots was achieved on MS basal medium supplemented with IAA/IBA (2.0 mg/L). Tissue culture plantlets survived (95%) after hardening under greenhouse conditions.
- To establish the protocol for micro propagation /clonal propagation using nodal and shoot tip explants.
- High percentages of protoplast were formed from Leaf explants in protoplast isolation.

Present works was under taken with a view to study and evaluate the effect of plant hormones, amino acids and coconut milk on micro propagation, induction of callus and regenerating ability from different explants of *Cucumis melo* L. on induction of callus, direct organogenesis and micro propagation was also studied.

During the present investigation, *Cucumis melo* var. Utilissimus commonly known as snake cucumber was choosen to study the micropropagation, callogenesis and regeneration.

Cucumis melo var Utilissimus is very popular in Maharastra resembles cucumber and used as a vegetable. The fruits are slender and alongated, the length varying from a few inches to about 3 ft. the tender frits are eaten raw or with salt and salads. They are also pickled and cooked into curries. The fruits form an important food during the hot weather. They are nourishing and useful in quenching thirst. The seeds are small and edible and are used in confectionery. A refreshing drink is prepared from ground kernels. The seeds are cooling and diuretic *Cucumis melo* Cv Bathasa is popular in Andra Pradesh it is a climbing or creeping herb.

After selecting the medium abroad spectrum of media containing various growth hormones in different concentrations and combinations were studied by culturing different explants Viz. cotyledon, hypocotyls, and leaf *Cucumis melo* var *Utilissimus* and also CV Bathasa under investigation.

In the present investigation on micro propagation studies were carried out by using the explants Viz. Cotyledon, hypocotyls and leaf. A simple and reproducible procedure was devised to obtain multiple shoots and regeneration of complete plantlets from different explants of *Cucumis melo*. among all the explants used cotyledon, hypocotyls and leaf explants were the best for multiple shoot induction.

MS medium supplemented with BAP and IAA initiated formation of shoots from leaf cultures. Addition of Kinetin along with L-Glutamic acid and 20% coconut milk induced multiple shoots (6) from shoot tip culture. The cotyledon hypocotyl and leaf explants on MS medium containing 2,4-D, IAA and NAA initiated callus.

Addition of 2.0 mg/L BAP 3.0 mg/L Kinetin to the MS medium induced shoot regeneration from hypocotyls, cotyledon and leaf cultures with in a period of two

weeks in culture. With an increase in the level of BAP (1.0 -6.0 mg/L) the percentage of shoot tip and nodal explants producing shoots also increased. The maximum number of shoots from shoot tip explants (4.0) were observed at 4.0 mg/L BAP.

In the present studies, the inductions of multiple shoots were reported from cotyledon, shoot tip, nodal, hypocotyls and leaf explants. To gain success in tissue culture experiments production of callus and its proliferation is highly essential. Callus inducing ability of explants like cotyledon, hypocotyls and leaf of *Cucumis melo* were tested on different growth media i.e., MS medium supplemented with different concentrations of 2,4-D, IAA and NAA. It was observed that 2,4-D was efficient in producing callus. 2,4-D increased the efficiency of callus induction in such explants. During the present investigations callus was found to be mostly friable, may be due to 2,4-D that has been used for callus induction. Concentrations of the growth hormones above 3.0 mg/L reduced the callus production.

In the present findings the methods of micro propagation, callus induction, regeneration are the fundamentals for the application of various technologies. These methods can be utilized for the improvement of *Cucumis melo* and also other vegetable Crops.

Conclusions

- ❖ Based on the experimental data the following conclusions can be made.
- ❖ Callusing efficiency from different explants in *Cucumis melo* was studied and the protocols have been established.
- ❖ Callusing efficiency from different explants in *Cucumis melo* was studied and the protocols have been established.
- ❖ Among the auxin tested IAA or NAA or induced the high quantity of callus in all the explants used.
- ❖ Leaf explants were proved to be better in inducing high yield of callus.
- ❖ The combination of IAA + BAP induced more number of shoots than IAA + Kn.
- ❖ Protocols have been established for efficient regeneration from different explants via direct organogenesis.
- ❖ High frequencies of shoots were formed directly on medium containing IAA + BAP.

- ❖ High frequencies of adventitious shoots were formed on medium containing both auxin and cytokinins in callus mediated regeneration.
- ❖ High frequency of plant regeneration or protocol was optimized in cotyledon explants.
- ❖ Cotyledon explants were found to be efficient in producing maximum number of shoots per explants.
- ❖ High reproducible protocol was developed for colonel propagation/ micro propagation.

Since the species *Cucumis melo*. Vegetative yielding plant were commercial significance it can be utilized for bio production of useful secondary metabolites and its multiplication and propagation using the protocols established in the present investigations provide an opportunity to study the bio synthesis of important metabolites with the possibility of year round production of bio active compounds. Thus from the foregoing it can be concluded that *Cucumis melo* can also be used as a modal system for introducing different types of foreign genes using the high frequency regeneration protocols established in the present investigations.

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ISSN:2349-8870

