IN VITRO MULTIPLICATION STUDIES IN BAEL [*Aegle marmelos* (L.) Corr.]

By

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IN THE MEMORY OF MY FATHER----------

DEDICATED

TO
MY BELOVED MAA

ABBREVIATIONS USED IN THE TEXT

BAP/BA	:	6-benzylaminopurine/benzyladenine
cm(s)	:	Centimetre (s)
^0C	:	Degree elsius
cv. (s)	:	Cultivar (s)
EDTA	:	Ethylene diamine tetracetic acid
et al.	:	Et alia = and other
Fig.	:	Figure
GA_3	:	Gibberellic acid
HCl	:	Hydrochloric acid
$HgCl_2$	:	Mercuric chloride
IBA	:	Indole-3-butyric acid
in vitro	:	Under aseptic conditions
MgL^{-1}	:	Miligram per litre
KIN	:	Kinetin (6-furfurylamino purine)
Min.	:	Minute
ml	:	Millilitre (s)
MS	:	Murashige and Skoog's (1962) basal medium
MM	:	Millimolar
µM	:	Micro molar
N	:	Normal solution
NAA	:	α-naphthaleneacetic acid
NaOH	:	Sodium hydroxide
NM	:	Nano meter
%	:	Per cent
pH	:	Negative logarithm of hydrogen ion concentration
Sec.	:	Second
SE	:	Standard error
v/v	:	Volume/volume
viz.	:	Namely
w/v	:	Weight/volume
2,4-D	:	2,4-dichlorophenoxy acetic acid

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CERTIFICATE - I

This is to certify that this dissertation entitled "***IN VITRO* MULTIPLICATION STUDIES IN BAEL [*Aegle marmelos* (L.) Corr.]**", submitted for the degree of Doctor of Philosophy in the subject of Horticulture to the Chaudhary Charan Singh Haryana Agricultural University, Hisar, is a bonafide research work carried out by **Mr. MURARI LAL** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

Dr. S. S. Sindhu
Major Advisor

CERTIFICATE - II

This is to certify that this dissertation entitled " ***IN VITRO*** **MULTIPLICATION STUDIES IN BAEL [*Aegle marmelos* (L.) Corr.]**", submitted by **Mr. MURARI LAL**, (Admn. No. 98A46D) to Chaudhary Charan Singh Haryana Agricultural University, Hisar, in partial fulfillment of the requirements for the degree of Ph.D., in the subject of Horticulture has been approved by the Student's Advisory Committee, after an oral examination on the same, in collaboration with an external examiner.

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MK

Place : Hisar **[Murari Lal]**

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CHAPTER : I

INTRODUCTION

Aegle marmelos (L.) Corr., locally called *bael* is a tree species belonging to the family Rutaceae. In English, it is known as Bengal Quince and comes under hard shelled citroid fruit tree group. It is medium sized, slow growing, deciduous, spiny woody fruit tree with trifoliate aromatic leaves of the tropics. Its flowers are sweet-scented and bloom during the spring. It takes 10-12 months from flowering to fruit ripening. Fruits are large, globose, ovoid or pyriform, green when unripe and turn yellow brown when ripe. Seeds are embedded in aromatic, sweet, thick pulp. The testa is woody and the seeds are oblong, compressed, the embryo has a thick fleshy cotyledon.

Bael is a native of the Indo-Burma sub continent (Hooker, 1975) and is now cultivated as a backyard tree in the South East Asia, especially in India, Pakistan, Bangladesh, Sri Lanka, Burma (Myanamer) and Thailand (Zaman, 1988). The crop is widely cultivated in U. P., particularly in Mirzapuri, Varanasi, Gorakhpur, Basti, Gonda, Faizabad, Etawah districts and also in Seven district of Bihar (Teaotia *et al.,* 1963). So far there are no

organized orchards of bael in our country. The plant has the capacity to adapt successfully to a wide range of habitats from arid, semi-arid to mesophytic conditions (Singh *et al.,* 1976; Arya, 1986).

Bael is a multipurpose fruit crop with considerable traditional socio-cultural values. Leaves, flowers and fruits have been used in various rituals of the Hindu religion in India since ancient times (Sharma, 1980). The fruit is the most important part of the plant which has a number of uses. Fruit pulp is rich in starch, calcium, iron and vitamin C (Hassan, 1988). Ripe fruit pulp is tasty and can be consumed fresh or made into 'Sharbat', one of the most popular drinks in the Indo-Bangla Sub-continent. Roasted or dried pulp is purgative, astringent, digestive tonic and stomachic (Heywood and Chant, 1982). In pharmacological trials, both fruits and roots have shown antiamoebic and hypoglycaemic activities (Kirtikar and Basu, 1975; Ponnachan *et al.,* 1993). The alkaloid aegeline present in the leaf is a potent antasthmatic agent (Haravey, 1968). It is one of the ingredients in the 'Dasamul' or ten roots, which is an important preparation in Ayurvedic system of medicine. The active principle in this plant is 'marmelosin' which acts as a laxative and diuretic (Nadkarni, 1954). The plant produces timber and is especially valuable for afforestation programme in arid and semi-arid areas because of its high drought tolerance (Singh *et al.,* 1976; Arya, 1986).

Bael is propagated either by seeds or root suckers. Propagation by grafting, budding and cutting are feasible for commercial propagation. However, it is slow, difficult and cumbersome. Since it is a cross-pollinated plant, maintenance of varietal purity is one of the important problems, because of enormous heterozygosity, sibling pollination may produce

inbreeding depression. *In vitro* propagation is an important tool in this direction to have true to type, disease-free and quality plants throughout the year. Singh (1963) was the first to report the *in vitro* culture of *bael* but regeneration of plants from nucellar tissue was not possible. Arya *et al.* (1981) described callus formation and some organogenesis from cultures developed from cotyledon and hypocotyl explants. The nucellus is a non-vascularized tissue very suitable for experimental studies on xylogenesis and it holds great potential for applied research in horticulture, development of nucellar seedlings and induction of polyembryony. Recovery of plants from nucellar tissues would eliminate systemic disease and facilitate storage and international exchange of germplasm (Rangaswamy, 1981). Recently, regeneration of multiple shoots through organogenesis was achieved from seedling leaves (Islam *et al.*, 1992, 1993), cotyledons (Hossain *et al.*,1994b), nucellus from developing fruits (Hossain *et al.*, 1993, 1994b) and root tips of intack seedlings (Islam *et al.*, 1996). Ajith Kumar and Seeni (1998) described callus free micropropagation system for producing a large number of plants from stem node explants of selected mature trees. Therefore, the present study was planned with the following objectives :-

i) To standardize the optimal culture conditions for shoot formation and plantlets regeneration from cotyledon explant.

ii) To standardize protocol for callus induction and its regeneration.

CHAPTER : II

REVIEW OF LITERATURE

The work done in India and abroad on the problem entitled "*In vitro* multiplication studies in bael [*Aegle marmelos* (L.) Corr.]" is reviewed as under with appropriate heads and sub-heads.

The term tissue culture technique colloquially covers a wide range of techniques including *in vitro* culture of organs (shoot tips, root tips, runner tips, stem segments, cotyledons, epicotyls, hypocotyls, leaf discs, flowers, anthers, ovaries, ovules, emryos etc.), tissues, cells and protoplasts. These techniques are becoming useful tools for rapid and clonal multiplication of plants. Since 1902, when Haberlandt conceived the idea of 'Totipotency', considerable success has been achieved in the field of plant tissue culture. The commercial feasibility of this technique has been demonstrated in many diverse plant species including fruit plants. White (1934) and Gautheret (1939), for the first time succeeded in raising continuously growing cultures with explanted tissues of chosen dicotyledonous plants. A breakthrough which has made tissue cultures applicable to the propagation of diverse

plants has been the discovery of Skoog and Miller (1957) that root and shoot initiation was basically regulated by interactions between two hormonal substances of auxins and cytokinins. Their work with tobacco callus cultures showed that both substances are necessary for tissue growth, the pattern of organogenesis depends upon their relative concentrations and sequence of application in the nutrient medium. A relatively high concentration of auxins favours root initiation, while suppressing shoot formation. In contrast, relatively high concentration of cytokinin induced shoot initiation and suppressed root initiation (Murashige, 1974). Steward *et al.* (1958) showed that certain specialized cells of plant body can revert back to meristematic state and subsequently regenerate into whole plant under defined hormonal, nutritional and physical conditions. One of the applications of this technique for rapid clonal propagation originated with the work on orchids by Morel (1960). Murashige and Skoog (1962) added another dimension to all cell culture techniques by standardising basic nutrient medium using tobacco cell cultures and their medium is one of very widely used media in the present day *in vitro* experiments. Winton (1970) was first to obtain fully organized plantlets of a tree species (*Populus tremuloides*) under aseptic conditions.

The progress in tissue culture work has been impressive and its application can be considered as under :

i) Rapid multiplication of selected genotypes and virus-free plants by meristem culture (Murashige, 1974, 1977, 1978; Rybalko and Kharuta 1978; Pena *et al.*, 1979; Fawzy *et al.*, 1992; Waithaka *et al.*, 1992; Denkova *et al.*, 1995; Raja *et al.*, 1999).

ii) Recovery of difficult to produce hybrids via proembryo culture (Bhojwani and Bhatnagar, 1978; Raghavan, 1977).

iii) Culturing pollen and producing androgenic haploids (Guha and Mahe Shwari, 1966).

iv) Production of mutant cell lines (Shirvin, 1978; Chaleff, 1981 and Larkins and Blowrroff, 1981).

v) Production of secondary metabolites (Carew and Staba, 1965; Street., 1973 and Constable *et al.,* 1974).

The regulation of growth and the development of a plant are complicated processes and are affected by various factors like genetic make up of the plant, explant type, media regimes, physical cultures environment and their interactions (Pierik, 1987).

In general, the efficiency of micropropagation depends on the complex interaction of factors which can be broadly classified into following categories :

a) Factors related to the source of explant and the explant itself (including their treatment while preparing them for *in vitro* culture).

b) Factors related to constituents of culture media not the culture conditions (culture environment).

c) Routes of micropropagation followed.

d) Performance of regenerated plantlets (including number of regenerated plantlets transferred to pots, number of regenerated plantlets survived in pots and survival percentage).

The review of literature that follows is aimed at highlighting the

factors and problems related to *in vitro* propagation of *bael* in particular and other woody plants in general. The relevant literature related to present studies is reviewed as under :

2.1 SELECTION OF PARENT MATERIAL:

Plant material with proven silvi-cultural traits is selected for the micropropagation. This can either be a selected phenotype (a specific tree), or the source of elite seeds (Debergh and Read, 1991). Sometimes, the explants from mature trees growing in the field are avoided due to the problem of endophytic microorganisms in vegetative explants. Instead the propagules (vegetative or sexual) from such trees are grown in more hygenic conditions in the green house (Debergh and Maene, 1981) to eliminate the contamination.

2.2 SELECTION OF EXPLANTS :

A judicious selection of type of explants could go a long way in achieving the eventual success in micropropagation. Murashige (1974) described the different factors that influence the response of explants, which include size and source of explants, season of collection, age of parent etc. Many workers have confirmed that the quality of explants primarily determines the establishment of *in vitro* cultures (John and Murray, 1981; Kim *et al.*, 1981; Keathley, 1984).

2.2.1 Source and type of explant :

Selection of explant is important in micropropagation (Williams and Maheswaran, 1986). Regeneration of explants depends on number of factors. These involve following factors decide the suitability of an explant :

i) The selection of organs that serve as tissue source.

ii) The season in which the explant is being obtained

iii) The size of explant.

iv) Original position of the explant in the plant.

v) Age of the plant and overall quality of the plant from which explants are to be obtained (Murashige, 1974b).

Hossain *et al.* (1994a) observed that cotyledons from seedlings of various ages of *bael* were cultured on Murashige and Skoog (MS) medium supplemented with different combinations of phytohormones. The optimum seedling age was 10 days for shoot induction response and benzyladenine (BA) was superior to either kinetin, isopentenyladenine or Zeatin. The optimum cytokinin (BA) concentration for bud induction was 2 mgL^{-1}, which improved shoot regeneration efficiency. The proximal part of the cotyledon had the highest regeneration potential. Arumugam and Rao (1996) reported that cotyledonary node explants, excised from 15-day-old seedlings of *bael*, were placed on Murashige and Skoog (MS) medium supplemented with BAP (Benzyl adenine), IBA or NAA. BAP induced the production of multiple shoots and subsequent plant regeneration. The highest number of shoots (75.2/explant) was observed on MS medium supplemented with BAP at 3 mgL^{-1}. The number of shoots was further enhanced by (i) using nodal explants of *in vitro* regenerated shoots as micro cutting and (ii) repeated subculture of the original explants on the same medium after excising the shoots. More that 12376 shoots were obtained from a singLe explant within 5 months. Islam *et al.* (1994) found a protocol for excising and culturing cotyledon explants from seeds of different ages was developed for the purpose of mass propagation. Cotyledon explant formed callus and shoot

buds on supplemented MS agar medium. The highest frequency of explants forming adventitious buds and the maximum number of shoots per explant were obtained with cotyledons 110-150 days old.

2.3 GENOTYPE OF PARENT :

Genotype is one of the important factor in determining the regeneration and culturability of plant cells, tissues and organs and has considerable bearing on the success of tissue culture programme. There are certain varieties which can be easily micropropagated with high success compared to others which exhibit low success.

Multiple shoots were obtained from shoot tips (2 to 3 mm) derived from mature plants (5 to 6 years old) of *Citrus reticulata* Blanco cv. Khasi mandarin and *Citrus limon* Burm. f. cv. Assam lemon when cultured on Murashige and Skoog (MS) medium, supplemented with 1.0 mgL^{-1} BAP, 0.5 mgL^{-1}KIN and 0.5 mgL^{-1} NAA. Root induction was observed when 7 week-old single shoots (2 cm long) of both citrus species were cultured on MS medium supplemented with 0.25 mgL^{-1} BAP, 0.5 mgL^{-1} NAA and 0.5 mgL^{-1} IBA (Singh *et al.*, 1994). Ghorbel *et al.* (1998) defined tissue culture techniques for morphogenesis and production of whole plants of grapefruit, sour orange and alemow. The NAA and BA concentrations to induce root formation and bud and shoot regeneration in stem segments were determined. No roots formed in sour orange over the range of 0-162 μM NAA. All species regenerated buds in response to BA but only those of alemow elongated sufficiently to root them.

Hazarika *et al.* (1996) reported that *in vitro* grown shoots of *bael* were cultured in MS medium supplemented with BAP (benzyladenine) (0,

0.25, 0.5, 0.75, 1.0, 1.25 or 1.5 mgL^{-1}). The highest shoot number and weight were observed after 8 weeks of culture on a medium supplemented with BAP at 0.5 mgL^{-1}. Shoots were rooted on half strength MS medium supplemented with 0.5 mgL^{-1} IBA. Arumugam and Rao (1996) observed that cotyledonary node explants excised from 15-day-old seedling of *bael,* were placed on MS medium supplemented with BAP, IBA, IAA or NAA. The highest number of shoots (75.2/explant) was observed on MS medium supplemented with BAP at 3 mgL^{-1}. More than 12376 shoots were obtained from a singLe explant with in 5 months. Regenerated shoots produced roots in 30% of cultures when transferred to a medium containing IBA at 4 mgL^{-1}.

A protocol for organogenesis from leaf explants of *in vitro* grown seedlings of *AegLe marmelos* is presented. Adventitious buds were initiated on MS medium containing various concentrations and combinations of BA and IAA. The maximum frequency of shoot organogenesis (61.2%) and the highest number of shoot per explant (38.4) were obtained when 1.5 mg BA and 0.5 mgL^{-1} IAA were used. Twenty day old seedlings proved to be the optimum source for explants. Shoots were elongated on the same basal medium without auxin and supplemented with 0.5 mgL^{-1} BA. Elongated shoots were rooted on half strength MS medium supplemented with 0.1 mgL^{-1} IBA (Islam *et al.* 1993a).

2.4 METHODS OF DISINFECTION OF THE EXPLANTS

Successful disinfection of explants is a pre-requisite for *in vitro* culture and often involves a standard set of treatments which vary with the type of explant and species in question (Thorpe and Patel, 1984).

Contaminations in tissue culture can originate from two sources, either through carry over of microorganism on the surface of the explant or in the tissue itself (endophytic microorganisms). Although in meristem culture, depending on meristem size most of microorganisms are eliminated where as in leaf, petiole and stem explants, the infection is carried over to the cultures (Cassells, 1991).

There are a few reports of the use of antibiotics and fungicide to in habit growth and bacteria in plant tissue cultures. Pollock *et al.* (1983) tested derivatives of penicillin G and cephalosporine and found that they were non toxic to the plant cells even upto 100 µg/ml.

Careful attention should be paid in explant selection to avoid sources that are more likely to harbour microorganisms and that by virtue of explant surface or character may escape the effects of surface disinfectants (Cassells, 1991). In addition, Debergh and Maene (1981) have proposed the stage 0 in microporpagation, aimed at reducing plant microbial contaminations by growing stock plants in more hygienic condition.

2.5 NUTRITION

2.5.1 Culture media and growth regulation :

Composition of media used for culturing is an important factor in successful establishment of a tissue culture conditions favouring callus growth may not be suitable for organ differentiation. Gautheret (1955) emphasized the importance of nutrition in plant tissue culture. Since the nutrition requirements differ with the type of tissue and objective to follow, each tissue type requires a different formulation depending on whether the objective is to obtain optimum growth rate or induce differentiation.

Several media have been developed by various workers to suit particular requirements of a cultured tissue. The basis of all nutrient media is a mixture of mineral salts combining the essential macro and micro nutrient elements together with a source of carbon which is always a sugar usually sucrose. Very few plant tissue can, however, be established or maintained on such a simple medium for the majority of tissues various additions are essential. The usual supplements are vitamins, amino acids, sugar, alcohol and plant growth regulators together with any supplementary compounds like coconut milk, casein hydrolysate, malt extract, yeast extract, adenine sulphate etc. It is important to recognize that a culture medium suitable for callus growth may not be suitable for organ differentiation. Depending upon the over all salt concentration divergent nutrient media have been formulated (Knop's, 1865; Nitsch, 1951; Murashige and Skoog, 1962; Linsmair and Skoog, 1965; Gamborg, 1968; McCrown and Lloyd, 1981). MS (Murashige and Skoog, 1962) medium is the medium most frequently used. A perusal of literature published during the last 2-3 decades shows that this has been widely used throughout the world for *in vitro* studies. It is a purely synthetic and chemically defined medium.

Hossain *et al.* (1994a) reported that benzyl adenine (BA) was superior to either kinetin, isopentenyl adenine or zeatin. The optimum cytokinin (BA) concentration for bud induction was 2 mgL^{-1}. The addition of indole-3-acetic acid (IAA; 0.2 mgL^{-1}) improved shoot regeneration efficiency. Adventitious shoots were elongated on MS medium containing 0.5 mgL^{-1} kinetin and 0.1 mgL^{-1} gibberellic acid. Approximately 25% of regenerated shoots were

induced to differentiate roots on half-strength MS medium with 0.5 mgL^{-1} indole-3-butyric acid.

Kumar and Seeni (1998) observed rapid clonal multiplication of *bael* by enhanced axillary bud proliferation in young single-node segments of a 25 year old tree cultured in Murashige and Skoog (MS) nutrient medium. Bud break was dependent on cytokinin supply, but the synergistic combination of 2.5 mgL^{-1} 6-benzyaminopurine (BAP) and 1.0 mgL^{-1} indole-3-acetic acid (IAA) induced the formation of 12.1 shoots of upto 5.2 cm length in 48% of the explants after 7 weeks of culture. Explants of *in vitro* grown shoot node, whole leaf, shoot tip and internode were subcultured in the presence of 0.05-2.5 mgL^{-1} BAP to produce 11.3, 18.4, 5.3 and 3.2 shoots and shoot buds at a 100%, 70%, 95% and 40% rate respectively, in 7 weeks. Nodal explants responded most favourably at low BAP (0.05-0.1 mgL^{-1}) and produced uniform (3.8-5.3 cm) shoots facilitating their simultaneous harvest for rooting. Shoot cuttings (3.0-5.2 cm) were best rooted in half-strength MS medium with 0.5 mgL^{-1} IAA (70%) or 10.0 mgL^{-1} indole-3 butyric acid (90%). Eighty-eight per cent of the rooted plants were established in polybags after hardening.

Hossain *et al.* (1993) was able to initiate adventitious buds on Murashige and Skoog's (MS) medium containing various combinations of 6-benzyl aminopurine (BA), α-naphthalene-acetic acid (NAA), 3-indole acetic acid and gibberellic acid. Medium containing 4.4 μM BA and 2.7 μM NAA produced the maximum number of adventitious buds per explant. Shoots were elongated by transferring explants with shoot buds to medium with a low concentration of BA (0.44 μM). Rooting of *in vitro* regenerated shoots

was obtained in half-strength MS medium with 4.9 μM indole-3-butyric acid.

Varghese *et al.* (1993) reported that callus of *bael* was initiated from stem explants on Murashige and Skoog's (MS) medium supplemented with different concentration of kinetin, 2, 4-D and naphthalene acetic acid in *bael.* Meristemoids developed in the callus when subcultured on medium supplemented with kinetin and naphthalene acetic acid. In the presence of only benzylaminopurine, or in combination with naphthalene acetic acid, these calli shoot development. Multiple shoot induction from nodal explants were achieved on MS medium augmented with different concentrations of benzylaminopurine, kinetin and naphthalene acetic acid. The shoot buds that developed from nodal explants were found in maximum number in the medium supplemented with kinetin and naphthalene acetic acid. Rhizogenesis of the shoots was achieved in presence of indole-3-acetic acid.

Islam *et al.* (1993a) presented a protocol for organogenesis from leaf explants of *in vitro* growth seedlings of *bael.* Adventitious buds were initiated on MS medium containing various concentrations and combinations of 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA). The maximum frequency of shoot organogenesis and the highest number of shoots per explant was obtained when 1.5 mgL^{-1} BAP and 0.5 mgL^{-1} IAA were applied. Twenty day old seedlings proved to be the optimum source of culture. Shoots were elongated on the same basal medium without auxin and supplemented with 0.5 mgL^{-1} BAP. Elongated shoots were rooted on half strength MS medium supplemented with 0.1 mgL^{-1} indole-3-butyric acid (IBA).

Hossain *et al.* (1994b) reported that Murashige and Skoog formulation containing 40 g/l sucrose, 400 mgL^{-1} casein hydrolasate, 5 mgL^{-1} naphthalene acetic acid and 1 mgL^{-1} kinetin. The basal medium with high concentration (1.5 mgL^{-1}) of N6-benzyadenine (BA) and low concentration (0.1 mgL^{-1}) of NAA was suitable for regeneration of shoots from 3-month-old calli. Addition of 1 mgL^{-1} gibberellic acid (GA_3) favoured shoot growth. Callus derived shoots produced roots and developed into plantlets when transferred to half-strength MS medium supplemented with 0.5 mgL^{-1} indole - 3-butyric acid (IBA) and 0.5 mgL^{-1} NAA.

Islam *et al.* (1996) developed a procedure which allows high frequency adventitious shoot regeneration from radical tissues of *Aegle marmelos*. Adventitious buds were initiated on Murashige and Skoog medium containing various concentrations of benzyladenine (BA) and NAA. The medium containing 1.0 mgL^{-1} BA and 0.2 mgL^{-1} NAA produced the highest number of shoots per explant with maximum frequency of regeneration. Shoots were elongated by transferring explants with shoot buds to a medium with a low concentration (0.1 mgL^{-1}) of BA. The shoots grown in medium containing 25 mgL^{-1} IBA for 1 week, when transferred to basal medium, produced adventitious roots. Maximum rooting (80%) with 3-6 roots per shoot was achieved. Islam *et al.* (1995) observed successful regeneration of plantlets from callus cultures derived from zygotic embryos is described for this multipurpose tree species. Details are given of the most appropriate culture media [Murashige and Skoog basal medium + various combinations of cytokinins (BA and kinetin) and auxins (IAA and NAA)]

for the different regeneration stages (callus induction and differentiation, shoot formation, rooting).

Impact of nutrients and temperature on apple callus tissue was studied by Saad and Boone (1964). Different cultivars of *Malus pumila* and *Malus robusta* were raised on a medium containing Sorbitol (D-glucitol) as a carbon source (Chong and Taper, 1972). Their relative growth was dependent on the cultivars. Lavee and Adiri (1973) studied the effect of abscisic acid and GA_3 on the development of apple callus *in vitro*. Shoots which were proliferated in Linsmaier and Skoog (LS) medium containing 15 gL^{-1} of sucrose and indole butyric acid (IBA). Mittal *et al.*, (1989) obtained multiple shoots from axillary buds of *Acacia auriculiformis* on Gamborg's (B_5) basal medium supplemented with coconut milk and BAP.

Nodal explants of Guava 'Banaras Local' were cultured *in vitro* by Amin and Jaiswal (1987). Multiple shoots were produced by enhancement of axillary branching on modified MS media containing 6-BA only. Rooting was observed on half strength MS media containing a low concentration of sucrose, IBA, NAA and activated charcoal. Amin and Jaiswal, 1988 in *Psidium guagava* L. cv. 'Chittidar' on agitation of explants in polyvinyl polyphyrrolidone (PVP) and 2-3 changes at medium was essential for culture establishment. 'Chittidar' also showed the same requirement as 'Banaras Local' for multiple shooting and rooting. The only difference was that the former did not require activated charcoal in the medium.

Hazarika *et al.* (1996) reported that *in vitro* grown shoots of *A. marmelos* were cultured in MS medium supplemented with BAP (benzyladenine) (0, 0.25, 0.3, 0.75, 1.0, 1.25 or 1.5 mgL^{-1}). Observations

were recorded 4 and 8 weeks after culture initiation. The highest shoot number and weight were observed after 8 weeks of culture on a medium supplemented with BAP at 0.5 mgL-1. Shoots were rooted on half strength MS medium supplemented IBA at 0.5 mgL^{-1}.

2.5.2 Sucrose :

The majority of media used for *in vitro* shoot regeneration and rooting in variably contains 2-3% sucrose as a source of energy (for heterotrophic shoots in culture) and osmotic agent. Root induction has been known to be inhibited when sucrose is omitted from the medium (Zimerman, 1983a). Adventitious root formation is a high energy requiring process and inhibition of rooting by sucrose omission can be explained by starvation of heterotrophic shoots (Nemeth, 1985). Rahaman *et al.* (1992) reported increased rooting with 40 gL^{-1} of sucrose as compared to 20 g and 60 gL^{-1} of sucrose.

2.5.3 Agar :

Agar which is used in *in vitro* cultures usually solidified the media. The concentration of agar used in most of the media is 8.0 gL^{-1}. Raising the level of agar in the medium can over come the vitrification (Debergh *et al.*, 1981) and increased the proportion of normal shoots developing from 46-77% (Leshem, 1983) but increased concentration of agar in the medium also reduced the availability of cytokinins and quxins to the explant was reported by Debergh (1983).

The concentration of agar was found to be a limiting factor in shoot tip culture of mulberry trees (Ohyama and Oka, 1976). Higher concentration (0.8-1.0%) of agar gave poor growth, with only a few leaves opening and

without further shoot development, where as concentration as low as 0.4% supported good shoot growth. Similar results were observed with winter bud cultures in mulberry (Oka and Ohyama, 1976). A promotive effect of agar at low concentration on tissue growth has also been reported in Malus (Romberger and Tabor, 1971) and Iris (Stoltz, 1971). This effect might be explained on the basis of increased uptake and availability of nutrients through leaves or tissues submerged in the medium.

Rahaman *et al.* (1992) reported decrease in rooting performance with increasing agar concentration and 15 gL^{-1} of agar was completely inhibitory. Agar concentration of 6 gL^{-1} was optimum for root induction in rose. Similarly, Skirvin *et al.* (1980a) reported that the lowering of agar concentration to 5-6 gL^{-1} resulted in good rooting in plum. High agar concentration reduced the relative humidity in the culture vessel and most roots from high agar concentration were damaged at the time of transplanting (Rahaman *et al.,* 1992). Similar results were obtained in jack fruit (Rahaman and Blake, 1988).

2.5.4 Other additives

Activated charcoal (AC) is reported to absorb toxic components released by the inoculum (Fridborg *et al.,* 1978). It may also affected the availability of various components of nutrient medium. This inhibition may be due to partial absorption of auxin and other nutrients (Weather-Heard *et al.,* 1979). Activated charcoal has been used in medium to prevent browning of cultures due to phenolic oxidation in explants from mature trees (Dawra et al., 1984; Raghavaswamy *et al.,* 1992). Similarly, PVP has been included in

medium to avoid explant browning (Mathur and Chandra, 1983; Rai and Chandra, 1988; Raghavaswamy *et al.,* 1992)

2.6 CULTURE ENVIRONMENT

The culture environment, which is the result of the interaction between the plant material, culture media, type of culture vessel and the external environment of the culture room, has tremendous influence on a tissue culture system (Debergh, 1988). These may in due date the physical form of the medium, pH, humidity and gaseous atmosphere, light and temperature. The major factors of the tissue culture environment are light and temperature. Frequently, the literature also presents data on relative humidity. The illumination of plant cultures must be considered in terms of the intensity, length of the daily exposure period and the quality.

2.6.1 Physical form of the medium :

Most success in tissue culture has been achieved with agar solidified medium, but the use of liquid media was increasingLy evaluated for tissue culture purposes (Keathley, 1983; Chun *et al.,* 1986). During the development of commercial micropropagation eg. towards automated systems, it is likely that liquid rather than agar cultures will be preferred (Haissing *et al.,* 1987). In such system, the repeated subculturing of explant to fresh media will be unnecessary as the liquid medium may be easily replaced or replenished by the addition of fresh nutrients. Chun *et al.* (1986) reported that use of stationary phase liquid media produced double the number of shoots as compare to agar medium. However, the liquid medium had adverse effect of vitrification of upto 23% of shoots (Chun *et al.,* 1986).

Keathley (1983) could obtain higher multiplication rates in spruce buds grown in liquid medium than on agar solidified medium.

Semi solid media have also been used for rooting of rose (Rahaman *et al.,* 1992) and for shoot growth in mulberry (Ohyama and Oka, 1976).

2.6.2 pH :

Generally, the pH of the culture medium is kept at and around 5.8. Low pH has been shown to induce roots in a few species. Rahamn *et al.* (1992) reported that the best pH for rooting was 5.5 in rose and at pH 5.7 root development was completely inhibited. Similarly, Williams *et al.* (1985) found that pH of 4.0 and dark period of 2 week was optimum for root induction of *Prostanthera striatiflora* and *Corea decumbens*. Low pH stimulated moderate rooting where as the dark treatment alone did not.

2.6.3 Humidity and gaseous atmosphere :

The rate of gaseous exchange in culture vessels is important. In more or less hermetically closed vessels, De proft *et al.,* (1985) obtained supra-optimal concentration of CO_2 and ethylene on sucrose containing medium, which had adverse effect on the micropropagation of *Magholia soulangeana.* This problem could be overcome by the use of more aerated containers. Humidity in the vessel and osmotic potential of the medium influence the water relations of plantlets *in vitro* and thus affect the growth, development and photosynthesis of plantlets *in vitro* in different ways Brown *et al.,* 1979; Ziv *et al.,* 1983.

2.6.4 Light :

Plants cultured *in vitro* are supplied with carbohydrates and therefore, are heterotrophic. However, the quality duration and intensity of light affect

shoot growth and morphogenesis in addition to having a role in photosynthesis (Hughes, 1981). Light regulates the size of leaves and stems, as well as morphogenic path way and therefore, ismplicated in pigment formation and vitrification (Ziv, 1991). Effect of photoperiod may or may not be pronounced in all the species. But DougLas (1989) reported that light grown (16 hr photoperiod) leaf explants from female mulberry tree showed low morphogenic capability while those in dark were highly morphogenic.

Apple stem callus culture of different cvs. of *Malus pumila* were grown under light intensities of 0, 850, 3350 and 7800 lux., cultivar Cortland grew better in dark where as growth of Robusta was not influence by light. Light did not influence the growth of 'Mclnthosh' callus between and 3350 lux, although stimulation of growth occurred at 7800 lux. The chlorophyll content of the cultures increased light intensity (Chong and Taper, 1974). Dark treatment in apple promoted rooting; raising the temperature to 30^0C simultaneously further increases it (Zimmerman and Fordham, 1983). Leaf and cotyledon explants of apple seedlings cultured in the dark for on initial three weeks, then transferred to light for four weeks, produced 5 to 20 fold more adventitious shoots than those cultured for seven weeks in the light. Conversely light did not significantly influence the number of adventitious shoots formed on hypocoty explants. Five minutes daily exposure of leaf explants to red light (651 nm) suppressed adventitious shoots formation by 80 per cent and five minutes exposure to infra-red light (729 nm) immediately following the red light counteracted the infra-fed suppression. Light inhibited callus formation on leaf and cotyledon explants, but not on hypocotyl explants (Lin *et al.*, 1983).

2.6.5 Temperature :

Just as the temperature influence morphogenesis *in vitro* it certainly controls growth and development *in vitro*. Temperature controls the RH in the culture vessel, so it has an indirect effect on vitrification (Maene and Debergh, 1987).

Maximum rooting of shoots *in vitro* in *prunus persica* occurred when shoots were placed on half strength MS medium stored in dark at 4^0C for 35-40 days and than incubated on rooting medium in the dark at 26^0C for 14 days (Hammerschlog *et al.*, 1987).

During acclimatization, the temperature of root zone is important to enhance root growth. The medium should be warmer than the air for good root activity and to increase the humidity around the cuttings (Dunstan and Turner, 1984; McCown, 1986).

2.7 Adventitious shoot production :

Adventitious shoot production is also of greater potential for multiplication because shoot induction occurs from sites other than bud meristems. The method, recently reviewed by Thorpe and Patel (1984), involves the induction of localized meristematic activity by phytohormone treatment, leading to primordium differentiation and eventually to shoot development often under phytoharmone free condition. The most frequently used explants that have led to successful regeneration have been the seed or seedling parts including the cotyledons, hypocotyl, epicotyl and embryognic axis.

Islam *et al.* (1993) presented a protocol for organogenesis from leaf explants of *in vitro* growth seedlings of *AegLe marmelos*. Adventitious buds

were initiated on MS medium containing various concentrations and combinations of 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA). The maximum frequency of shoot organogenesis and the highest number of shoots per explant was obtained when 1.5 mgL-1 BAP and 0.5 mgL^{-1} IAA were applied. Twenty day old seedlings proved to be the optimum source for culture. Shoots were elongated on the same basal medium without auxin and supplemented with 0.5 mgL^{-1} BAP. Elongated shoots were rooted on half strength MS medium supplemented with 0.1 mgL^{-1} indole-3-butyric acid (IBA).

Hossain *et al.* (1993) found a protocol for organogenesis from nucellar explants excised from fertilized Ovules of immature fruits of *bael.* Adventitious buds were initiated on Murashige and Skoog's (MS) medium containing various combinations of 6-bengyadenine (BA), α-naphthalene-acetic acid (NAA), 3-ndole acetic acid and gibberellic acid. Medium containing 4.4 μM BA and 2.7 μM NAA produced the maximum number of adventitious buds per explant. Shoots were elongated by transferring explants with shoot. Buds to medium with a low concentration of BA (0.44 μM). Rooting of *in vitro* regenerated shoots was obtained in half strength MS medium with 4.9 μM indole-3-butyric acid. Hossain *et al.* (1994) cultured cotyledons from seedlings at various ages of *AegLe marmelos* on Muroshige and Skoog (MS) medium supplemented with different combinations of phyto hormones. The optimum seedling age was 10 days for shoot induction response and benzyadenine (BA) was superior to either kinetin, isopentenyladenine or zeatin. The optimum cytokinin (BA) concentration for bud induction was 2 mgL^{-1}. The addition of indole-3-acetic

acid (IAA; 0.2 mgL^{-1}) improved shoot regeneration efficiency. Proximal part of the cotyledon had the highest regeneration potential. Adventitious shoots were elongated on MS medium containing 0.25 mgL^{-1} kinetin and 0.1 mgL^{-1} gibberellic acid. Approximately 25% of regenerated shoots were induced to differentiate roots on half-strength MS Medium with 0.5 mgL^{-1} indole-3-butyric acid. The rooted platnlets were successfully transplanted to soil.

Islam *et al.* (1996) tested intact seedlings of *AegLe marmelos* Corr. for their ability to produce adventitious shoots by direct culture of mature seeds on Murashige and Skoog (MS) medium supplemented with different concentrations of benzyladenine (BA); 1-2 mgL^{-1} BA was found to be the optimum concentration. Addition of 0.1 mgL^{-1} indole acetic acid (IAA) further increased shoot proliferation efficiency. Shoot buds originated from regions adjacent to the bases of cotyledonary axils and roots. In many cases, adventitious shoots were produced from the enlarged apical region of root. Shoots were rooted on MS medium supplemented with indole butyric acid. Rooted plantlets were successfully established in soil.

2.8 NUCELLUS CULTURE

AegLe marmelos was found difficult to propagate *in vitro* from mature plant tissues, due to problem of high phenolic exudation and rejuvenation of adult explants. Therefore, efforts were made to find a suitable alternative explant source and nucellus tissues from developing fruits.

Like other members of the Rutaceae, especially citus species, *AegLe marmelos* possesses a bulk mass of nucellar tissues at certain stages of ovule development which are amenable for excision and *in vitro* culture (Rangas-

ωamy, 1981). Nucellar-derived plants are thought to be true to type because nucellar tissues are of maternal origin (Button and Kochba, 1977).

Hossain et al. (1993) developed a protocol for organogenesis from nucellar explants excised from fertilized ovules of immature fruits of *A. marmelos* Corr. Adventitious buds were initiated on Murashige and Skoog's (MS) medium containing various combinations of 6-benzyladenine (BA), α-naphtholene-aceitc acid (NAA), 3-indole acetic acid and gibberellic acid. Medium containing 4.4 μM BA and 2.7 μM NAA produced the maximum number of adventitious buds per explant. Shoots were elongated by transferring explants with shoot buds to medium with a low concentration of BA (0.44 μM). Hossain *et al.* (1994b) developed techniques for the regeneration of *Aegle marmelos* from nucellar explants. Slow-growing calli were induced from nucellar explants excised from 90-120 d-old developing fruits. The medium consisted of MS formulation containing 40 gL^{-1} sucrose, 400 mgL^{-1} casein hydrolysate , 5 mgL^{-1} 1-naphthalene acetic acid and 1 mgL^{-1} kinetin. The basal medium with high concentration (1.5 mgL^{-1}) of N6-benzyladenine (BA) and low concentration (0.1 mgL^{-1}) of NAA was suitable for regeneration of shoots from 3-month-old calli. Addition of 1 mgL^{-1} gibberellic acid (GA_3) favoured shoot growth.

2.9 REGENERATION

Asexual embryogenesis is distinct from organogenesis because bipolar embryos are induced having both a shoot and root pole (Haccius, 1971). Such embryo result from a more precisely defined series of regular divisions, generally from single cell precursors in a manner analogous to sexual, zygotic embryogenesis is important from vegetative propagation and plant

improvement. Direct somatic embryogenesis involves the formation of an embryo from single cell or group of cells directly on a part of explant tissue without callus formation where as in indirect somatic embryogenesis first callus is formed from explant then somatic embryos formed (Ammirato, 1983, 1984).

2.9.1 Organogenesis :

The axillary bud production system is described as conservative and correspondingly of relatively low multiplication potential in comparison to the adventitious buds or asexual embryogenesis routes. It is considered conservative because of its relative ability to produce true-to-type plants without genetic change. For this segments of *Santalum album* (Sandal wood) were observed by Rao and Bapat (1978). Development of axillary shoots was induced when isolated embryogenic axes of chestnut (*Castanea sativa*) were cultured (Vieitez and Vieitez, 1980). Internodal pieces of Quandong (*Santalum acuminatum*) produced a sexual growth (Barless *et al.* 1980.).

Clonal propagation of *Tamarindus indica* (Tamarind) was achieved by direct adventitious buds (Kul Karni *et al.*, 1981).

Islam *et al.* (1993) reported a protocol for organogenesis from leaf explants of *in vitro* grown seedlings of *Aegle marmelos*. Adventitious buds were initiated on medium containing various concentrations and combinations 6-benzyl-amino purine (BAP) and indole-3-acetic acid (IAA). The maximum frequency of shoot organogenesis and the highest number of shoots per explant was obtained when 1.5 mgL^{-1} BAP and 0.5 mgL^{-1} IAA were applied. Twenty-day-old seedlings proved to be the optimum source of culture. Shoots were elongated with 0.5 mgL^{-1} BAP. Elongated shoots were

rooted on half strength MS medium supplemented with 0.1 mgL^{-1} indole-3-butyric acid (IBA). Hossain *et al.* (1993) developed a protocol for organogenesis from nucellar explants excised from fertilized ovules of immature fruits of *AegLe marmelos* Corr. Adventitious buds were initiated on MS medium containing various combinations of 6-benzyladenine (BA), α-naphthalene-acetic acid (NAA), 3-indole acetic acid and gibberllic acid. Medium containing 4.4 μM BA and 2.7 μM NAA produced the maximum number of adventitious buds per explant. Shoots were elongated by transferring explants with shoot buds to medium with a low concentration of BA (0.44 μM). Rooting of in vitro regenerated shoots was obtained in half strength MS medium with 4.9 μM indole-3-butyric acid.

Rapid clonal multiplication of *AegLe marmelos* was achieved by enhanced axillary bud proliferation in young singLe-node segments of a 25 year-old tree cultured in MS nutrient medium. Bud break was dependent on cytokinin supply but the synergistic combination of 2.5 mgL^{-1} 6-benzylaminopurine and 1.0 mgL^{-1} IAA induced the formation of 12.1 shoots of upto 5.2 cm in length in 48% of the explants after 7 weeks of culture. Explants of *in vitro* grown shoots (node, whole leaf, shoot tip and internode) were subcultured in the presence of 0.05-2.5 mgL^{-1} BAP to produce 11.3, 18.4, 5.3 and 3.2 shoots and shoot buds at a rate of 100, 70, 95 and 40%, respectively, in 7 weeks. Different shoot nodes and leaves were equally regenerative and adventitious organogenesis in the later was confined to cut petiolar ends. Nodal explants responded most favourably at low BAP (0.05-0.1 mgL^{-1}) and produced uniform (3.8-5.3 cm) shoots facilitating their simultaneous harves for rooting (Kumar and Seeni, 1998).

The reduced organogenic capacity of mature embryos could be loss of competence associated with maturation (Hu and Sussex, 1971). The immature embryos are known to be a good source for high regeneration capacity (Chalupa, 1990). Like embryoaxes, cotyledons from immature and mature seeds of *A. marmelos* showed high morphogenetic potential. Maximum shoot regeneration was achieved using fully grown cotyledons prior to full maturity and not from mature cotyledons. In cotyledon cultures of *A. marmelos*, shoot formation was highest in the region proximal to the embryo axis, indicating a polarity in the regeneration potential of cotyledon explants. Two-third distal parts of cotyledons produced less shoots than one-third proximal parts of cotyledon, where prolific shoot regeneration occurred. Similar observations were made in apple and pea cotyledons (Kouider *et al.*, 1985; Ozcan *et al.*, 1992).

Adventitious shoots were also induced on hypocotyl and leaf explants of *A. marmelos* seedlings. The regeneration ability at the leaf explants was significantly reduced when the abaxial rather than the adaxial surface was in contact with the medium on effect similar to that observed in quince (Baker and Bhatia, 1993). The position of the leaf on the seedling affected adventitious shoot regeneration. A gradient in response exists from the tip to the base of the seedling, with the distal leaves being more responsive and tending to form most shoots per regenerating leaf, as also observed by Fasolo *et al.* (1989) in apple.

2.9.2 Indirect organogenesis Via callus :

Historically, callus culture were the systems most adherent to Haberlandt's original stated theory of totipotency (Haberlandt, 1902), but

many researchers were led away from callus culture when, in the late 1970s, Organ cultures began to be increasingly successful in the regeneration from callus, as with axillary and adventitious bud cultures, involves separate shoot and root induction phase, similar to adventitious shoot induction. Callus organogenesis involved meristemoid induction.

Clonal propagation was achieved in citrus via callus. Isolated stem and leaf callus of *Citrus sinensis* (Chaturvedi and Mitra, 1974) and *C. paradisi* (Bhansali and Arya, 1978) regenerated plantlets. Stem callus tissue of *Citrus grandis* in long term callus cultures gave rise to plantlets (Chaturved *et al.,* 1974) callus and shoot formation was observed in hypocotyl and stem explants of *Aegle marmelos* (Arya *et al.,* 1981). Plant regeneration was obtained in different Prunus specis via callus using various explants (Mehra and Mehra, 1974; Druart, 1980).

Calli derived from hypocotyl explants of apple (*Malus domestica*) regenerated into shoot and roots (Lin *et al.,* 1983). Guli (1979) induced callus in stem segments and subsequently obtained plantlets in Chinese gooseberry.

Noerhadi (1981) could get only roots from callus of *Tectona grandis*. Shoot differentiation and root induction was obtained in *Annona squamosa* (Nair, 1986).

White globular somatic embryos appeared as loose structures after 30 days on explants. The highest response in number of explants producing somatic embryos per expant (12) was observed in presence of 2, 4-D (μM) and BA (μM) as per observation recorded after 42 days in *Aegle marmelos* (Islam *et al.,* 1996).

2.10 ROOTING AND ACCLIMATIZATION

No general technique can be used for in vitro rooting because of variation in response obtained (Williams *et al.,* 1985). A combination of auxins and cytokinins has been used for some woody legume (Harris *et al.,* 1989; Puri *et al.,* 1992; Raghavaswamy *et al.,* 1992). Rooting has already been induced in a few woody species by an initial incubation of shoots in auxin rich medium (NAA and/or IBA) followed by transfer to hormone free medium (Rai and Chandra, 1988; Yadav *et al.,* 1990).

Microshoots produced from various explants were excised and placed on MS medium supplemented with various concentrations of NAA and IBA for induction of roots. Differences in genotypic responses to root initiation in *bael* were observed. No rooting was induced on unsupplemented MS medium in any case. Formation of callus at the cut bases, malformation and slow growth of roots were observed on NAA-supplemented medium. Therefore, IBA was used for root induction and 1-2 mgL^{-1} IBA was found to be effective in some genotypes, where 55-60% of the cultured shoots produced roots. On the other hand, only 10-20% rooting frequency was observed in other genotypes in the same media formulation. However, when microshoots of these genotypes were treated for 10 days in 20-30 mgL^{-1} IBA, 75-85% of them produced roots on transfer of hormone-free MS medium. All these result suggest that rooting response in *bael* is genotype dependent. In *bael*, NAA was found to be unsuitable for root formation (Hossain *et al.,* 1994a). Hutchinson (1981) reported that preferred auxin for root initiation in tree species in IBA. Pretreatment of shoots in high level of

IBA (100 mgL^{-1}) induced roots in 80-90% of cultures in tea (Jha and Sen, 1992).

The term acclimatization is defined as the climatic adaptation of an organism, especially a plant, that has been moved to a new environment.It take place under the active guidance of human beings. The term acclimation has a similar meaning but it is a process of nature. Hossain *et al.* (1994a) reported that the rooted plantlets of *bael* were hardened-off by keeping them in the culture room for another 1-2 weeks until the colour of the roots became brown. The plantlets with brown roots were taken out from culture tubes, washed thoroughly to remove any remains of medium and planted in small plastic pots containing 2:1 garden soil and compost treated with 0.1% Agrason (fungicide) solution. For the initial period of transfer, potted plantlets were kept in culture room conditions and high humidity was maintained by covering the plantlets with transparent beakers. With in 4-5 weeks, the potted plants began to form new leaves and resumed new growth. The transplanted plants adapted in the soil with in 4-5 weeks. Plantlets were subsequently transferred to larger pots and gradually acclimatized to outdoor conditions. Transplantation success was 60%.

Hardening of shoots prior to placement in the rooting beds increases survival rates of transferred plantlets. So plantlets or shoots produced *in vitro* must be acclimatized gradually to with stand the harsh natural environment. Misting, spraying or covering the pots may serve to fulfil the above objective. Different types of substrates have been used during the acclimatization period such as soil-vermiculite mixture (Goyal and Arya, 1981), sterilized sand (Bhansali *et al.*, 1988) and soil (Kurten *et al.*, 1990).

During acclimatization the temperature of root zone is important for better root growth. The medium should be warmer than the air for good root activity and to increase the humidity around the cuttings (Dunstan and Turner, 1984; McCown, 1986).

Rooting and subsequent hardening can be carried out together if the rooting is to be carried out *ex vitro*. As reported by Hutchinson (1981), an economy in the cost of micropropagated plantlets could be achieved if the *in vitro* rooting stage could be omitted and shoots treated as tender cutting and rooted in the glass house. Hazarika *et al.*, (1996) developed a protocol for the acclimatization and rooting of *Aegle marmelos* microshoots *ex vitro*. Shoots (2-cm-long) were obtained from proliferating cultures grown in benzylaminopurine (benzyladenin) – supplemented media. Shoots were pulsed with either IBA or NAA (10 ppm for 2 min.) before being place to root in sterile soilrite or soilrite with sand for 6 weeks. Plant growth regulator treated cuttings rooted better than untreated cuttings (63.58-79.46%) compared with (18.12-21.62% rooting). Pulsing was successfull without any transplant shock in the soil.

2.11 Applications

The plant tissue culture technique has emerged as an essential research production and a significant commercial practice (Murashige, 1980). The very possibility of using plant tissue culture technique in commercial production of trees is no more a for stretched imagination to plant scientists today. The last one decade has witnessed a remarkable progress in improvement of plant tissue culture which added greater interest among the

researchers to extend the scope of the technique in a variety of species of tree useful afforastation, social, farm forestry and orchard establishment.

Tips from lateral and terminal shoots of gooseberry were used *in vitro* for eliminating virus. But only plantlets derived from terminal tips survived in pots (Jones and Vine, 1968). Saad (1965) used apple callus tissue to study the pathogenicity of *Venturia inaequalis*. Waitherka (1981) developed virus resistant *Ficus carcia* through shoot tip culture grown in heat therapy chambers.

Hosomi *et al.* (1984) studied the effect of plant growth regulators on production of triterpene ester in *Achras sapota*.

Tissue culture also can be used as a method to study the vigour of the trees and also on many other phenotypic changes. Messer and Lavee (1969) studied the vigour and dwarfism of apple trees.

Plantlets regenerated from cells and protoplasts and genetic transformation have openend the door to biotechnology and genetic engineering of fruit trees. The existing superior genotypes can be exploited for the production of novel and commercially valuable genotype.

CHAPTER : III

MATERIALS AND METHODS

3.1 MATERIAL

3.1.1 Plant material :

The present study was planned with the objectives to standardize the optimal culture conditions for shoot formation and plantlet regeneration from cotyledon explant and to standardize protocol for callus induction and its regeneration in three *bael* [*Aegle marmelos* (L.) Corr.] cultivars. The cultivars selected for experiment were Local, Gonda Selection and Mirzapuri. The explants viz. shoot tip, epicotyl, hypocotyl and leaf disc were excised from *in vitro* raised plantlets whereas, cotyledon were collected from unripe fruits of *bael* growing at experimental orchard of the Department of Horticulture, CCS Haryana Agricultural University, Hisar.

The explants used were shoot tip, epicotyl, hypocotyl (0.5 cm length), cotylendon and leaf disc of (4-7 mm diameter) (Table 3.1.1). The explants were collected and cultured in the months of December, January and February.

Table 3.1.1 : Sources and description of different explants from *bael* cvs.

Explant	Source	Age of plantlets/ plants	Place	Length/diameter (cm/mm)	Description
Cotyledons	Adult plants of *bael* cvs., Local, Gonda Selection and Mirzapuri	14-15 years	Orchard, Department of Horticulture CCS, HAU, Hisar	4-7 mm (diameter)	Cotyledons with/without embryo axis were collected from unripe green fruits.
Shoot tips	*In vitro* raised plantlets of Local, Gonda Selection and Mirzapuri	25 days	Tissue culture laboratory, Department of Hort., CCS HAU, Hisar	0.5 cm (length)	Unsprouted shoot tips were collected from *in vitro* raised plantlets from cotyledon (with embryo axis) explants on Knop's media supplemented with 0.5 mgl^{-1} BAP
Epicotyls	"	"	"	"	Epicotyls were collected from *in vitro* raised plantlets from cotyledon (with embryo axis) explants on Knop's media supplemented with 0.5 mgl^{-1} BAP.
Hypocotys	"	"	"	"	Hypocotyls were also collected from *in vitro* raised plantlets from cotyledon (with embryo axis) explants on Knop's + 0.5 mgl^{-1} BAP
Leaf discs	"	"	"	4-7 mm (diameter)	Leaf discs were excised from *in vitro* raised plantlets from cotyledon (with embryo axis) explants on Knop's + 0.5 mgl^{-1} BAP from the middle portion of the unfolded leaflets.

3.1.2 Chemicals :

The chemicals of high purity were used throughout the course of investigation. The chemicals viz., growth regulators, vitamins, inositol etc., were obtained from Sigma chemicals company, St. louis (USA) whereas, EDTA sodium salt, sucrose, agar-agar and other reagents were obtained from several companies viz., BDH, Glaxo, E'Merck, Sisco Research Laboratories and Hi-media (India).

3.1.3 Glassware :

All the glasswares used during the course of investigation were of boro-silicate quality and obtained from either Borosil India Ltd. or Corning Glass Company (India) and Sever Company Conical flasks of specified volume of 100 and 150 ml and culture bottles of 100 ml volume were used for culturing of explants. Test tubes of 25 mm x 150 mm and 25 mm x 200 mm dimensions were also used. Amber coloured bottles were used to eliminate the effect of light, whenever required.

3.2 METHODS

3.2.1 Sterilization of glassware and culture media :

Glasswares (pipettes, beakers, petri dishes, flasks and culture bottles) were thoroughly washed with liquid soap solution (Teepol) followed by sufficient washings with running tap water to remove the residues of detergent and then rinsed with distilled water. After that, these glasswares were dried in hot air oven for over night at 60^0C. Flask containing culture medium or distilled water were plugged with cotton and steam sterilized in an autoclave at 1.2 kg cm^{-2} pressure at 121^0C temperature for 15 minutes. Pipettes, scalepels and forceps were heat sterilized at 180^0C for 2-3 hours in

hot air oven. Scalpels and forceps were flame sterilized till they become red hot prior to use during inoculation and kept dipped in spirit inside the laminar airflow box. Petridishes wrapped in aluminum foil were sterilized in an autoclave at 1.2 kg cm^{-2} pressure (121^0C) for 15 minutes.

3.2.2 Surface disinfection of explants :

Seeds were separated from the fruits and thoroughly washed in tap water with few drops of 'Teepol'. Cotyledons were separated from seeds and treated with 70 per cent aqueous solution of ethanol (v/v) for 30 seconds followed by 0.1 per cent aqueous solution of $HgCl_2$ (w/v) for 4 minutes. Finally the explants were thoroughly washed in sterilized single distilled water to remove all the traces of mercuric chloride. All the disinfection operations were carried out in the horizontal laminar flow cabinet.

3.2.3 Culture media :

The Knop's (1865) basal media with some modifications were used for callus induction as well as plant regeneration. The composition of different media formulations are given in Table 3.2.3. In case of cotyledon with embryo axis explants cultured on media based on Knop's basal medium containing 0.5 mgl^{-1} BAP, no rooting media were used, as shoots and radicle were formed on the same medium. However, for shoot tip, epicotyl, hypocotyl, leaf disc and cotyledon (with or without embryo axis) explants cultured on media based on knop's basal medium containing BAP, KIN, NAA and 2,4-D were used for callus induction and shoot formation (Table 3.2.3.1), whereas, for rooting half or full strength Knop's media concentrations of IBA were used (Table 3.2.3.2).

Table 3.2.3. Composition of Knop's (1865) medium

Constituent		Concentration (mgL^{-1})
Calcium nitrate	$Ca(NO_3)_2.4H_2O$	800.0
Potassium nitrate	KNO_3	200.0
Potassium dihydrogen orthophosphate	KH_2PO_4	200.0
Magnesium suphate heptahydrate	$MgSO_4.7H_2O$	200.0
Ferrous sulphate	$FeSO_4$	10.0
Ethylene diaminetetracetic acid	EDTA	10.0
Plus the minor elements and vitamins of the Murashige and Skoog (1962) medium		
Glucose		30000.0
Agar-agar		10000.0
pH		5.7

Table 3.2.3.1 : List of media tested for callus induction/shoot regeneration in different explants of *bael*

Sr. No.	Media	Basal medium	Growth regulators (mgL^{-1})			
			Cytokinin		Auxin	
			BAP	KIN	NAA	2,4-D
1.	K_0	Knop's	0	0	0	0
2.	K_1	Knop's	0.25	0	0	0
3.	K_2	Knop's	0.50	0	0	0
4.	K_3	Knop's	1.00	0	0	0
5.	K_4	Knop's	2.00	0	0	0
6.	K_5	Knop's	0	0.25	0	0
7.	K_6	Knop's	0	0.50	0	0
8.	K_7	Knop's	0	1.00	0	0
9.	K_8	Knop's	0	2.00	0.5	0
10.	K_9	Knop's	0.50	0.50	0.5	0
11.	K_{10}	Knop's	0.50	1.00	0.5	0
12.	K_{11}	Knop's	1.00	0.50	0.5	0
13.	K_{12}	Knop's	1.00	1.00	0.5	0
14.	K_{13}	Knop's	0.20	0	0	0.20
15.	K_{14}	Knop's	0.20	0	0	0.50
16.	K_{15}	Knop's	0.20	0	0	1.00
17.	K_{16}	Knop's	0	0.50	5.00	0
18.	K_{17}	Knop's	0	0.50	2.00	2.00

Table 3.2.3.2 : Root induction media for shoot regenerated in *bael*

Sr. No.	Media	Basal medium	Growth regulators (mgL^{-1})		
			BAP	IBA	NAA
1.	R_0	Knop's	0	0	0
2.	R_1	Knop's	0	0.5	0
3.	R_2	Knop's	0	1.0	0
4.	R_3	Knop's	0	3.0	0
5.	R_4	Knop's	0	4.0	0
6.	R_5	Knop's	0	5.0	0
7.	R_6	Knop's	0	8.0	0
8.	R_7	Knop's	0	10.0	0
9.	R_8	Knop's	0	15.0	0
10.	R_9	½Knop's	0	----	0
11.	R_{10}	½Knop's	0	0.5	0
12.	R_{11}	½Knop's	0	1.0	0
13.	R_{12}	½Knop's	0	3.0	0
14.	R_{13}	½Knop's	0	4.0	0
15.	R_{14}	½Knop's	0	5.0	0
16.	R_{15}	½Knop's	0	8.0	0
17.	R_{16}	½Knop's	0	10.0	0
18.	R_{17}	½Knop's	0	15.0	0
19.	R_{18}	Knop's	0.25	0.50	0.50
20.	R_{19}	Knop's	0.50	1.00	1.00

3.2.4 Preparation of culture medium :

Various explant viz. cotyledons with or without embryo axis, shoot tips, epicotyls, hypocotyls and leaf discs were used and cultured on a medium containing 0.8 per cent agar and 3.0 per cent sucrose in Knop's medium supplemented with different concentrations of growth regulators.

Standard methods were followed in preparing stock solutions. Growth regulators viz., IBA, NAA, BAP and KIN were first dissolved in small volume of ethanol, however, 2,4-D was first dissolved in small volume of distilled water and then the volume was made with distilled water. To prepare specific medium, desirable concentrations of stock solutions were mixed in sequence mentioned. Growth regulators were added to medium as per the requirement and then the desired volume was made up by single distilled water. The pH of the media was adjusted to 5.7 with IN HCl and IN NaOH and after that 0.8 per cent agar was added to the medium. The media were melted at 1·2kg cm^{-2} pressure for 15 min. in the autoclave; melted media were dispensed in 100 ml culture bottle and 100 ml or 150 ml conical flasks then the culture bottles with lid and the flasks were closed with cotton plugs and sterilized at 1.2 kg cm^2 pressure at 121^0C for 15 minutes.

3.2.5 Storage of culture media and stock solution ;

Stock solutions of major and minor salts, vitamins, chelating agents and growth regulators were prepared and stored in a refrigerator at $4^0C \pm 2^0C$ and used whenever required. The autoclaved media in flasks were stored at 25 ± 2^0C.

3.2.6 Collection and inoculation of explants on culture media :

The explants were collected from *in vitro* raised plantlets and mature plants growing at the orchard of the Department of Horticulture, CCS Haryana Agricultural University, Hisar, during December, January and February in all the three cultivars. The cotyledons (with/without embryo axis) were collected from the unripe fruits and shoot tips, epicotyls, hypocotyls, leaf discs from *in vitro* raised plantlets (Table 3.1.1). The unripe fruits were used for seed extraction. Fruits were collected during morning hours (930-1000 hours) and brought to the tissue culture laboratory of the Department of Horticulture, CCS Haryana Agricultural University, Hisar and processed for culturing. The processed explants and flasks containing sterilized media were kept in laminar air flow cabinet under UV light for 20 minutes for disinfection of working area & flasks. The explant disinfection and inoculation was performed under fluorescent white light. One to five explants were inoculated per flask and about 100-225 explants were kept in every set of experiment. Explants were aseptically inoculated on culture medium and incubated under fluorescent white light. All the cultures were maintained at 25 ± 2^0C under 16/8 hours cycle of light (2000 lux fluorescent tubes) and dark leaf disc explants were cultured with their abaxial surface touching the medium.

3.2.7 Sub-culturing :

A two step micropropagation system (shoot generation/root induction) was adopted. The calli regenerated shoots were sub-cultured on a fresh medium (similar to or different from the first medium). The sub-culturing was done at every 20-25 days interval to promote callus/shoot proliferation

3.2.8 Observations and record of data in case of callusing :

From time to time, the callus cultures were observed in fluorescent white light for the following attributes :

i) Number of days taken for callus initiation.

ii) Per cent response = $\frac{\text{Number of explant showing callus growth}}{\text{Total number of explants}} \times 100$

iii) Per cent organogenesis = $\frac{\text{Number of calli showing shoot \& root}}{\text{Total number of calli}} \times 100$

iv) Average number of plantlets obtained through intermediate stage of callus.

v) Rooting percentage = $\frac{\text{Number of plantlet showing rooting}}{\text{Total number of plantlets}} \times 100$

3.3 MAINTENANCE OF CALLUS CULTURE

All the calli derived from cotyledon explants were sub-cultured on fresh medium after every 20-25 days for callus growth and maintanence of callus cultures.

3.4 DIRECT PLANTLET REGENERATION AND ROOT INDUCTION

Direct plant regeneration was tried in cotyladon shoot tip, epicotyl hypocotyl and leaf disc explants using several media formulations based on Knop's basal media. After 2-3 subculturing, the regenerated shoots were used for rooting induction by inoculation, of shoots on to the media

formulations based on full and half strength Knop's basal medium (Table 3.2.3.1). In all the experiments intended for root induction, the regenerate shoot were excised from the explant and any accompanying callus was removed from the shoots before keeping on the rooting medium.

The following data were recorded for plant regeneration :

i) Per cent regeneration = $\frac{\text{Number of explant regenerated}}{\text{Total number of explants}} \times 100$

ii) Number of days required for shoot initiation.

iii) Mean number of shoots per explant.

iv) Proportion (%) of shoots showing rooting = $\frac{\text{Number of shoots responding to rooting}}{\text{Total number of shoots cultured}} \times 100$

v) Mean number of roots per shoots.

vi) Mean root length.

3.5 TRANSFER OF REGENERATED PLANTLETS TO SOIL MEDIUM IN POTS AND THEN TO GREEN HOUSE

The rooted plantlets were separated gently from the medium and washed thoroughly in tap water to remove the traces of agar medium. Then the plantlets were planted in sterile soil + sand (1:1), soil + river sand (1:1) and soil + sand + FYM (1:1:1) potting mixtures with a polythene bag inverted on to it to keep humidity level high enough for the plant to avoid any undesirable desiccation. The plant were kept for 10-15 days in culture room for hardening irrigated with distilled water daily. Initially 0.5-1.0 ml Hoagland's solution per plant was given for one weeks at alternate days.

After that, 1 ml Hoagland's solution per plant was provided, whenever it was required for growth. The inverted polythene bags on the plants were removed daily for 5-10 minutes to provide fresh air to the plants. After 10-15 days of hardening in culture room conditions, the plants were transferred to green house. Drenching of soil with 0.15% Bavistin (w/v) aqueous solution once a month was done.

Observations:

i) Number of plants transferred to pots.

ii) Number of plants survived after transferring to pots.

iii) Proportion (%) of plant Survived = $\frac{\text{Number of plants survived}}{\text{Total number of plants transferred to pots}} \times 100$

STATISTICAL ANALYSIS OF DATA :

The data of all the experiments, recorded during the present investigation were subjected to statistical analysis in the following way :

a) Mean $(\bar{x}) = \frac{\sum xi}{n} =$ Sum of i^{th} treatment / Number of observations

b) Standard Deviation (σ) :

$$\sqrt{\frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n}}$$

c) Standard error of mean (SEm) = $\frac{\sigma(SD)}{\sqrt{n}}$

CHAPTER : IV

RESULTS

To develop efficient protocols for callus induction and plant regeneration in bael [*Aegle marmelos* (L.) Corr.] from cotyledon, shoot tip, epicotyl, hypocotyl and leaf disc explants, various media formulations were tested in the present investigation. The observations recorded during the course of investigation are presented below :

4.1 SURFACE DISINFECTION

4.1.1 Effectiveness of surface disinfection treatments on cotyledon explants :

Cotyledons disinfected with 70% aqueous solution of ethanol (v/v) for 30 seconds and then followed by 0.1% aqueous solution of $HgCl_2$ for 3 min. showed 20-40% browning of explants with no contamination. Seventy per cent browning of explants with no contamination were observed when cotyledon explants disinfected with 70% aqueous solution of ethanol (v/v) for 30 seconds followed by 0.1% aqueous solution $HgCl_2$ for 4 min.

Cotyledon explant disinfected with 70% aqueous solution of ethanol (v/v) for 30 seconds followed by 0.1% aqueous solution of $HgCl_2$ (w/v) for 2 min. showed less then 25 per cent contamination with no browning of explants. This treatment was found best for disinfecting of cotyledon explants. However, in cotyledons disinfected with 70% aqueous solution of ethanol (v/v) for 30 seconds and then followed by 0.1% aqueous solution of $HgCl_2$ (w/v) for 1 min., 25-49 per cent contamination was observed. Fifty to ninety nine per cent contamination was observed when cotyledons were disinfected with 70% aqueous solution of ethanol (v/v) for 30 seconds followed by 0.1% aqueous solution of $HgCl_2$ (w/v) for ½ min. Cent per cent contamination was observed when cotyledons were disinfected with 70% aqueous solution of ethanol (v/v) for 30 seconds only (Table 4.1.1).

4.2 CALLUS INDUCTION

4.2.1 Effect of different media combinations on per cent callus induction in *bael* cvs. from cotyledon explants :

Callus induction was observed in cotyledon explants on various media formulations within 10-21 days of inoculation. Callus induction was seen usually at cut end and on the abaxial surface of explants. In cultivar Local maximum (65.55%) callus induction was observed on K_{14} and K_{15} media followed by 62.22 per cent on K_{15} medium. Minimum (48.88%) callus induction was observed on K_{16} medium. No callus induction was observed on K_0 medium. In cultivar Gonda Selection maximum (76.66%) callus induction was found on K_{17} medium followed by 63.33 per cent on medium K_{13} and K_{16} media, while minimum (51.11%) callus induction was observed on K_{13} and K_{14} media. No callus induction was observed on K_0 medium. In

Table 4.1.1 : Comparative effectiveness of different surface disinfection treatments on explants of *bael* cvs.

Explant	Duration of treatment (min.)		Effectiveness
	70% aqueous solutions of Ethanol (v/v)	0.1% aqueous solution of $HgCl_2$ (w/v)	
Cotyledons	½	0	100% contamination
Cotyledons	½	½	50-99% contamination
Cotyledons	½	1	25-49% contamination
Cotyledons	½	2	No browning of explants and less than 25% contamination
Cotyledons	½	3	20-40% browning of explants with no contamination
Cotyledons	½	4	70% browning of explants with no contamination

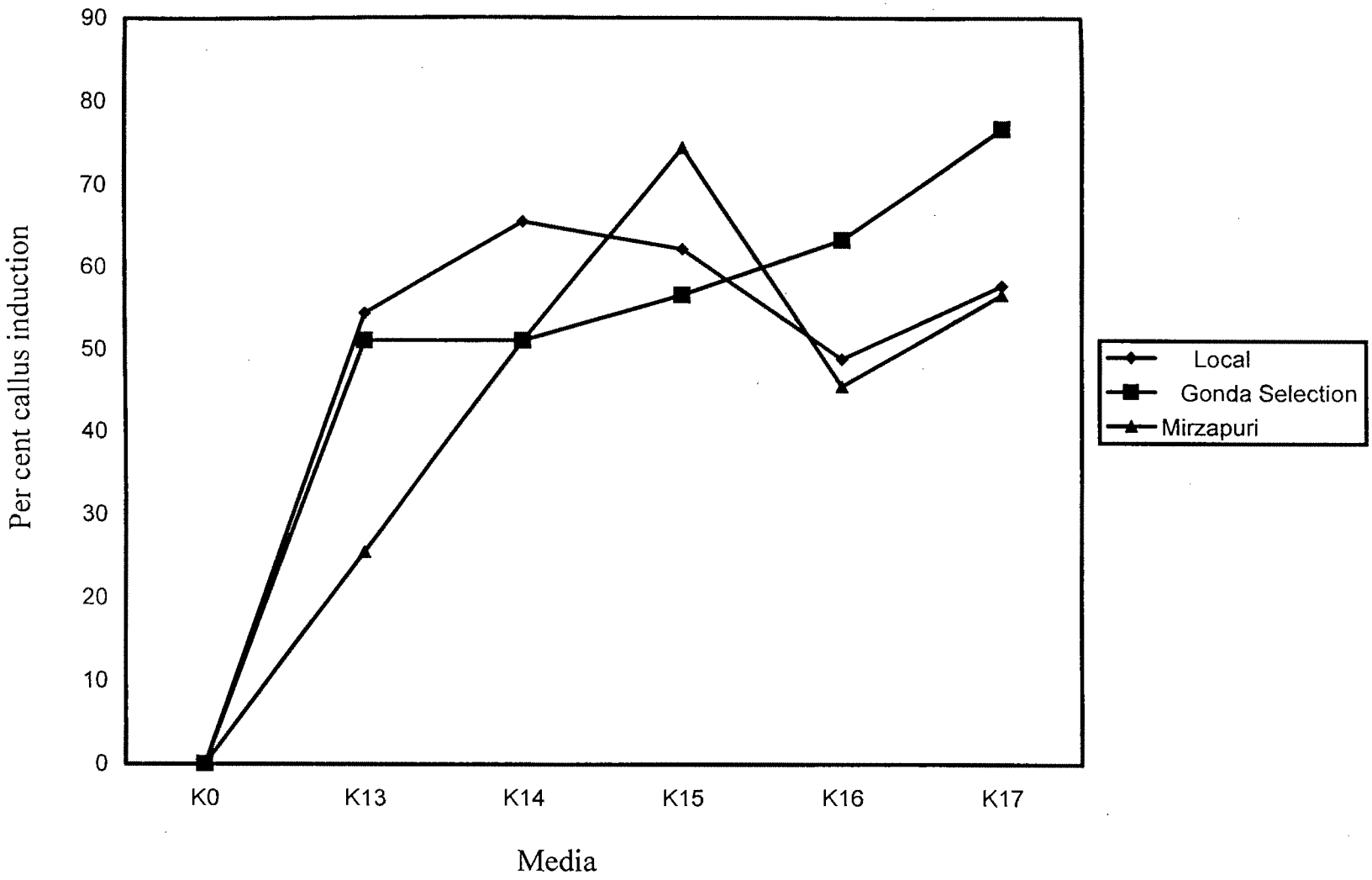


Fig. 1 : Effect of different media combinations on callus induction from cotyledon expalnts of *bael* cvs.

cultivar Mirzapuri, maximum (74.44%) callus induction was observed on K_{15} medium followed by 56.66 per cent on medium K_{17}. Minimum (25.55%) callus induction was observed on K_{13} medium. However, no callus induction was observed on K_0 medium in Fig. 1.

On the basis of all the three cultivars used in the present investigation (Table 4.2.1), cultivar Gonda Selection showed maximum (76.66%) callus induction on K_{17} medium followed by 74.44 per cent on K_{15} medium in cv. Mirzapuri.

4.3 ORGANOGENESIS

4.3.1 Effect of different media combinations on per cent organogenesis from various calli induced in *bael* cvs. from cotyledon explants:

In Local cv., maximum (62.50%) organogenesis was observed on K_3 medium followed by 57.14% on K_{12} medium. Minimum (28.57%) organogenesis was observed on K_6 medium, whereas, in cv. Gonda Selection, maximum (50.00%) organogenesis was observed on K_3 and K_{12} media followed by 28.57% on K_2 and K_{10} media. Minimum (12.50%) organogenesis was observed on K_7 medium. However, in cv. Mirzapuri maximum (57.14%) organogenesis was observed on K_{12} medium followed by 50.00% on K_3 medium. Minimum (16.66%) organogenesis was observed on K_6 and K_{10} media in Fig. 2.

On the basis of all the three cultivars used in the present investigation (Table 4.3.1), cultivar Local showed maximum (62.50%) organogenesis on K_3 medium followed by cv. Mirzapuri (57.14%) on K_{12} medium.

Table 4.2.1. Effect of different media combinations on *in vitro* callus induction of *bael* cvs. from cotyledon explants

Media	Per cent shoot regeneration		
	Local	Gonda Selection	Mirzapuri
K_0	0	0	0
K_{13}	54.44±2.93	51.11±1.11	25.55±2.93
K_{14}	65.55±2.93	51.11±5.87	51.11±1.11
K_{15}	62.22±2.22	56.66±3.33	74.44±2.93
K_{16}	48.88±1.11	63.33±3.33	45.55±2.93
K_{17}	57.77±2.22	76.66±3.33	56.66±3.33

Table 4.3.1. Effect of different media combinations on per cent organogenesis from various calli induced of *bael* cvs.

Media	Per cent organogenesis		
	Local	Gonda Selection	Mirzapuri
K_0	0	0	0
K_2	42.85	28.57	33.33
K_3	62.50	50.00	50.00
K_6	28.57	14.28	16.66
K_7	37.50	12.50	33.33
K_{10}	42.85	28.57	16.66
K_{12}	57.14	50.00	57.14

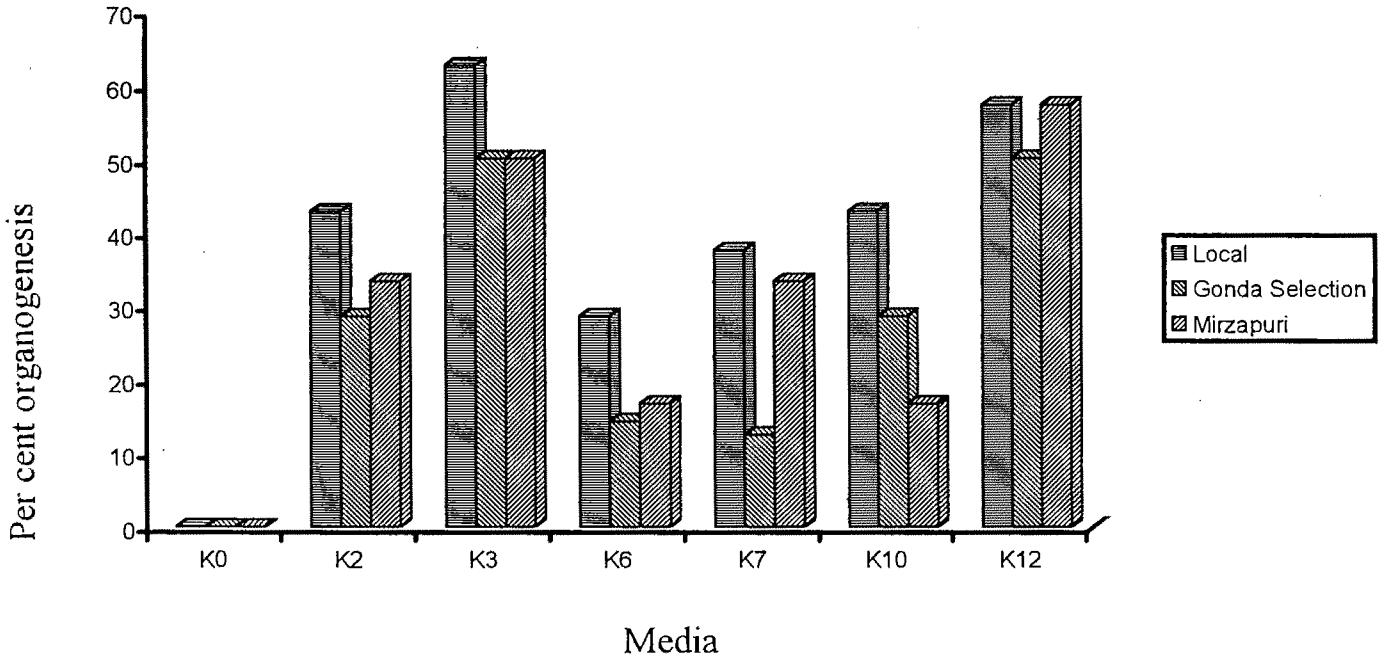


Fig. 2 : Effect of different media combinations on organogenesis from various calli induced of *bael* cvs.

4.3.2 Effect of different media combinations on mean number of plantlets obtained through intermediate stage of callus in *bael* cvs. from cotyledon explants :

The response of different media combinations on number of plantlets obtained through intermediate stage of callus have been presented in (Table 4.3.2). A perusal of the data revealed that in Local cultivar showed maximum (3.42) mean number of platnlets per calli on K_{12} medium followed by K_2 medium 2.28. Minimum (1.71) mean number of plantlets per calli was observed on K_{10} medium. In cultivar Gonda Selection maximum (3.25) mean number of plantlets per calli on K_3 medium followed by K_{12} medium (3.00). Minimum (1.28) mean number of plantlets per calli were observed was observed on K_2 medium. In cultivar Mirzapuri maximum (3.85) mean number of plantlets per calli were observed on K_{12} medium followed by K_3 medium 2.0 and minimum (0.66) mean number of plantlets were observed on K_{10} medium.

On the basis of result given in Fig. 3 it is clear that among three cultivars, Mirzapuri showed maximum (3.85) mean number of plantlets per calli on K_{12} medium followed by Local 3.42 on K_{12} medium. However, minimum (0.66) mean number of plantlets per calli was observed in cv. Mirzapuri on K_{10} medium.

4.3.3 Effect of different media combinations on mean shoot length from various calli induced in *bael* cvs. :

The data on the mean shoot length using cotyledon explants of different cvs. on different combinations of media are presented in (Table 4.3.3).

Table 4.3.2. Effect of different media combinations on mean number of plantlets from various calli induced of *bael* cvs.

Media	Mean number of plantlets per calli		
	Local	**Gonda Selection**	**Mirzapuri**
K_0	0	0	0
K_2	2.28±1.12	1.28±0.89	2.0±1.29
K_3	2.37±0.80	3.25±1.46	2.0±1.36
K_6	1.57±1.06	1.14±1.14	0.83±0.83
K_7	2.12±1.14	1.12±1.12	1.66±1.17
K_{10}	1.71±0.91	1.85±1.24	0.66±0.66
K_{12}	3.42±1.39	3.00±1.19	3.85±1.66

± SE

Table 4.3.3. Effect of different media combinations on mean shoot length from various calli induced of *bael* cvs.

Media	Mean shoot length (cm)		
	Local	**Gonda Selection**	**Mirzapuri**
K_0	0	0	0
K_2	1.20±0.22	0.97±0.18	1.26±0.18
K_3	1.54±0.15	1.36±0.16	1.47±0.19
K_6	1.21±0.18	0.87±0.13	1.33±0.35
K_7	1.17±0.17	0.83±0.10	1.53±0.21
K_{10}	1.32±0.20	1.14±0.16	1.01±0.37
K_{12}	1.32±0.14	1.18±0.10	1.51±0.14

± SE

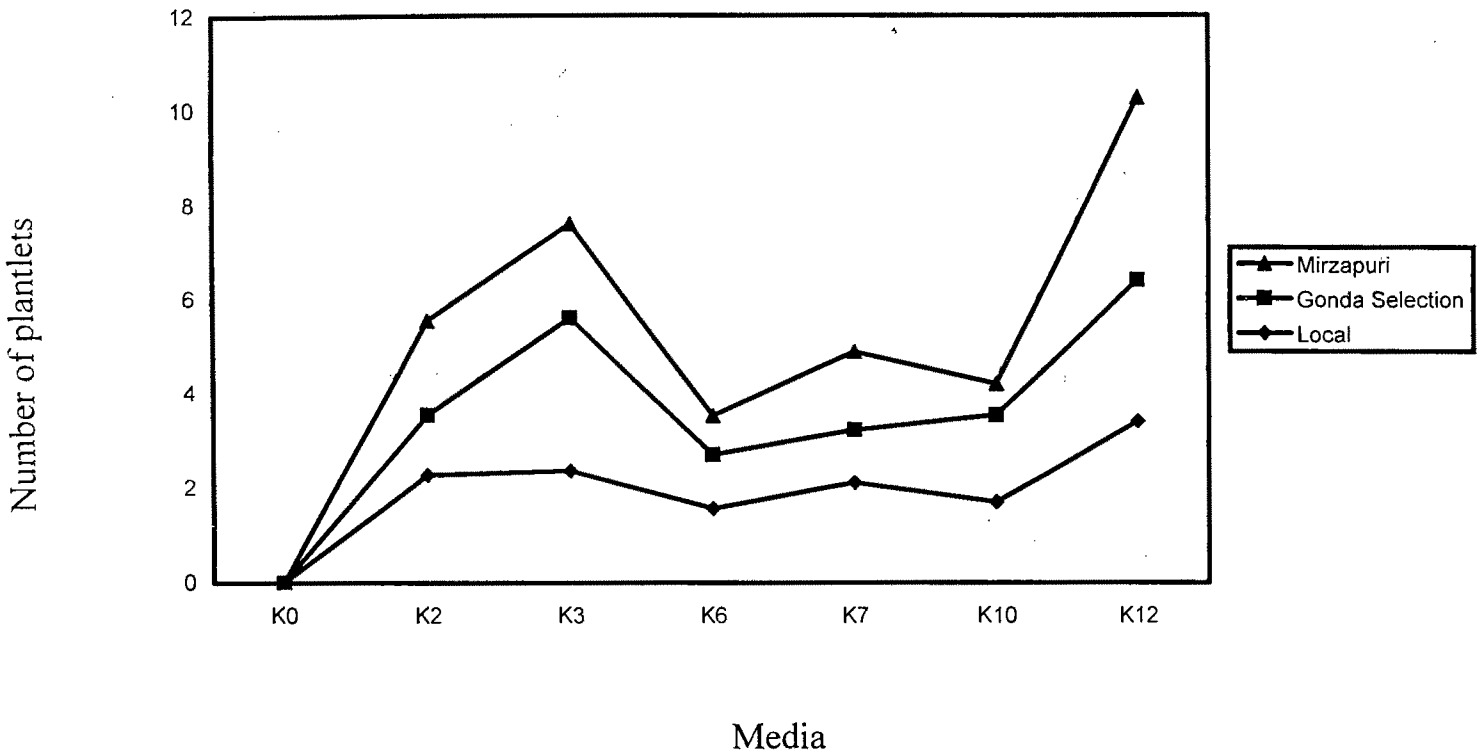
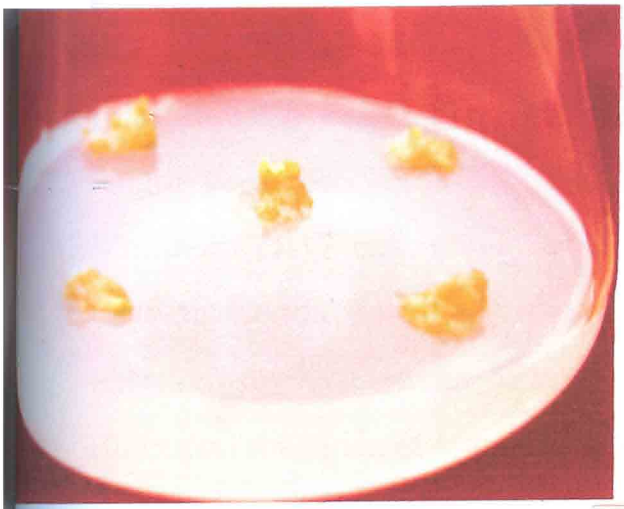


Fig. 3 : Effect of different media combinations on number of plantlets obtained through intermediate stage of callus.



Shoot regeneration in callus cultures derived from cotyledon explants of cv. Local on K_3 medium.

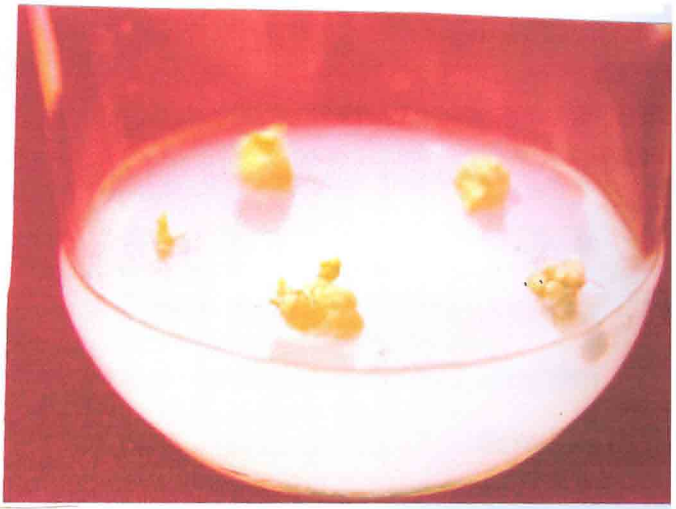


Plate - 2. **Shoot regeneration in callus cultures derived from cotyledon explants of cv. Mirzapuri on K_{12} medium.**

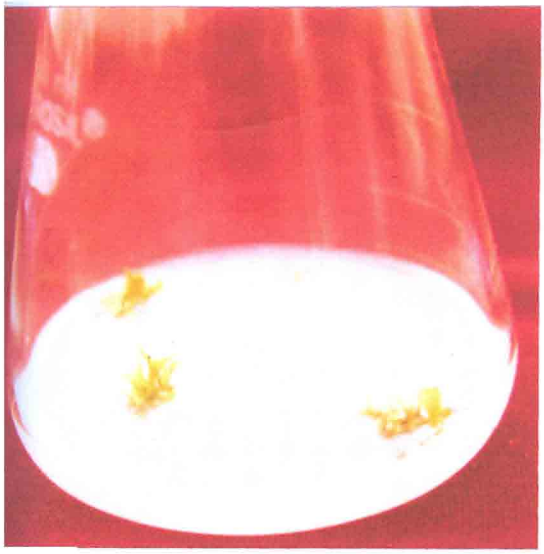


Plate - 3. **Multiple shoot regeneration in callus culture derived from cotyledon explants of cv. Mirzapuri on K_{12} medium.**

It is evident from the data that in cv. Local maximum (1.54 cm) mean shoot length was observed on K_3 medium followed by K_{10} medium 1.32 cm. Minimum (1.17 cm) shoot length was observed on K_7 medium, while in cultivar Gonda Selection, maximum (1.36 cm) mean shoot length was observed on K_3 medium followed by 1.14 cm on K_{10} medium. Minimum (0.83 cm) mean shoot length was observed on K_7 medium. In cv. Mirzapuri maximum (1.53 cm) shoot length was observed on K_7 medium followed by K_{12} medium 1.51 cm. Minimum (1.01 cm) mean shoot length was observed on K_{10} medium.

On the basis of the results given in Table 4.3.3, it is clear that among all the three cultivars, maximum (1.53 cm) mean shoot length was observed in cv. Mirzapuri on K_7 medium followed by Local 1.54 cm on K_3 medium, whereas, minimum (0.83 cm) mean shoot length was observed in cv. Gonda Selection on K_7 medium.

4.3.4 Effect of different media combinations on percentage of rooting in plantlets obtained through intermediate stage of callus :

The response of different media combinations on percentage of rooting have been presented in Fig. 4. A perusal of table revealed that in cv. Local maximum (22.5%) percentage of rooting was observed on R_{16} medium followed by R_{17} medium 20.0 per cent, while minimum (14.20%) rooting was observed on R_7 medium. In Gonda Selection, maximum (25.0%) percentage rooting was observed on R_{17} medium followed by R_{16} medium 22.20 per cent, while minimum (10.0%) rooting was observed on R_8 medium. In case of cv. Mirzapuri, maximum (37.50%) rooting was observed on R_{16} medium followed by R_{17} medium

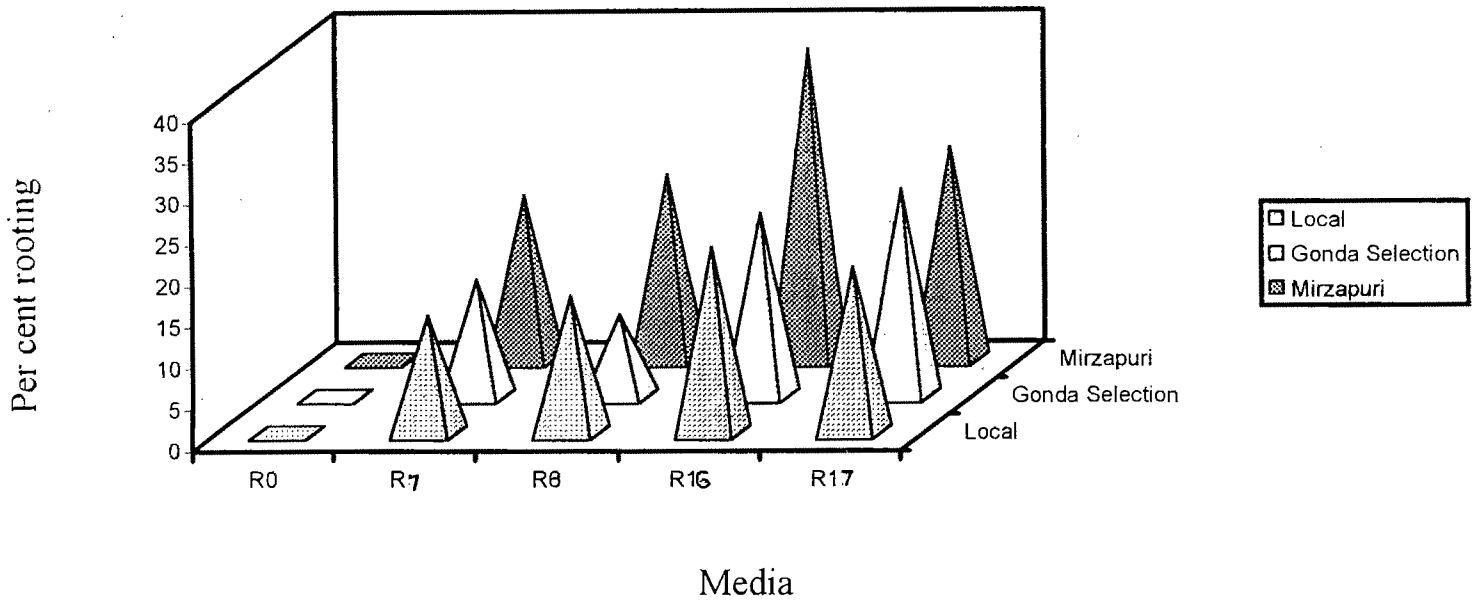


Fig. 4 : Effect of different media combinations on rooting of plantlets obtained through intermediate stage of callus.

25.70 per cent, whereas, minimum (20.00%) rooting was observed on R_7 medium.

On the basis of the results presented in Table 4.3.4, it is clear that among all the three cultivars, Mirzapuri showed maximum percentage of rooting (37.50%) on R_{16} medium followed by Gonda Selection 25.00 per cent on R_{17} medium. Minimum (10.00%) rooting was observed in cv. Gonda Selection.

Table 4.3.4. Effect of different media combinations on percentage of rootingin plantlets from various calli induced of *bael* cvs.

Media	Per cent rooting		
	Local	**Gonda Selection**	**Mirzapuri**
R_0	0	0	0
R_7	14.2	14.2	20.0
R_8	16.6	10.0	22.50
R_{16}	22.50	22.20	37.50
R_{17}	20.0	25.0	25.70

± SE

4.4 DIRECT SHOOT REGENERATION

4.4.1 Shoot regeneration from cotyledon explants in different media :

Shoot regeneration from cotyledon explant from all cvs. was observed on all the media tried viz. K_0, K_1, K_2, K_3 K_4, K_5, K_6, K_7, K_8, K_9, K_{10}, K_{11} and K_{12} (Table 4.4.1). Shoot regeneration was observed within 10-25 days of culture. It is evident from the data in cv. Local that maximum (97.22%)

Table 4.4.1. Effect of different media combinations on per cent shoot regeneration of *bael* cultivars from cotyledon explants

Media	Per cent shoot regeneration		
	Local	Gonda Selection	Mirzapuri
K_0	42.82±3.61	43.33±3.33	51.11±1.11
K_1	43.88±3.09	64.90±2.88	59.44±0.55
K_2	87.22±3.64	90.54±5.79	79.99±1.92
K_3	87.77±2.22	81.11±5.87	78.33±1.66
K_4	73.33±3.85	73.33±3.33	64.44±7.28
K_5	41.11±4.84	43.33±3.33	54.44±2.93
K_6	65.55±2.93	50.00±5.77	49.44±5.47
K_7	81.66±4.40	60.00±5.77	63.33±5.09
K_8	71.11±3.88	46.66±6.66	48.33±3.47
K_9	87.22±3.64	60.00±5.77	76.66±5.09
K_{10}	93.88±3.09	96.66±3.33	72.22±4.00
K_{11}	97.22±2.78	90.76±0.76	85.55±1.11
K_{12}	75.00±2.88	74.44±2.93	76.66±1.66

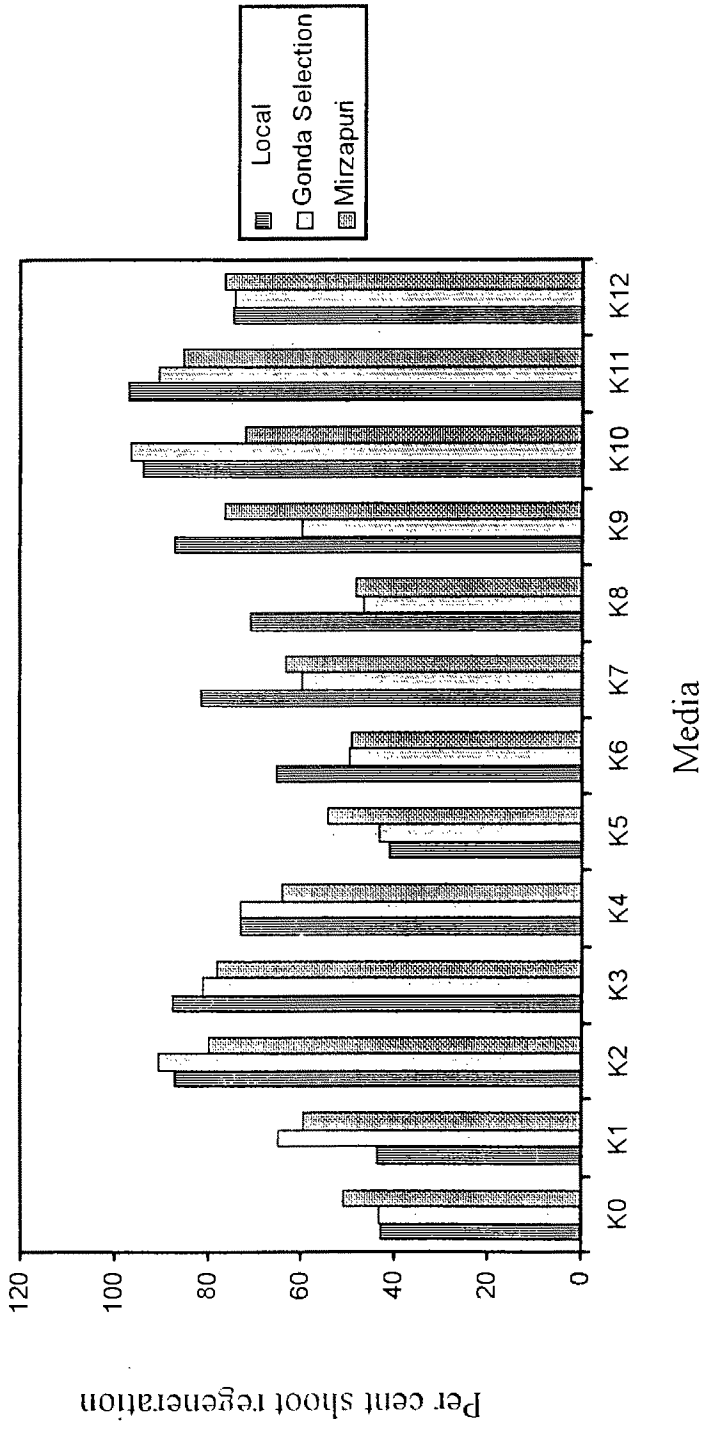


Fig. 5 : Effect of different media combinations on shoot regeneration from cotyledonary explants of *bael* cvs.

shoot regeneration was observed on K_{11} medium followed by 93.30 per cent shoot regeneration on K_{10} medium. However, the minimum (41.11%) shoot regeneration was observed on K_5 medium. Similarly in cultivar Gonda Selection maximum (96.66%) shoot regeneration was observed on K_{10} medium, followed by 90.76 per cent and 90.54 per cent shoot regeneration on K_{11} and K_3 media, respectively. Minimum (43.33%) shoot regeneration was observed on K_0 medium. In Mirzapuri cultivar, maximum (85.55%) shoot regeneration was observed on K_{11} medium followed by 78.73 per cent shoot regeneration on K_3 medium. Minimum (49.44%) shoot regeneration was observed on K_6 medium.

Among all the three cultivars, cv. Local showed maximum (97.22%) shoot regeneration on K_{11} medium followed by Gonda Selection 96.66 per cent on K_{11} medium. Minimum (41.11%) shoot regeneration frequency was recorded on K_5 medium in cv. Local (Fig. 5).

4.4.2 Shoot regeneration frequency from shoot tip explants of *in vitro* regenerated shoots in different media :

Shoot tip explants excised from all the three cultivars viz., Gonda Selection, Mirzapuri and Local of *in vitro* regeneration shoots showed 100% shoot regeneration in all the media tried.

4.4.3 Shoot regeneration from epicotyl explant of *in vitro* regenerated shoots in different media :

The data revealed that cultivar cultivar Local maximum (90.0%) shoot regeneration was recorded on K_{11} medium followed by 73.33 per cent shoot regeneration on K_3 medium. Minimum (40.0%) shoot regeneration was obtained on K_0 medium. In Gonda selection showed maximum (79.25%)

shoot regeneration on K_{12} medium followed by 73.33 per cent shoot regeneration on K_{11} medium. However, minimum (32.22%) shoot regeneration was observed on K_0 medium. Similarly, cultivar Mirzapuri showed maximum (90.0%) shoot regeneration on K_3 medium followed by 80.0 per cent shoot regeneration on K_5 medium. Minimum (43.33%) shoot regeneration was observed on K_0 medium.

The data presented in Table 4.4.3 in relation to comparative results revealed that maximum (90.0%) shoot regeneration was found in cultivar Mirzapuri and Local on K_3 and K_{11} media, respectively followed by cultivar Gonda selection 79.25 per cent on K_{12} medium. Minimum (32.22%) shoot regeneration was observed on K_0 medium in cv. Gonda Selection.

4.4.4 Shoot regeneration from hypocotyl explants in different media :

Hypocotyl explants were placed horizontal on different media tested in all the three cultivars. It is evident from the data in cultivar Local revealed that maximum (76.11%) shoot regeneration was found on K_3 medium followed by 68.33 per cent shoot regeneration K_{11} medium. Minimum (26.66%) shoot regeneration was observed on K_0 medium. In Gonda Selection, maximum (81.11%) shoot regeneration was observed on K_5 medium followed by 56.66 per cent shoot regeneration on K_1, K_2 and K_6 media. Minimum (28.33%) shoot regeneration was observed on K_0 medium. The results pertaining to cultivar Mirzapuri, maximum (96.66%) shoot regeneration was observed on K_2 medium followed by 83.33 per cent shoot regeneration on K_9 medium. Minimum (34.44%) shoot regeneration was observed on K_0 medium.

Table 4.4.3. Effect of different media combinations on per cent shoot regeneration of *bael* cvs. from epicotyl explants

Media	Per cent shoot regeneration		
	Local	Gonda Selection	Mirzapuri
K_0	40.00±5.77	32.22±4.00	43.33±3.33
K_1	53.33±3.33	55.41±2.91	76.66±8.81
K_2	50.00±5.77	60.00±5.77	76.66±3.33
K_3	73.33±8.81	63.33±3.33	90.0±5.77
K_4	40.00±5.77	43.33±3.33	53.33±3.33
K_5	43.33±3.33	43.33±3.33	80.00±5.77
K_6	53.33±3.33	50.00±5.77	76.66±3.33
K_7	53.33±8.81	53.33±3.33	76.66±8.81
K_8	50.00±5.77	33.33±3.33	36.66±3.33
K_9	66.66±3.33	56.66±3.33	50.00±5.77
K_{10}	56.66±3.33	66.66±3.33	56.00±3.33
K_{11}	90.00±5.77	73.33±3.33	76.00±3.33
K_{12}	63.33±3.33	79.25±5.82	73.33±3.33

Table 4.4.4. Effect of different media combinations on per cent shoot regeneration of *bael* cvs. from hypocotyl explants

Media	Per cent shoot regeneration		
	Local	Gonda Selection	Mirzapuri
K_0	26.66±3.33	28.33±1.66	34.44±2.93
K_1	54.44±2.93	56.66±8.81	43.88±3.09
K_2	57.77±2.22	56.66±3.33	96.66±3.33
K_3	73.11±2.00	56.66±3.33	75.00±2.88
K_4	43.88±3.09	43.33±3.33	43.88±3.09
K_5	48.88±1.11	81.11±1.11	62.22±2.22
K_6	50.55±5.47	56.66±3.33	75.00±2.88
K_7	62.77±.364	43.33±3.33	56.11±3.09
K_8	32.22±1.11	40.0±0.00	43.88±3.09
K_9	38.33±2.54	43.33±3.33	83.33±3.33
K_{10}	46.10±2.42	43.88±3.09	59.44±0.55
K_{11}	68.33±4.40	47.22±2.78	75.00±2.88
K_{12}	41.11±4.84	34.44±2.93	65.55±2.93

Among all the three cvs., the data revealed that maximum (96.66%) shoot regeneration was found in cultivar Mirzapuri on K_2 medium followed by cv. Gonda Selection 81.11 per cent on K_5 medium (Table 4.4.4).

4.4.5 Shoot regeneration from leaf disc explants in different media :

Leaf disc explants were placed abaxial on different media tried in all the three cultivars. The data revealed that in cultivar Local, the maximum (23.33%) shoot regeneration was observed on K_{12} medium followed by 16.66 per cent shoot regeneration on K_4 medium. Minimum (13.33%) shoot regeneration was observed on K_{11} medium. No shoot regeneration was observed on K_0, K_1, K_2, K_5, K_6, K_7, K_8, K_9 and K_{10} media. Cultivar Gonda Selection showed maximum (36.15%) shoot regeneration on K_{11} medium followed by 23.80 per cent shoot regeneration on K_{12} medium. However, minimum (15.55%) shoot regeneration was observed on K_9 medium. No shoot regeneration was observed on K_0, K_1, K_2, K_3, K_4, K_5, K_6, K_7 and K_8 media, whereas, in cultivar Mirzapuri, maximum (46.66%) shoot regeneration was observed on K_{11} medium followed by 43.33 per cent shoot on K_3 and K_{10} media. Minimum (33.33%) shoot regeneration was observed on K_7 and K_9 media. No shoot regeneration was found on K_0, K_1, K_2, K_5 and K_6 media.

The data presented in Table 4.4.5 revealed that maximum (46.66%) shoot regeneration was observed in cultivar Mirzapuri on K_{11} medium followed by cultivar Gonda Selection 36.15 per cent on K_{11} medium. Minimum (13.33%) shoot regeneration was observed on K_{11} medium in cultivar Local.

Table 4.4.5. Effect of different media combinations on per cent shoot regeneration of *bael* cvs. from leaf disc explants

Media	Per cent shoot regeneration		
	Local	Gonda Selection	Mirzapuri
K_0	0	0	0
K_1	0	0	0
K_2	0	0	0
K_3	14.44±2.93	0	43.33±3.33
K_4	16.66±3.33	0	36.66±3.33
K_5	0	0	0
K_6	0	0	0
K_7	0	0	33.33±3.33
K_8	0	0	36.66±3.33
K_9	0	15.55±2.93	33.33±3.33
K_{10}	0	18.88±1.11	43.33±3.33
K_{11}	13.33±3.33	36.15±3.88	46.66±3.33
K_{12}	23.33±3.33	23.80±3.15	40.00±5.77

Shoot regeneration from different explants of Gonda selection cv. placed on K_{11} medium (Knop's+1.0 mgL^{-1} BAP+0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA)

Multiple shoot regeneration from leaf disc explants of cv. Mirzapuri on K_{11} medium.

Plate 6. Solitary shoot regeneration from shoot tip explants of cv. Mirzapuri on K_1 medium (Knop's + 0.25 mgL^{-1} BAP)

4.5 SHOOT PROLIFERATION

4.5.1 Per cent multiple shoots from cotyledon explants in different media combinations :

The data presented in (Table 4.5.1) revealed that in cultivar Local revealed maximum (94.10%) multiple shoots on K_{10} medium followed by 86.66% multiple shoots on K_3 medium. Minimum (24.56%) multiple shoots were observed on K_0 medium. In cultivar Gonda Selection, maximum (84.87%) multiple shoots were recorded on K_{11} medium followed by 81.11 per cent on K_2 medium. Minimum (23.33%) multiple shoots were observed on K_0 medium, whereas, in cultivar Mirzapuri, maximum (83.33%) multiple shoots were observed on K_{11} medium followed by 76.66 per cent on K_3 medium. Minimum (23.33%) multiple shoots were observed on K_0 medium.

Among all the three cvs. studied, maximum (94.10%) multiple shoots were recorded in cultivar Local on K_{10} medium followed by cultivar Gonda Selection(84.87 per cent)and Mirzapuri(83.33 per cent)on K_{11} medium (Fig. 6).

4.5.2 Per cent multiple shoots from epicotyl explants in different media combinations :

The data on the per cent multiple shoots using epicotyls of different cvs. on different media combinations are presented in (Table 4.5.2). It is evident from the data that in cultivar Local, maximum (70.0%) multiple shoots were observed on K_{11} medium followed by 60.0 per cent multiple shoots on K_3 medium. However, minimum (10.0%) multiple shoots were observed on K_0 medium. In cv. Gonda Selection, maximum (55.18%) multiple shoots were observed on K_{12} medium followed by 46.66 per cent on K_{11} medium and minimum (14.44%) multiple shoots were recorded on K_0

Table 4.5.1. Effect of different media combinations on per cent shoot regeneration of *bael* cvs. from leaf disc explants

Media	Per cent shoot regeneration			Mean
	Local	Gonda Selection	Mirzapuri	
K_0	24.56±2.91	23.33±3.33	23.33±3.33	
K_1	46.66±3.33	43.72±3.15	33.33±3.33	
K_2	66.66±3.33	81.11±1.11	53.33±3.33	
K_3	86.66±3.33	65.55±2.93	76.66±3.33	
K_4	74.44±2.93	53.33±3.33	60.00±5.77	
K_5	33.33±3.33	43.33±3.33	63.33±3.33	
K_6	56.66±3.33	53.33±3.33	53.33±3.33	
K_7	63.33±3.33	43.33±3.33	56.66±6.66	
K_8	65.55±2.93	50.00±5.77	33.33±3.33	
K_9	76.66±3.33	60.00±5.77	70.00±5.77	
K_{10}	94.10±3.09	80.60±0.60	60.00±5.77	
K_{11}	81.11±1.11	84.87±2.88	83.33±3.33	
K_{12}	73.33±3.33	45.55±2.93	65.55±2.93	

Table 4.5.2. Effect of different media combinations on per cent multiple shoots of *bael* cvs. from epicotyl explants

Media	Per cent shoot regeneration		
	Local	Gonda Selection	Mirzapuri
K_0	13.33±3.33	14.44±2.93	20.00±5.77
K_1	26.66±3.33	33.75±3.14	40.00±5.77
K_2	40.00±5.77	43.43±3.33	43.33±3.33
K_3	60.00±5.77	40.55±5.79	70.00±5.77
K_4	33.33±3.33	23.33±3.33	33.33±3.33
K_5	23.33±3.33	33.33±3.33	23.33±3.33
K_6	26.66±6.66	34.44±2.93	33.33±8.81
K_7	33.33±3.33	37.77±2.22	33.33±3.33
K_8	36.66±3.33	23.33±3.33	23.33±3.33
K_9	33.33±3.33	31.27±1.27	26.66±6.66
K_{10}	43.43±3.33	43.33±3.33	33.33±3.33
K_{11}	70.00±5.77	46.66±3.33	60.00±5.77
K_{12}	53.33±3.33	55.18±2.89	46.66±3.33

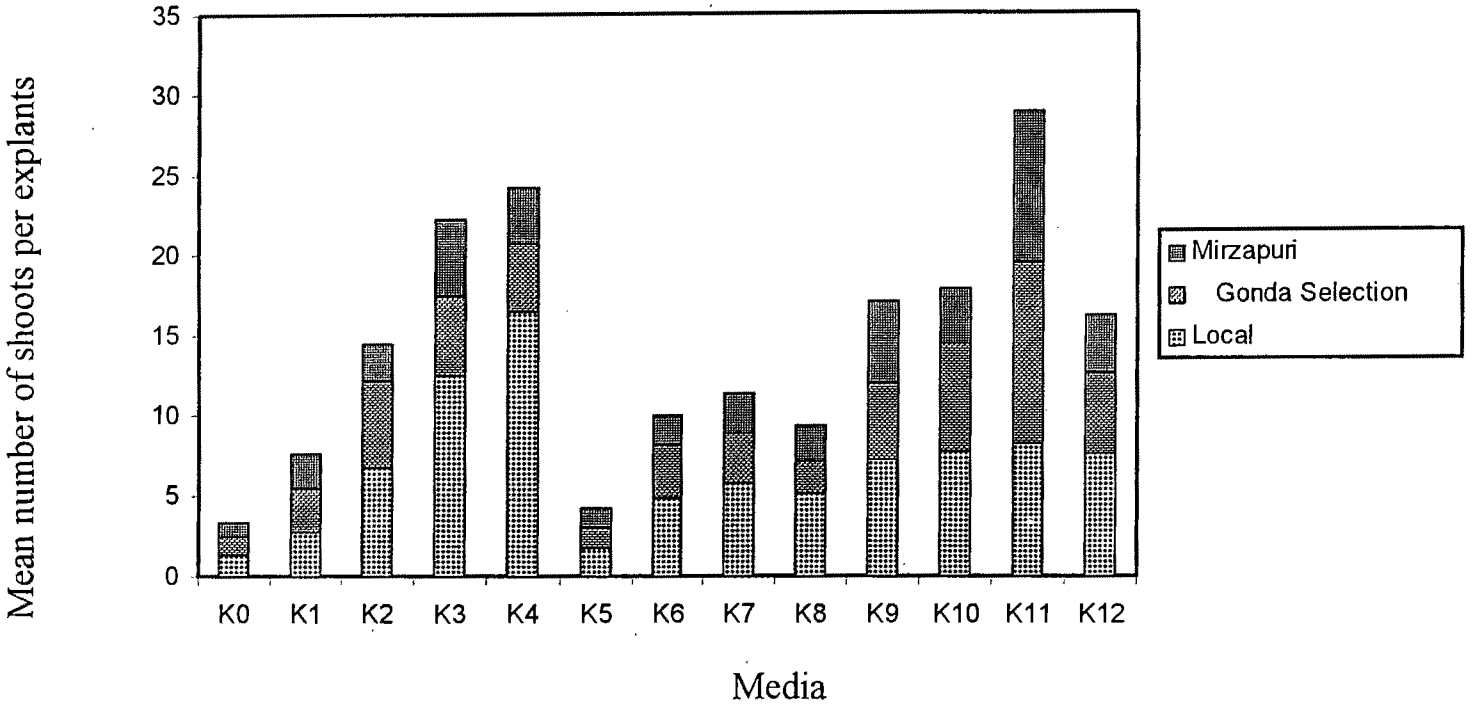


Fig. 6 : Effect of different media combinations on shoot proliferation from cotyledonary explants of *bael* cvs.

medium, whereas, in cultivar Mirzapuri maximum (70.0%) multiple shoots were observed on K_3 medium followed by 60.00 per cent multiple shoots on K_{11} medium. Minimum (20.0%) multiple shoots were observed on K_0 medium.

The data presented in Table 4.5.2 revealed that maximum (70.0%) multiple shoots were observed in cultivars Mirzapuri and Local on K_3 and K_{11} media, respectively.

4.5.3 Per cent multiple shoots from hypocotyl explants in different media combinations :

The data revealed that in cultivar Local the maximum (63.33%) multiple shoots were observed on K_3 medium followed by 43.33 per cent multiple shoots on K_7 medium. Minimum (23.33 %) multiple shoots were observed on K_4 and K_{10} media. No multiple shoots were observed on K_0 medium. In cultivar Gonda Selection, maximum (65.55%) multiple shoots were observed on K_5 medium followed by 63.33 per cent multiple shoots on K_1 medium. However, minimum (9.44%) multiple shoots were observed on K_0 medium (Table 4.5.3), whereas, in cultivar Mirzapuri , maximum (77.77%) multiple shoots were observed on K_2 medium followed by 63.33 per cent multiple shoots on K_9 medium. Minimum (22.22%) multiple shoots were observed on K_0 medium.

Among all the three cvs., cv. Mirzapuri produced maximum (77.77%) multiple shoot on K_2 medium followed cv. Gonda Selection 65.55 per cent on K_5 medium. Minimum (9.44%) multiple shoots per explant were observed in cv. Gonda Selection on K_0 medium.

Table 4.5.3. Effect of different media combinations on per cent multiple shoots of *bael* cvs. from hypocotyl explants

Media	Per cent shoot regeneration		
	Local	Gonda Selection	Mirzapuri
K_0	0	9.44±0.55	22.22±4.00
K_1	34.44±2.93	63.33±3.33	34.44±2.93
K_2	36.66±3.33	53.33±3.33	77.77±4.00
K_3	63.33±3.33	40.00±5.77	62.22±2.22
K_4	28.88±1.11	33.33±3.33	28.33±1.66
K_5	34.44±2.93	65.55±2.93	34.44±2.93
K_6	37.94±2.05	53.33±3.33	53.33±3.33
K_7	43.33±3.33	23.33±3.33	43.88±3.09
K_8	23.33±3.33	13.33±3.33	25.00±2.88
K_9	26.66±6.66	23.33±3.33	63.33±3.33
K_{10}	28.88±1.11	22.22±4.00	59.44±0.55
K_{11}	41.11±4.84	31.66±4.40	62.77±3.64
K_{12}	30.00±5.77	34.44±2.93	59.44±0.55

4.5.4 Per cent multiple shoots from leaf disc explants in different media combinations :

The data presented in (Table 4.5.4) revealed that in cultivar Local maximum (23.33%) multiple shoots were observed on K_{12} medium followed by 20.0 per cent multiple shoots on K_{11} medium. Minimum (13.33%) multiple shoots were observed on K_4 medium. No solitary and multiple shoots were observed on K_0, K_1, K_2, K_5, K_6, K_7, K_8, K_9 and K_{10} media. In cultivar Gonda Selection, maximum (21.79%) multiple shoots were observed on K_{11} medium followed by 12.77 per cent multiple shoots on K_{10} medium. Minimum (9.44%) multiple shoots were observed on K_9 medium. No solitary and multiple shoots were observed on K_0, K_1, K_2, K_3, K_4, K_5, K_6, K_7 and K_8 media, whereas, in cultivar Mirzapuri maximum (33.33%) multiple shoots were observed on K_{11} medium followed by 23.33 per cent multiple shoots on K_3, K_{10} and K_{12} media. Minimum (13.33%) multiple shoots were observed on K_4 and K_7 media. No solitary and multiple shoots were observed on K_0, K_1, K_2, K_5, K_6 and K_8 media.

The data pertaining to comparative pattern regarding per cent multiple shoots is presented in Table 4.5.4. which indicated that maximum (33.33%) multiple shoots were found in cultivar Mirzapuri on K_{11} medium followed by 23.33 per cent multiple shoots in cultivar Local on K_{12} medium.

4.5.5 Effect of different media combinations on mean number of multiple shoots per explant in different cvs. of *bael* from cotyledon explants :

Shoot proliferation from cotyledon explants was observed after seven weeks of culture on all media tested (Table 4.5.5). Solitary as well as

Table 4.5.4. Effect of different media combinations on per cent multiple shoots of *bael* cvs. from leaf disc explants

Media	Per cent shoot regeneration		
	Local	Gonda Selection	Mirzapuri
K_0	0	0	0
K_1	0	0	0
K_2	0	0	0
K_3	14.44±2.93	0	23.33±3.33
K_4	13.33±3.33	0	13.33±3.33
K_5	0	0	0
K_6	0	0	0
K_7	0	0	13.33±3.33
K_8	0	0	0
K_9	0	9.44±0.55	16.67±6.77
K_{10}	0	12.77±3.64	23.33±3.33
K_{11}	20.00±5.77	21.79±4.31	33.33±3.33
K_{12}	23.33±3.33	12.38±3.90	23.33±3.33

Table 4.5.5. Effect of different media combinations on mean number of shoots per explant of *bael* cvs. from cotyledon explants

Media	Mean number of shoots per explant		
	Local	Gonda Selection	Mirzapuri
K_0	1.20±0.66	1.33±0.60	0.90±0.40
K_1	2.76±1.01	2.70±1.13	2.20±1.09
K_2	5.33±1.01	6.80±1.93	2.40±0.68
K_3	5.00±1.75	12.50±2.20	4.70±1.13
K_4	4.30±1.61	6.46±1.91	3.50±1.26
K_5	1.20±0.80	1.80±0.96	1.30±0.44
K_6	3.25±1.97	4.90±1.47	1.80±0.64
K_7	3.20±1.77	5.70±1.73	2.50±0.89
K_8	2.0±1.26	5.15±1.03	2.20±1.04
K_9	4.80±2.05	7.22±1.56	5.10±1.55
K_{10}	6.81±1.41	7.72±1.80	3.40±1.12
K_{11}	11.25±2.09	8.30±2.06	9.38±1.33
K_{12}	5.06±1.59	7.61±1.51	3.58±0.86

± SE

multiple shoots were observed on Knop's basal media. Solitary and multiple shoot initiation was observed within 12 to 25 days of inoculation. The data indicated that in cultivar Local, maximum (11.25) mean number of multiple shoots per explant were observed on K_{11} medium followed by 6.81 mean number of multiple shoots on K_{10} medium while minimum (1.20) mean number of multiple shoots were observed on K_0 medium, whereas, in cultivar Mirzapuri, maximum (9.38) mean number of multiple shoos on K_{11} medium. Minimum (0.90) mean number of multiple shoots were found on K_0 medium. In cultivar Gonda Selection, maximum (12.50) mean number of multiple shoots were observed on K_3 medium followed by 8.30 mean number of multiple shoots on K_{11} medium. However, minimum (1.33) mean number of multiple shoots were observed on K_0 medium.

On the basis of results given in Table 4.5.5 it is clear that among all the three cultivars maximum (12.50) mean number of multiple shoots per explant were observed in cultivar Gonda Selection on K_3 medium followed by cv. Local 11.25 on K_{11} medium.

4.5.6 Effect of different media combinations on mean number of multiple shoots per explant in different cultivars of *bael* from shoot tip explants :

The data revealed that only solitary shoots per explant were observed in all the three cultivars on all the media tested. Solitary shoot initiation was observed within 2-5 days of inoculation.

4.5.7 Effect of different media combinations on mean number of multiple shoots per explant in different cvs. of *bael* from epicotyl explants :

The data presented in Table (4.5.7) indicated that in cultivar Gonda Selection, maximum (3.77) mean number of multiple shoots per explant were observed on K_{12} medium followed by 3.30 mean number of multiple shoots per explant on K_{11} medium while minimum (0.50) mean number of multiple shoots per explant were observed on K_8 medium. The data further revealed that in cultivar Mirzapuri, maximum (2.50) mean number of multiple shoots were observed on K_3 medium followed by 2.0 mean number of multiple shoots on K_{11} medium. However, minimum (0.90) mean number of multiple shoots per explant were observed on K_0 medium. In cultivar Local, maximum (2.80) mean number of multiple shoots per explant were observed on K_{11} medium followed by 2.20 mean number of multiple shoots per explant on K_3 medium. Minimum (0.40) mean number of multiple shoots per explant were observed on K_0 medium.

Among all the three cultivars used during the present investigation, cultivar Gonda Selection produced maximum (3.77) mean number of shoots per explant on K_{12} medium followed by cultivar Local 2.80 on K_{11} medium. Minimum (0.40) mean number of multiple shoots per explant were observed in cv. Local on K_0 medium.

4.5.8 Effect of different media combinations on mean number of multiple shoots per explant in *bael* cvs. from hypocotyl explants :

Multiple shoot initiation were observed within 22 to 38 days of inoculation. Shoot proliferation from hypocotyl explants were observed after

seven weeks of culture on all the media tested (Table 4.5.8). Solitary as well as multiple number of shoots per explant were observed on Knop's based media. Data revealed that maximum (2.50) mean number of shoots per explant were observed in case of cultivar Gonda Selection on K_1 medium followed by 2.30 mean number of multiple shoots per explant on K_2 medium while minimum (0.33) mean number of shoots were observed on K_0 medium.

In cultivar Mirzapuri maximum (2.50) mean number of multiple shoots per explant were observed on K_2 medium followed by 2.25 and 2.25 mean number of multiple shoots per explant on K_{11} and K_3 media, respectively. However, minimum (0.50) mean number of multiple shoots per explant were observed on K_0 medium.

The response in relation to cv. Local revealed that maximum (1.73) mean number of multiple shoots per explant were observed on K_3 medium followed by 1.53 and 1.53 mean number of multiple shoots per explant on K_{11} and K_7 media, respectively, while minimum (0.66) mean number of multiple shoots per explant were observed on K_9 and K_{12} media. No multiple shoots per explant were observed on K_0 medium but only solitary shoots per explant were observed.

Among all the three cultivar used during the present investigation, mean number of multiple shoots per explant from hypocotyls in different combinations of media, cv. Gonda Selection showed maximum (2.50) mean number of multiple shoots per explant on K_1 medium followed by 2.50 mean number of multiple shoots per explant on K_2 medium. However, cultivar

Table 4.5.8. Effect of different media combinations on mean number of shoots per explant of *bael* cvs. from hypocotyl explants

Media	Mean number of shoots per explant		
	Local	Gonda Selection	Mirzapuri
K_0	0.20±0.10	0.33±0.18	0.50±0.23
K_1	1.40±0.47	2.50±0.84	0.91±0.39
K_2	1.33±0.47	2.30±0.74	2.50±0.28
K_3	1.73±0.49	1.60±0.70	2.25±0.53
K_4	0.86±0.33	1.20±0.62	1.08±0.49
K_5	1.26±0.44	2.33±0.51	1.16±0.32
K_6	1.33±0.43	1.70±0.63	2.00±0.50
K_7	1.53±0.42	0.80±0.41	1.33±0.41
K_8	0.66±0.30	0.70±0.39	0.91±0.39
K_9	0.66±0.28	0.70±0.33	2.10±0.52
K_{10}	0.86±0.29	0.91±0.43	1.66±0.46
K_{11}	1.53±0.46	1.41±0.64	2.25±0.55
K_{12}	0.66±0.28	1.25±0.56	1.83±0.45

± SE

Local showed minimum (0.73) mean number of multiple shoots on K_0 medium among all the three cultivars tried.

4.5.9 Effect of different media combination on mean number of multiple shoots per explant in *bael* cvs. from leaf disc explants :

Shoot proliferation was observed in all cultivars tested viz. Gonda Selection, Mirzapuri and Local on Knop's basal media formulations (Table 4.5.9). Data revealed that maximum (0.53) mean number of multiple shoots per explant in cultivar Gonda Selection were observed on K_{11} followed by 0.35 mean number of multiple shoots per explant on K_{12} medium. However, minimum (0.25) mean number of multiple shoots per explant were observed on K_9 and K_{10} media. No solitary as well as multiple shoots were observed on K_1, K_2, K_3, K_4, K_5, K_6, K_7 and K_8 media.

In cultivar Mirzapuri, maximum (1.20) mean number of multiple shoots per explant were observed on K_{11} medium followed by 1.00 mean number of multiple shoots per explant on K_{12} medium while minimum (0.3) mean number of multiple shoots per explant were observed on K_8 medium. No solitary as well as multiple shoots per explant were observed on K_0, K_1, K_2, K_5 and K_6 media. While studying the response of various combinations in Local cultivar, maximum (0.66) mean number of multiple shoots per explant were observed on K_{12} medium followed by 0.40 mean number of multiple shoots per explant on K_{11} medium. Minimum (0.33) mean number of multiple shoots per explant were observed on K_3 medium. No solitary as well as multiple shoots per explant were observed on K_0, K_1, K_2, K_5, K_6, K_7, K_8, K_9 and K_{10} media.

Table 4.5.9. Effect of different media combinations on mean number of shoots per explant of *bael* cvs. from leaf disc explants

Media	Mean number of shoots per explant		
	Local	Gonda Selection	Mirzapuri
K_0	0	0	0
K_1	0	0	0
K_2	0	0	0
K_3	0.33±0.23	0	0.60±0.26
K_4	0.30±0.30	0	0.40±0.22
K_5	0	0	0
K_6	0	0	0
K_7	0	0	0.40±0.22
K_8	0	0	0.30±0.15
K_9	0	0.25±0.17	0.50±0.30
K_{10}	0	0.25±0.17	0.90±0.45
K_{11}	0.40±0.40	0.53±0.26	1.20±0.53
K_{12}	0.66±0.38	0.35±0.16	1.00±0.53

± SE

It is clear from the Table 4.5.9 that among all the three cvs., Mirzapuri cultivar showed maximum (1.20) mean number of multiple shoots per explant on K_{11} medium followed by Local 0.66 on K_{12} medium.

4.6 MEAN SHOOT LENGTH

4.6.1 Effect of different media combinations on mean shoot length in *bael* cvs. from cotyledon explants :

The data given in (Table 4.6.1) indicted that in cultivar Local maximum (1.29 cm) mean shoot length was observed on K_1 medium followed by 1.28 cm mean shoot length per culture on K_{12} medium. Minimum (0.82 cm) mean shoot length was observed on K_4 medium. In cultivar Gonda Selection, maximum (1.00 cm) mean shoot length was observed on K_{11} medium followed by 0.93 cm mean shoot length on K_8 medium. Minimum (0.35 cm) mean shoot length was observed on K_5 medium. In cultivar Mirzapuri, maximum (1.49 cm) mean shoot length was observed on K_{12} medium followed by 1.02 cm mean shoot length on K_9 medium while minimum (0.42 cm) mean shoot length was observed on K_6 medium.

Among all the three cultivars used during the present investigation, cultivar Mirzapuri showed maximum (1.49) shoot length on K_{12} medium followed by Local cv. 1.29 cm on K_1 medium.

4.6.2 Effect of different media combinations on mean shoot length in *bael* cvs. from shoot tip explants :

It is clear from the data given in (Table 4.6.2) that in cultivar Local maximum (1.83 cm) mean shoot length was observed on K_7 medium followed by 1.76 cm and 1.74 cm mean shoot length on K_3 and K_4 media,

Table 4.6.1. Effect of different media combinations on mean shoot length of *bael* cvs. from cotyledon explants

Media	Mean shoot length (cm)		
	Local	Gonda Selection	Mirzapuri
K_0	0.81±0.15	0.62±0.10	0.47±0.09
K_1	1.29±0.13	0.71±0.05	0.54±0.08
K_2	1.23±0.10	0.65±0.06	0.65±0.08
K_3	1.23±0.07	0.75±0.08	0.73±0.06
K_4	0.82±0.05	0.50±0.06	0.44±0.06
K_5	1.08±0.14	0.35±0.13	0.44±0.06
K_6	1.08±0.11	0.53±0.09	0.42±0.05
K_7	1.12±0.10	0.52±0.10	0.82±0.09
K_8	1.09±0.07	0.93±0.10	0.68±0.09
K_9	1.07±0.08	0.72±0.11	1.02±0.09
K_{10}	0.87±0.04	0.93±0.07	0.83±0.22
K_{11}	1.13±0.05	1.00±0.05	0.62±0.05
K_{12}	1.28±0.08	0.73±0.07	1.49±0.18

± SE

Table 4.6.2. Effect of different media combinations on mean shoot length of *bael* cvs. from shoot tip explants

Media	Mean shoots length (cm)		
	Local	Gonda Selection	Mirzapuri
K_0	1.07±0.09	1.16±0.12	1.80±0.13
K_1	1.59±0.14	1.63±0.10	1.92±0.14
K_2	1.56±0.14	1.63±0.10	1.77±0.12
K_3	1.76±0.18	1.41±0.09	1.83±0.14
K_4	1.74±0.20	1.75±0.10	1.81±0.16
K_5	1.27±0.09	1.36±1.00	1.81±0.17
K_6	1.54±0.10	2.43±0.11	1.91±0.11
K_7	1.83±0.15	1.57±0.15	1.47±0.18
K_8	1.64±0.21	1.28±0.10	1.62±0.19
K_9	1.37±0.13	1.83±0.07	1.66±0.20
K_{10}	1.16±0.08	2.27±0.20	1.65±0.11
K_{11}	1.02±0.08	1.60±0.10	1.62±0.19

± SE

respectively. Minimum (1.02 cm) mean shoot length was observed on K_{12} medium. Cultivar Gonda Selection showed maximum (2.43 cm) mean shoot length on K_6 medium followed by 2.27 cm mean shoot length on K_{10} medium, while minimum (1.16 cm) mean shoot length was observed on K_0 medium. In cultivar Mirzapuri, maximum (1.92 cm) mean shoot length was observed on K_1 medium followed by 1.91 cm mean shoot length on K_6 medium. However, minimum (1.47 cm) mean shoot length was observed on K_7 medium.

Among all the three cultivars tried, Gonda Selection showed maximum (2.43 cm) mean shoot length on K_6 medium followed by Mirzapuri 1.92 cm on K_1 medium.

4.6.3 Effect of different media combinations on shoot length in *bael* cvs. from epicotyl explants :

It is clear from the data given in (Table 4.6.3) that in cultivar Local, maximum (1.96 cm) mean shoot length was observed on K_{11} medium followed by 1.22 cm on K_3 medium. However, minimum (0.63 cm) mean shoot length was observed on K_0 medium. Maximum (1.89 cm) mean shoot length was observed in cv. Gonda Selection on K_7 medium followed by 1.84 cm shoot length on K_5 medium. However, minimum shoot length (0.81 cm) was observed on K_0 medium. In cultivar Mirzapuri maximum (2.46 cm) mean shoot length was observed on K_3 medium followed by 2.39 cm mean shoot length on K_4 medium while minimum (1.20 cm) mean shoot length was observed on K_0 medium.

Table 4.6.3. Effect of different media combinations on mean shoot length of *bael* cvs. from epicotyl explants

Media	Mean shoots length (cm)		
	Local	Gonda Selection	Mirzapuri
K_0	0.63±0.25	0.81±0.14	1.20±0.17
K_1	0.85±0.29	1.45±0.10	2.34±0.12
K_2	0.98±0.33	1.62±0.08	2.28±0.15
K_3	1.22±0.35	1.51±0.12	2.46±0.18
K_4	0.76±0.32	1.46±0.25	2.39±0.16
K_5	0.81±0.28	1.84±0.09	2.33±0.27
K_6	0.78±0.27	3.64±2.04	2.10±0.20
K_7	0.78±0.32	1.89±0.08	2.20±0.18
K_8	1.06±0.31	1.09±0.37	2.10±0.28
K_9	1.33±0.31	1.60±0.08	2.27±0.17
K_{10}	1.16±0.33	1.70±0.12	1.70±0.10
K_{11}	1.96±0.27	1.48±0.08	1.32±0.06
K_{12}	1.17±0.35	1.49±0.09	1.81±0.14

± SE

On the basis of result given in (Table 4.6.3). It is clear that among all the three cultivars, Mirzapuri showed maximum (2.46 cm) mean shoot length on K_3 medium followed by cv. Local 1.96 cm on K_{11} medium.

4.6.4 Effect of different media combinations on mean shoot length in *bael* cvs. from hypocotyl explants :

The response of different media combinations on mean shoot length have been presented in (Table 4.6.4). A perusal of the Table revealed that in cultivar Local, maximum (0.645 cm) mean shoot length was observed on K_7 medium followed by 0.64 cm on K_3 medium. In cv. Gonda Selection, maximum (0.89 cm) mean shoot length was observed on K_4 medium followed by K_{12} medium with 0.84 cm. Minimum (0.16 cm) mean shoot length was observed on K_0 medium. In cultivar Mirzapuri, maximum (2.06 cm) mean shoot length was observed on K_{11} medium followed by 2.01 cm on K_7 medium while minimum (0.82 cm) mean shoot length was observed on K_0 medium.

On the basis of the results of the all the three cultivars presented in (Table 4.6.4), cultivar Mirzapuri showed maximum (2.06 cm) mean shoot length on K_{11} medium followed by Gonda Selection 0.89 cm on K_4 medium.

4.6.5 Effect of different media combinations on mean shoot length in *bael* from leaf disc explants :

The effect of different media combinations on mean shoot length have been presented in (Table 4.6.5). The data revealed that in cultivar Local, maximum (0.38 cm) mean shoot length was observed on K_{12} medium followed by 0.17 cm on K_{11} medium. Minimum (0.08 cm) mean shoot length was observed on K_3 medium, while maximum (0.38 cm) mean shoot length

Table 4.6.4. Effect of different media combinations on mean shoot length of *bael* cvs. from hypocotyl explants

Media	Mean shoot length (cm)		
	Local	Gonda Selection	Mirzapuri
K_0	0.10±0.06	0.16±0.07	0.82±0.25
K_1	0.28±0.08	0.24±0.02	1.80±0.17
K_2	0.29±0.09	0.30±0.04	1.75±0.12
K_3	0.64±0.16	0.58±0.25	1.36±0.10
K_4	0.34±0.13	0.89±0.16	1.80±0.09
K_5	0.40±0.15	0.30±0.04	1.50±0.13
K_6	0.52±0.17	0.60±0.10	1.89±0.19
K_7	0.64±0.17	0.63±0.13	2.01±0.13
K_8	0.38±0.16	0.48±0.14	1.68±0.17
K_9	0.30±0.14	0.23±0.07	1.96±0.12
K_{10}	0.40±0.15	0.52±0.10	1.86±0.11
K_{11}	0.57±0.17	0.65±0.09	2.06±0.11
K_{12}	0.24±0.10	0.84±0.12	1.67±0.14

± SE

Table 4.6.5. Effect of different media combinations on mean shoot length of *bael* cvs. from leaf disc explants

Media	Mean shoot length (cm)		
	Local	Gonda Selection	Mirzapuri
K_0	0	0	0
K_1	0	0	0
K_2	0	0	0
K_3	0.08±0.05	0	0.62±0.13
K_4	0.13±0.07	0	0.25±0.11
K_5	0	0	0
K_6	0	0	0
K_7	0	0	0.34±0.14
K_8	0	0	0.22±0.11
K_9	0	0.11±0.06	0.42±0.15
K_{10}	0	0.14±0.07	0.64±0.12
K_{11}	0.17±0.07	0.38±0.12	0.95±0.09
K_{12}	0.38±0.09	0.21±0.08	0.73±0.11

± SE

Plate - 9. **Multiple shoot regeneration from hypocotyl explants of cv. Gonda Selection on K_1 medium. (Knop's + 0.25 mgL^{-1} BAP)**

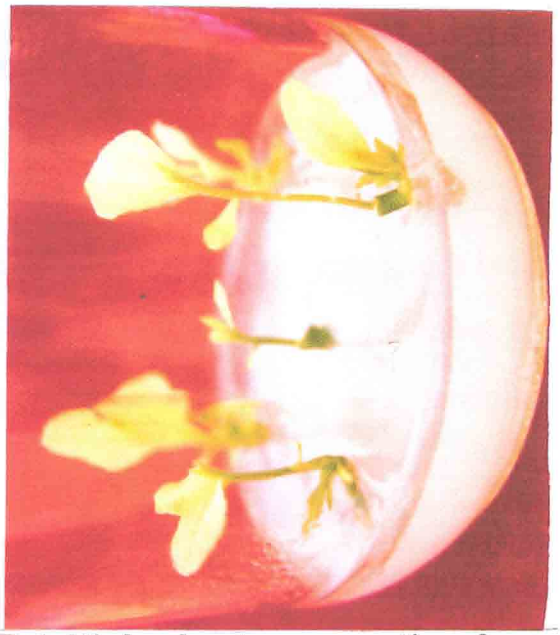


Plate - 10. **Multiple shoot regeneration from epicotyl explants of cv. Gonda Selection on K_{12} medium. (Knop's + 1.0 mgL^{-1} BAP + 1.0 mgL^{-1} KIN + 0.5 mg L^{-1} NAA)**

Plate - 8. **Multiple shoot regeneration from cotyledon explants of cv. Mirzapuri on K_{11} medium.**

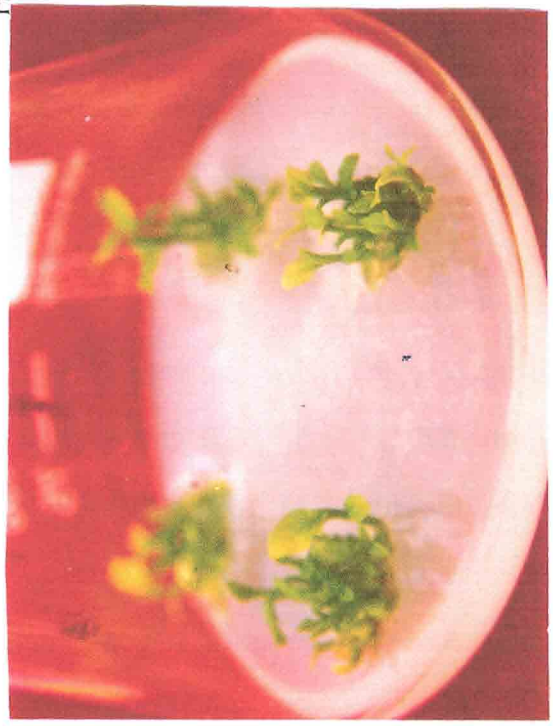


Plate - 7. **Multiple shoot regeneration from cotyledon explants of cv. Gonda Selection on K_3 medium.**

was recorded in cv. Gonda Selection on K_{11} medium followed by 0.21 cm on K_{12} medium, while minimum (0.11 cm) mean shoot length was observed on K_9 medium. No shoot length was observed on K_0, K_1, K_2, K_3, K_4, K_5, K_6, K_7 and K_8 media. In cultivar Mirzapuri, maximum (0.95 cm) mean shoot length was observed on K_{11} medium followed by 0.73 cm on medium K_{12}. However, minimum (0.22) mean shoot length was observed on K_8 medium. No shoot was length was observed on K_0, K_1, K_2, K_5 and K_6 media.

4.7 ROOT INDUCTION

A large number of media formulations were used to induce roots in the regenerated shoots from various explants viz. cotyledon, shoot tip, epicotyl, hypocotyl and leaf disc. Roots initiation were observed with in 34-45 days of inoculation.

4.7.1 Effect of different media combinations on per cent rooting of plantlets in *bael* cvs. :

Data on the effect of different media combinations on per cent rooting of *bael* plantlets have been presented in Table 4.7.1. A perusal of the Table revealed that in cv. Local showed maximum (43.33%) rooting on R_8 medium followed by 34.44 per cent rooting on R_{17} medium. However, minimum (20.0%) rooting was observed on R_7 medium. No rooting was found on R_0, R_1, R_2, R_3, R_4, R_5, R_6, R_9, R_{10}, R_{11}, R_{12}, R_{13}, R_{14}, R_{15}, R_{18} and R_{19} media. In Gonda Selection cultivar, maximum (43.89%) rooting was observed on R_8 medium followed by 34.44 per cent rooting on R_{17} medium. Minimum (13.33%) rooting was observed on R_{15} medium. No rooting was observed on R_0, R_1, R_2, R_3, R_4, R_5, R_9, R_{10}, R_{11}, R_{12}, R_{13}, R_{14}, R_{18} and R_{19} media. In cultivar Mirzapuri, maximum (34.44%) rooting was observed on R_8 and R_{17}

Table 4.7.1. Effect of different media combinations on per cent rooting of *bael* cvs.

Media	Percentage of shoot showing rooting		
	Local	Gonda Selection	Mirzapuri
R_0	0	0	0
R_1	0	0	0
R_2	0	0	0
R_3	0	0	0
R_4	0	0	0
R_5	0	0	0
R_6	0	18.89±1.11	0
R_7	20.0±5.77	25.0±0.89	25.55±2.93
R_8	43.33±3.33	43.89±3.09	34.44±2.93
R_9	0	0	0
R_{10}	0	0	0
R_{11}	0	0	0
R_{12}	0	0	0
R_{13}	0	0	0
R_{14}	0	0	0
R_{15}	0	13.33±3.33	0
R_{16}	23.33±3.33	23.33±3.33	23.33±3.33
R_{17}	34.44±2.94	34.44±2.94	34.44±2.93

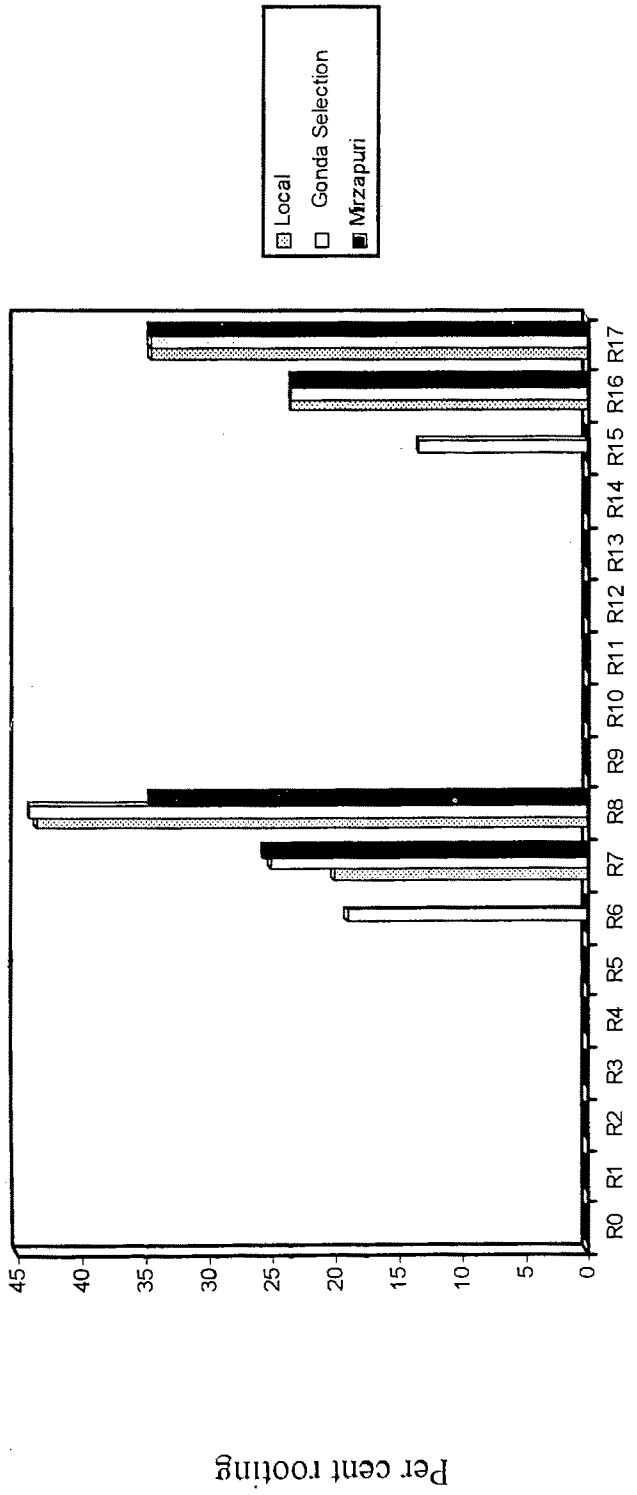


Fig. 7 : Effect of different media combinations on rooting of regenerated plantlets of *bael* cvs.

media followed by 25.55 per cent rooting on R_7 medium, while minimum (23.33%) rooting was recorded on R_{16} medium. No rooting was recorded on R_0, R_1, R_2, R_3, R_4, R_6, R_6, R_9, R_{10}, R_{11}, R_{12}, R_{13}, R_{14}, R_{15}, R_{18} and R_{19} media.

The data presented in Table 4.6.1 revealed that among all the three cultivars, Gonda Selection showed maximum (43.89%) rooting on R_8 medium followed by Local 43.33 per cent on same medium.

4.7.2 Effect of different media combinations on mean number of roots per plantlet :

The effect of different media combinations on mean number of roots per shoot in different cvs. of *bael* have been presented in (Table 4.7.2). A perusal of the Table revealed that in cultivar Local maximum (2.46) mean number of roots per shoot were recorded on R_6 medium followed by 1.6 mean number of roots per shoot on R_{15} medium. In cv. Gonda Selection showed maximum (3.0) mean number of roots per shoot on R_6 medium followed by 2.16 mean number of roots per shoot on R_{15} medium. However, minimum (0.75) mean number of roots per shoots was observed on R_4 medium, whereas, in Mirzapuri maximum (2.20) mean number of roots per shoot was observed on R_6 medium followed by 2.13 mean number of roots per shoot on R_{15} medium while minimum (0.73) mean number of roots per shoot was observed on R_{14} medium.

Among all the three cultivars tried, cv. Gonda Selection, showed maximum (3.0) mean number of roots per shoot followed by 2.46 mean number of roots per shoot in cv. Local.

Table 4.7.2. Effect of different media combinations on mean number of roots per shoot of *bael* cvs.

Media	Mean number of roots per shoot		
	Local	Gonda Selection	Mirzapuri
R_0	0	0	0
R_1	0	0	0
R_2	0	0	0
R_3	0	0	0
R_4	0	0	0
R_5	0	0	0
R_6	0	0.75±0.50	0
R_7	0.90±0.60	1.50±0.82	1.06±0.50
R_8	2.46±0.88	3.0±1.19	2.20±0.89
R_9	0	0	0
R_{10}	0	0	0
R_{11}	0	0	0
R_{12}	0	0	0
R_{13}	0	0	0
R_{14}	0	0	0
R_{15}	0	0.40±0.40	0
R_{16}	0.70±0.47	1.33±0.71	0.73±0.39
R_{17}	1.6±0.67	2.16±0.97	2.13±0.83
R_{18}	0	0	0
R_{19}	0	0	0

± S E

4.7.3 Effect of different media combinations on mean length of root in *bael* cvs. :

The data on mean length of longest root as affected by different media combinations have been presented in (Table 4.7.3). In cultivar Local maximum (1.31 cm) mean root length was found on R_8 and R_{17} medium followed by 1.37 cm mean root length on R_7 medium. Minimum (0.77 cm) mean root length was observed on R_{16} medium. In cultivar Gonda Selection, maximum (1.24 cm) mean root length was observed on R_7 medium followed by 1.15 cm mean root length on R_8 medium while minimum (0.57 cm) mean root length was observed on R_{15} medium, whereas, in cultivar Mirzapuri, maximum (1.25 cm) mean root length was observed on R_{17} medium followed by 1.13 cm mean root length on R_8 medium. However, minimum (0.66 cm) mean root length was observed on R_{16} medium.

On the basis of all the three cultivar used in the present investigation, cultivar Local showed maximum (1.31 cm) root length followed by 1.30 cm mean root length in cultivar Local.

4.12 TRANSFER OF PLANT TO POTTING MIXTURES

Regenerated plantlets with well developed roots were taken out along with the medium from the culture vessels and the traces of agar medium was removed which might provide a substrate for microorganisms. These plantlets were transferred to pots containing potting mixtures [soil + sand (1:1), soil + river sand (1:1) and soil + sand + FYM (1:1:1)]. Drenching of potting mixtures with Bavistin 0.15% (w/v) aqueous suspension, at 30 days interval was done. For the initial period of transfer, potted plantlets were kept in culture room conditions and high humidity was maintained by covering the plantlets with transparent polythene bags or beakers helped to prevent desiccation of plantlets under soil conditions within 4-6 weeks, the potted plants began to form new leaves and resumed new growth. The transplanted plants adapted to the soil condition within 4-6 weeks. Plantlets were subsequently transferred to larger pots (Fig. 8) gradually acclimatized

Table 4.7.3. Effect of different media combinations on mean length of roots of *bael* cvs.

Media	Mean length of roots (cm)		
	Local	Gonda Selection	Mirzapuri
R_0	0	0	0
R_1	0	0	0
R_2	0	0	0
R_3	0	0	0
R_4	0	0	0
R_5	0	0	0
R_6	0	1.08±0.23	0
R_7	1.30±0.21	1.24±0.28	0.98±0.13
R_8	1.31±0.11	1.15±0.12	1.13±0.27
R_9	0	0	0
R_{10}	0	0	0
R_{11}	0	0	0
R_{12}	0	0	0
R_{13}	0	0	0
R_{14}	0	0	0
R_{15}	0	0.57±0.24	0
R16	0.77±0.21	0.86±0.20	0.66±0.15
R17	1.31±0.11	1.13±0.12	1.25±0.20

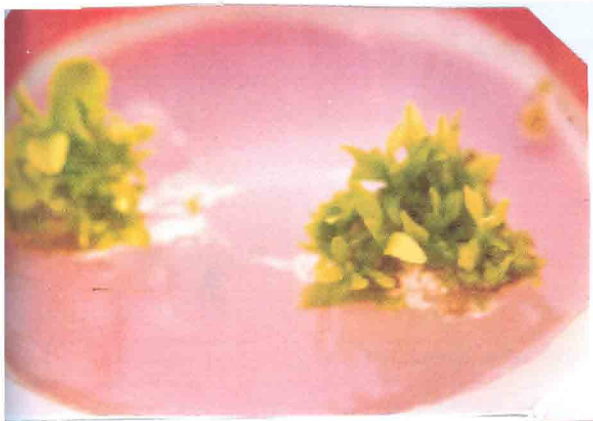
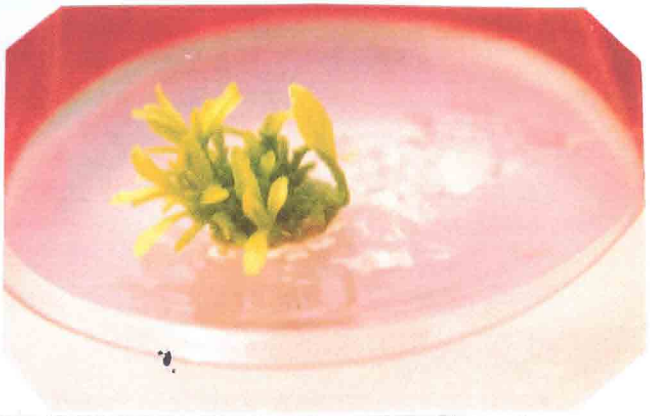


Plate - 12. **Multiple shoot regeneration from cotyledon explants of cv. Gonda Selection on K_3 medium.**



Multiple shoot regeneration from cotleydon explants of cv. Local on K_{11} medium.

Plate - 13. ***In vitro* rooting of *bael* plantlets on R_8 medium (Knop's + 15 mgL^{-1} IBA)**

Plate - 14. **Regenerated plants raised fro different explants of *bael* cv transferred to the pots.**

to outdoor conditions. The data regarding survival percentage of rooted plantlets of *bael* in different potting mixtures is presented in Table 4.8. The data revealed that in the potting mixture soil + sand (1 : 1) maximum (66.66%) survivability was observed in cv. Gonda Selection followed by 65.0 per cent in cv. Local. Minimum (64%) survivability was observed in cv. Mirzapuri. In potting mixture soil + river sand (1 : 1) maximum (73.33%) survivability was observed in cvs., Local followed by Gonda Selection 72.0 per cent. Minimum (64.70%) survivability was observed in cv. Mirzapuri, whereas, in soil + sand + FYM (1 : 1 : 1) potting mixture, maximum (75.0%) survivability was observed in cvs. Local and Gonda Selection followed by 65.0 per cent in cv. Mirzapuri.

On the basis of overall mean, maximum (71.67%) survivability was observed in soil + sand + FYM (1 : 1 : 1) potting mixture followed by 70.01% soil + river sand (1 : 1).

Table 4.8. Effect of different potting mixtures on the survivability of *in vitro* raised plantlets in pots

Potting mixture	Per cent survial of rooted plantlets			Mean
	Local	Gonda Selection	Mirzapuri	
Soil + sand (1:1)	65.00	66.66	64.00	65.22±0.78
Soil + river sand (1:1)	73.33	72.00	64.70	70.01±2.68
Soil + sand + FYM (1:1:1)	75.00	75.00	65.00	71.67±3.38

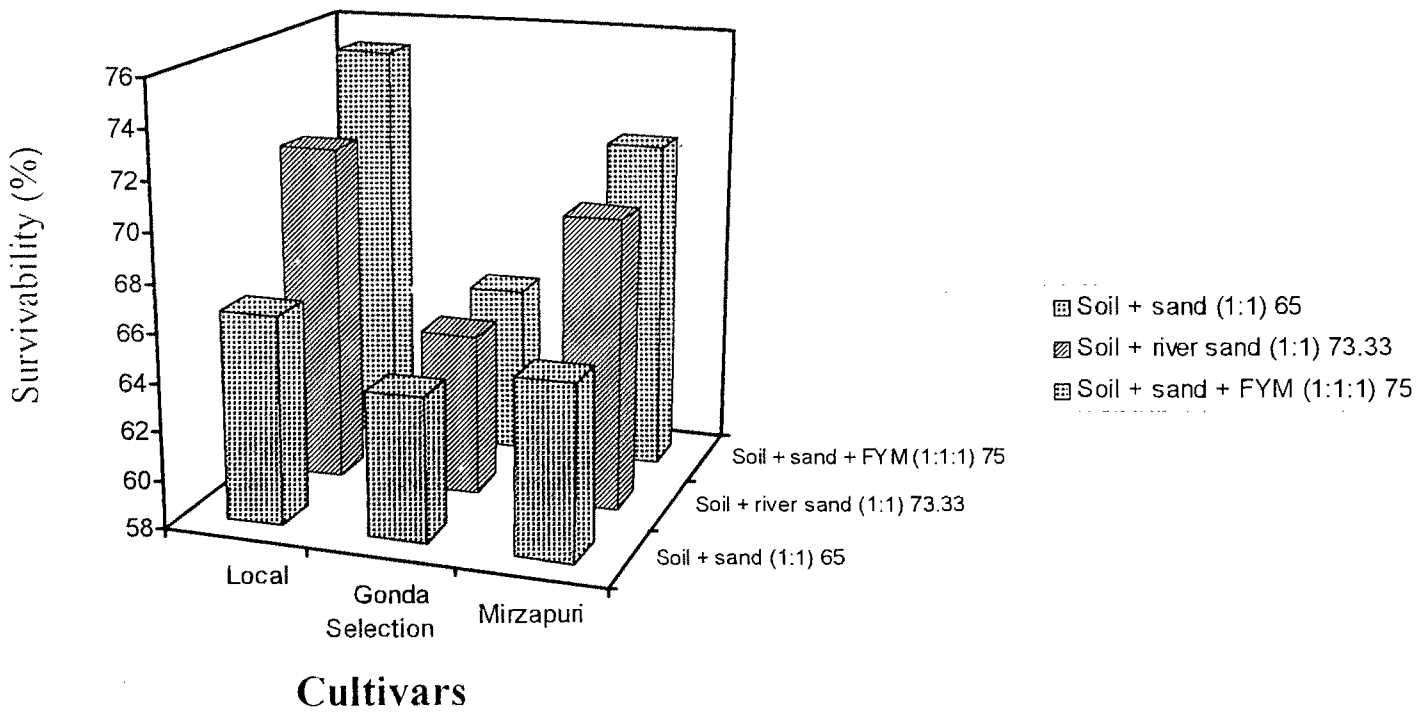


Fig. 8 : Effect of potting mixtures on survivability of *in vitro* raised *bael* plantlets in pots.

CHAPTER : V

DISCUSSION

In the present investigation on "*In vitro* multiplication studies of *bael* [*Aegle marmelos* (L.) Corr.], an attempt has been made to develop efficient protocols for callus induction and plant regeneration in three cultivars of bael viz. Local, Gonda Selection and Mirzapuri. Results obtained in different experiments of the present investigation are discussed in this chapter under suitable heads :

5.1 SURFACE DISINFECTION OF EXPLANTS

Treatment of cotyledon explants with 70% aqueous solution of ethanol (v/v) for 30 seconds followed by 0.1% aqueous solution of $HgCl_2$ (w/v) for 2 minutes and 2 drops of teepol per 100 ml solution resulted in less than 25% contamination with no browning of explants (Table 4.1.1). Treatment of $HgCl_2$ checked the growth of fungus and bacteria responsible for contamination. Similar, results have also been reported by Varghese *et al.*, (1993), Hossain *et al.* (1994a) and Islam *et al.* (1996) in *bael.* Contaminations in tissue culture can originate from two sources, either

through carry over of microorganism on the surface of the explant or in the tissue itself (endophytic microorganisms). Although in meristem culture, depending on meristem size most of microorganisms are eliminated where as in leaf, petiole and stem explants, the infection is carried over to the cultures (Cassells, 1991).

5.2 CALLUS INDUCITON

5.2.1 Per cent callus induction :

Standardization of media compositions for callus induction and establishment of cultures of the experimental crops is the first step in the application of any tissue culture technique. The specific combination and concentration of growth regulators, nutrients and incubation conditions modify the normal physiology of explants and induces dedifferentiation and redifferentiation of tissues. Thus it is necessary to understand the nutrient requirements and physical factors influencing callus induction. In the present investigation, callus induction was observed in all the media tested except K_0 medium (Knop's medium without growth regulators) in *bael.* callus can easily be induced from cotyledon explants on Knop's medium. Auxins are required essentially, whereas cytokinins are not necessarily important. Cotyledon explants after 7 weeks of culture revealed maximum (76.66%) callus induction in cv. Gonda Selection on K_{17} medium (Knop's + 2.0 mgL^{-1} NAA + 2.0 mgL^{-1} 2, 4-D + 0.5 mgL^{-1} KIN) followed by cv. Mirzapuri 74.44 per cent on K_{15} medium (Knop's + 1.0 mgL^{-1} 2, 4-D + 0.2 mgL^{-1} BAP), whereas, Local cv. showed maximum (65.55%) callus induction on K_{14} medium (Knop's + 0.50 mgL^{-1} 2, 4-D + 0.20 mgL^{-1} BAP) in Table 4.2.1.

These results are found in close conformity with the findings of Hossain *et al.*, (1994b) and Varghese *et al.*, (1993) in *bael*. Arya *et al.* (1981) reported callus induction from cotyledonary explants of *bael* in presence of NAA and KIN.

The basal Knop's medium was supplemented with different combinations of auxins and cytokinins. In combinations, auxin promote cell enlargement, cells elongation and root initiation while cytokinin promotes cell division and shoot initiation. So it is important that tissue culture media contain the correct ratio and right kind of growth regulators.

Moreover, the choice of genotype beside explant is also very important which plays a definite role in callus induction.

5.3 ORGANOGENESIS

5.3.1 Per cent organogenesis from various calli induced :

Regeneration of complete plants from single cells and tissues is of great importance for the application of biotechnology in crop improvement. Application of biotechnology techniques (*in vitro* mutant selection and protoplast fusion to cell culture) had limitation in many crop species because of the instability to regenerate plants. Only few species have been exploited fully to such studies.

In present organogenesis studies, among all the three cultivars, cv. Local showed maximum (62.50%) organogenesis on K_3 medium (Knop's + 1.0 mgL^{-1} BAP) followed by cvs. Mirzapuri 57.14 per cent on K_{12} medium and Gonda Selection 50.0 per cent on K_3 and K_{12} media. No organogenesis was observed on K_0 medium (Knop's medium without growth regulators) in Table 4.3.1. During present investigation KIN along with 2, 4-D and NAA

was found to be best suited for callus initiation and its growth in shoot apices.

Arya *et al.* (1981) reported shoot development from meristemoids occurred only when they were transferred to BAP and KIN alone or BAP + NAA, whereas KIN was quite suitable for shoot induction from cotyledonary explant callus in *bael.* Similarly Hossain *et al.* (1994b) obtained regeneration from undifferentiated callus was to maintain the callus on basal medium with a low concentration of NAA together with various concentration of BA in *bael.* Thus, the concentration of NAA was fixed at 0.1 mgL-1, which by itself was capable of sustaining a good rate of growth of callus.

Generally, low auxin and high cytokinin concentration in the medium resulted in induction of shoot morphogenesis in the present study.

5.3.2 Number of plantlets obtained through intermediate stage of callus:

Maximum (3.85) number of plantlets per calli were observed in cv. Mirzapuri from cotyledon explant derived callus on K_{12} medium (Knop's + 1.00 mgL^{-1} + BAP + 1.00 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) followed by Local 3.42 and Gonda Selection 3.25 on K_{12} (Knop's + 1.00 mgL^{-1} + BAP + 1.00 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) and K_3 (Knop's + 1.0 mgL^{-1} BAP) media. No plantlets were observed in all the three cvs. on K_0 medium (Knop's medium without growth regulators) in Table 4.3.2. It might be due to higher concentration of cytokinin which enhances the shoot initiation and auxin elongates the shoot primordial.

Similarly, Rao and Lee (1982) achieved multiple shoot formation in *Calophyllum Eugenia* and *Fragaria* in presence of BAP, while in *Syzygium aromaticum*, Mathew and Hariharan (1990) reported multiple shoot regeneration in presence of BAP and NAA. The findings of present studies which is in contrast with the findings of Varghese *et al.* (1993) reported that BAP also induced multiple shoots though in less frequency compared to KIN and NAA. The maximum number of multiple shoot induction from nodal segments was achieved in the medium containing KIN and NAA. Barna and Wakhlu (1998) reported similar results of shoot induction in *Plantago ovata*. Hossain *et al.* (1994b) reported that *in vitro* regeneration of complete plantlets is possible using callus cultures from nucellar tissues of *bael*. Nucellus derived plants are supposed to be true to type because nucellar tissues are of maternal origin (Rangaswamy, 1981). Moreover, nucellus derived plants generally are free of virus and other disease causing microorganisms, due to the absence of vascular connections between the surrounding maternal tissue and the nucellus (Button and Kochba, 1977).

5.3.3 Mean shoot length :

Maximum (1.54 cm) and (1.53 cm) mean shoot length was observed in cvs. Local and Mirzapuri on K_3 medium (Knop's + 1.0 mgL^{-1} BAP), respectively, followed by Gonda Selection 1.36 cm on K_7 (Knop's + 1.0 mgL^{-1} KIN) and K_3 (Knop's + 1.0 mgL^{-1} BAP) media. No shoot length was observed in all the cvs. tried on K_0 medium (Knop's medium without growth regulators) in Table 4.3.3. Hossain *et al.* (1994a) reported maximum mean shoot length (7.2) on MS medium supplemented with 5 mgL^{-1} BAP + 0.1 mgL^{-1} NAA + 1.0 mgL^{-1} GA_3 in *bael*.

5.4 ROOTING PERCENTAGE IN PLANTLETS OBTAINED THROUGH INTERMEDIATE STAGE OF CALLUS

Maximum (37.50%) rooting was observed in cv. Mirzapur on R_{16} medium (½Knop's + 10.0 mgL^{-1} IBA) followed by cv. Gonda Selection 25.0 per cent on R_{17} medium (½Knop's + 15 mgL^{-1} IBA). No rooting was observed in all the cultivar tried on R_0 medium (Table 4.3.4). The findings of present investigations which are in contrast with findings of Varghese *et al.* (1993)ω reported that roots were formed in shoots regenerated from callus as well as multiple shoots from nodal explants when transferred to low concentration of IAA in *bael*. Arya *et al.* (1981) achieved rooting of shoots in medium containing KIN along with IBA in *bael*. Hossain *et al.* (1994b) reported that when IBA and NAA (0.5 mgL^{-1} each) were used in combination, the rooting percentage and number of roots per cutting increased significantly in *bael*. However, rooting was poor with a maximum of 25% of cultures showing root formation.

5.5 DIRECT SHOOT REGENERATION

5.5.1 Per cent shoot regeneration from various explants in *bael* cvs. on different media

Hundred per cent shoot regeneration was observed in shoot tip explants excised from *in vitro* regenerated shoots in all the three cvs. on all the tested media. In cotyledon explants, cv. Local showed maximum (97.22%) shoot regeneration on K_{11} medium (Knop's + 1.0 mgL^{-1} BAP + 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) followed by Gonda Selection 96.66 per cent on the same medium. Minimum (41.11%) shoot regeneration frequency was observed on K_5 medium in cv. Local (Table 4.4.1).

Epicotyl explants showed maximum (90%) shoot regeneration potential on K_3 (Knop's + 1.0 mgL^{-1} BAP) and K_{11} (Knop's + 1.0 mgL^{-1} BAP + 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) media in cultivars Mirzapuri and Local respectively followed by cv. Gonda Selection 79.25 per cent on K_{12} medium (Knop's + 1.0 mgL^{-1} BAP + 1.0 mgL^{-1} KIN + 0.5 mgL^{-1} NAA). Minimum (32.22%) shoot regeneration was observed on K_0 medium in cv. Gonda Selection in Table 4.4.3.

In hypocotyl explants, (96.66%) shoot regeneration potential was recorded in cv. Mirzapuri on K_2 medium (Knop's + 0.5 mgL^{-1} BAP) followed by Gonda Selection 81.11 per cent on K_5 medium (Knop's + 0.25 mgL^{-1} KIN) in Table 4.4.4.

In leaf disc explant, maximum (50.0%) shoot regeneration was observed in cv. Mirzapuri on K_{11} (Knop's + 1.0 mgL^{-1} BAP + 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) medium followed by cv. Gonda Selection 36.46 per cent on same medium. Minimum (10.0%) shoot regeneration was observed on K_4 (Knop's + 2.0 mgL^{-1} BAP) and K_{11} (Knop's + 1.0 mgL^{-1} BAP + 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) media in cv. Local (Table 4.4.5). Similarly, Kumar and Seeni (1998) also reported that explants viz. single node, whole leaf, shoot tip and internode were regenerative at frequencies of 100%, 70%, 95% and 40% respectively, after 7 weeks of culture in *bael*. This differential morphogenetic response could be due to differences between the physiological states of the buds on different regions of a stem (Vieitez *et al.*, 1985). Similar results were also reported in *Syzygium cuminii* and *Morus australis* (Yadav *et al.*, 1990; Pattnaik *et al.*, 1996). Hossain *et al.* (1994a) also reported similar result in cotyledon explants from 10-day-old seedlings

of *bael*. Shoot differentiation efficiency varied remarkably when cotyledons from seedlings of different ages were cultured on medium supplemented with 2 mgL^{-1} BA and 0.2 mgL^{-1} IAA. Islam *et al.* (1993a) reported that the frequency of buds producing explants increased with the increase of culture period and became optimum at the end of eight weeks of culture. The maximum frequency of regenerating explants (61%) was obtained when leaf explant were cultured on MS medium supplemented with 1.5 mgL^{-1} BAP and 0.5 mgL^{-1} IAA in *bael*.

5.6 SHOOT PROLIFERATION

5.6.1 Per cent shoot proliferation :

No multiple shoots per explant were observed in shoot tip explants after seven weeks of culture in all the three cultivars and on all the media tried. Cotyledon explants showed maximum (94.10%) multiple shoots in cultivar Local on K_{10} medium (Knop's + 0.5 mgL^{-1} BAP 1.0 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) followed by cultivar Gonda Selection 84.87 per cent on K_{11} medium (Knop's + 1.0 mgL^{-1} BAP 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA). Mirzapuri cv. showed maximum (83.33%) multiple shoots on K_{11} medium (Knop's + 1.0 mgL^{-1} BAP + 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) in Table 4.5.1. However, seventy per cent shoot proliferation was observed from epicotyl explant on K_{11} (Knop's + 1.0 mgL^{-1} BAP 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) and K_3 (Knop's + 1.0 mgL^{-1} BAP) media in cvs. Local and Mirzapuri respectively. Gonda Selection cv. showed maximum (55.18%) shoot proliferation on K_{12} medium (Knop's + 1.0 mgL^{-1} BAP 1.0 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) in Table 4.5.2.

In hypocotyl explants, cv. Mirzapuri showed maximum (77.77%) shoot proliferation on K_2 medium (Knop's + 0.5 mgL^{-1} BAP). Gonda Selection and Local cvs. showed (65.55%) and (63.33%) shoot proliferation on K_5 (Knop's + 0.25 mgL^{-1} KIN) and K_3 (Knop's + 1.0 mgL^{-1} BAP) media respectively in Table 4.5.3.

In leaf disc explants cv. Mirzapuri showed maximum (33.33%) shoot proliferation on K_{11} (Knop's + 1.0 mgL^{-1} BAP 0.5 mgL^{-1} + KIN + 0.5 mgL^{-1} NAA) medium, whereas, cv. Local responded better on K_{12} (Knop's + 1.0 mgL^{-1} BAP 1.0 mgL^{-1} + KIN + 0.5 mgL^{-1} NAA) medium and Gonda Selection cv. better on K_{11} (Knop's + 1.0 mgL^{-1} BAP 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) medium i. e. (23.33%) and (21.79%) respectively in Table 4.5.4. Hossain *et al.* (1993) found maximum frequency of adventitious bud proliferating cultures on MS medium supplemented with 4.4 µM BA + 2.7 µM NAA among the different treatment combinations in *bael*. Similarly, Islam *et al.* (1993a) also reported shoot differentiation from whole leaf plant collected from 25 days old seedlings of *bael*. The findings of the present investigation are in close conformity with the above findings. Cytokinin was essential for shoot proliferation, it enhanced the multiple shoot formation in explants. Of the two cytokinins tested, BAP was found to be superior to KIN in shoot induction. The superiority of BA on adventitious shoot proliferation from cotyledon explants have also been reported in apple (Kouider *et al.*, 1985), water melon (Dong and Jia, 1991), *Phaseolus* (Malik and Saxena, 1992) and *Picea* (Toivonen and Kartha, 1988). Cytokinin in addition to auxin (NAA) caused maximum proliferation as well as elongation of shoots.

5.6.2 Mean number of multiple shoots per explant :

Solitary shoots per explant were observed in all the three cultivars on all the media tested from shoot tip explants. The possible reason for the solitary shoot was that the apical dominance was more prominent in shoot tip explants. However in cotyledon explants, cv. "Gonda Selection" showed maximum (12.50) mean number of shoots per explant on K_3 medium (Knop's + 1.0 mgL^{-1} BAP) followed by cv. Local 11.25 on K_{11} medium (Knop's + 1.0 mgL^{-1} BAP 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA). Mirzapuri cv. showed maximum (9.38) mean number of multiple shoots per explant on K_9 medium (Knop's + 0.5 mgL^{-1} BAP + 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) in Table 4.5.5, while in cultivar Gonda Selection maximum (3.77) mean number of shoots per explant was observed on K_{12} medium (Knop's + 1.0 mgL^{-1} BAP 1.0 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) followed by cultivar Local 2.80 on K_{11} medium (Knop's + 1.0 mgL^{-1} BAP 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA). Mirzapuri cultivar showed maximum mean (2.50) number of multiple shoots on K_3 medium (Knop's + 1.0 mgL^{-1} BAP) in epicotyl explants (Table 4.5.7). Whereas, in hypocotyl explants, Gonda Selection showed maximum (2.50) mean number of multiple shoot per explant on K_1 medium (Knop's + 0.25 mgL^{-1} BAP). Local cv. showed maximum (1.73) mean number of shoots per explant on K_3 medium (Knop's + 1.0 mgL^{-1} BAP) in Table 4.5.8, whereas, in leaf disc explants cv. Mirzapuri responded best in mean number of shoots per explant (1.20) on K_{11} (Knop's + 1.0 mgL^{-1} BAP 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) medium followed by Local 0.66 on K_{12} (Knop's + 1.0 mgL^{-1} BAP 1.0 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) medium. Gonda Selection cv. was better on K_{11} (Knop's + 1.0 mgL^{-1} BAP 0.5 mgL^{-1}

KIN + 0.5 mgL^{-1} NAA) medium with (0.53) mean number of shoots per explant (Table 4.5.9).

Cotyledon explants excised from seeds collected from unripe fruits of field grown plant and shoot tip, epicotyl, hypocotyl and leaf disc explants excised from *in vitro* raised plantlets from cotyledon (with embryo axis) explants were induced to form adventitious buds directly without an intervening callus stage. Adventitious shoots developed in a range of media supplemented with cytokinin and auxin. Cytokinin alone was suitable for differentiation of adventitious shoots. However, cytokinin (0.5-1.0 mgL^{-1}) along with an auxin (0.5 mgL^{-1}) was also suitable for differentiation of adventitious shoots in all the explants. Cytokinin in presence of auxin increased cell division efficiency and cell division enhanced the shoot multiplication per explant. This synergistic effect of cytokinin auxin in multiple shoot formation was also reported in *bael* by Hossain *et al.* (1993, 1994b), Scaria *et al.* (1993), Islam *et al.* (1993a) and Kumar and Seeni (1998).

In cotyledon cultures of *bael,* shoot formation was highest in the region of proximal to the embryo axis, indicating a polarity regeneration potential cotyledon explants. Two-third distal parts of cotyledons produced less shoots than one-third proximal parts of cotyledon, where prolific shoot regeneration occurred. Similar observation were recorded in bael, apple and pea cotyledons (Hossain *et al.,* 1993; Kouider *et al.,* 1985; Ozcan *et al.,* 1992). This was in contrast to the maximum number of multiple shoots induction from nodal segments was achieved in the medium containing KIN and IAA. Barna and Wakhlu (1998) reported similar results of shoot

induction in *Plantago ovata*. Rao and Lee (1982) also achieved multiple shoot formation in calophyllum, *Eugenia* and *Fragaria* in presence of BAP, while in *Syzygium aromaticum,* Mathew and Hariharan (1990) reported multiple shoot regeneration in presence of BAP and NAA.

Ajith Kumar and Seeni (1998) reported the varied concentrations of BAP required for maximum shoot formation in different explants of shoot cultures in *bael* was not anticipated and may be related to differential endogenous levels of cytokinin. This was in contrast to the less frequent shoot initiation was preceded by callusing in internodal segments, direct shoot formation was observed for all other explants. In such cases, the possibility of genotypic differences between individuals, contributing to varied shoot multiplication, rates cannot be ruled out. Shoot tip cultures were less desirable as the harvestable shoots were few (one to two) and the remaining 0.1 to 0.5 mm buds did not develop into shoots, similar to the presented investigation. Similarly, direct adventitious shoot formation observed in leaf explants cultures was confined to cut petiolar ends. The age independent formation of shoots in both the nodal and leaf explants, seldom observed in arborescent taxa, offered the opportunity to a mass, large number of shoots. A similar phenomenon was also observed in *M. australis* (Pattnaik *et al.,* 1996). Islam *et al.,* (1993a) observed variation in number of regenerating shoot buds between leaves of different ages.

Direct organogenesis had been reported from roots of intact microplants (Jones *et al.,* 1984), hypocotyl (Liu *et al.,* 1983), cotyledon (Kouider *et al.,* 1985) and leaf (Fasolo *et al.,* 1989) of apple and seedling explants (Amin and Akhtar, 1993) of citrus. Organogenesis from leaf tissues

of woody dicot has been reported previously by (Liu *et al.*, 1993; Jones *et al.*, 1984; Kim *et al.*, 1985; Simola, 1985; Srivastava *et al.*, 1985; Charles *et al.*, 1986). Arya *et al.*, (1981) obtained plant regeneration from hypocotyl explants through intermediate callus stage. Overall, the results suggested that maximum multiple shoot formation was observed from cotyledon explants in all the three cultivars on a defined Knop's medium. It might be due to the lack of apial dominance and the cumulative effect of endogenous and exogenous level of cytokinin, which affects the shoot multiplication rate.

5.6.3 Mean shoot length per culture :

In cotyledonary explants, Cultivar Mirzapur showed maximum (1.49 cm) shoot length on K_{12} (Knop's + 1.0 mgL^{-1} BAP 1.0 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) followed by Local 1.29 cm and Gonda Selection 1.00 cm on K_1 (Knop's + 0.25 mgL^{-1} BAP) and K_{11} (Knop's + 1.0 mgL^{-1} BAP 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) media respectively (Table 4.6.1), while in shoot tip explants, cultivar Gonda Selection showed maximum (2.43 cm) mean shoot length on K_6 medium (Knop's + 0.5 mgL^{-1} KIN) followed by cvs. Mizapuri 1.92 cm on K_1 medium (Knop's + 0.25 mgL^{-1} BAP) and Local 1.83 cm on K_7 medium (Knop's + 1.0 mgL^{-1} KIN) in Table 4.6.2. In epicotyl explants, cv. Mirzapuri showed maximum mean shoot length (2.46 cm) on K_3 (Knop's + 1.0 mgL^{-1} BAP) medium followed by cvs. Local 1.96 cm on K_{11} medium and Gonda Selection 1.89 cm on K_7 medium (Knop's + 1.0 mgL^{-1} KIN) in Table 4.6.3.

In hypocotyl explants cv. Mirzapuri showed maximum (0.06 cm) mean shoot length on K_{11} medium followed by cvs. Gonda Selection 0.89 cm and Local 0.64 cm on K_4 (Knop's + 2.0 mgL^{-1} BAP) and K_7 (Knop's +

1.0 mgL^{-1} KIN) media respectively (Table 4.6.4). In leaf discs explants, cv. Mirzapuri showed maximum (0.95 cm) mean shoot length per culture on K_{11} medium followed by cvs. Gonda Selection 0.38 cm on K_{11} and Local 0.38 cm on K_{12} media (Table 4.6.5). This increase in mean shoot length per culture as a result of BAP and NAA treatment might be due to the fact that cytokinin and auxin promotes vegetative growth by inducing cells division, cell enlargement and cell elongation which might have resulted in increasing the mean shoot length.

Plantlets from epicotyl and shoot tip explants showed maximum mean shoot length per culture as compare to hypocotyl, cotyledon and leaf disc explants. It might be due to apical region which is the active site of auxin synthesis and it enhances the apical dominance. These findings were in conformity with the findings of Ajith Kumar and Seeni (1998) in *bael.*

5.7 *IN VITRO* ROOTING

5.7.1 Rooting percentage :

Root initiation was observed within 30-40 days of culture in all cvs. of *bael.* The maximum (43.89%) rooting percentage was observed on full strength Knop's medium supplemented with 15.0 mgL^{-1} IBA in cultivar Gonda Selection followed by 43.33 per cent on similar medium. No rooting was observed on R_0, R_1, R_2 R_3, R_4, R_5, R_9, R_{10}, R_{11}, R_{12}, R_{13}, R_{14}, R_{18} and R_{19} media in all the three cultivars tested (Table 4.7.1). Rooting has already been induced in a few woody species by an initial incubation of shoots in auxin rich medium (NAA and/or IBA) followed by transfer to hormone-free medium (Rai and Chandra, 1988; Yadav *et al.*, 1990).

Similarly Kumar and Seeni (1998) also reported that markedly different concentrations of IAA (0.5 mgL^{-1}) and IBA (10.0 $mgLl^{-1}$) were required to induce formation of one to three branched roots in 3 weeks at a frequency of 70% and 90% respectively. The shoots that failed to form roots during this period did not respond after words.

Micro shoots produced from various explants were excised and placed on Knop's medium supplemented with different concentrations of IBA for induction of roots. Differences in genotypic responses to root initiation in *bael* were observed. No rooting was induced on un-supplemented Knop's medium in either case.

Roots were formed in shoots regenerated from callus as well as multiple shoots from nodal explants when transferred to low concentration of IAA (Varghese *et al.*, 1993). Arya *et al.* (1981) recorded rooting of shoots in medium containing KIN along with IBA.

The findings of present investigation in contrast to the 50-60% rooting responses of *A. marmelos* in 1-2 mgL^{-1} IBA supplemented media (Hossain *et al.*, 1993). However, in a separate experiment when another genotype was used, rooting frequencies were found to be only 10-25% (Hossain *et al.*, 1994a).

5.7.2 Mean number of roots per shoot :

Higher concentrations of IBA (10-15 mgL^{-1}) although producing many roots whereas, no rooting was observed with lower concentration (1-5 mgL^{-1}) during present investigation, IBA stimulated maximum (3.0) mean number of roots per shoot in cv. Gonda Selection on full strength Knop's medium supplemented with 15 mgL^{-1} IBA concentration followed by 2.46

and 2.20, roots per shoot in cvs. Local and Mirzapuri on same media, respectively (Table 4.7.2).

These findings of present investigation which are in contrast to the findings of higher concentration of IBA (1-2 mgL^{-1}) although producing many roots, inhibited shoot growth and produced a substantial amount of callus formation at the base of the shoots. At 0.1 mgL^{-1} IBA, fewer but healthier and more vigorously growing roots formed directly at the base of the shoots with no callus formation in *bael* (Islam *et al.*, 1993). Similarly, Hossain *et al.* (1994a) reported that when IBA (0.5 mgL^{-1}) and NAA (0.5 mgL^{-1}) were used in combinations, the rooting percentage and number of roots per cutting increased significantly in *bael*. However, rooting was poor with a maximum of 25% of cultures showing root formation. Similarly, Kumar and Seeni (1998) reported that the IAA-induced roots grow vigorously but remained thin and slender while those on IBA were slow to grow and remained robust and short. Medium fortified with NAA induced maximum root formation preceded by callusing and the later increased with increasing auxin concentration in *bael*. Reports of concentration dependent callus inducing activity of NAA during root initiation in shoot cuttings are not uncommon (Demeke and Hughes 1990; Toussaint *et al.*, 1992). However, most of the roots differentiated from the calli did not have a vascular connection with the stem and establishment of the plants after hardening was poor.

5.7.3 Mean length of roots :

Maximum (1.28 cm) mean length of the root per shoot was observed in cv. Gonda Selection on full strength Knop's medium supplemented with

10 mgL^{-1} IBA followed by 1.30 cm and 1.25 cm in cvs. Local and Mirzapuri on Knop's medium with 15 mgL^{-1} respectively (Table 4.7.3).

IBA have also been reported by Ajith Kumar and Seeni (1998). Hossain *et al.* (1994a) and Islam *et al.* (1993a) also obtained the maximum mean root length on same media composition in *bael*, which is in contrast with the findings of present investigation. Similarly, maximum mean root length (3.5 cm) was observed on MS medium supplemented in combinations with 0.5 mgL^{-1} IBA and 0.5 mgL^{-1} NAA in *bael* (Hossain *et al.*, 1994b). The possible reason for the production of maximum mean root length per shoot with IBA alone or in combination with IAA or NAA might be due to early initiation of adventitious roots and there by having more total period for root growth. Secondly, there could be more growth of roots with IBA and IAA application. These results are in accordance with the work of Hossain *et al.*, (1993) and Kumar and Seeni (1998) in *bael*.

5.8 TRANSFER OF PLANTS TO SOIL MEDIUM IN POTS

Maximum (70%) survivability was observed in the potting mixture containing soil + sand + FYM (1:1:1) followed by 73.33 per cent in soil + river sand (1:1). Survivability of plantlets transferred in potting mixtures depends on the manipulation of internal factors of growth chambers and external environmental factors like temperature, erature humidity and soil moisture, rather than on the composition of culture media from which the plants were multiplied. The standard potting mixtures consisting soil + sand + FYM (1:1:1) responded best in which maximum survivability of plantlets was observed (Table 4.8). It might be due to its ability to maintain optimum moisture aeration and nutrient availability for sufficient time. Similar findings were also reported by Hossain *et al.* (1994a) in *bael*.

CHAPTER : VI

SUMMARY

The present investigation entitled "*In vitro* multiplication studies in bael [*Aegle marmelos* (L.) Corr.]" was carried out in tissue culture laboratory, Department of Horticulture at CCS HAU, Hisar during the years of 2000-2002. The objectives of the investigation were (i) to standardize the optimal culture conditions for shoot formation and plantlet regeneration from cotyledon explant and (ii) to standardize protocol for callus induction and its regeneration in the three cultivars of bael viz. Local, Gonda Selection and Mirzapuri. The five explants viz. cotyledon, shoot tip, epicotyl, hypocotyl and leaf disc were selected to study the *in vitro* multiplication on different media formulations for callus induction and plant regeneration. The salient features of the results achieved are as follows :

The surface disinfecting treatment of the explants by 70% aqueous solution of ethanol (v/v) for 30 seconds followed by 0.1 per cent aqueous solution of $HgCl_2$ (w/v) for 2 min. was found to be best (Table 4.1.1).

2. Cotyledon explant showed maximum (76.66%) callus growth in cv. Gonda Selection on K_{17} medium (Knop's + 5.0 mgL^{-1} NAA + + 0.5 mgL^{-1} KIN) followed by cv. Mirzapuri 74.44 per cent on K_{15} (Knop's + 0.2 mgL^{-1} BAP + 1.0 mgL^{-1} 2, 4-D) where cv. Local showed maximum (66.66%) callus growth on K_{15} and K_{16} media. Minimum (25.55%) callus growth was recorded on K_{13} in cv. Mirzapuri. No callusing was observed on K_0 medium in all the three cultivars (Table 4.2.1).

3. Maximum (62.50%) organogenesis was recorded in calli obtained from cotyledon explants in cv. Local on K_3 medium (Knop's + 1.0 mgL^{-1} BAP) followed by Mirzapuri (57.14) on K_{12} medium (Knop's + 1.0 mgL^{-1} BAP + 1.0 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) whereas, cv. Gonda Selection showed maximum (50.0%) organogenesis on K_3 and K_{12} media. Minimum (12.50%) organogenesis was observed in cv. Gonda Selection on K_7 medium (Knop's + 1.0 mgL^{-1} KIN) in Table 4.3.1.

4. K_{12} medium showed maximum (3.85) mean number of plantlets per calli in cv. Mirzapuri followed by 3.42 and 3.00 cvs. Local and Gonda Selection. Whereas, K_{10} medium showed minimum (0.66) mean number of plantlets per calli in cv. Mirzapuri (Table 4.3.2).

5. Maximum (1.53 cm) mean shoot length in cv. Mirzapuri was observed on K_7 medium followed by Local (1.54 cm) on K_3 medium whereas, cv. Gonda Selection showed maximum (1.36 cm) mean shoot length on K_3 medium. Minimum (0.83 cm) mean

shoot length was observed in cv. Gonda Selection on K_7 medium (Table 4.3.3).

6. Maximum (37.50%) rooting in plantlets obtained through intermediate stage of callus was found on R_{16} (½Knop's + 10 mgL^{-1} IBA) in cv. Mirzapuri followed by Gonda Selection 25.0 per cent on R_{17} ((½Knop's + 15 mgL^{-1} IBA). Minimum (10.0%) rooting was observed in cv. Gonda Selection on R8 (Knop's + 15 mgl L^{-1} IBA). No rooting was observed in all the three cvs. of *bael* on R_0 medium (Table 4.3.4).

7. Cotyledon explants showed maximum (97.22%) shoot regeneration frequency on K_{11} medium (Knop's + 1.0 mgL^{-1} BAP + 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) followed by cv. Gonda Selection 96.66 per cent on K_{11} medium (Knop's + 1.0 mgL^{-1} BAP + 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA). Minimum (41.11%) shoot regeneration frequency was recorded on K_5 medium (Knop's + 0.25 mgL^{-1} KIN) in cv. Local (Table 4.4.1).

8. Hundred per cent shoot regeneration frequency was observed in all the three cultivars and all the media tried from shoot tip explants.

9. Maximum (90.0%) shoot regeneration frequency from epicotyl explants was observed on K_3 (Knop's + 1.0 mgL^{-1} BAP) and K_{11} (Knop's + 1.0 mgL^{-1} BAP + 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) media in cvs. Mirzapuri and Local respectively. Minimum (32.22%) shoot regeneration was observed on K_0 medium in cv. Gonda Selection (Table 4.4.3).

10. Hypocotyl explants showed maximum (96.66%) shoot regeneration frequency in cv. Mirzapuri on K_2 medium (Knop's + 0.5 mgL^{-1} BAP). Minimum (26.66%) shoot regeneration frequency was observed in cv. Local on K_0 medium (Table 4.4.4).

11. Leaf disc explant showed maximum (50.0%) shoot regeneration frequency in cv. Mirzapuri on K_{11} medium (Knop's + 1.0 mgL^{-1} BAP + 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA). Minimum (10.0%) shoot regeneration frequency was observed on K_4 (Knop's + 2.0 mgL^{-1} BAP) and K_{11} (Knop's + 1.0 mgL^{-1} BAP + 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) media in cv. Local (Table 4.4.5).

12. Maximum (94.10%) multiple shoot from cotyledon explants was recorded in cv. Local on K_{10} medium (Knop's + 0.5 mgL^{-1} BAP + 1.0 mgL^{-1} KIN + 0.5 mgL^{-1} NAA). Minimum (23.33%) multiple shoot was observed in cvs. Gonda Selection and Mirzapuri on K_0 medium (Table 4.5.1).

13. All the cvs. showed solitary shoot development in all the tested media based on Knop's basal medium from shoot tip explants.

14. Among all the cvs. tried, cvs., Mirzapuri and Local showed maximum (70.0%) multiple shoots on K_3 and K_{11} media respectively, whereas, Gonda Selection showed maximum (55.18%) multiple shoots on K_{12} medium (Knop's + 1.0 mgL^{-1} BAP + 1.0 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) from epicotyl explants (Table 4.5.2).

15. Hypocotyl explants showed maximum (77.77%) multiple shoots in cv. Mirzapuri on K_2 medium (Knop's + 0.5 mgL^{-1} BAP) followed

by 65.55 per cent multiple shoots in cv. Gonda Selection on K_5 medium (Knop's + 0.25 mgL^{-1} KIN) in Table 4.5.3.

16. Among all the cvs. from leaf disc explants, cv. Mirzapuri showed maximum (33.33%) multiple shoots on K_{11} medium followed by 23.33 per cent multiple shoots in cv. Local on K_{12} medium (Table 4.5.4).

17. Cotyledon explants showed maximum (12.50) mean number of shoots per explant in cv. Gonda Selection on K_3 medium followed by 11.25 mean number of multiple shoots on K_{11} medium in cv. Local where as in cv. Mirzapuri (9.38) on K_{11} medium. Minimum (0.90) mean number of shoots were found in cv. Mirzapuri on K_0 medium (4.5.5).

18. Maximum (3.77) mean number of shoots per explant from epicotyl in cv. Gonda Selection on K_{12} medium followed by cv. Local 2.80 on K_{11} medium, whereas, Mirzapuri showed maximum (2.50) mean number of multiple shoots on K_3 medium. Minimum (0.40) mean number of multiple shoots was observed in cv. Local on K_0 medium (Table 4.5.7).

19. Maximum (2.50) mean number of multiple shoots from hypocotyl explants in cv. Gonda Selection on K_1 medium followed by 2.50 mean number of multiple shoots per explant on K_2 medium in cv. Mirzapuri. Minimum (0.33) mean number of multiple shoots per explant on K_0 medium in cv. Gonda Selection (Table 4.5.8).

20. Maximum (1.20) mean number of multiple shoots per explant from leaf disc explant in cv. Mirzapuri on K_{11} medium followed by

Local cv. 0.66 in K_{12} medium. Minimum (0.25) mean number of multiple shoots per explant was observed on K_9 and K_{10} media (Table 4.5.9).

21. Cotyledon explants showed maximum (1.49 cm) mean shoot length in cv. Mirzapuri on K_{12} medium followed by 1.29 cm in cv. Local on K_1 medium. Minimum (0.42 cm) mean shoot length in cv. Mirzapuri was observed on K_6 medium (Table 4.6.1).

22. Shoot tip explant showed maximum (2.43 cm) mean shoot length on K_6 medium in cv. Gonda Selection. Minimum (1.02 cm) mean shoot length was observed on K_{12} medium in cv. Local (Table 4.6.2).

23. Maximum (2.46 cm) mean shoot length from epicotyl explants in cv. Mirzapuri on K_3 medium, whereas, minimum (0.63 cm) shoot length was observed on K_0 medium in cv Local (Table 4.6.3).

24. Maximum (2.06 cm) mean shoot length in cv. Mirzapuri on K_{11} medium from hypocotyl explants. Minimum (0.10 cm) mean shoot length on K_0 medium in cv. Local (Table 4.6.4).

25. Leaf discs explants showed maximum (0.95 cm) mean shoot length in cv. Mirzapuri, whereas, cv. Local showed minimum (0.08 cm) shoot length on K_3 medium (Table 4.6.5).

26. Maximum (43.89%) rooting was observed in cvs., Local and Gonda Selection on R_8 medium (Knop's + 15 mgL^{-1} IBA). Minimum (13.33%) rooting was observed in cv. Gonda Selection on R_{15} (Table 4.7.1).

27. Maximum (3.0) mean number of roots per shoots in cv. Gonda Selection on R_6 medium (Knop's + 15 mgL^{-1} IBA) was observed whereas, minimum (0.73) mean number of roots per shoots in cv. Mirzapuri on K_{12} medium (½ Knop's + 10 mgL^{-1} IBA) in Table 4.7.2.

28. Maximum (1.28 cm) mean root length per shoot in cv. Gonda Selection on R_5 medium (Knop's + 10 mgL^{-1} IBA) was observed Minimum (0.57 cm) mean root length per shoot in cv. Gonda Selection on R_{11} medium (Table 4.7.3).

29. Potting mixtures consisting soil + sand + FYM (1:1:1) was found to be the best with reference to maximum survival percentage of transferred platnlets in cultivars Local (75.0%) and Gonda Selection (75.0%) followed by Mirzapuri (65.0%) in Table 4.8.

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ABSTRACT

Title of thesis	:	***IN VITRO* MULTIPLICATION STUDIES IN BAEL [*Aegle marmelos* (L.) Corr.]**
Full name of the degree holder	:	**MURARI LAL**
Title of degree	:	Doctor of Philosophy
Name and Address of Major Advisor	:	**Dr. S. S. Sindhu,** Professor Department of Horticulture, CCS HAU, Hisar – 125 004, India
Degree awarding University	:	CCS Haryana Agricultural University, Hisar – 125 004, India
Year of award of degree	:	**2002**
Major subject	:	Horticulture (Pomology)
Total number of pages in thesis	:	127 + xx
Number words in abstract	:	Approximately 600

The present investigation were carried out in Plant Tissue Culture Laboratory, Department of Horticulture CCS Haryana Agricultural University, Hisar during the years of 2000-2002. The five explants cotyledon, shoot tip, epicotyl, hypocotyl and leaf disc were selected to study the *in vitro* multiplication in different cvs. of bael on different media formulations. All the five explants were cultured on Knop's medium with and without growth regulators (auxins, cytokinins and 2,4-D) at different concentrations alone and incombinations.

Hundred per cent shoot regeneration frequency was observed in all the three cultivars and the media tried from shoot tip explants. Cotyledon explants showed maximum percentage of shoot regeneration in cvs. Local (97.22%), Gonda Selection (96.66%) and Mirzapuri (85.55%) on K_{11} medium (Knop's + 1.0 mgL^{-1} BAP + 0.5 mgL^{-1} KIN + 0.5 mgL^{-1} NAA) while, hypocotyl explants produced maximum percentage of shoot regeneration in cvs. Mirzapuri (96.66%), Gonda Selection (81.11%) and Local (76.11%) on K_2 medium

(Knop's + 0.5 mgL^{-1} BAP), K_5 medium (Knop's + 0.25 mgL^{-1} KIN) and K_3 medium (Knop's + 1.0 mgL^{-1} BAP) respectively. In case of epicotyl explants, maximum percentage of shoot regeneration were recorded in cvs. Mirzapuri (90.0%), Local (90.0%) and Gonda Selection (79.25%) on K_3, K_{11} and K_{12} media respectively where as, leaf disc explants gave maximum percentage of shoot regeneration in cvs. Mirzapuri (46.66%), Gonda Selection (36.15%) on K_{11} medium and Local (23.33%) on K_{12} medium. Maximum mean number of shoots per explant were obtained in cvs. Gonda Selection (12.50) on K_3 medium, Local (11.25) and Mirzapuri (9.38) on K_{11} medium from cotyledon explants. Knop's medium supplemented with 15 mgL^{-1} IBA produced maximum rooting percentage in cvs. Gonda Selection (43.89%), Local (43.33%) and Mirzapuri (34.44%). Cotyledon explants showed maximum per cent callusing in cvs. Gonda Selection (76.66), Mirzapuri (74.44) and Local (65.55) on K_{17}, K_{15} and K_{14} as well as K_{15} media respectively. Maximum percentage of organogenesis was observed in cvs. Local (62.50%), Mirzapuri (57.14) and Gonda Selection (50.0%) on K_3, K_{12} and K_3 as well as K_{12} media respectively while, maximum mean number of plantlets per calli was recorded in cvs. Mirzapuri (3.85), Local (3.42) on K_{12} medium and Gonda Selection (3.25) on K_3 medium. Maximum percentage of rooting in plantlets obtained through intermediate stage of callus was found in cvs. Mirzapuri (37.50%), Gonda Selection (25.0%) and Local (22.50%) on R_{16} (½ Knop's + 10 mgL^{-1} IBA), R_{17} (½ Knop's + 15 mgL^{-1} IBA) and R_{16} media respectively. Potting mixture consisting soil + sand + FYM (1:1:1) was found to be the best with reference to maximum survival percentage of transferred plants in pots of cvs. Local (75.0%), Gonda Selection (75.0%) and Mirzapuri (65.0%).

Overall it can be concluded that among all the explants, explant cotyledons showed maximum mean number of shoots per explant in cvs. Gonda Selection on K_3 medium, Local and Mirzapuri on K_{11} medium. Knop's medium supplemented with 15 mgL^{-1} IBA was found to be best for *in vitro* rooting. Potting mixture consisting soil + sand + FYM (1:1:1) was found to be best.

MK

SIGNATURE OF THE STUDENT

MAJOR ADVISOR & CHAIRMAN

HEAD OF THE DEPARTMENT