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In Vitro P:Ropagation and Somatic Embryogenesis of Morussp.

Zaman, Ather-uz-

University of Rajshahi

IN VITRO PROPAGATION AND SOMATIC EMBRYOGENESIS OF *MORUS* SP.

A Thesis Submitted to The University of Rajshahi, Bangladesh, In Fulfillment of The Requirements for The Degree of

DOCTOR OF PHILOSOPHY

By

ATHER-UZ-ZAMAN

Department of Botany
University of Rajshahi
Bangladesh

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Dedicated to
Most Revered and Beloved

Mirza Tahir Ahmad

whose blessings and support enabled me to fulfill my humble task

DECLARATION

I hereby declare that the whole of the research work now submitted as a thesis for the degree of Doctor of Philosophy in Botany of the University of Rajshahi, Bangladesh is the result of my own investigation.

28/9/95

(Professor, O.I. Joarder)

Supervisor

28.9.95

(Ather-uz-zaman)

Candidate

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INTRODUCTION

1. INTRODUCTION

In modern agriculture, only about 160 plants are extensively cultivated. Many of these are reaching the limits of their improvement by traditional methods. Thus one can no longer expect the gains generated by the green revolution. The next agriculture revolution is near and will be based to a large extent on biotechnology. Tissue culture technology will play a pivotal role, as it is an important vehicle for carrying out micropropagation system, parasexual hybridization and genetic engineering. Thus the prospects of this decade are particularly exciting!

The term "plant tissue culture" is commonly used to describe the *in vitro* and aseptic cultivation of any plant part on a nutrient medium. Practically plant tissue culture technology is based on three fundamental objectives: First, the plant part or explant must be isolated from the plant body, this effectively disrupts the cellular, tissue, and /or organ interactions that may occur in the intact plant. Second, the explant must be maintained in a controlled and preferably defined midia. Both the physical and chemical composition of the medium should effectively control the expression of any genotypic or phenotypic potential in the cultured plant part. Third, aseptic and controlled environmental conditions (light, humidity and temperature) must be maintained. The term "shoot culture" describes rootless sprouts growing on agar media. Shoot cultures are being widely used for micropropagation of ferns,

trees, and ornamentals for conservation of genotypically defined stocks, and as experimental material in biochemical, physiological, and genetic investigations.

The use of *in vitro* techniques for clonal or asexual mass propagation is the most advanced application of plant tissue culture. Rapid asexual multiplication can be achieved by (1) enhancing axillary bud breaking, (2) production of adventitious buds and (3) somatic embryogenesis.

Biotechnology together with knowledge obtained from whole plant systems offer novel and powerful model experimental systems to study major problems in plant physiology, biochemistry and genetics which had previously remained inaccessible and unsolved (Gautheret, 1985). But today's progress of plant tissue culture is based on numerous milestone discoveries made through unpretentious research of numerous scientists over centuries (Gautheret, 1985).

1.1 HISTORICAL BACKGROUND OF PLANT TISSUE CULTURE AND ITS IMPORTANCE

It is now some 80 years since Haberlandt (1902) first began experiments which could truly be described as cell culture in that he attempted to grow single cells isolated from a variety of plant tissues in simple nutrient solutions. Since these early observations of Haberlandt, plant cell, tissue and organ culture has progressed

a long way. Rapid developments in the 1950s and 1960s (Murashige, 1978) converted tissue and organ culture from an area primarily of academic interest into a major tool for the horticultural industry through the mass propagation of house plants and the development of unique lines with desirable characteristics.

Plant tissues were first successfully cultured by White (1934). White *et al.*, (1939) reported the first successful callus culture of carrot and tobacco. Tissue culture of plants is based on the concept of totipotency, which is inherent in the cell theory of Schleiden (1838) and Schwann (1839).

The demonstration of cellular totipotency in carrot cell culture by Steward *et al.,* (1958) and Reinert (1958) opened up a tremendous potentiality for the application of tissue culture technique . Murashige and Skoog (1962) published a defined medium for tobacco tissue culture which has probably been cited more than other for culture of wide range of plant species , including both dicot and monocot.

The first major steps into experimental plant tissue culture were made independently by Robbins and Katte, (Robbins, 1922a, b; Kotte, 1922). They developed aseptic culture technique to culture intact meristem. The most important discovery during 1960s was the isolation and identification of cytokinin "Kinetin" (Miller *et al.,* 1955). The culture of isolated embryos of *Datura* (Van overbeek *et al.,* 1941) and Skoog's experiment (1944) on organogenesis of tobacco callus culture

were two substantial contributions towards the present day knowledge on tissue culture. Plant regenerate from various organs of plants *in vitro* (Murashige, 1974;1978; Vasil and Vasil, 1980), through androgenesis (Clapham, 1977), and the demonstration of protoplast isolation and fusion (Sharp, Vasil and Vasil, 1980) advanced a new step in tissue culture technology.

The advancement of tissue and cell culture for last few decades has become much enriched and some applications are gradually showing immense potential such as (i) shoot apex culture producing large numbers of plantlets of homogenous nature, (ii) disease elimination and conservation of disease free stock, (iii) embryo rescue and *in vitro* fertilization (iv) to create new plant type through (a) soma and gametoclonal variation (b) somatic hybridization and (v) production and extraction of secondary metabolites from cultured cells instead of growing the plants in the field. Experiments also include (a) protoplast fusion within species, between species, between monocot and dicot and even between plants and animals, (b) introduction of mitochondria and plastids into protoplasts. (c) uptake of blue green algae, bacteria and virus by protoplasts and (d) transfer of chromosomes, nucleic acids (DNA, RNA) into isolated protoplasts.

Plant cell and tissue culture techniques have tools for propagation of many ornamental and woody species. It has been estimated that about 50% bio-products

are accelerated by cell and tissue culture method. It also forecasts the world wide tissue culture as a tool of biotechnology in agriculture, food and beverage will be worth US 103 billions by 1995. A report from L. William Towels and company (an International seed and plant science consulting company) that world food production will rise 5-10 % in the next 25 years as a result of biotechnology.

In the early experiments on plant tissue cultures, the basic nutrient media were often supplemented with complex mixture of natural origin (coconut milk, juice of various fruits, casein hydrolysate, yeast extract etc.) in order to obtain optimal growth and to induce organogenesis (Stewart, 1968; Hildeberdant, 1962). Most of the plant tissue culture media were modifications of the formulations used by White (1934, 1963), Gautheret (1934, 1939) and Heller (1953). Up to now MS medium (1962) is the concerned.

Propagation via *in vitro* techniques may have several advantages over conventional methods. Most of these advantages over conventional methods depend on the speed of tissue culture propagation. Tissue culture can be used for plants that are normally propagated slowly. The basic advantages of *in vitro* propagation lie mainly in the rapidity with which plant multiplication can be achieved and the relatively short period of time, with conservation of space and often at a lower cost. Plants produced asexually though organogenesis *in vitro* are of uniform quality and

can be produced much more rapidly than is possible through sexual reproduction or by conventional methods. Clonally propagated plants produce uniformly superior seeds, improve progeny and evaluation of breeding experiments and show improved vigor and quality. The richness and fullness of clonally propagated plants gives them an enhanced esthetic value.

Hussey and de Fossard (1975) have reliable vegetative propagation which may utilize plant tissue culture technique. These include:

a) Rapid multiplication of new hybrid cultivars, which arise as single plants for testing and eventual commercial production.

b) Elimination of viruses from infected stocks.

c) Vegetative propagation of difficult to propagate species.

d) Year around propagation of clones.

e) Propagation of genetically uniform parent plants in large numbers for large scale hybrid seed production. A micropropagation method could assist in building up the clonal stock of elite genotypes by enabling rapid multiplication after their selection in a breeding programme. Micropropagation is being used extensively for rapid clonal propagation of many fruit trees (Zimmerman, 1985) since it enables rapid propagation and hastens the availability of new cultivars (Zimmerman, 1981). Using this method, a million fold increase per year in the rate of clonal

multiplication over conventional methods is possible (Murashige, 1974).

Micropropagation of plants using tissue culture techniques has many useful applications in agronomy also (Murashige, 1974; Winton, 1978). This is especially in woody plants, some of which are difficult to propagate by means of conventional methods (Conger, 1981). Moreover, *in vitro* techniques are important because it provides opportunity of large scale production of clones throughout the year which is not possible in conventional way (Hussey, 1978; Fossard, 1976). The expanding use of tissue culture in developmental biology, biochemistry, physiology, genetics and molecular biology is providing new knowledge about fundamental characteristics of plants. Furthermore, it has become an integral part of "Plant biotechnology". Scientists in universities, research institutes and companies pursue the development of improved plants for agriculture, horticulture and forestry.

Micropropagation, which is carried out under aseptic conditions, facilitates the International transport and exchange of germplasm without the inherent risks of spreading pests and pathogens. There are two pathways to obtain propagules through tissue culture. One is adventitious initiation of shoots, or embryoids from callus or explants, the other is enhanced axillary branching by means of bud or in species where plant regeneration from cultured tissues is difficult to obtain.

The tree that shows great genetic diversity, selection and multiplication of elite trees by vegetative methods might lead to even higher yields (Morgan and Shultz, 1981). Numerous plant species can be cultured satisfactorily *in vitro*. This has been achieved mainly by studying the composition of culture media and, in particular, the level of growth substances (Boxus, 1974).

One of the primary objectives of most micropropagation system is the maximization of shoot proliferation to obtain numerous microcuttings. Considerable attention has been focused on achieving high shoot proliferation rate through manipulation of growth regulators in the culture medium (Lundergan and Janick, 1980).

One of the basic requirements for employing the technique of tissue, cell and protoplast culture in the plant breeding is to either make use of or devise an *in vitro* systems which would permit whole plant regeneration. In recent years the methods of *in vitro* cultivation of excised organs and tissues have been applied to the study of shoot and root growth of woody plants by several investigators. The application of tissue culture are not only limited to solving the problems of plant physiology but also considered as an important means to develop the plant breeding method. The emerging application of tissue culture technology of clonal propagation provides an alternative to the routine vegetative propagation of woody species with such

desirable traits (Biondi and Thorpe, 1982; Thorpe, 1983). In fact, the *in vitro* culture of cells, tissues and organs offers unparallel opportunities for tree improvement (Karnosky, 1981).

Adventitious organogenesis and embryogenesis can induce high levels of somaclonal variation and are useful tools in breeding programs (Sharp and Evans, 1986). High frequency regeneration is useful in evaluating the influence of different factors (James *et al.*, 1984) and of mutagenic agents on regenerative process. Furthermore it allows the application of transformation techniques (James *et al.*, 1988).

1.2. MULBERRY (*Morus* sp.) : BOTANICAL AND AGRONOMICAL PERSPECTIVE

Mulberry belongs to the family Moraceae. *Morus* is the Latin word for mulberry (France: muris; Italian: gelso; Japanese: lewwa; Bangali: tunt). The plant is highly heterozygous, fast growing deciduous trees or shrubs, perennial, profuse branched. Plant habit, branching and height of mulberry depends upon the type of cultivation. Mulberry is a multipurpose social tree of great economic importance. The leaves constitutes the chief source of food for the silkworms (*Bombyx mor*i L). It is reported that, silkworm is attracted to some fragrance and is equipped with

special organs which respond to the taste of leaves (Ishikawa and Hirao, 1963, 1965). Protein of mulberry leaves is the source for the silkworms to biosynthesis the two silk proteins, fibroin and sericin.

1.2.1. Origin and Distribution

Genus *Morus* is native to Indo- China and found widely distributed in the lower sub- Himalayan region upto the elevation of 2100 m covering both the temperate and subtropical regions of the northern hemisphere. Towards the year 2800 B.C. Chin - Nong, one of the successors of Empires FO-HI tought cultivation of mulberry in China . Silk industry took its origin in the province of Chang- tong. According to Western historians mulberry culture spread by about 140 B.C. A global survey of sericulture industry reveals that there are about 56 countries where mulberry is cultivated. Mulberry plants are cultivated in the north zone of Bangladesh, particularly at Rajshahi, where Sericulture Research Institute has already been established since 1962. Of about 68 species so far recognized in the genus, a greater number occur in Asia , especially in China and Japan but the continental America is also rich in its *Morus* species. The genus is poorly represented in Africa, Europe and Middle East, while it is unrepresented in Australia.

In Asia, 24 species are represented in China, 19 species (including hybrid species in cultivation) in Japan, 6 in Korea, 4 each in Taiwan and India, 3 each in Burma and Indonesia, 2 each in Thailand, Vietnam and Muscat. In the Americas, out of 21 species as many as 14 species are found in North America and the remaining 7 species in central South America

Soviet biogeographer N.I. Vavilov while reviewing the centers of origin of cultivated plants divided phytogeography into 8 "gene centers" and placed *Morus* in "China - Japan" which includes East China, Korea and Japan.

Presently, *Morus* grows in warm and moist climatic zones between 50°N Lat. and 10°S Lat. which includes south Eastern tip of Asia, and Japan, Java and Sumatra Islands, Oman, Cacasia, Persia and West Asia; West Africa, North and South America including Mexico.

1.2.2. Taxonomic Description

The identity and nomenclature of the species are confusing as they are highly heterozygous and show great diversity of morphological characters. The genus *Morus* belongs to the Fig family Moraceac and comprises of about 65 species occurring in the tropic and subtropic belt. Difficulty in classifying the genus *Morus* and to draw conclusion on distribution of its species is mainly because of the non

availability of clear documents on early introduced economically important species and cultivars in different countries. The other reasons being highly cross pollinated nature of the genus *Morus*. As a result of cross pollination, enormous genotypic/phenotypic variation has been created naturally representing continuous type of variation. Another important point which deserves mention is the out breeding nature and natural crossability.

Linnaeus (1753) established the genus *Morus* with 7 species, viz. *M. tartarica, M.papyrifera, and M. tinctoria.* The last two species were later considered to represent distinct genera *Broussonetia* and *Chlorophora* respectively. Today *Morus* comprises of about 68 recognised species and almost the identity and nomenclature of the species of *Morus* is so confusing, the number of species growing in the world keep fluctuation. Airyshaw (1973) recognized less then 10 species under the genus *Morus*. The species of *Morus* being highly heterozygous and as unisexual plants produce a number of natural hybrids with many intermediate forms which create difficulties with regard to their correct delimitations.

Plants are dioecious or monoecious. Inflorescence are catkin. Male catkin are usually lager than female measuring 2.5-5 cm.

Flowers are small, sessile or shortly pedicillate, regular, unisexual and cross pollinated mainly by wind. The male flower consists of four perianth leaves arranged

in two whorls with imbricate aestivation; perianths are green homochlanydous. The androecium consists of four stamens arranged opposite to perianth. Filaments are broad with narrow top. Authors are dithecaus with longitudinal dehiscence, the pollengrains are round, dry, light and dust like with smooth exine.

Female flowers consist of four perianth arranged as male flower. Style is single . Ovary is bicarpellary, syncarpous, superior or unilocular. Seed is oval, dark purple in colour. Seed coat consists of two layers. The embryo consists of a primary axis (plumule and radicle) and endosperm. The seeds of polyploid species weigh more than those of diploids.

Plant habit, branching and height of mulberry depends upon the type of cultivation. Leaves are simple, alternate, entire, toothed or three lobed, stipulate, petiolate. Lamina is glabrous. Leaf tip is acute, double acute, acuminate and long acuminate, teeth are cerenate, serrate, dentate, dentate-serrate, serrate-dentate. Venation is multicostate reticulate. Phyllotaxy is of 1/2, 1/5 or 2/5. Buds (explants) can be excised from mulberry branches grown in the field at any time during the winter, but branches, if one wants to store them as long as possible, are preferably harvested just after the cessation of dormancy and prior to the ascent of sap.

1.2.3. Soil and Climate

Bangladesh experiences a subtropical monsoon climate with flat, fertile and alluvial land and is suitable for mulberry cultivation. The temperature in Bangladesh varies between 11°C - 29°C in the winter and between 21°C - 36°C in the summer . The average rainfall varies from 127 cm to 508 cm. All these factors together constitute favorable climatic conditions for luxurious growth of mulberry plant in the country. Still the leaf yield of mulberry in Bangladesh is very low. The leaf production is presently estimated in the country at about 16-18 tones / hac. (Bari, 1988).

Generally mulberry plants grow well in loam, Sandy loam, silt- loam and sandy-clay loam soils, which contains 40% minerals, 30% water, 20% air and 10% organic matter in order to better growth and yield. However, the texture of soil greatly influence the fertilizer of soil. Texture of soil means the ratio of sand, clay and silt particles present in the soil. It is well known that nutrient content of soil has a very profound influence not only on the yield of mulberry but also on their quality. So quality of soil ultimately affects the growth of silkworms and quantity and quality of silk produced. Optimum pH level of soil for handsome growth of mulberry is around 6.5, i.e., mulberry likes slightly acidic condition of soil.

Mulberry is a perennial woody plant and grows in various climatic conditions ranging from temperate to tropical (Rangaswami, 1976). The growth and development of mulberry plant is largely determined by its surrounding environmental factors. External environmental factors are interrelated with one another and they act altogether on the growth and development of mulberry plant (Anon, 1985). Mulberry belongs to the sunny plants requiring a strong sun-shine for its normal growth. The strength of the sun-shine largely affects the leaf quality of mulberry. Mulberry branches and twigs, under sufficient sun-shine, become stronger, its leaves become thicker and dark green in colour. Under insufficient sunlight the branches, twigs and leaves become soft, photosynthesis diminishes, moisture content in leaves increases, causing the leaves of poor nutritive status for the silkworm and more prone to diseases.

The optimum temperature for the growth of mulberry plant is about 25°c (range 23- 30°C) and it varies according to the mulberry genotypes, day length and rainfall (Anon, 1985). The growth of mulberry plants slow down when ambient temperature falls below 20°C and it will stops its all growth activities and enter into the dormant period when the temperature drops to 10°C or below. The winter buds begin sproutings when ambient temperature is about 12°C. High temperature about 40°C is likely to be detrimental to its normal growth, when respiration goes on

vigorously and the rate of photosynthesis decreases causing the plant to wither (Anon, 1975). Water plays an important role in the growth activities of mulberry plants. Mulberry plants require comparatively more water than other crop plants and it requires 280-400 ml water to synthesize 1g dry matter while that of Paddy plant requires 278-284 ml (Anon, 1975). Water content of mulberry leaves ranges from 64% - 83% in weight which makes the leaves succulent for silkworms. The climatic particulars of mulberry growing countries in the world are given below:

Climatic types of the mulberry growing regions:

1. Countries with Mediterranean type of climate	France, Italy, Greece, Cyprus, Turkey and Yugoslavia.
2. Countries with continental type of climate	Spain, Hungary, and Poland.
3. Countries with extreme variations in climate	U.S.S.R and China.
4. Countries with monsoon type of climate	Japan, India, Srilanka. Burma, Bangladesh etc.

Altitude range up to about 700 m above MSL (Marine surface level) is considered to be desirable for luxuriant growth of mulberry.

1.2.4. Propagation

Based on the method of reproduction all the crop/ plant species can be grouped into three major categories viz., crops reproducing only through seed, crops reproducing only vegetatively and crops reproducing both by sexual and asexual means. Mulberry belongs to the third category where both sexual propagation by seed and asexual propagation by stem cuttings and grafting are commonly used. Choice of the propagation method mainly depends on the genotypic specificity and purpose of propagation. Accordingly following methods are used to suit various conditions in mulberry a) Seed propagation b) Vegetative propagation.

Improved strains of mulberry are obtained by adopting the conventional breeding methods and they are selection, hybridization and selection, polyploidy, breeding and mutation breeding.

a) Seed propagation:

This is mainly used for improvement purpose through hybridization and selection and to raise root stocks for grafting. Some of the species like *M. laevigata* and some exotic varieties which are poor in rooting can be propagated by seeds if the seed setting is from a homozygous population.

b) Vegetative method:

This is used to propagate the crops which do not produce seeds or when seeds are non-viable. By this method parental characters can be kept intact. This method is followed to harvest early crop and to keep crop uniformity and homogeneity. Several types like stem cuttings, graftings, budding and layering are used depending on the rooting of genotype and the ability of regenerate. In Japan, 70% of nursery plants are produced by means of grafting. The problem with the technique is that, it takes 2 whole years to get nursery plants. The cuttings have the advantage of getting nursery plants in a year. In the softwood cutting, successful rooting depends on so many factors regarding physiological states of the cutting and environmental conditions that the results tend to have great variability. In the hard wood cuttings, only a limited number of varieties have enough rooting ability, whereas in tropical regions cutting is the easiest method of propagation. In Bangladesh this method is popular and widely used but is restricted in a single season and usually done just prior to winter.

1.2.5. Importance

Mulberry plant is not only associated with silk, but is also useful as a fodder source, timber for building houses and boats, for sports goods, furniture, fancy items and as medicine. In China and Europe, the bark stripped from waste and pruned

branches of mulberry is used in paper industry. Moreover, the bark of mulberry branches on rotting yields white fibers of a quality required in a textile industry (Bose, 1987). The seed also contain 25-30% oil and it is reported , in Ankara Province of Turkey mulberry seed oil is preferred to butter oil and olive oil (Rangaswami, 1976). Mulberry, being a heavy leaf producing plant, is found to be ideal to raise as shade trees in the tea and coffee plantation and a number of 109 and 435 mulberry trees per acre can be raised in tea and coffee respectively (Jolly, 1987). Along the coastal area of South India a large quantity of mulberry is cultivated as an intercrops in coconut plantations (Pillae *et al.*, 1987).

The pearl river delta in South China offers human a variety of land use alternative giving a complex ecosystem (mulberry dike-fish pond ecosystem) which has been greatly contributes to the regions agricultural productivity (Gongfu, 1982).

In India, sericulture emerged as the highest remunerative income source compared to other agricultural crops (Chakrabarty *et al.*, 1987) and the comparative account is given below:

Agricultural crops	Annual earnings Rupees/bigha.
Sericulture	5,996.00
Sugarcane	1,500.00
Mustard	935.00
Aman Paddy	910.00
Jute	844.00
Boro paddy	689.00

1.2.6. Role of Tissue Culture in Mulberry Plants

Mulberry is an important plant in sericulture industry as the foliage constitute the chief feed for the mulberry silkworm *Bombyx mori.* Enormous heterozygosity is very common in mulberry due to cross pollination habit. As a result, true-to-types of these plants can not be maintained through seed propagation and their improvement through conventional breeding methods is very difficult (Das, 1983). Conventionally mulberry is propagated by stem cutting and root grafts. Perennial nature of the plant coupled with prolonged juvenile period limits the speed of improvement in this crop. Further, dioecious nature of the genus in which the important species /varieties are represented by one sex only (either male or female) is a barrier to genetic improvement by conventional hybridization technique. There is a paucity of information about the inheritance pattern of yield contributing characters which is a limiting factor in choosing the parents. Moreover, in vegetatively propagated plants like mulberry it takes many years to evolve a desirable clone from economic and commercial point of view by conventional hybridization methods. Non-conventional breeding methods such as polyploidy and induction of mutations too have some long range problems such as poor response to mutagenic agents and reversion to the original parental characters with the passage of time.

In vitro techniques such as tissue and organ culture offer the plant breeder new openings in the clonal propagation, genetic manipulation and production of homozygous inbred lines (Rao *et. al.,* 1989).

Plant cell and tissue culture techniques have, in recent years developed into very powerful tools to overcome and minimizing the said problems of mulberry and according to Murashige and Skoog (1978) there were at least 100 facilities engaged in commercially propagating a variety of plants through tissue culture.

1.3. TISSUE CULTURE RESEARCH IN MULBERRY - PAST AND PRESENT

In 1962, Fosset first reported *in vitro* cultures of organs and tissues of mulberry trees in 2nd International Technical Sericultural Conference of Marrcia. But successful *in vitro* culture of mulberry was experienced by Ohyama (1970) when he succeeded in culturing the excised explants of mulberry in *in vitro*. Ghugale *et al.,* (1971) studied the effect of auxins and gibberellic acid on growth and differentiation of *M. alba* and *Populus nigra* tissues *in vitro*. He found that, root differentiation took place in the presence of IAA, IPA, α- NAA and β-NAA and not in 2,4-D and GA_3.

Minamizawa and Hirano (1973) worked on the effect of the concentrations of several sugars on differentiation in excised stem segments of mulberry trees cultured *in vitro*. They found that in green wood segments multiplication of callus was promoted remarkably on the medium containing 30 g/l of sucrose, 10 g/l of fructose, 40 g/l of galactose or 10 g/l of glucose. Root initiation from callus tissues was particularly conspicuous on medium containing 10 g/l of any of the sugars. Neither induction of callus tissues or root initiation from callus tissues was noticeable on the medium without sugars. Oka and Ohynama (1973) examined the effects of constituents of medium on callus formation in mulberry. They found that, compact callus was comparatively well formed when inorganic salts especially nitrate of them was high simultaneously high concentration of auxin and yeast extract or casein hydrolysate was added. He found 2,4-D for better callus induction than IAA or NAA, and KN or BA did not show any additional effects on callus formation. He also found that, a large amount of compact callus was formed on modified MS (Murashige and Skoog, 1962) medium.

Oka and Ohyama, (1974) the pioneer workers of mulberry tissue culture succeeded in culturing the winterbuds in MS medium enriched in cytokinin. Oka and Ohyama (1975) studied on *in vitro* culture of excised buds in mulberry tree and examined the effect of growth substances on the development of shoots from buds.

They concluded their results as follows:

a) Young greenish buds with stem grew into shoots on MS + NAA, MS + NAA + BA.

b) Excised greenish buds did not grow in var. Ichinose, whereas, they sometimes grew in medium with BA in var. Kenmochi.

c) Excised brownish buds that were more aged than greenish buds did not grow in both varieties .

d) Both auxin and cytokinin had to be added to basal medium for bud sprouting .

e) Flowers rarely appeared together with shoot growth.

After a year Oka and Ohyama (1976) reported a suitable growth media for subculture of mulberry callus. According to them, modified MS + NAA 10^{-5} M + 10^{-6} M was the best medium for subculturing and supported 12 fold growth of mulberry callus per month. In the same year they reported that an exogenous supply of BA and NAA is necessary for the morphogenesis of mulberry explants in *in vitro* conditions.

In 1978, Oka and Ohyama experienced best shoot formation at the lower pH range of 4-5 and fructose as the best carbon source for *in vitro* mulberry culture.

In 1981, Oka and Ohyama cultured leaf explants of mulberry derived from aseptically grown shoots and seedlings on MS medium. They regenerated normal

and abnormal leaves in the culture media by varying the concentrations of BA. These leaves differed in the way of forming adventitious buds.

Multiple shoot formation was first reported by Ohyama and Oka in 1982 from mulberry hypocotyls. Patel *et al.*, (1983) experienced morphogenetic responses of axillary buds of *Morus india* in MS media with different concentrations and combinations of cytokinin and auxins.

Mhatre *et al.*, (1985) could regenerate mulberry plantlets by culturing leaves and axillary buds of *M. indica* by pretreating the explants in liquid MS containing BA 0.5. 1.0 and 2.0 mg/l prior to the culture in the semisolid media which also contained BA with the same concentrations. Kim *et al.*, (1985) reported morphogenic response of cotyledonary, leaf, hopocotyl and shoot tip explants of mulberry in MS media supplemented with BA in conjunction with or without low concentrations of NAA. In 1986, Oka and Ohyama for the first time were able to regenerate complete plantlets of mulberry through *in vitro* techniques successfully. Jain *et al.*, (1990) multiplied 5 genotypes of mulberry belonging to 4 species by culturing axillary buds in MS media with different concentrations and combinations of cytokinin and auxin. Yadav *et al.*, (1990) regenerated mulberry plantlets (*Morus nigra*) through *in vitro* techniques by culturing shoot tip and nodal explants in MS media supplemented with different concentrations of BA and Kn.

In 1991, Zaman *et al.,* tried to findout a suitable *in vitro* technique for mass propagation of mulberry. The transplanted mulberry plants survived well in the field but they did not study the morphological and nutritional status of *in vitro* raised plants. One year later, Islam *et al.,*(1992) worked on callogenesis of *Morus laevigata* where, they were able to produce different types of callus but could not observe adventitious bud or somatic embryo. In the same year, Zaman *et al.,* (1992a) observed the effect of sugars, agar and pH on *in vitro* shoot proliferation of mulberry. Zaman *et al.,* (1992b) also experienced the *in vitro* response of different mulberry genotypes and observed different mulberry genotypes responded differentially on *in vitro* condition.

Hossain *et al.,* (1992) for the first time was able to micropropagate *Morus laevigata*, while one year later Zaman *et al., (1993)* observed effects of sucrose, pH and temperature on *in vitro* rooting of mulberry using *Morus alba* cv S_1.

Islam *et al.,* (1994) worked on cloning of mulberry microshoots and simultaneously observed the *in vitro* microbial interruptions. In the same year Zaman *et al.,* (1994) observed effects of aminoacids on cloning of mulberry microshoots and reported that, among the aminoacids, tyrosine was found to be best additive for shoot proliferation.

1.4. OBJECTIVES

During the last two decades plant tissue culture techniques have been used successfully in the propagation of many herbaceous plants (Conger, 1981). Considerable amount of work has also been done in tree propagation (Mascarenhas *et al.*, 1988) but in comparison to herbaceous, ornamental and crop species, most hard wood and soft wood tree species of forest value have proven difficult to propagate. In view of their highly heterozygous nature and long duration, it is very important to develop methods of clonal propagation of superior trees. The traditional methods of vegetative propagation of woody perennials by rooting of cutting or grafting is difficult. Since seed propagation results in great variability, tissue culture seems to be the only method which offers some promise to the solution of the above problem.

Mulberry (*Morus* sp.) is a woody tree of great economic importance in the sericulture industry because its leaves constitute the chief feed for the silkworms (*Bombyx mori*). Delicious jelly is prepared from its sweet edible fruits which is rich in vitamins `C' (ascorbic acid). High quality timber is prepared from some of the species like *M. laevigata.* Since mulberry is rapid growing tree, it is planted along the road side and for afforestation purposes. Its roots are used for medicinal preparation and that is why the mulberry is called the multipurpose `social' tree.

From the biological point of view mulberry plants are highly heterozygous due to its cross pollination habit (Das, 1983). In the Indo-Pakistan-Bangladesh subcontinent this plant is cultivated by means of cutting which is easiest and popular. Inspite of that this conventional method has some draw backs. For example, cuttings include soft wood and hard wood cutting. Both cuttings have the advantage of getting nursery plants in a year. However, in the soft wood cutting, successful rooting depends on so many factors regarding the physiological states of the cuttings, and environmental conditions that the results tend to have great variability. On the other hand, in the hard wood cutting, only a limited number of varieties have enough rooting ability. Besides, only 30 to 40% cuttings of mulberry survive the time period between pruning, transportation and final transplantation (Rao *et. al.*, 1989). Another major problem of this conventional method is, this form of mulberry cultivation is restricted to a single season and usually it is done just prior to winter (September-October) in Bangladesh. In other seasons if the cutting is planted, it does not sprout usually, and if it does, the growth and vigor of plant is hampered. For the said reasons, the yield of mulberry does not meet the demand of silkworms every year in Bangladesh and consequently, the leaf yield and synthesis of silk are reduced.

Therefore, the present investigation was undertaken to find out a suitable and reliable method for mulberry propagation through *in vitro* technique . Main objectives are :

i) proliferate single and multiple shoots of different genotypes as per our desires by manipulating different hormonal combinations and concentrations,

ii) see *in vitro* rooting ability of microshoots belonging to different genotypes,

iii) Year round sapling production by culturing nodal segments and shoot apices of different genotypes for choosing best genotype and getting true-to-type plants,

iv) acclimatize *in vitro* derived plants and study morphogenic behavior and nutritional status in comparison to cutting derived plants of the same age, as it is very important, because, synthesis of silk fibre by the silk worms totally depends on the nutritional status of mulberry leaves,

v) induce callus from different parts of mulberry plants for somatic embryogenesis.

2

MATERIALS & METHODS

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Plant Material:

Leaf, nodal segment, shoot apex, cotyledon and internode from seven different genotypes of *Morus alba* L. viz., BSRM-1, BSRM-2, BSRM-3, BSRM-8, BSRM-17, BSRM-18, BSRM-19 and one genotype of *M. laevigata,* BSRM-14 were used as experimental materials. Explants (node, internode and shoot apex) were collected from field grown mature plants. Leaf explants were collected from *in vitro* grown microshoots. While cotyledones were collected from aseptically germinated seedlings. The explants from different genotypes of *M. alba* and *M. laevigata* were collected from the germplasm bank of Bangladesh Sericulture Research and Training Institute, Rajshahi. The characteristics of *M. alba* and *M. laevigita* are arranged in table 1.

Table 1.

Plant Material Used

Genus	Species	Genotypes	Origin	Sex	Rooting (%) of cuttings	Leaf yield mt/ha/yr.
Morus	***alba***	BSRM-1	Bangladesh	♀	80	13-14
		BSRM-2	Bangladesh	♂	75	12-13
		BSRM-3	Bangladesh	♂	85	15-18
		BSRM-8	Bangladesh	♀	90	10-12
		BSRM-17	India	♂	80	30-32
		BSRM-18	India	♂	80	29-30
		BSRM-19	India	♂	85	34-35
Morus	***laevigata***	BSRM-14	China	♀	10	20-21

2.1.2. Nutrition Media:

MS basal medium (Murashige and Skoog, 1962) was used in conducting most of the experiments. However, other media composition used in the present study were Whites media (White, 1934), N_6 (Chu *et.al.*, 1975), LS (Linsmaier and Skoog ,1975) and Woody plant media (Mc. Cown and Lloyd, 1981) to evaluate their effects on *in vitro* response of mulberry.

2.1.3. Plant Growth Regulators:

The following plant growth regulators were used in present investigation.

Auxins:

- Indole-3-acetic acid (IAA)
- Indole-3-butyric acid (IBA)
- α - naphthaleneacetic acid (NAA)
- 2,4- Dichlorophenoxyacetic acid (2,4-D)

Cytokinins:

- 6- Benzyladenine (BA)
- 6- Furfurylaminopurine (KN)
- 2- Isopentenyladenine (2iP)

Gibberellic acid (GA_3)

2.1.4. Additives:

Casein hydrolysate (CH), malt extract (ME), coconut milk (CM) and tyrosine were added where necessary. Sucrose and sigma agar were used as carbon source and gelling agent respectively. In one experiment other sugars, like glucose, lactose and fructose were used.

2.1.5. Sterilent and Surficant:

Mercuric chloride ($HgCl_2$) and ethanol were used as sterilizing agents. Tween`80 and savlon (commercial product of 20% cetrimide an antiseptic plus detergent) were used as detergent and surficant respectively.

2.2. METHODS

Experiments were carried out in Plant Breeding Laboratory of the Department of Botany, University of Rajshahi and in Plant Biotechnology Division, Bangladesh Sericulture Research and Training Institute, Rajshahi, Bangladesh.The different procedures followed in different experiments are described below under separate heads.

2.2.1. Preparation of Stock Solutions:

The different constituents of culture media were prepared into stock solution for ready use during preparation of media. As different constituents were required in different concentrations, separate stock solutions for macronutrients, micronutrients, vitamins and hormones were prepared.

2.2.1.1. Stock solution `A` (Macronutrients):

It was made upto ten times the final strength of the medium. They were weighed accurately in electronic balance, dissolved separately in glass beaker containing 750 ml of distilled water and then made upto 1 litre. The stock solation was filtered through a Whatman NO.1 filter paper, if it contained any solid contaminants like cellulose, dust, cotton etc. The stock solution was stored in a refrigerator at 4°C.

2.2.1.2. Stock Solution `B' (Micronutrients):

The separate stock solutions of microsalts were prepared as follows:

a) Stock Solution of $FeSO_4$ and Na-EDTA:

This solution was prepared at 100X to that of the required concentrations. Requisite amount of $FeSO_4$ and Na-EDTA were taken and dissolved separately in a clean glass beaker containing 225 ml of DDW. Na-EDTA solution was then transferred to a 500 ml volumetric flask. Subsequently, the solution of $FeSO_4$ was poured to a volumetric flask slowly with constant stirring. The final volume of the solution was made upto 500 ml and chelated for 24h at 58°C by placing in an incubator. pH of the solution was adjusted to 5.6 and after filtering stored at 4°C in a refrigerator.

b) Stock Solution of the Rest of the Micronutrients (Solution `C'):

This was made at 100X in 500 ml DDW as described for solution `A'. All components were weighed (except $CaCl_2$) separately and dissolved in 400 of DDW. $CaCl_2$ was dissolved separately and added to the solution. Finally the volume of the solution was adjusted upto 500 ml and after filtering was stored at 0°C in a plastic bottle.

2.2.1.3. Stock Solution `D' (Organic Components):

This stock solution was also prepared at 100X, dissolved in 100 ml DDW as described for stock solution `A'. The solution was stored in a deep freezer in a plastic bottle. Casein hydrolysate, yeast extract, malt extract, aspergine, glutamine and tyrosine were directly added to the medium whenever necessary.

2.2.1.4. Hormonal Stock Solution:

Stock solution of different hormones were prepared separately. Phytohormones namely, naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-furfurylaminopurine (Kinetin/KN), benzyladenine (BA) were dissolved in appropriate solvent. The solvents used for dissolving these growth regulators are shown as against each of the hormones.

Hormone :	**Solvent :**
NAA	: 0.1 N NaoH
IAA	: 70% ethyl alcohol
IBA	: 70% ethyl alcohol
2.4-D	: 70% ethyl alcohol
Kinetin	: 0.1 N HCl
BA	: 0.1 N NaOH
2iP	: 70% ethyl alcohol
Tyrosine	: 0.1 N NaOH.

To prepare a stock solution of any of the above hormones, 10 mg of the hormone was placed on a clean watch glass and dissolved in 1 ml of the particular solvent. The mixture was then washed off with distilled water and poured into a 100 ml measuring cylinder. Sufficient

distilled water was added to make the volume upto the desired level. Thus stock solution of all hormones were made and stored in deep freeze.

2.2.2. Sterilent Solution:

$HgCl_2$ solution of 0.1% was used for surface sterilization.. To prepare 0.1% solution, 0.1g of $HgCl_2$ was taken in a litre bottle and dissolved in 100 ml DW. Freshly prepared $HgCl_2$ was always used. $HgCl_2$ solution was prepared 1 h before use.

2.2.3. Preparation of Culture Media :

To make one litre of media the following steps were followed:

(i) 100 ml of stock solution `A' and 10 ml each of the stock solutions B and C were poured in a conical flask.

(ii) Desired amount of sucrose, generally 3% (30 g/l), were dissolved in 500 ml of distilled water and added to the stock solution that was taken in the conical flask.

(iii) Different concentrations of hormonal supplements as required was added singly or in different combinations to this solution and thoroughly mixed.

Since each hormonal stock solution contained 10 mg of the hormone in 100 ml of solution, the addition of 10 ml of any hormonal stock solution of one litre of medium resulted in 1 mg/l concentration of that hormonal supplement. Similarly for different concentrations of hormones, different amount of hormonal stock solutions was added to the medium.

(iv) The whole mixture was then poured into a one litre measuring cylinder and made upto the desired volume with the addition of distilled water. The mixture was then poured into a 2 litre flask and again mixed well.

(v) Other supplements such as aspergine, glutamine tyrosine, casein hydrolysate, malt extract, adenine sulphate etc., if required, were added.

(vi) The pH of the medium was adjusted to required level using pH meter by means of 0.1 N NaOH and 0.1 N HCl, whichever necessary.

(vii) 0.7% of sigma agar was added to the medium and heated to melt the agar completely.. Proper care was taken so as not to boil the solution while melting agar.

(viii) The medium was then poured into test tubes or conical flasks while still hot and the culture vessels were plugged tightly with non-absorbent cotton or heavy duty aluminum foil. The test tubes / conical flasks containing different hormone supplemented medium were properly marked by a glass marker to indicate specific supplementation.

(ix)Finally the medium was autoclaved at 15 lbs pressure per square inch at temperature of 121°C for 15 minutes to ensure sterilization and used within a week.

2.2.4.Collection of Explants and Surface Sterilization:

In the present investigation the explants collected from the following sources :

a) Field growing mature plants of age 10 to 15 years:

For this purpose young greenish and healthy twigs were excised and brought in the laboratory. Leaves and scales were trimmed. Young twigs were then cut into pieces each containing 2-3 nodes and a shoot apex. These segmented twigs were then placed in running tap water for about 1 h to washout the bacterial and fungal spores adhered on its surface. After thorough washing the explants were submerged in distilled water containing Tween 80

+savlon (100 ml distilled water + 1 ml Tween 80 and 1 ml savlon) for 15-20 minutes. After that, the explants were washed about 10 times with distilled water to washout the Tween 80 and savlon. The next step of sterilization was done on laminar air flow cabinet. Shoot apices were excised, separated and dipped in 70% ethanol for only about 1-2 seconds, rinsed with sterile distilled water and then treated with 0.1% $HgCl_2$ solution (w/v) for 9-10 minutes. After that, these explants were washed with sterile distilled water for 6-7 times to washout the remains of $HgCl_2$. The explants (shoot apices) were then placed in the appropriate media. The sterilization process of nodal explants were same as shoot apex explants. The difference was only the duration of ethanol and $HgCl_2$ solution. In this case duration of ethanol was 2-3 seconds and disinfected with 0.1% $HgCl_2$ for 10-15 minutes. Single nodal explants were then implanted in the appropriate media.

b) *In vitro* grown microshoots:

Explants from *in vitro* grown microshoots were 100% sterile, no sterilization procedure was followed as in case of field grown plants. In this case shoot apices, nodal segments and leaf explants were directly excised and were used for different experiments. Unless otherwise mentioned shoots having 2-3 cm long were harvested from *in vitro* proliferated shoot mass and further cultured singly in auxin riched media for root induction.

2.2.5.Culture Environment:

All cultures were allowed to grow in an air conditioned room having ambient temperature of 28±1°C and light intensity of 2000 lux provided by white Phillips fluorescent

light fitted at a distance of 33 cm from the culture racks. Most of the cultures were incubated at a photoperiod of 16 h light and 8 h dark.

2.2.6. Setting and Designing of Experiments:

To experience diverse morphogenic potentialities of different explants, several experiments were designed and conducted with various growth regulators having different concentration and combinations. To ensure the optimum condition of a specific factor, experiments were carriedout with different degrees of modifications of these factors (cytokinin- auxin ratio etc.) keeping other constant. Most of the experiments were arranged in randomized block design. In an experiment each treatment had 3 replications with 10-15 culture tubes per replication.

2.2.7. Inoculation and Maintenance of cultures:

2.2.7.1. Shoot proliferating culture:

Within 4-5 weeks of culture, the cultured explants produced multiple shoots. Inorder to increase number of shoots the proliferated cultures were subcultured in the same media composition and sometimes in media with different concentrations of cytokinin (BA). After 6-8 weeks of subculture multiple shoots were formed. From these cultures individual microshoots were harvested and subcultured in the some way. Plenty of microshoots were obtained from a single explant. Some of these *in vitro* derived shoots were used as the source of explants for different rooting experiments.

2.2.7.2. Microcutting preparation and root induction:

The usable healthy microshoots (usually 3 cm in length) were harvested aseptically from the proliferating cultures during different stages of subcultures. Before transferring into the rooting media, the shoots were soaked in sterile moistened filter paper for avoiding dessication. The microcutting from these proliferated shoots were prepared by trimming the basal portion of the shoot and unhealthy leaves and cultured individually in test tubes (25 x 150 mm diameter) containing 15-20 ml of rooting media. The cultures were then incubated for root induction.

2.2.7.3. Callus culture and somatic embryogenesis :

Different explants like leaf, petiole and internode were used for callus induction in auxin enriched media. When the calli attained a size of about 20-25 mm in diameter they were subcultured onto the fresh media for further growth and proliferation. For this purpose, the calli were removed aseptically from the test tubes on a sterile petridesh; cut into 0.4-0.5 cm diameter piece by a sterile scalpel and placed on suitable media in culture tubes or conical flasks for further growth and somatic embryogenesis.

2.2.8. Precaution :

During culture operation all dissecting instruments (which were autoclaved earlier namely razor, knives, needles and forceps) were dipped in 70% alcohol and flamed over a spirit lamp before each time use. Glasswares such as petridishes, bottles, conical flasks etc. were sterilized by hot air treatment by putting them in an over at 160°C for h. Media, cotton, cotton plugs, filter papers etc. were sterilized by autoclaving at 15 lbs per square inch pressure at the temperature of 121°C for 15 minutes. $HgCl_2$ treatment for disinfecting explants (taken

from field grown plants) was done very carefully, since, percentage (w/v) of the $HgCl_2$ solution and duration of treatment (usually 10-12 minutes) were very critical factors.. The floor of the laminar airflow cabinet and hands were washed with 70% alcohol before starting the procedure.

2.2.9. Transplantation of *in vitro* Derived Plantlets to Soil :

Rooted microshoots were taken out from the culture vessels and washed thoroughly under running tap water to remove remains of the medium adhered to the surface of the root. These plants were then transferred to polybags filled with garden soil, organic compost and sand or saw dust in a ratio of 2:1:1.

Before removing, the test tubes with the plantlets inside were kept for a week out side the culture room (in another room, where temperature and light is not controlled) for primary adaptation till the roots became brown. Soon after the transfer of plantlets to the polybag, the plantlets were covered with polybags to maintain high humidity. After a week of transplantation, the covered polybags were perforated to allow free air circulation to the transplanted clones. The polybags were finally removed after the second week. After one month, the plantlets were exposed to sunlight periodically, ie. in the first day 1 h exposure, in the second day one and half hour exposure and so on. This practice made the transplanted plants acclimatized in *ex vitro* natural environment slowly and steadily. After 2-3 months, the plants were transferred to the field directly. The plants were watered periodically, manured and mulched occasionally whenever necessary. Two to three months after transferring in the field, the plants resumed vigorous growth and within 6 months the plants reached upto 2-2.5 m hight. When these plants attained maturity (1-year-old), different morphological parameters

like plant height, internodal distance, leaf number per branch, weight of 100 leaves, stomatal frequency etc. were recorded to compare with the cutting derived plants of the same age.

2.2.10. Evaluation of morphogenetic parameters:

Different growth parameters were considered to score data on diverse morphogenic responses of different explants under different culture media and external factors. The parameters considered in the present investigation were as fallows:

A. *In vitro* Parameters :

i) Percentage (%) of shoot proliferation and root formation :

These parameters were taken for different shooting and rooting experiments and data were scored after 4-5 weeks and 5-6 weeks of culture respectively.

ii) Number of shoot and root per explant :

Number of shoots were counted usually after 6-7 weeks of culture whereas those of roots were recorded after 5-6 weeks of incubation.

iii) Length of shoot and root :

Shoot height was measured in cm after 5 weeks of culture. The longest shoot was considered if more than one shoot was present in a culture. Length of roots was also measured in cm before transplanting the plantlets in the soil.

B. *In vivo* Parameters :

i) Plant height and internodal distance :

After one year of transplantation in the field, plant height and internodal distance were measured in cm. which was followed for cutting derived plants of the same age for comparative analysis.

ii) Weight of leaves :

When the plants were 1-year old after transplantation, leaves were plucked and weighted in`g'. This parameter was also considered for cutting derived plants of the same age for comparison.

iii) Stomatal frequency :

Stomatal density was measured in *in vitro* derived plants as well as for cutting derived plants for comparison. Peeling was taken from the leaves (*in vitro* and cutting derived plants of the same age) and stomata were counted by compound microscope through 60X lense power.

2.2.11. Evaluation of Biochemical and Nutritional Status of *In Vitro* Regenerated Plants and Cutting Derived Plants.

Total Mineral :

Total mineral content was determined by the following method (**AOAC, 1980**)

Equipments :

a) Porcelain crucible.

b) Muffle furnace.

c) Electrical balance (Mettler H 18).

d) Desiccator.

Procedure :

Two grams of leaf powder were taken in porcelain crucible (previously weighed). The crucible was placed in a muffle furnace for about six hours at about 600°C. It was then cooled in desiccator and weighed. To ensure completion of ashing, the crucible was again heated in the muffle furnace for half an hour, cooled and weighed again. It was repeated till two consecutive weights were the same.

Calculation :

Total mineral % (g per 100 g of mulberry leaf powder)

$$= \frac{\text{Weight of the total mineral obtained}}{\text{Weight of mulberry leaf powder}} \times 100$$

Determination of Nitrogen, Phosphorus and Potassium Content of Mulberry Leaves:

Nitrogen :

Nitrogen content of different mulberry leaves was determined by the method of micro kjeldhal method (Wong, 1993).

Reagents and equipments:

a) Solid potassium sulphate.

b) Concentrated sulfuric acid.

c) 5% $CuSO_4$, 5 H_2O in distilled water.

d) 0.01N H_2SO_4 solution.

e) NaOH (5N).

f) Boric acid solution containing bromocresol green (receiving fluid) : 10 g of boric acid was dissolved in hot water (250 ml) and cooled. 1ml of 0.1% bromocresol green in alcohol was added and diluted to 500 ml with distilled water.

g) Few quartz chips.

h) Nitrogen determination apparatus (micro model) according to Paranas-wagner, made of JENA Glass-all connections with interchangeable ground joints.

Procedure :

a) Digestion :

4 to 6 ml conc. H_2SO_4, 1 g K_2SO_4 and one or two drops of 5% $CuSO_4$ solution (catalyst) and some quartz chips (to avoid bumping) were added to 0.5 -1 g of leaf powder in a Kjeldhal flask. The mixture was heated till it become light green (2-3 hours).

b) **Collection of ammonia :**

The digestion was carried out in a steam distillation chamber of the nitrogen determination apparatus. The chamber was designated to act as a micro kjeldhal flask and could be easily detached when needed. After the completion of digestion, the steam distillation chamber containing the digested mixture was fitted back to the nitrogen determination apparatus. Boric acid solution (15 ml) in a small flask was so placed that the tip of the condenser outlet dipped below the surface of the boric acid solution. Sufficient amount of concentrated sodium hydroxide solution (approximately 30-40 ml) was added to the digest in the chamber to neutralize the amount of acid present. Steam was generated from the steam generating flask and the sample in the chamber was steam distilled until 20 ml of distillate was collected in the boric acid solution. The condenser outlet was then rinsed with little distilled water and the receiving flask was removed.

(c) **Titrimetric estimation of ammonia :**

The ammonia in the boric acid solution was titrated with 0.01N H_2SO_4 until the solution had been brought back to its original yellow green colour. The titration was repeated with a control containing only 15 ml of boric acid solution diluted to approximately the final volume of the titrated sample. The volume of acid required was noted.

Calculation :

The total nitrogen was calculated using the formula given below:

1000 ml of IN acid + 14 g of nitrogen

Nitrogen content (g per 100 g of mulberry leaf powder)

$$= \frac{\text{Weight of the nitrogen obtained}}{\text{Weight of the mulberry leaf powder}} \times 100$$

Phosphorus :

Phosphorus content of mulberry leaf was determined by the method of Vogel (1961).

Preparation of Molybdate Solution :

12.50 g of A. R. grade sodium molybdate (Na_2MoO_4, $2H_2O$) was dissolved in 10 N H_2SO_4 and diluted to 500 ml with 10N H_2SO_4.

Preparation of hydrazine sulphate solution :

0.75 g of A.R. grade hydrazine sulphate was dissolved in deionized water and diluted to 500 ml.

Preparation of standard phosphate solution :

Exactly 0.219 g of A. R. grade potassium dihydrogen phosphate was dissolved in deionized water and diluted to 1 litre.

Then, 1 ml solution = 0.05 mg phosphorous.

Preparation of stock solution :

The total mineral ash obtained, as described earlier was moistened with a small amount of distilled water (0.5-1.0ml) and then 5ml of conc. HCl was added to it. The mixture was evaporated to dryness of a boiling water bath. Another 5 ml of conc. HCl was added and the solution was evaporated to dryness as before, 4 ml of HCl and a few ml of distilled water were then added to the dry ash, and the solution was warmed on a boiling water bath. The warmed solution was then filtered into a 100 ml volumetric flask using Whatman no. 40 filter paper. After cooling, the volume was made upto 100 ml with distilled water and suitable aliquots were used for the estimation of phosphorus, potassium, iron, boron, sulphur, manganese and chloride.

Procedure :

One ml leaf extract, 2 ml of hydrazine sulphate and 5 ml of molybdate reagent were taken in a 50 ml volumetric flask and made upto the mark with deionized water. The mixture was mixed well. The flask kept immersed in a boiling water bath for 10 minutes, then it was removed and cooled rapidly. The absorbance for each solution was measured at 830 nm against reagent blank.

Construction of calibration curve :

A calibration curve was constructed in the usual process by using six standard phosphorus solution containing 1, 2, 3, 4, 5, and 6 ppm of phosphorus. The mg percentage of phosphorus present in each different mulberry leaf powder was calculated by using the standard curve of phosphorus.

Calculation :

mg percent of phosphorus present (mg per 100 g of mulberry leaf powder)

$$= \frac{\text{mg of phosphorus obtained}}{\text{Wt. of leaf powder}} \times 100$$

Potassium :

Potassium content was measured by flame emission spectroscopy (FES) following the method of Jackson (1973).

Preparation of standard potassium solution :

1.909 g of A. R. grade potassium chloride was dissolved in deionised water and made upto 1 liter in a volumetric flask. This solution contains the equivalent of 1.00 mg K per ml. This stock solution was diluted to obtained required conc. of K (5, 10, 15, 20, 25, 30, 35 40, 45 and 50 ppm).

Preparation of stock solution :

Stock solution of potassium was perpared as describe before for phosphorus.

Procedure :

The intensity of emission (read out) of each of different mulberry leaf aliquot is measured for potassium with the help of a flame photometer using potassium filter.

Calibration curve of emission intensity (read out) vs concentration for potassium was constructed by using ten standard potassium chloride solution containing 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 ppm of potassium ions.

The mg percent of potassium present in each mulberry leaf powder was calculated by using the standard curve of potassium.

Calculation :

mg percent of potassium (mg per 100 g of mulberry leaf powder)

$$= \frac{\text{mg of potassium obtained}}{\text{weight of leaf powder}} \quad \text{x } 100$$

Construction of calibration curve :

A calibration curve for manganese was prepared in the usual manner using 0, 1, 2, 3, 4, 5 and 6 ppm of manganese (II).

Calculation:

mg percentage of manganese (mg per 100 gm of mulberry leaf powder).

$$= \frac{\text{mg of manganese obtained}}{\text{Weight of leaf powder}} \times 100$$

Calcium :

Calcium content was determined by titrimetric method (Bernard, 1965).

Reagents :

(a) Hydrochloric acid (concentrated)

(b) Ammonium oxalate (6%)

(c) Methyl red indicator

(d) Dilute sulphuric acid (2N)

(e) Strong ammonia

(f) Potassium permanganate (Jam brand, West Germany) solution (N/100)

Preparation of stock solution :

The ash/mineral obtained as described earlier was moistened with a small amount of distilled water (0.5 - 1.0 ml) and then 5 ml concentrated HCl was added to it. The mixture was evaporated to dryness on a boiling water bath. 5 ml of concentrated HCl was added again and the solution was evaporated to dryness. The residue was dissolved in about 4 ml HCl and a few ml of water, and the solution was warmed on a boiling water bath. The warmed solution was then filtered using Whatman no. 40 filter paper. After cooling, the volume was made upto 100 ml with distilled water and suitable aliquots were used for the estimation of calcium.

Procedure :

25 ml of the stock solution was taken in a conical flask and 125 ml of double distilled water was added to it. A few drops of methyl red indicator was added and mixture was neutralized with ammonia till the pink colour changed to yellow. The solution was heated to boiling and 10 ml of ammonium oxalate was added. The mixture was then allowed to boil for a few minutes and glacial acetic acid was added to it till the colour became distinctly pink. The mixture was kept in dark at room temperature for 1h. When the precipitate settled down, the supernatant was tested with a drop of ammonium oxalate solution to ensure the completion of the precipitation. The precipitate was then filtered through Whatman no. 40 filter paper

and washed with warm water till the precipitate became free of oxalate (tested with $CaCl_2$). The precipitate was transferred to a beaker by piercing a hole in the filter paper and about 5 to 10 ml dilute H_2SO_4 (2N) was poured over it. The solution was then heated to about 70°C and titrated with N/100 $KMnO_4$ solution.

Calculation :

1 ml N/100 $KMnO_4$ solution = 0.2004 mg of calcium

mg percent of calcium content (mg per 100 g of mulberry leaf powder)

$$= \frac{\text{mg of calcium obtained}}{\text{Weight of leaf powder}} \times 100$$

Determination of Moisture, Protein, and Carbohydrate Contents of Mulberry Leaves.

Moisture:

Moisture content was determined according to following method :

Materials:

(a) Electric weighing balance (Mettler H 18).

(b) Oven (W.C.HEARAEUS GMBH HANAU).

(C) Desiccator.

Procedurc:

Ten fresh mulberry leaves were weighed and dried in an oven at 70° C. After 10-12 h, the dried leaves were removed from the oven then kept in a desiccator and weighed. The process was repeated until constant weight was obtained.

Calculation :

Moisture (g per 100 g of mulberry leaves).

$$= \frac{\text{(wt. of fresh leaf- wt. of dried leaf)}}{\text{wt. of fresh leaf}} \times 100$$

Protein :

Protein content of different cultivars of mulberry leaf was determined by micro-kjeldhal method (Jayaraman, 1985).

Free Sugar : (Total Sugar)

Free sugar content of the mulberry leaf was determined colorimetrically by the Anthrone method (Morse, 1947).

Reagents :

(a) Anthrone reagent: The anthrone reagent was prepared by dissolving 200 mg of anthrone in 100 ml of conc. H_2SO_4.

(b) Standard glucose solution : A standard solution of glucose (BDH Chemicals Ltd., Poole, England) was prepared by dissolving 10 mg of glucose in 100 ml of distilled water.

Extraction of sugar from mulberry leaf:

Extraction of sugar from mulberry leaf was done following the method of Loomis (1947).

Five g of mulberry leaf powder were plunged into boiling ethyl alcohol and allowed to boil for 5 to 10 minutes. 10 ml of alcohol was used for every g of powder. The extract was cooled and pasted in a mortar with a pastle. Then the extract was filtered through two layers of muslin-cloth and re-extracted the powder for three minutes in hot 80 percent alcohol using 3 ml of alcohol for alcohol soluble substances. The extract was cooled and passed through muslin-cloth. Both the extracts were filtered through Whatman No. 41 filter paper.

The volume of the extract was evaporated to about 1/4th of its volume over a steam bath and cooled. The extract was then transferred to a 100 ml volumetric flask and made upto the mark with distilled water (working standard).

Procedure :

1 ml of the leaf-extract was pipetted into different test tubes and 4 ml of the anthrone reagent was added to each of this solution and mixed well. Glass marbles were placed on top of each tube to prevent loss of water by evaporation. The tubes were placed in a boiling water bath for 10 minutes, then removed and cooled. A reagent blank was prepared by taking 1 ml of water and 4 ml of anthrone reagent in a tube and treated similarly. The absorbance of the blue-green solution was measured at 680 nm in a colorimeter (Gallenkamp color spec.)

The standard curve of glucose was prepared by taking 0.0,0.1.0.2,0.4,0.6.0,8 and 1 ml of standard glucose solution in different test tubes containing 0.0 mg,10 mg, 20 mg, 40 mg, 60 mg, 80 mg and 100 mg of glucose, respectively and made the volume upto 1.0 ml with distilled water. 4 ml anthrone reagent was added to each test tube and mixed well. All the solutions were treated similarly as described above. The abosrbance was measured at 680 nm.

The amount of free sugar present in the extract was calculated from the standard curve of glucose. Finally, the percentage of free sugar present in the mulberry leaf was determined using the formula given below.

Calculation:

Percentage of free sugar (g per 100 g of mulberry leaf powder)

$$= \frac{\text{Weight of sugar obtained}}{\text{Weight of leaf powder}} \times 100$$

Reducing Sugar :

Reducing sugar content of the mulberry leaf powder was estimated by DNS (dinitrosalicylic acid) method (Miller, 1972).

Reagent:

(a) Dinitrosalicylic acid (DNS) reagent was prepared by simultaneously dissolving 1 g of dinitrosalicylic acid; 200 mg of crystaline phenol and 50 mg of sodium sulphite placed in a beaker with 100 ml of percent solution of NaOH by stirring. The reagent was stored in a stoppered bottle at 4°C. The reagent deteriorates during storage due to atmospheric oxidation of the sulphite present.

(b) Standard glucose solution (10 mg/100 ml).

(c) Rochelle salts (40% sodium potassium tartate).

Procedure :

3 ml extract was mixed uniformly with 3 ml DNS reagent in a test tube. The mixture was heated for 5 min in a boiling water bath. 1 ml of 40% Rochelle salts was added when the contents of the tubes were still warm. A reagent blank was

treated similarly. The absorbance of the solution was measured at 575 nm in a colorimeter.The amount of reducing sugar present in the extracts was calculated using the standard curve of glucose.

Calculation:

Percentage of reducing sugar (g per 100 g of mulberry leaf powder)

$$= \frac{\text{Weight of the reducing sugar obtained}}{\text{Weight of leaf powder}} \times 100$$

Starch :

The starch content of the mulberry leaf was determined by the anthrone method (Morse, 1947 and Loomis, 1937).

Reagents :

(a) Anthrone reagent (0.2% in conc. H_2SO_4).

(b) Standard glucose solution (10 mg/l 100 ml).

Procedure :

About 5 g of mulberry leaf powder was homogenized well with 10 ml of distilled water. The content was filtered through double layer of muslin cloth. The filtrate, twice the volume of ethanol was added to precipitate the polysaccharide, mainly starch. After kept it overnight in cold, the precipitate was collected by

centrifugation at 3000 rpm for 15 minutes. The precipitate was dried over a steam bath. 40 ml of 1M hydrochloric acid was added to the dried precipitate and heated to about 70°C . It was then transferred to a volumetric flask and diluted to 100 ml with 1M HCl. 1 ml of diluted solution was taken in another 100 ml volumetric flask and made upto 100 ml with 1M HCl.

Aliquot of 1 ml of the leaf-extract was pipetted into test-tubes in duplicate and treated in the same manner as described for free sugar estimation.

A standard curve of glucose was prepared and the amount of starch present in the leaf powder was calculated from the curve.

Calculation :

Percentage of starch content (g per 100 g of mulberry leaf powder)

$$= \frac{\text{Weight of starch obtained}}{\text{Weight of leaf powder}} \times 100$$

Soluble Carbohydrate :

The content of soluble carbohydrate was obtained on oven dry basis by addition, i.e.with the addition of free sugar and polysaccharide (starch).

RESULTS

3. RESULTS

A sum of trial and error were carried out searching over chemical and physical factors, culture conditions as well as nature and source of explants. Watching and experience regarding trial and error of different experiments are being narrated both interms of theoretical and statistical point of views.

3.1. Shoot Proliferation

3.1.1. Shoot proliferation ability of different explants

In order to see the organogenetic ability of different explants belonging to three genotypes BSRM-3, BSRM-18 and BSRM-19, this experiment was setup using three different basal media viz., MS, LS and B5. Three different explants, namely cotyledon, shoot apex and nodal segments belonging to three genotypes were cultured separately in the above stated media. All media contained 1mg/l BA + 30 g/l sucrose + 7.0 g/l agar. The results obtained are sumarized in table 1. Cent percent shoot proliferation was exhibited by nodal explants of BSRM-19 in MS followed by BSRM-18 in the same media while BSRM-3 displayed 81% shoot proliferation in LS media.

Cotyledonary explants of all genotypes used stimulated poorly in all the media. Exceptions were noticed only in case of BSRM-19 (59%) and BSRM-3 (51%) in MS and LS media respectively.

Frequency of shoot proliferation through shoot apex culture was better than that of cotyledonary explant. MS media was suitable followed by LS while B5 did not suit to the satisfactory level.

From the analysis of variance it is evident that, all the items viz., genotypes (G), explant (E) and media (M) were highly significant which indicates that a real differences existed among genotypes, explants and media in response to shoot proliferation.

The analysis further shows that, the genotypes (G) interacted significantly with the media (M). Similar interaction between explant (E) and media (M) was also present. However, interaction between G x E and G x M x E was nonsignificant.

3.1.2. Proliferation Efficiency of Shoot Tip and Nodal Explant From Different Genotypyes

Shoot tip and nodal explants from four genotypes namely BSRM-3, BSRM-8, BSRM-18 and BSRM-19 were cultured in MS media supplemented with 2mg/l BA. After 6 weeks of culture two parameters as, number of shoots per explant and percentage of explants producing shoots were observed and recorded in the table 2.

In general, nodal segments were found to be superior to shoot tip explants in terms of shoot number except in BSRM-18 where shoot tip explants dominated over nodal explants.

PLATE I. *In vitro* shoot proliferation of three genotypes of mulberry.

A. Multiple shoots produced from nodal explants of BSRM-19 in MS + 2 mg/l BA, after 6 weeks of culture.

B. Multiple shoots produced from shoot tip explants of BSRM-19 in MS + 2 mg/l BA, after 6 weeks of culture.

C. Shoots produced from shoot tip explants of BSRM- 8 in MS + 2 mg/l BA, after 6 weeks of culture.

D. Multiple shoots produced from nodal explants of BSRM-3 in MS + 2 mg/l BA, after 6 weeks of culture.

PLATE I

Highest number of shoots (10.5) (PLATE I.A) was obtained by culturing nodal explants of BSRM-19 followed by shoot tip explants (7.0) (PLATE I.B) of the same genotype. Lowest number of shoots (2.7) (PLATE I.C) was produced by BSRM-8 through shoot tip culture. However, nodal explants of BSRM-3 also produced good number (6.0) (PLATE I.D) of shoots.

As per frequency of explants producing shoot is concerned, BSRM-19 exhibited best performance (100%) in both explants followed by BSRM-18 and BSRM-3.

3.1.3. Effect of Different Basal Media on Shoot Proliferation

Effect of different basal media namely, 1/2 MS, MMS, MS, B5, WP, LS and N6 on shoot proliferation by culturing nodal explants from the three genotypes viz., BSRM-19, BSRM-18 and BSRM-3 were evaluated and tabulated in the table 3. Two itmes, percentage of shoot proliferation and number of shoots per culture were taken into account.

Highest shoot proliferation (91.69%) was exhibited by BSRM-19 followed by BSRM-18 (85.43%) and BSRM-3 (72.52%) in MS media. Rate of shoot proliferation was reduced to 40% and even lower than this in all the genotypes when nourished with N6 media. On the other hand, highest number (9.94) of shoots was

obtained from nodal explants of BSRM-19 followed by BSRM-18 (7.49) in MS media. Lowest number of shoots was produced in N6 and B5 media.

Analysis of variance shows that, genotype (G) differed significantly for rate of shoot proliferation whereas, they did not differ significantly in producing shoots per culture. Highly significant differences existed among the media in both the characters as the item media (M) was significant at 0.1% level.

The analysis further shows that, the genotypes and the media interacted (GxM) significantly at 5% in case of rate of shoot proliferation and 0.1% in number of shoots per culture respectively which indicated that the genotypes used responded differently in different basal media.

3.1.4. Shoot Proliferation Efficiency of Different Genotypes

In this experiment *in vitro* cloning of 7 mulberry genotypes was standardized. Nodal segments belonging to BSRM-1, BSRM-2, BSRM-3, BSRM-8, BSRM-14, BSRM-18 and BSRM-19 were cultured in MS + 1 mg/l BA + 0.5 mg/l IAA. Number of shoots per culture and shoot proliferation frequencies were noted and arranged in table 4.

The frequency of shoot proliferation was 97.0% in BSRM-19 followed by 89.3% in BSRM-18 while the lowest frequency (63.5%) was exhibited by BSRM-1.

On the other hand, where the number of shoots/culture is concerned, BSRM-19 dominated over all the genotypes followed by BSRM-18.

3.1.5. Effect of Different Cytokinins

In this experiment efficiency of different cytokinins (BA, Kn, 2iP and Zn) was standardized. Nodal explants of BSRM-19 were cultured separately in MS media each contained either of the four cytokinins at the rate of 2.0 mg/l. Results obtained are shown in table 5.

Among the four cytokinins, though the Zn was costly item, even then, BA proved to be the best for both shoot proliferation frequency as well as number of shoots per culture. Both 2iP and Zn had unsatisfactory performance while Kn was intermediate type.

3.1.6. Effect of Different Concentrations of Benzyladenine

Nodal explants from BSRM-19 were cultured on MS media supplemented with 10 different concentrations of BA (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mg/l) to findout the optimal concentration for the best cloning. The findings are tabulated in the table 6. With the increase of concentration of BA the rate of proliferation decreased and highest percentage of shoot proliferation (81.60%) was

PLATE II. Effects of different concentrations of BA singly and in combination (synergism) with Kn on shoot proliferation from nodal explants of BSRM-19.

A. Multiple shoot proliferation in MS + 2 mg/l BA after 6 weeks of culture.

B. Multiple shoot proliferation in MS + 2.5 mg/l BA after 6 weeks of culture.

C. Less number of shoots in high concentration of BA (MS + 3.0 mg/l BA) after 6 weeks of culture.

D. Highest number of shoots proliferated in MS + 1 mg/l BA + 1.0 mg/l Kn, after 6 weeks of culture.

PLATE II

recorded at the concentration of 1.5 mg/l followed by 2.0 mg/l (72.06%). Unlike rate of proliferation, highest number of shoot (8.00) (PLATE II.A) per culture was obtained in comparatively higher concentration of BA (2.0 mg/l) followed by 2.5 mg/l (7.50) (PLATE II.B). Number of shoots reduced (3.60) with the increase of BA concentration to 3.0 mg/l (PLATE II.C). Similarly concentration below 2.0 mg/l also reduced the number of shoots per culture. In case of length of shoots, comparatively lower concentration favoured growth of shoots. Highest length of shoot of 5.51 cm was achieved in media containing 1.0 mg/l followed by 5.09 cm in 1.5 mg/l of BA. Analysis of variance shows that, the item treatment (T) was highly significant for rate of shoot proliferation and number of shoots per culture while it was non-significant for shoot length (table 6).

3.1.7. Response of Nodal Explants of BSRM-18 in BA and KN

Effect of different concentrations of BA and Kn (0.5, 1.0,2.0 and 3.0 mg/l) was tested singly on shoot proliferation and recorded in table 7. Nodal explants of BSRM-18 was used as experimental material, while MS was used as basal media.

It is evident that, BA was found to be superior to KN. Shoot proliferation occured in 86-87% of culture in 1-2 mg/l BA which was the highest. Lower and higher concentrations of BA reduced proliferation rate. However, in case of Kn,

lower concentration promoted rate of shoot proliferation. In both the hormones there was no significant difference for the days taken for bud sprouting. Highest number of shoots (7.41) were produced when the basal media was supplemented with 2.0 mg/l BA. In other level of both the cytokinins the number of shoots per culture was the same. Longest shoot (5.89 cm) was achieved at 1.0 mg/l BA and in other concentrations of both cytokinins the length ranged from 3-4 cm.

Analysis of variance shows that, the item hormone (H) was highly significant in case of rate of shoot proliferation, while it was nonsignificant in other parameters. Similarly, the item concentration (C) was highly significant in case of rate of shoot proliferation as well as time taken for bud sprouting, while it was significant at 5% level in case of shoot length and nonsignificant in number of shoots per culture. The interaction item was significant only in number of shoots per culture.

3.1.8. *In Vitro* Responses of BSRM-19

Responses of nodal explants of BSRM-19 to different concentrations of BA and KN singly and in combination were tested. Results obtained are shown in table 8.

There was a significant difference between different concentrations of two cytokinins for days taken to bud sprouting. Days (17.13) required was highest in hormone free basal media. Rate of shoot proliferation was highest in 1.0 - 2.0 mg/l

of BA followed by combination of two cytokinins. BA and KN in combination (each 1.0 mg/l) produced highest number of shoots (16.22) (PLATE II.D) followed by 2.0 mg/l BA, where number of shoots per explant was 10.26. Number of shoots per culture decreased considerably when basal media was not supplemented with either of the hormones and in lower concentration of KN. Shoot length favoured lower concentrations of KN and was more than 6 cm in basal media supplemented with 0.5 or 1.0 mg/l of KN. Lowest length was obtained in hormone free MS media. However, in rest of the treatments the length of microshoots was moderate.

3.1.9. Seasonal Effect on *In Vitro* Shoot Proliferation of BSRM-19

Shoot apex and nodal segments of the genotype BSRM-19 were collected monthwise right from January' 93 to December'93 and were cultured in the respective month of collection. Culture media contained MS + 1.0 mg/l BA + 0.5 mg/l KN.

Parameters like, percentage of shoots proliferation, number of shoots per culture and length of shoots were taken into consideration for evaluation of culture response. Data collected are tabulated in the table 9.

The results show that, there was a substantial effect of explant collection season (months) on percentage of shoot proliferation. Explant type (shoot apex and

node) also displayed different response and in general nodal explants responded better over shoot apex interms of rate of shoot proliferation. Highest percentage (93%) of shoot proliferation was achieved from nodal explants during collection period of March followed by 90% and 89% proliferation in the month of February and April respectively. Highest percentage (76%) of shoot proliferation was recorded from shoot apex in the month of March followed by 73% in April and July and 72% in February. Rate of shoot proliferation gradually decreased with the advent of winter. In winter (December-January) the rate of shoot proliferation reduced to almost 50% for both the explants.

Number of shoots per culture was influenced by collection season and type of explants. Highest number of shoots (9.39) was obtained in March followed by 9.11 and 9.0 per explant in April and February respectively from the nodal explants. In case of number of shoots per explants, shoot apices were also found inferior to nodal explants, and highest number of shoots (5.22) were produced from shoot apices in July. Number of shoots per explants gradually decreased in pre-winter (November), and winter (December-January) for both the explants. In summer and rainy season (May-August), the number of shoots ranged from 5-6 shoots per explant. In late winter and spring (March-April) both the explants produced highest number of shoots per culture.

Shoot apex responded better over nodal explants in respect of shoot length. Shoot length was highest (7.11 cm) when the explants were collected in the month of May followed by 7.06 cm in June. Explant collected in late spring and summer was suitable for shoot elongation, while explant collected in winter gave poor shoot elongation.

From analysis of variance it is observed that, the items month (M), explant (E) and MxE interaction were highly significant for shoot proliferation, number of shoots per explants and shoot length. It indicated effect of month of collection of explant is important to get healthy culture growth. Explant produced different responses.

3.1.10. Effect of Collection Date of Explants From Different Genotypes.

Effect of collection date of explants of three genotypes on multiple shoot proliferation was evaluated. The medium used in the experiment was MS + 1.0 mg/l Kn + 0.5 mg/l BA.

Nodal explants were colleted from three genotypes of mulberry viz., BSRM-3, BSRM-18 and BSRM-19 in the month of February, March and April and placed on the culture media. Parameters like, number of shoots per culture, length of shoots

and degree of shoot growth were considered. Data were recorded after 6 weeks of culture and tabulated in table 10.

The result indicates that the collection date of explants had a remarkable effect on multiple shoot induction. The genotypes also varied in their response to different collection period of explants.

Highest number of shoots (9.61) was induced when nodal explants were collected in April from BSRM-19 followed by BSRM-18 (6.11) in March. Lowest number of shoots from BSRM-19 and BSRM-18 were induced in the month of February while BSRM-3 exhibited better shoot proliferation (5.43) in February. In March, BSRM-3 and BSRM-19 both produced almost same number of shoots (table 10). Similarly, BSRM-3 and BSRM-18 also produced same number of shoots in April.

Genotypes also varied in their response to shoot length. longest shoot (5.03 cm) was produced by BSRM-19 when nodal explants were collected in April followed by BSRM-3 (4.11 cm) when collected and cultured in February. The length of shoots in other genotypes during other collection period (months) was more or less same. As a whole, degree of growth was best in BSRM-19 (+ + + +) followed by BSRM-18 and BSRM-3 in the month of April. However, degree of growth was satisfactory during the Month of March while it was unsatisfactory in February especially for BSRM-18.

The results of analysis of variance indicates that, the item genotype (G) was non-significant in both the characters. However, the collection date (C) was highly significant for both the parameters. On the other hand, genotype (G) and collection date (C) interacted (GxC) significantly for number of shoots per culture and shoot length at 0.1% respectively.

3.1.11. Effects of Different Basal Media and Collection Date of Explants on Shoot Proliferation of Different Genotypes

In this experiment effects of basal media and collection date on shoot proliferation of different genotypes were evaluated. The culture media contained 1.0 mg/l BA + 0.5 mg/l Kn. Four basal media viz., MS, WP, LS and B5 and three genotypes viz., BSRM-3, BSRM-18 and BSRM-19 were tested. The only parameter, number of shoots per explant was considered in this experiment.Explants were collected on 25th of February, March and April. Data were scored after 6 weeks of culture and tabulated in the table 11.

On average, MS basal medium was proved to be the best followed by LS and WP. B5 was found to be inferior for multiple shoot induction in all the genotypes except in BSRM-3, where nodal explants cultured in February produced 4.09 number of shoots. Highest number of shoots (10.69) (PLATE III. A) was produced

PLATE III. Effect of collecting period of explants on shoot proliferation from nodal explants.

A. Highest number of shoots produced by BSRM-19 in MS + 1.0 mg/l BA + 0.5 mg/l Kn after 6 weeks of culture. Explants were collected on 25th April.

B. Multiple shoot proliferation in BSRM-19 after 6 weeks of culture in MS + 1.0 mg/l BA + 0.5 mg/l Kn. Explants were collected on 25th March.

C. Highest number of shoots produced by BSRM-3 in MS + 1.0 mg/l BA + 0.5 mg/l Kn after 6 weeks of culture. Explants were collected on 25th February.

D. BSRM-18 proliferated highest number of shoots in MS + 1.0 mg/l BA + 0.5 mg/l Kn after 6 weeks of culture. Explants were collected on 25th March.

PLATE III

by BSRM-19 when explants were collected in 25th April followed by 9.97 (PLATE III. B) when the explants were collected in 25th March and cultured in MS media. BSRM-3 produced 8.61 (PLATE III. C) shoots and BSRM-18 produced 8.03 (PLATE III. D) shoots in explant collected on 25th February and 25th March respectively.

Analysis of variance shows that, the item media (M) was highly significant against the experimental error which indicates that high differences existed among the media. No variation was observed for genotype and collection date as the items G and C were non-significant.Among the interactions only GxC was found to be significant.

3.1.12. Effect of Explant Collection Date on Shoot Proliferation of BSRM-19 in BA and KN

An experiment was conducted to see the effect of different explant collecting time on *in vitro* shoot proliferation of BSRM-19. Nodal explants were collected in the month of October, November, December, January, February, March, April, May, June and July and were cultured in 6 different media, viz.,

(i) MS + 1.0 mg/l BA + 30 g/l sucrose

(ii) MS + 2.0 mg/l BA + 30 g/l sucrose

(iii) MS + 3.0 mg/l BA + 30 g/l sucrose

(iv) MS + 1.0 mg/l KN + 30 g/l sucrose

(v) MS + 2.0 mg/l KN + 30 g/l sucrose

(vi) MS + 3.0 mg/l KN + 30 g/l sucrose

Data on days to bud sprouting, number of shoots per explant and shoot length were recorded after 6 weeks of inoculation and tabulated in tables 12 (A), 12 (B) and 12 (C).

From table 12 (A), it is evident that, there was a significant effect of collecting dates of explants as well as different cytokinins (BA and Kn) on days required to bud sprouting from nodal explants of BSRM-19.

In both the cytokinins, both explants took almost 7-9 days for bud sprouting when they were collected during October to February and in rest of the collecting months (March-July) days required for bud sprouting increased considerably. As many as 16.76 and 16.16 days were required when the explants were collected in May and cultured in 3.0 mg/l KN and 2.0 mg/l KN respectively. Analysis of variance reveals that, all the items were highly significant against the experimental error which indicating real differences existed among the months of collection (C) of explant type, hormone (H) and concentration (C). All the interactions, MxH, MxC and MxHxC were also highly significant.

Explant collection months also had tremendous effect on multiple shoot production from nodal explant cultured in different concentrations (1.0,2.0 and 3.0 mg/l) of BA and KN.

From table 12 (B) it is observed that, explants collected during the months of October-February produced higher number of shoots than those collected in other months (March-July).

Again among three concentrations of BA, 2.0 mg/l was the best for inducing highest number of shoots as this concentration yielded 10.71 and 10.55 shoots in January and February respectively. However, exceptions were also noticed in December, June and July where higher concentration(3.0 mg/l) enhanced shoot production. Lowest number of shoots were induced in explants collected during April to July and cultured in 1.0 mg/l BA. For multiple shoot proliferation BA was superior to Kn. Unlike BA, higher concentration of Kn (3.0 mg/l) was suitable for more shoot formation than lower concentration (1.0 and 2.0 mg/l).

Analysis of variance indicates that, the items months (M) and concentrations (C) were significant at 0.1% level while the hormone (H) was significant at 5% level. Among the interaction items only MxHxC was found to be significant at 5% level indicating existence of criss-cross interactions among the items.

From the table 12 (C), it is observed that, explant collection date, hormones and concentrations of BA and KN had substantial effect on shoot growth.

In most of the cases explants collected during pre-winter and winter (October-January) produced comparatively shorter height than those produced during the collection period of March-July. On average, BA was found to be superior to KN for better growth of microshoots. With the increase of concentrations of BA height of microshoots gradually reduced.

Highest length of microshoots were 46.55, 45.15 and 42.73 mm when explants were cultured in 1.0 mg/l BA and collected in the months of July, May and June respectively. However, 2.0 mg/l BA had also satisfactory response during summer (June-July).

Analysis of variance shows that, the collection months (M), hormones (H) and concentrations (C) were highly significant (0.1% level) against the experimental error. None of the interaction items was significant in this case.

3.1.13. *In Vitro* Contamination Rate of Different Genotypes in Different Months

An experiment was conducted to note the effect of explant collection months on *in vitro* contamination of mulberry microshoots belonging to three genotypes,

viz., BSRM-3, BSRM-18 and BSRM-19. All media contained MS + 2.0 mg/l BA. Nodal explants were used in this experiment. The only parameter, percentage of explant contamination was recorded after 2 weeks of inoculation.

It has been observed visually that, the micro organisms contaminated the basal portion of the explants as well as the surface of the media. The sign of contamination was visible within 1 week of inoculation. The colour of contamination was different types. Sometimes it was reddish and creamy while, other times, it was grayish. The contaminated portion of explants and the basal media were examined under high power (100 X) microscopic lense in microbiological laboratory. It was found that, most of the microorganisms belonged to *Aspergillus* and *Penicillium* species. However, rod shaped *Bacillus* was also common.

Quantitative data of this experiment were statistically analysed after angular transformation and tabulated in table 13. Highest rate of contamination was noticed during the month of July and August where 50% of the culture vessels were contaminated. Lowest contamination (%) was recorded when explants were collected in December folowed by January and February. Among the genotypes BSRM-19 showed highest survival in most of the months followed by BSRM-18 in *in vitro* condition.

Analysis of variance shows that, the only item month (M) was significant at 5% level and rest of the items including month-genotype interaction (MxG) were non-significant against the experimental error.

3.1.14. Effect of Different Aminoacids.

An experiment was conducted to observe the effect of different aminoacids of different concentrations on *in vitro* shoot proliferation of three mulberry genotypes.The aminoacids used were tyrosine, glutamine and aspergine each having three concentrations (25, 50 and 100 mg/l). A control (without amino acid) was kept to detect the effect of aminoacids. The genotypes used were BSRM-3, BSRM-18 and BSRM-19. Nodal explants and the MS media supplemented with 1.0 mg/l BA + 0.1 mg/l IAA were used in this investigation. Data recorded were analysed statistically and tabulated in the table 14.

Parameters considered were, percentage (%) of shoot proliferation, number of shoots per culture and length (cm) of shoot. From table 14 it is noted that, all the aminoacids had substantial effect on *in vitro* shoot proliferation and simultaneously all the genotypes responded differently in different aminoacids.

Higher percentage of shoot proliferation was recorded (93.5%) when explants from BSRM-19 were cultured in MS + 1.0 mg/l BA + 0.1 mg/l IAA + 50 mg/l

tyrosine folowed by BSRM-18 (88.1%) in the same media composition. In control shoot proliferation rate was 78.3% exhibited by BSRM-19.

With few exceptions (50 mg/l for BSRM-3 and 100 mg/l for BSRM-3) glutamine resulted unsatisfactory performance in case of rate of shoot proliferation.

Like rate of shoot proliferation, 50 mg/l tyrosine also induced highest (6.9) number of shoot per culture in BSRM-19. Number of shoots drastically reduced to 1.3 and 1.6 only in BSRM-18 when media were supplemented with 100 mg/l tyrosine and 50 mg/l glutamine respectively. Length of shoots was also favoured by the addition of 50 mg/l tyrosine (5.89 cm, highest).

Analysis of variance gives us a clear evaluation of the experiment. The anova table shows that, the item genotype (G), amino acid (A), concentrations (C) and the interactions, GxA, AxC and GxAxC were highly significant except GxC was non-significant for rate (%) of shoot proliferation.

3.1.15. Combined Effect of Different Cytokinins with Tyrosine

Responses of shoot apex and nodal explants of BSRM-3 were investigated by culturing on MS media in different cytokinins viz., BA, KN, 2iP and Zn (each 1.0 mg/l) with aminoacid-tyrosine. Three concentrations (20, 25 and 100 mg/l) of tyrosine were employed for all the treatments. Data were recorded after 6 weeks of culture and after statistical analysis they are arranged in table 15. Two parameters -

number of shoots and average length of shoots were taken into consideration. From table 15 it is observed that, both the explants varied in their response to different cytokinins and different concentrations of tyrosine.

For shoot tip explants, highest number of shoots produced were 2.1 and 2.0 shoots/culture in 2iP and Zn respectively where tyrosine at 100 mg/l was added. In other treatments shoot apex explants did not response satisfactorily. In all cases nodal explants were found to be superior to shoot tip explants. However, 2iP and Zn were not suitable for multiple shoot induction from nodal explants. BA suited best with 50 mg/l tyrosine to produce highest number (4.5) of shoots per culture, while BA with lower and higher (greater than 50 mg/l) concentrations of tyrosine did not induce higher number of shoots. KN exhibited best performance in combination with lower concentration of tyrosine (25 mg/l).

Both the explants resulted same length of microshoot in BA with 50 mg/l tyrosine followed by 2iP (4.1) with 50 mg/l tyrosine.

Analysis of variance shows that, in number of shoot per explant the items cytokinin (C), tyrosine (T) and explant (E) were significant at 1%, 5% and 0.1% respectively. Among the interaction items, CxT, CxE and TxE were significant.

In case of shoot length the items, cytokinin (C) and tyrosine (T) were highly significant (0.1% level).

3.1.16. *In Vitro* Shoot Proliferation of *Morus laevigata*

Morus laevigata is one of the important species of the genus *Morus* in respect of timber quality. It is very difficult to propagate this species by cutting and grafting. Therefore, an experiment was conducted to establish *in vitro* proliferation for this species.

Nodal explants were collected from 10-year-old *M. laevigata* and cultured on MS basal media supplemented with different concentraions of BA and KN, and in combination with 2,4-D and NAA. Parameters considered were days taken to bud sprouting, percentage of proliferating cultures, number of shoots per culture and shoot length (cm). Data were recorded after 6 weeks of culture have been summarized in table 16.

The result shows that, days required for bud sprouting were least in low concentraions of BA (0.5, 1.0 mg/l) followed by low concentrations of KN. With the increase of concentrations (greater than 1.0 mg/l) of BA or KN time taken for bud sprouting increased. On the other hand more than 12 days were required when cytokinins were combined with either of the auxins. Percentage of shoot proliferation was less than 70% in all the treatments employed. Highest percentage of shoot proliferation was exhibited (66.6) in 1.0 mg/l BA followed by 62.2% in 2.0 mg/l BA and 52.1% in 3.0 mg/l BA. In KN supplemented media percentage of shoot

proliferation were 51.6% and 56.2% in 2.0 mg/l and 0.5 mg/l respectively.

Highest number (8.91) (PLATE IV. A) of shoots was obtained in 1.0 mg/l BA followed by 5.71 (PLATE IV. B)in 2.0 mg/l KN. 2.0 mg/l BA also proliferated good number (5.61) (PLATE IV. C) of shoots in comparison to other treatments. In combinations of cytokinins and auxins, Kn with 2,4-D or NAA induced an average of 4 shoots per culture but the cultures in 2,4-D containing media displayed callus formation at the basal portion of the explant. Kn (1.0 mg/l) in combination with NAA (0.1 mg/l) produced 3.41 number of shoots (PLATE IV. D).

Better shoot length (5.39) was achieved in low concentration of (0.5 mg/l) BA followed by length of 5.16 cm in BA 1.0 mg/l + 2,4-D 0.1 mg/l. In other treatments shoot length was satisfactory (table 16).

Analysis of variance shows that, the item treatment (T) was highly significant for days required for bud sprouting and percentage of shoot proliferation at 1 % and 0.1% respectively.

PLATE IV. *In vitro* shoot proliferation of *Morus laevigata.*

A. Highest number of shoots proliferated from nodal explants cultured in MS + 1.0 mg/l BA, after 6 weeks of culture.

B. Multiple shoot proliferation on nodal explants in MS + 2.0 mg/l Kn, after 6 weeks of culture.

C. Multiple shoot proliferation on nodal explants in MS + 2.0 mg/l BA, after 6 weeks of culture.

D. Shoot proliferation from nodal explants in MS + 1.0 mg/l Kn + 0.1 mg/l NAA, after 6 weeks of culture.

A

B

C

D

PLATE IV

3.2. ROOT INDUCTION

3.2.1. Root Induction of *M. laevigata*

It is very difficult to propagate *M. laevigata* through cuttings due to its poor rooting ability. Therefore, an experiment was conducted to evaluate the rooting ability of *M. laevigata* in *in vitro* condition.

IBA and NAA singly and in combination were given in 1/2 MS basal media to induce roots in microshoots.

Microcuttings of the size 3-4 cm were harvested from *in vitro* proliferated shoot clumps. Healthy microshoots having 2-3 nodes were always selected for *in vitro* root induction. Data were recorded after 8 weeks of culture and are tabulated in table 17. Parameters considered were percentage of rooting, average number of roots per culture and average root length (cm).

Table 17 indicates that, medium with IBA and NAA (each 0.1 mg/l) in combination was the best for highest (85.77%) percentage of root induction followed by 1.0 mg/l IBA (82.10%). IBA was found to be superior for root induction to NAA. Less rooting was obtained with NAA in all the three concentrations tested compared to IBA supplemented medium. Formation of callus at cut bases, malformation and slow growth of roots, and smaller number of roots per microcutting were observed at

PLATE V. *In vitro* root induction on microshoots of *M. laevigata.*

A. Highest number of roots induced in ½ MS + 0.1 mg/l IBA + 0.1 mg/l NAA, after 8 weeks of culture.

B. Root initiation in ½ MS + 1.0 mg/l NAA, after 8 weeks of culture.

C. Root initiation in ½ MS + 1.0 mg/l IBA, after 8 weeks of culture.

PLATE V

NAA supplemented medium. IBA in the medium, on the other hand, promoted root length and number, and inhibited basal callusing.

Only 15% of the shoots produced roots on auxin free medium and they were ill developed and weak.

Number of roots was also higher (11.13) (PLATE V. A) where IBA and NAA given in combination (0.1 : 0.1) followed by 1.0 mg/l NAA (8.51) (PLATE V. B). IBA at 1.0 mg/l also induced good number (8.11) (PLATE V. C) of roots. Only 3-4 roots were produced by the microcutting in media where auxins were lacking.

Length of roots was enhanced by the synergistic effect of IBA and NAA (0.1 mg/l each) where average length was more than 6.0 cm. In rest of the treatments the length of roots ranged from 4-5 cm.

Analysis of variance shows that, item replication for number of roots per culture was significant at 5% level. On the other hand the item treatment was highly significant for the character percentage of root induction and number of roots per culture at 0.1% and 1% respectively which indicates that the different treatments produced significant variation on root induction from microshoots.

3.2.2. *In vitro* Rooting Response of BSRM-19

An experiment was set up to optimise the concentration of IBA for root

induction on microshoots of BSRM-19. All media contained 1/2 MS + 25 g/l sucrose while pH level was 5.5. Seven concentrations (0.1, 0.5, 1.0, 1.5, 2.0, 3.0 and 5.0 mg/l) of IBA were tested to evaluate the best concentration for root initiation. The parameters considered were mean number of roots per culture and length of roots. Data were recorded after 8 weeks of incubations. Microcuttings of the size 3-4 cm were used as the experimental material.

Results obtained are shown in table 18. It indicates that, the microshoots cultured in different concentrations of IBA exhibited different performances in regards to root induction.

Low concentrations favoured best root induction and highest number of roots (41.0) were induced in 0.5 mg/l IBA followed by (39.2) 1.0 mg/l IBA. However, 1.5 mg/l IBA also induced good number (32.6) of roots. Concentrations less than 0.5 mg/l and greater than 1.5 mg/l reduced root number per microshoot.

Length of roots was highest in 1.5 mg/l of IBA where 3.9 cm roots were induced followed by 1.0 mg/l (3.7 cm). On the other hand less than 0.5 mg/l and greater than 1.5 mg/l remarkably reduced the root length.

Analysis of variance shows that, the item replication was non-significant while the item treatment (T) was highly significant for number and length of roots at 0.1% and 1.0% level respectively.

3.2.3. Effect of Different pH Levels on Root Induction

Effect of different pH levels on *in vitro* root induction from microshoots of BSRM-19 were evaluated. For optimising pH level, microshoots having 2-3 cm in length were cultured in 1/2 MS media + 30 g/l sucrose + 6 g/l sigma agar + 1.0 mg/l IBA using different pH levels, viz., 4.0, 4.5, 5.0, 5.5, 6.0 and 7.0. For pH levels of 4.0 and 4.5 paper bridge was used instead of agar. The results obtained are summarized in table 19.

The results indicated that, different pH levels influenced root induction significantly. Percentage of root induction was highest (91.90%) at pH 6.0, followed by 87.8% at the pH 5.5. Lower and higher than 6.0 level of pH, the rate of root initiation reduced significantly. Similarly, number of roots per microcutting and root length were significantly higher at the pH levels of 5.5 and 6.0 than those induced at lower and higher pH levels (table 19).

3.2.4. Effects of Different Concentrations of Sucrose on Root Induction

Effect of different concentrations of sucrose on *in vitro* root induction from microshoots of BSRM-19 were examined and the results obtained are tabulated in table 19.

For determining the optimum sucrose concentration different concentrations, 10, 20, 25, 30, 40 and 50 g/l of sucrose were tested where pH of the medium was adjusted to 5.8. For each treatment, 1/2 MS basal media were supplemented with 6 g/l sigma agar and 1.0 mg/l IBA. Microshoots that reached upto 2-3 cm were harvested and used as experimental material.

The results indicated that, different concentrations of sucrose had substantial and significant effect on root induction. Highest rate (92.7%) of root initiation was induced at 30 g/l of sucrose followed by 40 g/l (76.5%) . More than 50% of root induction was obtained at the concentration of 25 or 50 g/l but below and higher to these concentrations, poor root induction was exhibited. Media lacking sucrose induced only 16.3% of rooting. Highest number (31.3) of roots were induced in 25 g/l sucrose followed by 22.9 number in 30 g/l sucrose. Higher and lower of these concentrations, number of roots per culture significantly reduced to a remarkable level. On contrary length of roots was enhanced at 40 g/l (4.6 cm) of sucrose followed by 30 g/l (3.9 cm).

3.2.5. Effect of Subcultures on Rooting Efficiency

An experiment was conducted to observe the root induction efficiency of microcuttings of BSRM-19 harvested from different subcultures. In all treatments

basal media (1/2 MS) was fortified with 0.1 mg/l IBA plus 25 g/l sucrose and 7.0 g/l sigma agar while pH level was 5.5. Parameters considered were frequency (%) of rooting, number of roots per microcutting and root length. Quantitative data were scored after 8 weeks of inoculation and arranged in table 20.

The result indicates that, frequency (%) of rooting was high (88.77%) among the microshoots taken from 1st subculture followed by (79.12%) 3rd subculture. Microshoots taken from 6th and onward subculture displayed lower rooting (42.41%) frequency. Similarly, number of roots gradually decreased with the increase of subculture beyond 5th passage. The number of roots per microcutting were 33.20 (PLATE VI. A), 19.56 (PLATE VI. B) and 17.23 when microcuttings were harvested form 1st, 2nd and 3rd subculture respectively. Lowest number of roots 15.60 and 13.43 were induced from shoots of initial culture and from microshoots of 7th subculture respectively which was significantly different from the root number that were induced on microcuttings harvested from 1st and 2nd subculture.

Root length was also affected by source of microcuttings from 7th and onward subculture. Highest length was exhibited (5.07 cm) by the microcutting derived from 1st subculture while that of lowest (3.03 cm) from the microshoots derived from 7th subculture.

3.2.6. Rooting Ability of BSRM-18

This experiment was conducted to observe the *in vitro* rooting ability of microshoot belongs to BSRM-18. IBA and NAA each having three concentrations (0.1, 0.5 and 1.0 mg/l) were employed singly along with 1/2 MS + 25 g/l sucrose, while pH level was 5.8. Percentage of cuttings rooted, days taken for root induction, number of roots per culture and length of root were considered in this investigation. Microshoots having 2-3 cm length were harvested from *in vitro* grown microshoots of BSRM-18 and used as the experimental material. Quantitative data were recorded after 8 weeks of culture are arranged in table 21.

Highest percentage (75.31%) of root induction was observed at 0.1 mg/l IBA followed by 74.26% at 1.0 mg/l IBA. Root initiation percentage ranged from 60-70% in NAA enriched media whereas, this percentage of initiation drastically reduced to less than 12% when the media was devoid of auxin.

There were no significant differences in days required for root initiation in different concentrations of both auxins except the treatment where auxin was lacking.

For number of roots, IBA was superior to NAA where highest number of roots (29.71) were induced when media was supplemented with 1.0 mg/l IBA followed by 0.5 mg/l IBA (17.03). In many microshoots NAA at 1.0 mg/l formed creamy hard callus at the base.

As per as the length of root is concerned IBA irrespective of concentrations used induced better root length. However, root length was satisfactory in 0.5 and 1.0 mg/l of NAA. On the other hand, length of root was only 2.27 cm in auxin free medium.

Analysis of variance indicates that, the item treatment (T) was highly significant for percentage of rooting and number of roots per culture at 0.1% and 1.0% level respectively.

3.2.7. Effect of Different Strength of MS and Auxin on Rooting of Different Genotypes

In vitro rooting ability of the genotypes BSRM-19, BSRM-18 and BSRM-3 was investigated by designing and conducting the following experiment. Different strength (1/4, 1/2 and Full) of MS, supplementation of different auxins (IAA, NAA and IBA) singly and in combination were the culture conditions for this investigation.

Microcuttings those reached upto 2-3 cm were harvested from *in vitro* grown clumps of the said genotypes and were implanted in the rooting media having different nuritional composition. For all the treatments, levels of sucrose (25 g/l) and pH (5.5) were constant. Quantitative data were recorded after 8 weeks of culture and are arranged in table 22.

The result as indicated in table 22 shows that, different strength of MS media as well as different auxins influenced remarkably root induction from *in vitro* grown microshoots of different genotypes used.

Highest percentage of root induction (79%) was exhibited by BSRM-19 in 1/2 MS plus 1.0 mg/l IBA. Full and 1/4 strength of MS did not enhance rate of root initiations as 1/2 MS did. However, in some cases, BSRM-18 and BSRM-3 induced better rooting than BSRM-19.

Average root number also varied in different genotypes, in different media compositions and in different MS strength. Highest number of roots (40.71) (PLATE VI. C) was initiated from the microshoots of BSRM-19 in 1/2 MS + 1.0 mg/l IBA followed by the same genotype (33.78) (PLATE VI. D) in 1/4 MS + 0.5 mg/l IBA + 0.5 mg/l NAA. In most of the cases, where NAA was added, little callus was formed at the base of the microcuttings simultaneously with the formation of roots. IBA 1.0 mg/l + 1/2 MS induced 40.71 (PLATE VI. C), 31.09 (PLATE VI. E) and 25.33 (PLATE VI. F) number of roots from the microcuttings of BSRM-19, BSRM-18 and BSRM-3 respectively. Number of roots was reduced in all genotypes when microshoots were implanted onto the full and 1/4 strength of MS media. Different hormonal treatments as well as MS strength did not have remarkable effect on root length of different genotypes.

Analysis of variance shows that, the item replication was significant (5% level) for root length. The item hormone (H) was highly significant for percentage and number of roots (0.1%) and also for root length (5% level). The item MS strength (S) was highly significant for rate of root induction as well as number of roots while it was non-significant for root length. The item genotype (G) was highly significant (0.1% level) for percentage of root initiation and root number and also significant (5% level) for root length. The item hormone and MS strength interaction (HxS) was significant for percentage of root induction (5%) and number of roots (1% level) while for length of root it was non-significant. Interaction between the item hormone (H) and genotype (G) (HxG) was significant (5% level) for percentage of root initiation and root length. SxG interaction was non significant for all the parameters. On the other hand,HxSxG interaction was highly significant (0.1%) for number and length of roots against the experimental error.

PLATE VI. Effect of subculture, different strength of MS, single and combined effect of auxins on root induction from microshoots of three mulberry genotypes.

A. Highest number of roots induced on microshoots of BSRM-19 harvested from 1st subculture in ½ MS + 0.1 mg/l IBA, after 8 weeks of culture.

B. Root induction on microshoots of BSRM-19 harvested from 2nd subculture in ½ MS + 0.1 mg/l IBA, after 8 weeks of culture.

C. Root induction on microshoots of BSRM-19 cultured in ½ MS + 1.0 mg/l IBA, after 8 weeks of culture.

D. Root induction on microshoots of BSRM-19 cultured in ¼ MS + 0.5 mg/l IBA + 0.5 mg/l NAA, after 8 weeks of culture.

E. Root induction on microshoots of BSRM-18 in ½ MS + 1.0 mg/l IBA, after 8 weeks of culture.

F. Root induction on microshoots of BSRM-3 in ½ MS + 1.0 mg/l IBA, after 8 weeks of culture.

PLATE VI

3.3. Acclimatization and Performance of In Vitro Derived Plantlets in Soil and Nature

3.3.1. Initial Establishment :

The first step of acclimatization was taken out of the test tube containing rooted plantlets from the culture room and kept them in normal temperature and humidity for about one week. This technique was very useful for hardening of roots and also enhanced the adaptability of plants at the the very initial stage of transfer from artificial to natural environment. After one week the roots of *in vitro* derived plantlets became light brown. At this stage the plantlets were ready for transfer into the soil.

Rooted microcuttings were initially planted in polybag containing non-sterile garden soil and compost (1:1). The transferred plantlets were covered with polythene bags for one week to keep the air saturated, with 16 h light and 8 h darkness. The plantlets were sprayed (twice in a week) with water and fungicide (Captan 0.1%) for the first month of transfer. Within 6-8 weeks of transplantation in polybags, the *in vitro* derived plantlets reached upto 10-14 inches height from the soil level (PLATE VII. A). Variation among the genotypes in terms of shoot length was also noticed.

After a few days of indoor acclimatization, the plantlets from polybags were transplanted directly in the field. It was observed that about 90 % of the planted cuttings survived during initial establishment. Transplation from *in vitro* to *ex vitro* condition was done throughout the year. It was observed that prevailing atmospheric condition had no effect on initial survival of the transplanted plants. However, plants transplanted during warm humid condition showed necrotic lesson on their leaves and shoot tips. Temporary wilting of plants was also common during initial period of transplantation. Necrosis and temporary wilting generally did not affect the survival of the plantlets. Neverthless, there was no difference in survibility of the transplanted plants among their sources of origin. However, survival of the plantlets on soil varied with duration of proliferation period. Plantlets originated from first to third harvest of microshoots showed the highest rate (90%) of survibility on soil. Whereas, survibility of plantlets decreased (not less than 50%) when microshoots were harvested from 5th or onward subcultures.

3.3.2. Adaptability and Survival Percentage of Different *In Vitro* Derived Mulberry Genotypes in Soil :

Five different genotypes of mulberry viz., BSRM-3, BSRM-8, BSRM-17, BSRM-18 and BSRM-19 were successfully transferred in soil and nature. These transplanted plants resumed growth and grew vigorously within three months. After

one year of transplantation in soil, survival percentage were 70, 52, 63, 80 and 82 for the genotypes BSRM-3, BSRM-8, BSRM-17, BSRM-18 and BSRM-19 respectively (table 23).

Comparison of morphogenic and nutritional status of leaves of *in vitro* and cutting derived plants were studied in the present investigation.

PLATE VII. Acclimatization of *in vitro* derived mulberry plants.

A. *In vitro* derived mulberry plantlets in polybag, 3 months after transplantation.

B. *In vitro* derived mulberry plants in field after 1-year of transplantation.

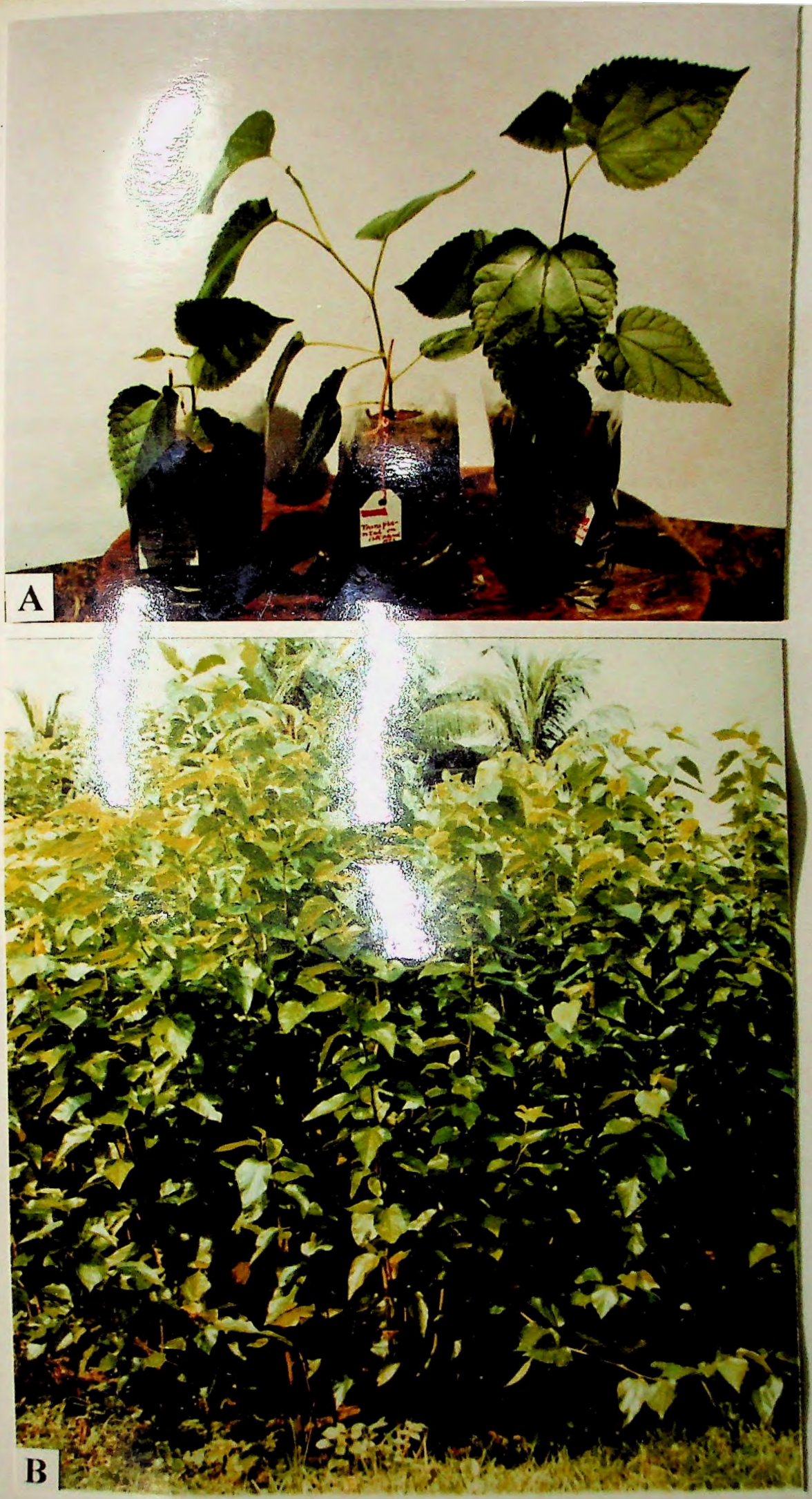


PLATE VII

3.4. Morphological and Nutritional Comparison of *In Vitro* Derived and Cutting Derived Plants

The *in vitro* raised mulberry plants belongs to the genotype BSRM-1, BSRM-3, BSRM-8, BSRM-17, BSRM-18, and BSRM-19 were acclimated successfully. Within 6 months of transplantation the transplanted plants exhibited vigorous growth and resumed juvenile twigs and leaves. After one year of transplantation these *in vitro* raised plants (PLATE VII. B) were compared morphologically and bio-chemically with the cutting raised plants of the same age.

3.4.1. Morphological comparison:

For morphological comparison following parameters were considered:

a. Plant height

b. Leaf number per plant.

c. Single leaf area

d. Weight of 100 leaves and

e. Internodal distance.

Results of morphological comparison of the genotypes BSRM-1,BSRM-3, BSRM-8, BSRM-17, BSRM-18 and BSRM-19 from both the sources (*in vitro* and cutting) are summarised in the table 24.

Plants height : In most cases, tissue culture derived plants exhibited more height which are significantly differed from cutting derived plants. highest height (99.6 cm) was displayed by BSRM-1 followed by BSRM-19 (98.7 cm) and BSRM-3 (97.1 cm) in tissue culture derived plants while the respective cutting derived plants had the height of 87.7, 92.4 and 85.2 cm respectively. However, BSRM-8 and BSRM-18 cutting derived plants dominated over tissue cultured plants in terms of height.

Leaf Number: Unlike plant height, leaf number/branch was high in cutting derived plants of the genotype BSRM-8 and BSRM-18 but is rest of the genotypes tissue cultured plants produced highest number of leaves in comparison to cutting derived plants (table 24).

Leaf area: Leaf area of both cutting and tissue culture derived plants was more or less similar. Highest leaf area was exhibited by tissue culture derived plants (71.4 mm) of the genotype BSRM-1 followed by the cutting derived plants (70.3 mm) of the same genotype. Minimum leaf area was displayed by BSRM-8 in both tissue culture derived and cutting derived plants (table 24).

Weight of leaves: As the leaves of tissue culture plants were thicker than those of conventionally grown plant, obviously weight of tissue culture derived leaves were greater than the cutting derived leaves. One exception was found in BSRM-8, where weight of 100 leaves was 69.9g in cutting derived plants while it was 62.8g in tissue cultured plants of the same genotype. In rest of the genotypes weight of leaves from tissue culture plants was greater than cutting derived plants (table 24).

Internodal distance: Internodal distance was more in tissue culture derived plants but there were also some exceptions. Internodal distance was maximum (4.97 cm) in tissue culture derived plants of the genotype BSRM-18 followed by cutting derived plants of the same genotype. Analysis of variance shows that, the only item replication for leaf area was significant at 5% level and rest of the factors were non-significant. Item genotype(G) was highly significant (0.1%) for plant height, leaf area and internodal distance. The item origin (O) was significant for plant height (5%), leaf number (1%) and for weight of leaves (5%). Analysis of variance further shows that, genotype-origin interaction (G x O) was also significant for the parameter plant height (1%), leaf number (5%) and leaf area (1%).

3.4.2. Nutritional Comparison

3.4.2.1. Comparison of nitrogen, potassium, phosphorus and magnesium content

A comparative study was carried out to measure the percentage of nitrogen (N), potassium (K), phosphorus (P), calcium (C) and magnesium (Mg) in the leaves of tissue culture and cutting derived plants of the genotypes BSRM-1, BSRM-3, BSRM-8, BSRM-17, BSRM-18 and BSRM-19. Results obtained are tabulated in table 25.

Nitrogen: Highest percentage of nitrogen (3.96%) was obtained in tissue culture derived plants of BSRM-17 followed by BSRM-19 (3.69%). In most of the cases *in vitro* derived plant possessed higher nitrogen in comparison to cutting derived plants.

Potassium: *In vitro* raised plants of BSRM-19 were rich in potassium (2.16%) whereas, cutting derived plants of the same genotype had lower amount of potassium (0.15%). In BSRM-3 and BSRM-8, percentage of potassium was more or less same in both tissue culture and cutting derived plants. On the other hand in BSRM-1, BSRM-17 and BSRM-18 potassium percentage was slightly higher in *in vitro* raised plants.

Phosphorus: Amount of phosphorus was almost same in BSRM-17, BSRM-18 and BSRM-19 whereas, BSRM-3 and BSRM-8 had higher amount of phosphorus when the plants were derived through *in vitro* method. On the other hand cutting derived plants of the same genotypes had poor amount of phosphorus.

Calcium: Percentage of calcium did not differ in BSRM-1, BSRM-3 and BSRM-8 in both cutting and *in vitro* derived plants. However, BSRM-19 of *in vitro* derived plants possessed higher amount of calcium (2.56%) while cutting derived of the same genotype had only 0.61 percent of calcium. *In vitro* derived plants of BSRM-17 and BSRM-18 had also more amount(%) of calcium than those of cutting derived plants (table 25).

Magnesium: Availability (%) of magnesium was almost same between the *in vitro* derived and cutting derived plants of the genotypes used. Analysis of variance shows that, replication item for all the factors was non-significant. The item genotype (G) was highly significant (0.1%) for nitrogen and significant (5%) for magnesium and for other element it was no-significant. Item origin (O) was non-significant for all the nutrients. However, genotype- origin interaction (G x 0) was significant (5%) only for magnesium and for other nutrient it was nonsignificant.

3.4.2.2. Comparative nutritional status at different stages of leaf maturity in different seasons

Nutritional and bio-chemical status of leaves (moisture, carbohydrate, reducing sugar, total sugar, protein and minerals) of tissue culture and cutting derived plants of the genotype BSRM-19 were evaluated and results are summarized in tables 26-31. Leaves were categorized into 'tender' (leaves present at the apical portion of the twig) 'mid-mature' (leaves present at the middle portion of the twig) and 'mature' (leaves present around the basal portion of the twig) which were collected in three seasons, viz., winter, summer and rainy.

Moisture: Comparative analysis of moisture is tabulated in table 26. Analysis of variance shows that the item replication was non-significant while seasons (S) and leaf maturity (M) items were highly significant at 0.1% level. Among rest of the items, season-maturity interaction(S x M) was highly significant (0.1%) against the experimental error. The item origin (O) was non-significant indicates that, the difference between the cutting and tissue culture derived plants was very little. Generally tender leaves during rainy season retained highest percentage of moisture. Percentage of moisture decreased during summer in mature leaves from cutting derived plants (51.4%) followed by tissue culture derived plants (51.8) (table 26).

Percentage of moisture was comparatively higher in winter than summer and there existed little differences between cutting and tissue culture derived plants.

Carbohydrate: Unlike moisture, percentage of carbohydrate was highest during summer season followed by winter. Analysis of variance shows that, the item replication, seasons (S), and maturity (M) were highly significant and a real difference was existed among the seasons and leaf maturity. The item origin (O) was nonsignificant indicates carbohydrate content was more or less same in both cutting and tissue culture derived plants. Among the interactions, S x M (season x maturity) was highly significant (0.1%) while the other interactions were non-significant against the experimental error. Highest percentage of carbohydrate (27.9%) was found in mature leaves of cutting derived plants during summer and winter while tissue culture derived plants had 27.6% (table 27). Tender leaves were found to have low percentage of carbohydrate. Howevere, tissue culture derived plants possessed 24.4% of carbohydrate in tender leaves during summer.

Reducing Sugar: Leaf maturity was immaterial for reducing sugar content since tender, mid-mature and mature leaves contained almost equal amount (%) of reducing sugars. On the other hand, percentage of reducing sugar varied with the

change of seasons. Leaves of both cutting and tissue culture derived plants contained higher amount of reducing sugars during summer season. Hihgest percentage of reducing sugar (13.8%) was found in mature leaves of tissue culture derived plants during summer. Reducing sugar reduced during rainy season while in winter it was intermediate (table 28). Analysis of variance shows that, all the items were non-significant except the item season (S) which was significant at 1% level.

Total Sugar: Like reducing sugar, total sugar was higher during summer and in general mature leaves contained slight higher amount (%) of total sugar. Analysis of variance shows that, among all the items only seasons (S) and leaf maturity (M) were significant at 0.1% and 5% respectively. Rest of the items were nonsignificant. The item origin (O) was non-significant which indicates that, tissue culture and cutting derived plants had no significant difference as per as the total sugar is concerned. Highest percentage (16.0%) of total sugar was present in mature leaves of tissue culture derived plants in summer followed by mature leaves of cutting derived plants in the same season. Amount (%) of total sugar was low in the leaves (tender & mid-mature) of cutting derived plants in the rainy season but the amount was higher in tissue culture derived plants (table 29).

Protein: Percentage of protein was higher in tender leaves followed by mid-mature while it was low in mature leaves. Analysis of variance shows that, the items seasons (S) and leaf maturity (M) were highly significant (0.1%) while rest of the items were non-significant. It indicates that real differences exist among the seasons and leaf maturity. Since, the item origin (O) was non-significant, it indicates no differences exist among the cutting and tissue culture derived plants. Winter and rainy seasons produce comparatively lower amount (%) of protein. Higher amount of protein (32.6%) was formed in tender leaves of tissue culture derived plants in summer followed by cutting derived plants (32.1%) of the same season (table 30). Lower amount (%) of protein (20.8%) was formed in the leaves of cutting derived plants in rainy seasons while the amount increased in the mature leaves (22.2%) of tissue culture derived plants in the same season. It is also evident that, in different types of leaves and in all seasons, the percentage of protein was little bit higher in the leaves of tissue cultured plants. However, the difference was non-significant.

Mineral: Comparison of mineral in different aged leaves of cutting and tissue culture derived plants was evaluated and tabulated in the table 32. From the table it is evident that, there was a remarkable effect of different seasons on the available of minerals in the leaves. Analysis of variance indicated that, the item season (S) was

significant at 5% level while the item origin (O) was highly significant (0.1%). It also indicated that, a real difference exists in respect of minerals among cutting and tissue culture derived plants. All other items were nonsignificant (table 31). In rainy season mineral content of leaves was higher and higher percentage of mineral was found in the mature and mid-mature leaves of cutting and tissue culture derived plants (22.2% and 22.1% respectively). Generally tender leaves contained low amount (%) of minerals. In summer the availability of minerals was low and consequently 13.3% of mineral was found in the tender leaves of cutting derived plants while it was increased a little bit (13.8%) in tender leaves of tissue culture derived plants (table 31). In winter, mineral content of different aged leaves of both cutting and tissue culture derived plants was higher than that of summer but little less than rainy season.

3.4.2.3. Comparative nutritional status of the leaves of different genotypes in different seasons

Comparative nutritional status of leaves of *in vitro* regenerated soil established plants with that of cutting derived plants of four genotypes, viz. BSRM-3, BSRM-8, BSRM-18 and BSRM-19 was evaluated. After one year of transplantation in the field, leaves from two sources were collected randomly (mixture of tender, mid-

mature and mature) and analysed biochemically. Three seasons viz. winter, summer and rainy were also considered to experience the effect of seasons on formation and accumulation of protein, mineral, reducing sugar, total sugar, soluble carbohydrate, starch, and moisture.

Protein: Results of protein analysis are summarized in table 32. Biochemical analysis shows that, accumulation of protein was higher during summer season in leaves of BSRM-18 and BSRM-19. Further highest percentage of protein (29.7%) was found in leaves of *in vitro* derived BSRM-19 followed by cutting derived BSRM-19 (29.4%) in the same season. However, in rainy season leaves of both cutting and *in vitro* derived plants of all genotypes possessed lower amount (%) of protein. Low percentage of protein (22.2%) was found in leaves of *in vitro* derived BSRM-3 followed by cutting derived BSRM-3 (22.3%) of the same season. In winter 28.3% of protein was found in leaves of *in vitro* derived BSRM-19.

Analysis variance reveals that, the items genotype (G) and seasons (S) were highly significant (1%) indicating differences existed among the genotypes and seasons. Among the interactions, only genotype-origin (G x O) was significant (0.1%). The items origin (O) was nonsignificant which indicates that the amount of protein in cutting and *in vitro* derived plants was the same.

Mineral: The results on nutritional status for mineral in leaves of cutting and *in vitro* derived genotypes of BSRM-3, BSRM-8 BSRM-18 and BSRM-19 are arranged in table 33. Analysis of variance shows that, among all the items, the item genotype(G) was significant at 5% level which means a real difference exists among the genotypes. The item season (S) was highly significant (0.1%) which reveals that there was a significant differences exist in different seasons on mineral formation in leaves. Rest of the items were nonsignificant. Among all other items, the origin (0) was nonsignificant which indicates that,no real difference existed for mineral among the genotypes originated through cutting and *in virtro* system. Mean table indicates that, unlike protein, minerals formed highest during rainy season followed by winter. However, summer was not so suitable for mineral accumulation in leaves. Leaves of cutting derived BSRM-8 and BSRM-3 were rich in minerals (24.4% and 24.3% respectively) in rainy season while the leaves of same genotypes in same season derived from *in vitro* system had the minerals of 23.8% and 23.6% respectively. In winter mineral percentage ranged from 18 - 20% approximately in both cutting and *in vitro* derived genotypes but leaves of *in vitro* derived BSRM-3 possessed higher amount (%) of mineral (22.3%). Lowest percentage of mineral was found in leaves of cutting derived BSRM-18 during summer. However in other genotypes, originated from cutting and *in vitro* system, mineral deposition decreased (table 33).

Reducing Sugar: Bio-chemical analysis of reducing sugar of different genotypes (BSRM-3, BSRM-8, BSRM-18 and BSRM-19) originated through cutting and *in vitro* system in different seasons (winter, summer and rainy) are summarized in the table 34. Season had substantial effect on reducing sugars in leaves of different genotypes. Leaves of different genotypes were rich in reducing sugar during summer followed by winter. Analysis of variance indicated that. the items genotype (G) and seasons (S) were highly significant which means there exist a real difference among the genotypes and seasons. Other items were non significant. No real difference was existed among the cutting and *in vitro* derived plants for reducing sugar since the item origin (O) was nonsignificant. Highest percentage of reducing sugar (12.1%) was available in leaves of *in vitro* derived BSRM-18 followed by 12.0% in leaves of cutting grown plants of BSRM-18 in summer. In winter leaves of *in vitro* originated BSRM-19 had 10.9% of reducing sugar while cutting grown plants had 10.8% (table 34). Minimum amount (%) of reducing sugar was available during rainy season and it was only 5.7% in leaves of cutting grown plant of BSRM-3 while in the same season and in the same genotype the amount slightly increased (6.3%) when raised through *in vitro* systems. In rainy season highest percentage of reducing sugar was found in *in vitro* raised BSRM-19 and BSRM-18 (8.9% and 8.7% respectively) (table 34).

Total Sugar: Comparative nutritive value for total sugar in different seasons (winter, summer and rainy) of different genotypes (BSRM-3, BSRM-8 BSRM-18 and BSRM-19) raised trough cutting and *in vitro* system was investigated. The results obtained for total sugar are summarized in the table 35.

Analysis of variance shows that, the item genotype (G) was significant at 5% level while the item season (S) was highly significant (0.1%) indicates that, a real difference existed among the seasons and the seasons had remarkable difference for availability of total sugar in leaves of different genotypes. The item origin (O) was non-significant which reveals that, there was no real difference between the cutting and *in vitro* derived plants for presence of total sugar in leaves. All other interactions were nonsignificant.

Bio-chemical analysis shows that, like reducing sugar, total sugar formed in higher percentage during summer followed by winter. During rainy season both cutting and *in vitro* derived plants had comparatively lower amount (%) of total sugar. However only exception was found in the leaves of *in vitro* raised plants (BSRM-19) where the amount of total sugar was 10.5% in rainy season (table 35). Highest percent (15.9%) was found in leaves of *in vitro* raised BSRM-19 in summer followed by (15.7%) cutting raised plants of BSRM-18. *In vitro* raised BSRM-8 had 14.9% of total sugar in summer where as cutting raised of the some genotype had comparatively lower amount of total sugar (11.7%) in the same season.

Soluble Carbohydrate: The results obtained for soluble carbohydrate for comparing cutting and *in vitro* raised genotypes of BSRM-3, BSRM-8, BSRM-18 and BSRM-19 are tabulated in the table 36. Analysis of variance shows that, the items genotype (G) and season (S) were highly significant (0.1%). This confirms that, among the genotypes a real difference exists and they differed from each other as per as the accumulation of soluble carbohydrate is concerned. The seasons also varied significantly as they had different influence on the availability of soluble carbohydrate in leaves. The item, origin (O) was nonsignificant indicating no real difference existed between the cutting and *in vitro* derived plants.

From the table 36, it can be assumed that, during summer formation of soluble carbohydrate was maximum in leaves of different genotypes and highest percentage of soluble carbohydrate (29.6%) was found in leaves of cutting derived BSRM-18 followed by *in vitro* derived plants (29.3%) (table 36). In winter highest percentage (27.8%) of soluble carbohydrate was found in *in vitro* raised BSRM-19, whereas, the same genotype in the same season had 26.3% of soluble carbohydrate (table 36). Soluble carbohydrate content was low in rainy season and minimum amount (19.1% and 19.5%) was found in leaves of cutting derived and *in vitro* derived plants of BSRM-3 respectively table 36.

Starch: Comparative nutritive value for starch in leaves of cutting and *in vitro* grown genotypes of BSRM-3, BSRM-8, BSRM-18 and BSRM-19 are shown in the table 37. Analysis of variance shows that, the item genotype (G) was significant (5% level), which means a difference existed among the genotypes considered. A real difference prevailed among the seasons and they differed in regard to the accumulation of starch in leaves of different genotypes, since the item season (S) was highly significant. The item origin (O) was non-significant which indicates, there was no real difference among cutting and *in vitro* grown plants in respect of starch. Anova table further shows that, the other items were nonsignificant. Like soluble carbohydrate, starch content was higher in leaves of different genotypes during summer. Highest percentage of starch (24.5%) was noted in *in vitro* derived BSRM-18 followed by cutting derived BSRM-18 (24.4%). In rainy season availability of starch was low in most of the genotypes whether derived from cutting or *in vitro* system. However, cutting grown BSRM-18 had the amount of 20.8% of starch in leaves during rainy season. Lower percentage of starch (17.0% and 17.1%) was found in leaves of *in vitro* and cutting derived BSRM-3 respectively during rainy season (table 37).

During winter, cutting and *in vitro* derived BSRM-3 and BSRM-8 had comparatively lower amount (18-19%) of starch while BSRM-18 and BSRM-19 possessed comparatively higher amount.

Moisture: The results obtained for moisture available in leaves of cutting and *in vitro* derived plants of the genotype BSRM-3, BSRM-8, BSRM-18 and BSRM-19 are shown in table 38. Analysis of variance indicated that, the item season (S) was highly significant (o.1%) which indicates that, a real difference exist among the seasons in respect % of moisture. Other items were nonsignificant except the item genotype(G) -season(S) -origin(O) interaction which was highly significant (0.1%). Since the item origin(O) was non-significant which indicates that there exist no real difference among the cutting and *in vitro* derived plants.

Percentage of moisture was high during rainy season. Highest percentage of moisture (62.8%) was found in leaves of cutting grown BSRM-3 followed by BSRM-19. *In vitro* raised BSRM-3 and BSRM-19 had the moisture percentage of 62.1% and 61.3% respectively (table 38).

In summer, moisture content for all the genotypes decreased and did not exceed 55% (table 38). Lowest percentage of moisture was available in leaves of *in vitro* grown BSRM-8 (51.2%) while the cutting grown had 51.4% in the summer.

In winter, moisture percentage was higher than that of summer. In all genotypes moisture percentage was more than 58%. *In vitro* derived BSRM-18 showed 61.7% moisture in winter while cutting grown BSRM-18 had 59.1% in the same season.

3.5. Somatic Embryogenesis

A callus consists of an amorphous mass of loosely arranged thin-walled parenchyma cells arising from the proliferating cells of the parent tissue. The most important characteristic of callus, from a functional view point, is that this abnormal growth has the potential to develop normal roots, shoots and embryoids that can form plants. Some callus growths are heavily lignifield and hard in texture, whereas, others break easily into small fragments. Fragile growths that readily separate termed "friable" cultures. Callus may be appear yellowish, white, cream, brown, green, or pigmented with authocyanin.

An investigation was carried out using different explants (cotyledon, leaf segment, internodal segment) of mulberry for induction of callus and somatic embryoids. The explants were cultured on MS medium supplemented with different concentrations of auxins, 2,4-D and NAA (0.5, 1.0, 2.0 and 3.0 mg/l). Different explants responded differentially in different hormonal treatments. Data on percentage of explants formed callus, nature of callus and formation of somatic embryo like structures (SELS) were recorded in this investigatons. Morphogenic responses of cotyledon, internodal segment and leaf segment for BSRM-19 were observed and tubulated in tables, 39-42.

3.5.1. Morphogenic responses of cotyledonary explants of BSRM-19

Seeds of BSRM-19 were allowed to germinate aseptically in MS media without any phytohormone. Well developed expanded cotyledones were excised and cultured in MS

media supplemented with different concentrations (each 0.5, 1.0, 2.0 and 3.0 mg/l) of 2,4-D and NAA singly. However, cotyledonary explants were also cultured in MS media supplemented with the combinaton of auxin (2,4-D and NAA each 1.0 mg/l) and cytokinin (BA and KN each 0.1 mg/l).

Morphogenic display of cotyledonary explants is summarized in table 39. Of all the media, no where cotyledonary explant induced 50 percent callus. Highest percentage (47%) of callogenesis was recorded in media having combination of NAA and KN (1.0 and 0.1 mg/l respectively) followed by (41%) in 1.0 mg/l 2,4-D + 0.1 mg/l BA. Without cytokinin, auxin (2,4-D or NAA) alone at any concentration could not induce even upto 35 percent of callus from cotyledonary explant. Lower as well as higher concentrations of auxins deteriorated callus induction. Percentage of callus reduced upto 19% when media was fortified with 3.0 mg/l NAA. Root initiation was also observed simultaneously with the induction of callus when 0.5 or 1.0 mg/l NAA was added. Callus induced in 2,4-D enriched media were cream colour and compact in nature. NAA at low concentration (0.5 and 1.0 mg/l) produced light brown amorphous callus while at high concentration (2.0 and 3.0 mg/l) the colour was brown and the texture remained amorphous.

Friable callus (PLATE VIII. A) was visible when BA was added in auxin supplemented media. However, amorphous callus (PLATE VIII. B) was induced when BA was replaced with KN. No sign of somatic embryo like structure (SELS) was visible on cotyledon derived callus in any of the media tested.

3.5.2. Induction of callus on internodal segments of BSRM-19

The same (employed for cotyledonary explants) auxins and cytokinins in same concentration and combinations were used for callus induction on internodal segments. Morphogenic responses were arranged in table 40. Internodal segments were excised from *in vitro* grown shoots. Auxin singly and in combination with cytokinins had substantial effect on callus induction. Percentage of callus induction was more than 60% in 2,4-D enriched media while in high contraction (3.0 mg/l) rate of callogenesis declined to 47 percent. Like cotyledonary explant, internodal explants also produced roots simultaneously with the formation of callus when treated with NAA alone but high concentration of NAA (3.0 mg/l) did not induce roots.

Percentage of callus induction was higher when auxin was combined with cytokinin. Higher percentage of callus induction (98%) was exhibited when internodal explants were cultured in MS supplemented with 1.0 mg/l 2,4-D + 0.1 mg/l BA. However, NAA (1.0 mg/l) or in combination with either BA (0.1 mg/l) or KN (o.1 mg/l) induced 79% of callus from internodal explants. On the other hand, KN (0.1 mg/l) in combination with 2,4-D (1.0 mg/l) also induced 74% of callus (table 41). Except 3.0 mg/l NAA other concentrations (0.5, 1.0 and 2.0 mg/l) induced roots from internodal explants.

Like cotyledonary explants, 2,4-D alone could induce brown and compact callus whereas, cream amorphous and yellowish amorphous callus was exhibited from internodal explants in different concentrations of NAA. Auxin in combination with cytokinin

displayed different nature of callus. 2,4-D and NAA in combination with BA induced green or light green friable callus. Loose and brown coloured callus was induced when KN was added with auxin.

Unlike cotyledonary explants formation of somatic embryo like structure (SELS) was visible in media having 1.0 mg/l 2,4-D + 0.1 mg/l BA (PLATE VIII. C) and 1.0 mg/l 2,4-D + 0.1 mg/l KN. In rest of the media composition SELS was not formed. These somatic embryo like structure (SELS) were oval or bottle shaped and were cream and light brown in colour.

With the help of sterio microscope the SELS(S) were excised aseptically and they were further subcultured in MS, LS, N6 and B5 media supplemented with different concentrations and combinations of auxins and cytokinin (data not shown). Unfortunately, no where SELS germinated into microshoot. Further experiments were conducted separately by adding adenine sulphate (Ads), different organic supplements like yeast extract, caseinhydrolysate for successful germination of (SELS) that formed by culturing internodal segments of the genotype BSRM-19 on MS + 1.0 mg/l 2,4-D + 0.1 mg/l BA. Moreover, aminoacids like aspergine, glutamine and tyrosine were added in different concentrations (25, 50 and 100 mg/l) in the above mentioned hormonal combination to accelarate the germination of SELS (data not shown) but no where even a single germination of SELS was noticed. The somatic embryo like structures (SELS) became necrotic and ultimately died within 2-3 weeks of subculture in all cases.

3.5.3. Morphogenic response of leaf explants

Leaves were plucked from *in vitro* grown plants of the genotype BSRM-19 and they were chopped to make convinient size (2-3 mm) and placed aseptically onto the media that were employed to experience the morphogenic effect of cotyledonary and internodal explants.

Morphogenic responses of leaf explants of BSRM-19 in touch with different auxin cytokinin enriched media were summarised in the table 41. Highest percentage (67%) of callogenesis was noticed in NAA (1.0 mg/l) supplemented media, while more than 1.0 mg/l or less than 1.0 mg/l NAA, percentage of callus induction reduced (table 42). Nature of callus produced through NAA supplementation in all concentrations were cream and friable. However, NAA (1.0 mg/l) in conjunction with BA (0.1 mg/l) or KN (0.1 mg/l) produced greenish friable callus (PLATE VIII. D). On the other hand 2,4-D alone produced more than 50% callus from the leaf explants except, high concentration (2,4-D 3.0 mg/l) deteriorated percentage of explants formed callus. Unlike NAA, 2,4-D induced brown and compact callus whereas, 2,4-D in combination with cytokinin displayed greenish callus which were amorphous in nature. Addition of cytokinin (BA or KN) in 2,4-D supplemented media enhanced the rate (%) of callogenesis but like internodal explants leaf explants could not able to produce any somatic embryo like structure (SELS) (table 41).

Callus from all mentioned media composition was dissected aseptically and subcultured in fresh media having different hormonal combination of cytokinins and auxins. Some of the media were lacking auxins. Besides, organic supplements (yeast extract, caseinhydrolysate etc.), aminoacids (glutamine, aspergine and tyrosine) were also incorporated for the proliferation of somatic embryo and ultimately the results were similar to cotyledonary explants and the calli were without any somatic embryo like structure. (data not shown).

3.5.4. Morphogenic response of internodal segments of 5 mulberry genotypes in MS + 1.0 mg/l 2,4-D + 0.1 mg/l BA

Since somatic embryo like structure (SELS) was formed in internodal explants of BSRM-19 only on MS + 1.0 mg/l 2,4-D + 0.1 mg/l BA, this reliable combination was used in this experiment. Consequently 5 other genotypes of mulberry (BSRM-1, BSRM-3, BSRM-8, BSRM-17 and BSRM-18) were also tested by culturing internodal segments on the above mentioned media composition. The results obtained are arranged in the table 42. Percentage of callogenesis was higher in BSRM-18 and BSRM-17 (67% and 63% respectively). In other 3 genotypes rate of callus induction was less than 50%. In BSRM-18, the texture of callus was friable while in other genotypes, it was compact. BSRM-1 and BSRM-3 induced light brown calli while cream coloured calli were induced only on explants of BSRM-18. Out of 5 genotypes, only BSRM-18 produced (1-2 SELS /explant)

somatic embryo like structure (SELS) (PLATE VIII. E). The SELS were separated aseptically and subcultured in different media composition (employed for culturing and subculturing internodal segments of BSRM-19) for their germination. In all treatments the SELS could not germinate and eventually became necrotic after 2-3 weeks of subculture (data not shown).

PLATE VIII. Response of different explants of mulberry (BSRM-19 and BSRM-18) in auxin-cytokinin combination.

A. Formation of friable callus on cotyledonary explants of BSRM-19 in MS + 1.0 mg/l 2,4-D + 0.1 mg/l BA after 4 weeks of culture.

B. Induction of amorphous callus on cotyledonary explants of BSRM-19 in MS + 1.0 mg/l 2,4-D + 0.1 mg/l Kn, after 4 weeks of culture.

C. Formation of somatic embryo like structure (SELS) simultaneously with the induction of callus on internodal explants of BSRM-19 in MS + 1.0 mg/l 2,4-D + 0.1 mg/l BA, after 4 weeks of culture.

D. Induction of greenish friable callus on leaf explants of BSRM-19 in MS + 1.0 mg/l NAA + 0.1 mg/l BA, after 4 weeks of culture.

E. Formation of somatic embryo like structure (SELS) simultaneously with the formation of callus on internodal explants of BSRM-18 in MS + 1.0 mg/l 2,4-D + 0.1 mg/l BA, after 4 weeks of culture.

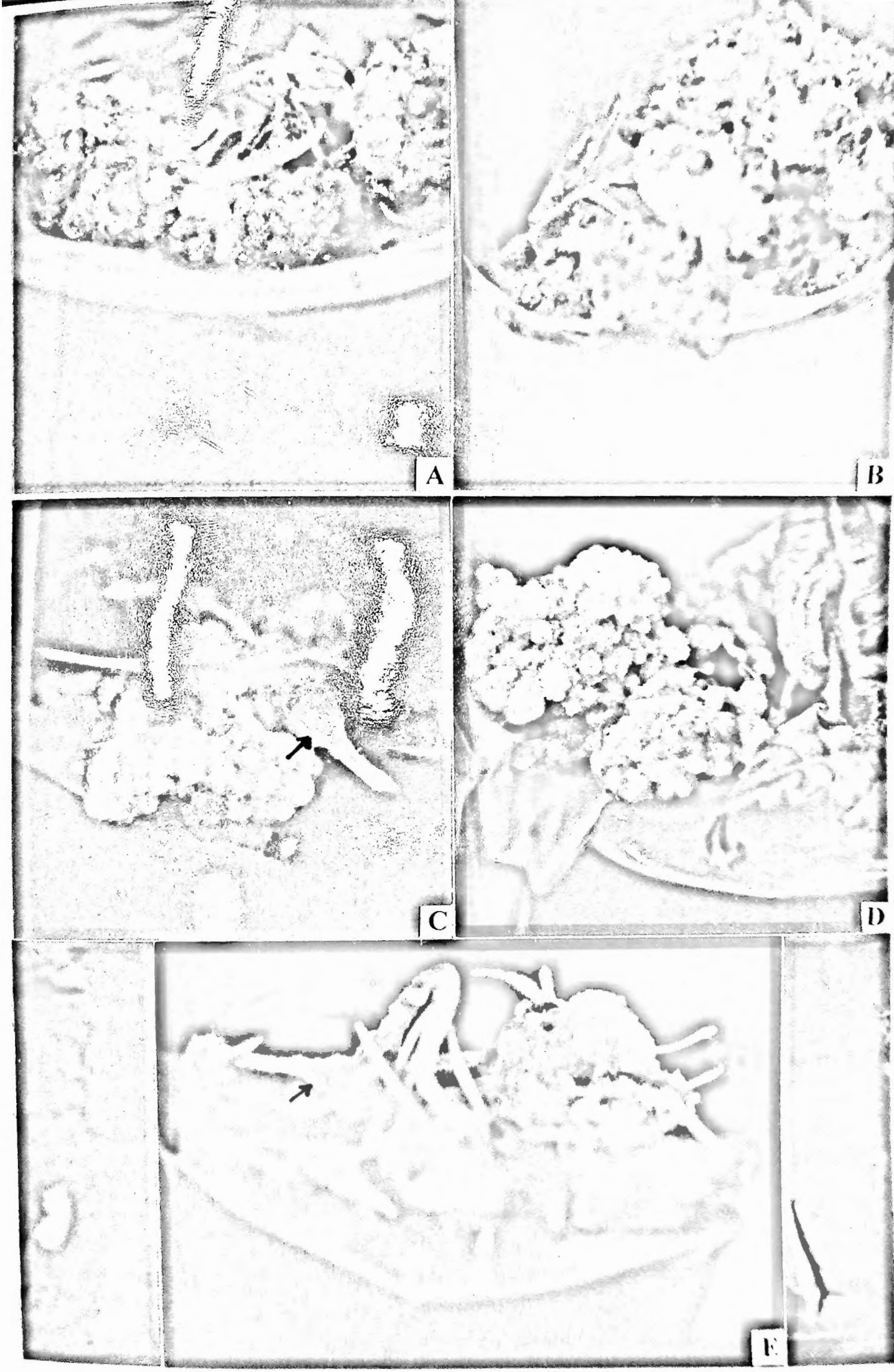
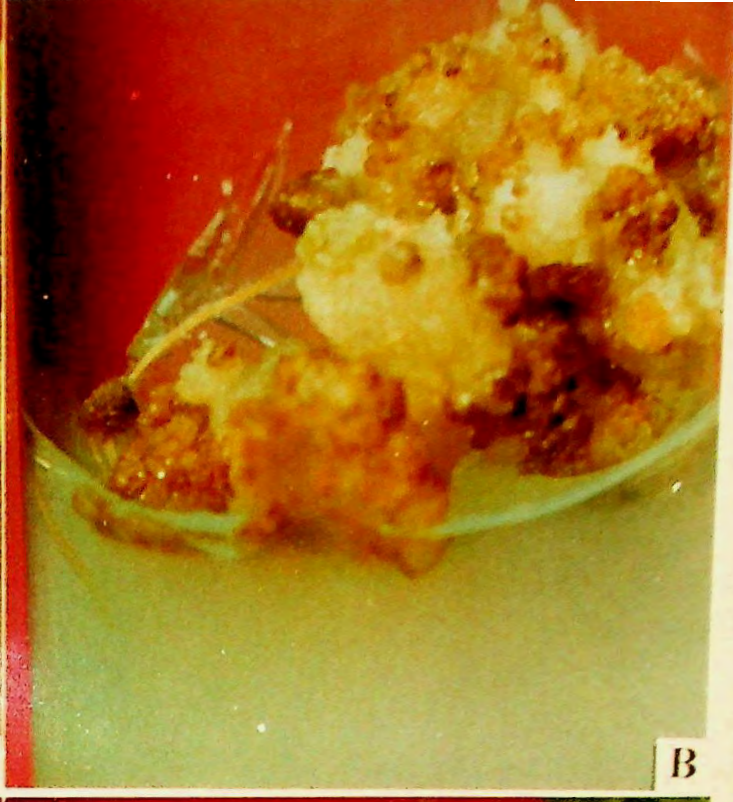


PLATE VIII



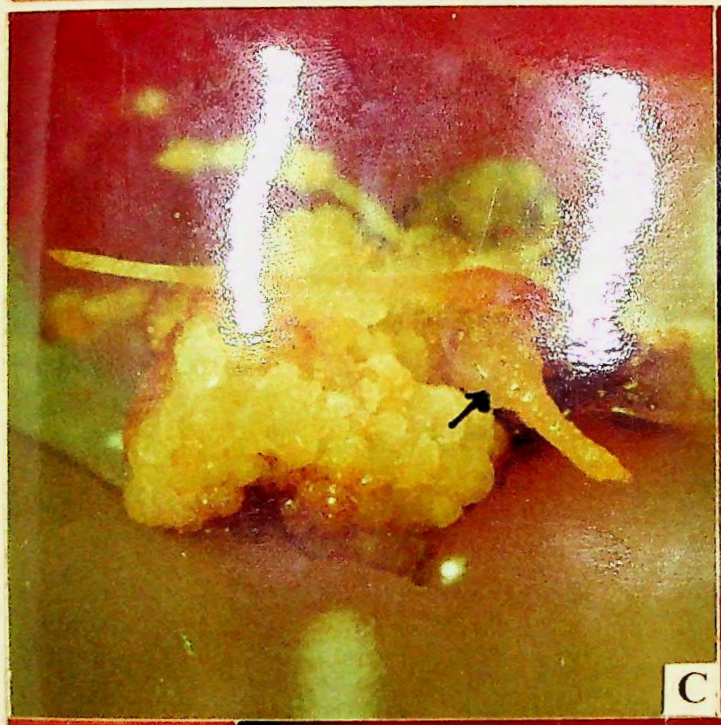


PLATE VIII

Table 1. Influence of different basal media on the frequency of shoot proliferation in three explants from three mulberry genotypes. All media contained 1.0 mg/l BA. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Explant	Genotype	Frequency of shoot proliferation		
		MS	LS	B5
Cotyledon	BSRM-3	42	51	15
	BSRM-18	37	30	19
	BSRM-19	59	41	32
	Mean	**42.68a**[1]	**39.53b**	**27.69b**
Shoot apex	BSRM-3	19	77	51
	BSRM-18	77	49	37
	BSRM-19	67	74	47
	Mean	**57.48b**	**55.03a**	**42.10a**
Node	BSRM-3	73	81	39
	BSRM-18	92	61	29
	BSRM-19	100	71	57
	Mean	**74.08c**	**57.64a**	**40.08a**

Item	DF	MS
Replication	2	50.8
Genotype	2	183.88 ***
Explant	2	1020.93 ***
Media	2	1070.38 ***
G x E	4	35.49
G x M	4	116.96 **
E x M	4	94.72 *
G x E x E	8	24.83
Error	52	20.80

*, **, *** Significant at 5%, 1%, 0.1% respectively.

[1] Means within a column followed by the same letters are not significantly different (P=0.05) according to Duncan's Multiple Range Test.

Table 2 : Effect of explant nature and genotype on shoot proliferation. In each treatment 2mg/l BA was used.

Genotype	Number of shoots per explant		Percentage of explants producing shoots	
	Shoot tip	Node	Shoot tip	Node
BSRM- 3	4.5b[1]	6.0b	84b	77b
BSRM- 8	2.7c	4.0c	37c	68c
BSRM-18	5.7b	2.9d	93ab	56d
BSRM-19	7.0a	10.5a	100a	98a

1. Means within a column followed by the same letters are not significantly different according to Duncan's Multiple Range Test (P = 0.05).

Table-3 : Effect of different basal media on shoot proliferation from nodal explants of three genotypes. All media contained 2 mg/l BA. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Media	Genotype	% of shoot proliferation A	Number of shoots per culture. B
1/2 MS	BSRM-19	47.00	1.89
	BSRM-18	39.37	2.01
	BSRM- 3	51.23	1.77
	Mean	**42.61b**	**1.89c**
MMS	BSRM-19	58.66	3.22
	BSRM-18	54.11	2.69
	BSRM- 3	33.89	1.83
	Mean	**44.30b**[1]	**2.58bc**
MS	BSRM-19	91.69	9.94
	BSRM-18	85.43	7.49
	BSRM- 3	72.52	4.89
	Mean	**66.39a**	**7.44a**
B5	BSRM-19	49.53	1.76
	BSRM-18	51.09	1.43
	BSRM- 3	37.67	2.06
	Mean	**42.74b**	**1.75c**
WP	BSRM-19	60.11	3.09
	BSRM-18	45.55	2.00
	BSRM- 3	31.62	1.22
	Mean	**42.49b**	**2.10c**
LS	BSRM-19	44.36	2.88
	BSRM-18	53.96	3.87
	BSRM- 3	62.11	4.49
	Mean	**47.0b**	**3.74b**
N6	BSRM-19	31.38	1.09
	BSRM-18	36.77	2.32
	BSRM- 3	23.29	1.22
	Mean	**33.43c**	**1.54c**

Item	DF	MS (A)	MS (B)
Total	62		
Replication	2	22,43	1.14
Genotype	2	64.82**	1.52
Medium	-6	244.49***	13.08***
G x M	12	32.27*	1.25***
Error	40	11.33	0.76

1. Means within a column followed by the same letters are not significantly different (P=0.05) according to Duncan's Multiple Range Test.

*, **, *** - significant at 5%, 1% and 0.1% respectively.

Table 4. Shoot regeneration ability of 7 mulberry genotypes. Nodal explants were cultured on MS media supplemented with 1.0 mg/l BA plus 0.5 mg/l IAA.

Genotypes	Total Number of explants cultured	Number of explants forming shoots	Number of shoots per explant	Shoot proliferation frequency
BSRM-1	52	33	1.6b[1]	63.5e
BSRM-2	50	39	1.1c	78.0d
BSRM-3	52	44	2.6b	84.bc
BSRM-8	49	41	1.9b	83.7c
BSRM-17	50	39	2.0b	78.0d
BSRM-18	56	50	4.3a	89.3ab
BSRM-19	50	46	5.1a	97.0a

1. Means within a column followed by the same letters are not significantly different (P=0.05) according to Duncan's Multiple Range Test.

Table 5. Effect of different cytokinins (BA, Kn, 2iP and Zn) (each 2.0 mg/l) on shoot proliferation capacity by culturing nodal explants of BSRM-19.

Cytokinins (mg/l)	Total number of explants	Number of explants cultured shoots	Number of shoots per producing culture	Shoot proliferation frequency
BA	49	47	8.71a[1]	95.9a
Kn	49	44	4.33b	70.8b
2iP	46	28	1.26c	60.9c
Zn	48	23	1.11c	47.9d

1. Means within the column followed by the same letters (P=0.05) are not significantly different according to Duncan's Multiple Range Test.

Table 6. Effect of different concentrations of BA on *in vitro* shoot proliferation of nodal explants of BSRM-19. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Growthregulator (mg/l)	% of proliferating cultures (A)	Average number of shoots/culture (B)	Average length of longest shoot (cm) (C)
0.5	45.30d[1]	3.95bc	4.13ab
1.0	65.51bc	4.19bc	5.51a
1.5	81.60a	5.50ab	5.09a
2.0	72.06b	8.00a	4.01ab
2.5	59.09c	7.50a	4.11ab
3.0	41.07de	3.60bc	3.09b
3.5	41.89de	2.17c	2.95bc
4.0	39.69de	2.16c	2.61bc
4.5	33.19e	2.06c	2.10bc
5.0	33.06e	2.01c	1.82c

Item	DF	MS (A)	MS (B)	MS (C)
Replication	2	22.56	1.10	0.54
Treatment	9	104.84***	4.32***	1.51
Error	18	11.30	0.55	0.44

1. Means within a column followed by the same letters (P=0.05) are not significantly different according to Duncan's Multiple Range Test.
*** - significant at 0.1%.

Table 7. Effect of BA and Kn on *in vitro* shoot proliferation from nodal explants of BSRM-18. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Hormonal concentrations (mg/l)		% of explants produced shoots (A)	Days taken to bud sprouting (B)	Number of shoots per culture (C)	Length (cm) of shoots (D)
BA	0.5	74.31	6.03	2.25	4.71
	1.0	87.02	7.45	3.30	5.89
	2.0	86.01	9.03	7.41	3.10
	3.0	57.11	11.21	3.11	3.00
	Mean	**61.38a**[1]	**8.43a**	**4.01a**	**4.17a**
Kn	0.5	79.91	7.10	2.95	3.50
	1.0	77.11	7.91	3.30	4.13
	2.0	71.91	8.95	2.53	3.03
	3.0	49.29	13.22	2.63	3.11
	Mean	**56.84b**	**9.29a**	**2.85a**	**3.44a**

Item	DF	MS (A)	MS (B)	MS (C)	MS (D)
Replication	2	8.92*	1.07	1.89	0.80
Hormone (H)	1	41.22***	1.49	2.71	1.08
Conc (C)	3	138.39***	11.95***	2.26	1.76*
H x C	3	18.11	0.40	3.19*	0.40
Error	14	2.33	0.38	0.69	0.40

1. Means within a column followed by the same letters are not significantly different (P=0.05) according to Duncan's Multiple Range Test.
* and *** - significant at 5% and 0.1% respectively.

Table 8. Effect of BA and KN on *in vitro* morphogenic response of BSRM-19. Nodal explants were cultured on MS basal media.

Hormonal conc (mg/l)		Days taken to bud sprouting	% of explant produced multiple shoots	Number of shoots per culture	Length of shoots (cm)
BA	0.5	7.43d[1]	57.27b	2.27e	5.29e
	1.0	7.23d	6.77a	4.09cd	4.97ab
	2.0	9.71c	85.28a	10.26b	4.33b
	3.0	10.27bc	54.76b	6.02c	4.20b
Kn	0.5	11.92a	47.23c	1.33e	6.29a
	1.0	11.11ab	46.11c	1.76e	6.77a
	2.0	11.32a	43.20c	4.31cd	5.20ab
	3.0	12.10a	48.98c	4.01cd	4.20b
BA1.0+Kn 1.0		7.29d	67.90ab	16.22a	5.09ab
Control		17.13e	14.21d	1.01e	2.01c

1 Means in a column followed by the same letter are not significantly different (P=0.05) according to Duncan's Multiple Range Test

Table 9. Effect of time of collection (months) of explants on *in vitro* cloning of BSRM-19. All media contained MS + 1.0 mg/l BA + 0.5 mg/l Kn. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Months[2]	Explant	% of shoot proliferation (A)	Number of shoots/culture (B)	Length of shoots (cm) (C)
JANUARY	Shoot apex	61	4.44	5.00
	Nodal segment	65	7.93	3.01
	Mean	**52.55ef[1]**	**6.18bc**	**4.00d**
FEBRUARY	Shoot apex	72	4.89	5.19
	Nodal segment	90	9.00	3.21
	Mean	**64.85a**	**6.94a**	**4.20d**
MARCH	Shoot apex	76	5.13	6.09
	Nodal segment	93	9.39	4.32
	Mean	**67.70a**	**7.26a**	**5.20c**
APRIL	Shoot apex	73	5.07	6.24
	Nodal segment	89	9.11	5.00
	Mean	**64.65a**	**7.09a**	**5.62bc**
MAY	Shoot apex	65	4.16	7.11
	Nodal segment	73	6.22	5.23
	Mean	**56.20cd**	**5.19cd**	**6.17a**
JUNE	Shoot apex	70	4.20	7.06
	Nodal segment	72	6.12	5.14
	Mean	**57.45cd**	**5.16cd**	**6.10a**
JULY	Shoot apex	73	5.22	5.66
	Nodal segment	70	6.00	6.00
	Mean	**57.75c**	**.61c**	**5.83ab**
AUGUST	Shoot apex	69	5.17	5.23
	Nodal segment	76	5.03	5.61
	Mean	**58.45bc**	**5.10cd**	**5.42c**
SEPTEMBER	Shoot apex	65	5.10	4.12
	Nodal segment	66	4.93	4.03
	Mean	**54.00de**	**5.01d**	**4.07d**

Table. 9 (continued)

OCTOBER	Shoot apex	52	4.71	3.91
	Nodal segment	50	4.86	4.11
	Mean	**45.55g**	**4.78d**	**4.01d**
NOVEMBER	Shoot apex	56	3.61	4.66
	Nodal segment	64	4.59	3.52
	Mean	**50.75f**	**4.10e**	**4.09d**
DECEMBER	Shoot apex	54	3.09	4.18
	Nodal segment	65	4.13	3.41
	Mean	**50.50f**	**3.61e**	**3.79e**

Item	DF	MS (A)	MS (B)	MS (C)
Replication	2	50.14	1.96*	38.2***
Months (M)	11	86.96***	2.69***	1.72***
Explants (E)	1	156.06***	21.13***	5.86***
M x E	11	14.92	1.45**	0.47**
Error	46	17.39	0.54	0.17

1 Means within a column folowed by the same letters are not significantly different (P=0.05) according to Duncan's Multiple Range Test.
*, **, *** - significant at 5%, 1% and 0.1% respectively.

2. NOV = early winter, DEC-JAN = winter, FEB = late winter, MAR-APR = spring, MAY-JUN = summer, JUL-AUG = rainy and SEP-OCT = autumn.
Percentage (%) data were analysed after angular transformation.

Table 10: Effect of collection date on nodal explants forming multiple shoot. All media contained MS + Kn 1.0 mg/l+ BA 0.5mg/l . Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Collection date	Date of inoculation	Genotype	Number of shoot/ culture(A)	Length of shoot (cm) (B)	Degree of growth (C)
25th FEB	26th FEB	BSRM-3	5.43	4.11	+++
		BSRM-18	3.36	2.96	+
		BSRM-19	2.40	3.01	++
Mean			**3.73c**[1]	**3.36b**	
25th MAR	26th MAR	BSRM-3	4.31	3.33	+++
		BSRM 18	6.11	3.19	++
		BSRM 19	4.34	2.70	+++
Mean			**4.92b**	**3.07b**	
25th APR	26th APR	BSRM-3	5.03	2.86	+++
		BSRM 18	5.79	3.49	+++
		BSRM 19	6.61	5.03	+++
Mean			**6.81a**	**3.97a**	

Item	DF	MS (A)	MS (B)
Replication	2	1.44*	0.45*
Genotype (G)	2	0.22	0.10
Collection date(C)	2	7.23***	0.39*
G x C	4	4.63***	0.84**
Error	16	0.33	0.09

1 Means within a column followed by the same letters (P=0.05) are not significantly different according to Duncan's Multiple Range Test.

*, **, *** - significant at 5%, 1% and 0.1% respectively.
'+' indicates the degree of growth.

Table 11. Effect of collection date and basal media on nodal explants forming multiple shoot from three mulberry genotypes. All media contained. 1.0 mg/l BA + 0.5 mg/l Kn. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Basal media	Genotypes	Date of explant collection		
		25th FEB	25th MAR	25th APR
MS	BSRM -3	8.61	6.66	5.42
	BSRM-18	4.12	8.03	5.24
	BSRM-19	4.33	9.97	10.69
	Mean	**5.68a**[1]	**8.22a**	**7.11a**
WP	BSRM -3	4.19	3.33	3.12
	BSRM-18	2.26	2.00	2.19
	BSRM-19	2.71	3.03	3.15
	Mean	**3.05a**	**2.78b**	**2.82ab**
LS	BSRM -3	1.02	3.00	4.01
	BSRM-18	2.29	2.87	3.13
	BSRM-19	3.63	5.83	7.71
	Mean	**2.31a**	**3.90ab**	**4.95ab**
B5	BSRM -3	4.09	1.21	1.00
	BSRM-18	1.06	1.13	1.00
	BSRM-19	1.42	1.37	2.13
	Mean	**2.19a**	**1.23b**	**1.37b**

Item	DF	MS
Replication	2	19.43
Genotype (G)	2	8.88
Media (M)	3	47.69***
Collection date(C)	2	2.19
G x M	6	2.52
G x C	4	51.39***
M x C	6	3.03
G x M x C	12	5.39
Error	70	7.22

1. Means within the column followed by the same letters are not significantly different (P=0.05) according to DMR Test.
*** - significant at 0.1%.

Table 12. Seasonal influence on *in vitro* shoot proliferation from nodal explants of BSRM-19 cultured in cytokinins (BA and Kn). Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

A. Effect on days required to bud sprouting.

Months	BA 1.0mg/l	BA 2.0 mg/l	BA 3.0 mg/l	Mean
OCTOBER	8.14	7.79	7.44	7.88d[1]
NOVEMBER	7.93	7.82	6.93	7.56d
DECEMBER	8.64	7.95	7.84	8.14d
JANUARY	8.15	8.04	8.01	8.06d
FEBRUARY	8.22	8.11	7.20	7.84d
MARCH	12.76	11.16	10.15	11.35c
APRIL	14.15	12.22	12.77	13.04ab
MAY	13.32	14.19	14.15	13.88a
JUNE	11.16	13.16	13.00	12.44bc
JULY	12.15	12.72	13.15	12.67b
	KN 1.0mg/l	**Kn 2.0 mg/l**	**Kn 3.0 mg/l**	**Mean**
OCTOBER	7.19	8.92	6.14	**7.41e**
NOVEMBER	6.93	8.31	7.92	**7.72e**
DECEMBER	8.21	6.59	8.55	**7.78e**
JANUARY	7.45	9.14	9.17	**8.58de**
FEBRUARY	7.83	8.43	7.32	**7.86**
MARCH	9.92	9.14	8.19	**9.08d**
APRIL	10.11	10.33	8.24	**9.56cd**
MAY	9.15	16.16	16.76	**14.02a**
JUNE	10.24	15.14	14.14	**13.17a**
JULY	10.67	12.13	10.32	**11.04b**

Item	DF	MS
Replication	2	2.56*
Months (M)	9	34.38***
Hormones (H)	1	6.45**
Conc (C)	2	2.96**
M x H	9	2.80***
M x C	18	23.14***
M x H x C	18	5.27***
Error	30	0.51

1 Means within a column followed by the same letters (P=0.05) are not significantly different according to Duncan's Multiple Range Test.

*, **, *** - Significant at 5%, 1% and 0.1% respectively.

Table 12 (B). Effect on number of shoot per explant. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Months	BA 1 mg/l	BA 2 mg/l	BA 3 mg/l	Mean
OCTOBER	3.16	9.64	7.69	**6.83a**[1]
NOVEMBER	3.29	7.19	7.29	**5.92a**
DECEMBER	2.76	6.03	8.14	**5.64ab**
JANUARY	2.34	10.71	6.32	**6.45a**
FEBRUARY	2.45	10.55	6.16	**6.38a**
MARCH	3.16	7.28	4.29	**4.91ac**
APRIL	1.16	4.76	3.14	**3.02c**
MAY	1.44	3.11	3.32	**2.62c**
JUNE	1.76	3.09	4.02	**2.95c**
JULY	1.32	3.17	4.83	**3.11bc**
	Kn 1 mg/l	**Kn 2 mg/l**	**Kn 3 mg/l**	**Mean**
OCTOBER	1.49	5.62	7.21	**4.77a**
NOVEMBER	2.06	4.89	6.89	**4.61ab**
DECEMBER	1.57	4.81	6.74	**4.37ab**
JANUARY	1.89	5.32	6.55	**4.59ab**
FEBRUARY	1.82	5.62	6.92	**4.78a**
MARCH	2.45	4.44	7.02	**4.63ab**
APRIL	1.66	3.03	2.19	**2.29ab**
MAY	1.49	3.19	4.64	**3.11ab**
JUNE	1.55	2.32	3.73	**2.53ab**
JULY	1.83	2.15	2.41	**2.13b**

Item	DF	MS
4.89		
Replication	2	11.37***
Months (M)	9	14.90*
Hormone (H)	1	76.07***
Conc (C)	2	0.93
M x H	9	2.37
M X C	18	4.74*
M x H x C	18	2.30
Error	30	

1 Means within a column followed by the same letters (P = 0.05) are not significantly different according to Duncan's Multiple Range Test.

*, *** - significant at 5 % and 0.1% respectively.

Table 12 (C). Effect on length of shoot (mm). Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Months	BA 1 mg/l	BA 2mg/l	BA 3 mg/l	Mean
OCTOBER	29.16	22.17	12.11	**21.14c**[1]
NOVEMBER	30.14	18.16	13.03	**20.44c**
DECEMBER	30.76	19.22	11.19	**20.39c**
JANUARY	31.44	21.45	10.66	**21.18c**
FEBRUARY	33.67	24.16	12.14	**23.32bc**
MARCH	39.11	28.19	16.32	**27.87abc**
APRIL	42.17	31.14	18.19	**30.50ab**
MAY	45.15	32.76	18.32	**32.07a**
JUNE	42.73	34.53	22.67	**33.31a**
JULY	46.55	32.16	22.14	**33.61a**
	Kn 1mg/l	**Kn 2mg/l**	**Kn 3mg/l**	**Mean**
OCTOBER	22.17	14.15	11.17	**15.83b**
NOVEMBER	20.19	15.04	12.32	**15.85b**
DECEMBER	19.67	16.07	14.15	**16.83ab**
JANUARY	21.13	15.11	13.39	**16.54ab**
FEBRUARY	22.73	16.23	11.16	**16.70ab**
MARCH	25.92	18.43	12.76	**19.03ab**
APRIL	29.11	21.44	14.39	**21.64ab**
MAY	30.36	23.76	15.44	**23.18ab**
JUNE	32.45	22.19	14.32	**22.98ab**
JULY	32.76	26.15	15.05	**24.65a**

Item	DF	MS
Replication	2	102.56*
Months (M)	9	121.54***
Hormone (H)	1	765.98***
Conc (C)	2	1432.83***
M x H	9	7.67
M x C	18	5.76
M x H x C	18	25.02NS
Error	30	25.02

1 Means within a column followed by the same letters (P=0.05) are significantly different according to Duncan's Multiple Range Test.

*,*** - significant at 5% and 0.1% respectively.

Table 13: Effect of collection date on explant contamination rate (%) of mulberry genotypes. All cultures contained BA 2mg/l with the same MS basal media. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Months	Genotypes	% of contamination	Months	Genotypes	% of contamination
JANUARY	BSRM -3	15.2	JULY	BSRM -3	47.2
	BSRM-18	21.4		BSRM-18	52.7
	BSRM-19	16.6		BSRM-19	49.3
	Mean	**24.33cd**[1]		**Mean**	**44.83a**
FEBRUARY	BSRM -3	15.0	AUGUST	BSRM -3	47.9
	BSRM-18	14.9		BSRM-18	53.0
	BSRM-19	12.2		BSRM-19	50.1
	Mean	**21.96cd**		**Mean**	**45.36a**
MARCH	BSRM -3	24.0	SEPTEMBER	BSRM -3	30.2
	BSRM-18	26.9		BSRM-18	26.5
	BSRM-19	22.2		BSRM-19	23.7
	Means	**29.53bcd**		**Mean**	**31.13bc**
APRIL	BSRM -3	28.6	OCTOBER	BSRM -3	26.
	BSRM-19	16.6		BSRM-19	22.5
	Mean	**28.60bcd**		**Mean**	**29.73bcd**
May	BSRM -3	31.6	NOVEMBER	BSRM -3	19.0
	BSRM-18	28.7		BSRM-18	17.6
	BSRM-19	29.3		BSRM-19	15.5
	Mean	**33.13abc**		**Mean**	**24.60cd**
JUNE	BSRM -3	39.7	DECEMBER	BSRM -3	07.9
	BSRM-18	42.6		BSRM-18	10.1
	BSRM-19	46.3		BSRM-19	09.0
	Mean	**40.90ab**		**Mean**	**17.43d**

Item	DF	MS
Replication	2	150.29
Month (M)	11	234.79*
Genotypes (G)	2	10.97
M x G	22	3.40
Error	70	58.69

1 Means within a same column followed by the same letters (P=0.05) are not significantly different according to Duncan's Multiple Range Test.

* Significant at 5% level.

Table 14. Effect of different amino acids on shoot proliferation from nodal explant culture of mulberry genotypes. All media contained 1.0 mg/l of BA + 0.1 mg/l IAA. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Amino acids (mg/l)		Genotypes	% of shoot proliferation (A)	Number of shoot/culture (B)	Length of shoot(cm) (C)
Tyrosine	0.0	BSRM -3	47.7	3.3	3.03
		BSRM-18	61.3	3.9	3.71
		BSRM-19	78.3	4.6	4.01
		Mean	**52.46b**[1]	**3.93b**	**3.58c**
	25	BSRM -3	39.3	3.0	3.10
		BSRM-18	62.6	3.1	3.91
		BSRM-19	59.8	4.9	4.33
		Mean	**47.06b**	**3.66b**	**3.78bc**
	50	BSRM -3	19.2	4.7	4.77
		BSRM-18	88.1	5.3	5.06
		BSRM-19	93.5	6.9	5.89
		Mean	**63.16a**	**5.63a**	**5,24a**
	100	BSRM -3	41.1	2.7	4.11
		BSRM-18	50.3	1.3	5.67
		BSRM-19	66.6	2.0	5.12
		Mean	**46.6b**	**2.0c**	**4.96a**
Glutamine	0.0	BSRM -3	47.1	3.1	3.10
		BSRM-18	63.0	3.7	3.82
		BSRM-19	50.3	4.4	4.22
		Mean	**47.0a**	**3.73a**	**3.71a**
	25	BSRM -3	22.3	3.0	2.78
		BSRM-18	39.9	3.9	2.03
		BSRM-19	37.1	3.4	3.12
		Mean	**34.96b**	**3.43ab**	**2.64ab**
	50	BSRM -3	69.7	2.7	2.03
		BSRM-18	33.3	1.6	2.16
		BSRM-19	47.0	3.5	2.59
		Mean	**45.03a**	**2.6b**	**2.26b**
	100	BSRM -3	62.3	2.1	2.30
		BSRM-18	29.9	4.7	3.77
		BSRM-19	55.4	3.6	4.11
		Mean	**44.43a**	**3.46ab**	**3.39**

Table 14. (Continued).

Aspergine 0		BSRM-3	46.2	3.0	2.99
		BSRM-18	63.0	3.8	3.67
		BSRM-19	52.2	4.5	4.09
		Mean	**47.2b**	**3.76a**	**3.58a**
	25	BSRM -3	71.6	4.0	3.37
		BSRM-18	65.4	4.2	3.69
		BSRM-19	87.3	3.6	2.03
		Mean	**60.95a**	**3.93a**	**3.03a**
	50	BSRM -3	52.4	2.6	3.61
		BSRM-18	62.9	3.1	2.01
		BSRM-19	68.7	4.9	2.21
		Mean	**51.63b**	**3.53a**	**2.61a**
	100	BSRM -3	43.3	4.6	4.33
		BSRM-18	58.7	2.8	2.13
		BSRM-19	51.3	3.7	2.11
		Mean	**45.6b**	**3.7a**	**2.85a**

Item	DF	MS (A)	MS (B)	MS (C)
Replication	2	63.65ns	0.98*	0.98
Genotype (G)	2	192.97***	2.86***	0.39
Amino acid(A)	2	325.35***	0.87	7.63***
Concentration (C)	3	95.80**	1.35**	0.63
G x A	4	126.80***	0.35	1.41*
G x C	6	18.03	0.67	4.06***
A x C	6	155.84***	3.04***	1.65**
G x A x C	12	60.70**	0.66*	1.84***
Error	70	21.55	0.30	0.44

1. Means within a column followed by the same letters (P=0.05) are not significantly different according to Duncan's Multiple Range Test.
*, **, *** - significant at 5%, 1% and 0.1% respectively.

Table 15. Effect of cytokinins and tyrosine on response of shoot tip and nodal explants of the genotype BSRM-3. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Cytokinins (mg/l)	Tyrosine (mg/l)	Shoot tip		Node	
		Number of shoots per culture	Average length of shoot(cm)	Number of shoots per culture	Average length of shoot (cm)
BA 1.0	0	1.1	3.5	1.5	4.0
	25	1.3	3.0	2.1	3.6
	50	1.7	5.1	4.5	5.0
	100	1.6	3.0	2.6	2.9
Mean		**1.42a**[1]	**3.65a**	**4.10a**	**3.87a**
Kn 1.0	0	1.0	2.5	3.1	2.3
	25	1.5	1.5	3.0	2.5
	50	1.0	3.5	2.1	4.0
	100	1.7	1.9	1.8	1.7
Mean		**1.30a**	**2.35b**	**2.50b**	**2.62b**
2iP 1.0	0	1.0	3.1	1.0	3.3
	25	1.2	2.5	1.3	2.7
	50	1.5	1.7	1.5	4.1
	100	2.1	1.0	1.7	1.9
Mean		**1.45a**	**2.07bc**	**1.37c**	**3.0ab**
Zn 1.0	0	1.0	2.0	1.2	3.1
	25	1.5	1.5	3.1	2.1
	50	1.5	1.9	1.0	2.3
	100	2.0	1.3	1.0	1.2
Mean		**1.50a**	**1.67c**	**1.57bc**	**2.17b**

Item	DF	MS (Number of shoot)	MS (Shoot length)
Replication	2	0.82**	1.80
Cytokinin (C)	3	0.72**	4.79***
Tyrosine (T)	3	0.48*	3.76***
Explant (E)	1	3.02***	1.85
C x T	9	0.59**	0.41
C x E	3	1.00***	0.21
T x E	3	0.45*	0.17
C x T x E	9	0.38	0.24
Error	63	0.16	0.60

1. Means within a column followed by the same letters (P=0.05) are not significantly different according to Duncan's Multiple Range Test.
*, **, *** - significant at 5%, 1% and 0.1% respectively.

Table 16. Effect of growth regulators on shoot proliferation from nodal explants of *Morus laevigata*. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Growth regulator (mg/l)		Days taken to bud sprouting (A)	% of proliferating culture(B)	Number of shoots per culture(C)	Shoot length (cm)(D)
BA	0.5	7.5f[1]	51.3de	2.11d	5.39a
	1.0	7.8ef	66.6a	8.91a	5.11a
	2.0	9.0bcdef	60.9bc	5.61b	5.55bc
	3.0	10.3bcdef	52.1de	2.09d	4.06abc
Kn	0.5	8.2def	56.2cd	1.09d	4.96ab
	1.0	8.7cdef	62.2ab	2.00d	4.11abc
	2.0	10.2bcdef	51.6de	5.71b	3.01c
	3.0	11.1bcd	48.6efgh	2.55cd	3.96abc
BA1.0+2.4-D 0.1		12.6ab	45.0h*	2.01d	5.16ab
BA1.0+NAA 0.1		13.0a	46.1fgh	2.00d	4.86ab
Kn1.0+2,4-D 0.1		11.3abc	49.3efgh*	3.91bcd	4.01abc
Kn1.0+NAA 0.1		12.0ab	45.9gh	3.41bcd	4.96ab

Item	DF	MS (A)	MS (B)	MS (C)	MS(D)
Replication	2	2.98	8.20	3.30	0.98
Treatment(T)	11	3.59**	16.69***	5.10NS	0.55
Error	22	1.10	3.10	1.02	0.32

* Callus was formed at the basal portion of microshoot.

1. Means within the column followed by the same letters (P=0.05) are not significantly different according to Duncan's Multiple Test.

, * - significant at 1% and 0.1% repectively.

Table 17. Rooting responses of *in vitro* raised shoots of *M. laevigata*. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Nutrient media (mg/l)		% of cuttings rooted (A)	Average number of roots/cul ture (B)	Average root length (cm) (C)
IBA	0.1	69.08**bc**[1]	5.71**bcde**	4.35**ab**
	1.0	82.10**ab**	8.11**ab**	5.12**ab**
	2.0	61.01**cd**	4.16**de**	5.90**a**
NAA	0.1	61.09**cd**	4.30**cde**	4.19**ab**
	1.0	69.79**abc**	8.51**ab**	4.83**ab**
	2.0	51.06**d**	Ψ6.89**bcd**	3.13**b**
IBA 0.1+NAA 0.1		85.77**a**	11.13**a**	6.09**a**
Without auxin		14.41**e**	3.31**e**	4.14**ab**

Item	DF	MS (A)	MS (B)	MS (C)
Replication	2	70.14	3.33*	1.14
Treatment	7	197.31***	7.04**	0.96
Error	14	28.41	1.14	0.76

Ψ Formation of callus was noticed at the basal portion of the micro cuttings.
1 Means within a column followed by the same letters (P=0.05) are not significantly different according to Duncan's Multiple Range Test.
*, **, *** - significant at 5%, 1% and 0.1% respectively.

Table 18. Effect of various concentrations of IBA on rooting response of *in vitro* derived microshoots of the genotype BSRM-19. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Concentrations of IBA (mg/l)	Number of roots (A)	Length of roots (cm) (B)
0.1	15.1**d**[1]	1.2**cd**
0.5	41.0**a**	2.4**bc**
1.0	39.2**a**	3.7**ab**
1.5	32.6**a**	3.9**a**
2.0	16.3**cd**	1.0**d**
3.0	19.1**bcd**	2.1**cd**
5.0	09.2**d**	1.1**cd**

Item	DF	MS (A)	MS (B)
Replication	2	39.40	0.54
Treatment	6	162.11***	1.47**
Error	12	13.46	0.18

1. Means within a column followed by the same letters (P=0.05) are not significantly different according to Duncan's Multiple Range Test.
, * - significant at 1% and 0.1% respectively.

Table 19. Effect of pH level and sucrose concentrations on root induction of BSRM-19. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Treatment		% of root induction	Number of roots/culture	Average root length (cm)
pH	4.0	29.3	4.0	1.9
	4.5	41.2	5.1	2.6
	5.0	49.9	10.7	3.2
	5.5	87.8	25.5	4.3
	6.0	91.9	22.3	4.1
	7.0	53.6	9.6	2.3
L.S.D	**(5%)**	**9.8**	**2.0**	**1.1**
Sucrose (g/l)	10	31.6	11.2	1.8
	20	47.1	16.6	2.1
	25	59.4	31.3	2.7
	30	92.7	22.9	3.9
	40	76.5	12.4	4.6
	50	51.2	7.3	2.2
	0	16.3	8.1	1.3
L.S.D	**(5%)**	**10.0**	**1.9**	**1.7**

Table 20. Induction of roots on microshoots of BSRM-19 obtained from different subcultures. Micro shoots were allowed to grow in 1/2 MS plus 0.1 mg/l IBA. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Source of micro cuttings	Frequency of rooting (%)	Number of roots/cutting	Root length (cm)
Shoot from initial culture	43.26a[1]	15.60a	4.56b
Shoots from 1st subculture	88.77d	33.20c	5.07c
Shoots from 3rd subculture	79.12c	19.56b	4.27b
Shoots from 5th subculture	70.57b	17.23b	5.00a
Shoots from 7th subculture	42.41a	13.43ab	3.03a

1. Means within a column followed by the same letters (P = 0.05) are not significantly different according to Duncan's Multiple Range Test.

Table 21. Effect of auxins on adventitious root formation on micro cuttings of BSRM-18. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Growth regulators (mg/l)		% of cutting rooted (A)	Days taken to root initiation (B)	Number of root perculture (C)	Length of root (cm) (D)
IBA	0.1	75.31a[1]	8.61b	16.35b	4.60ab
	0.5	67.32bc	8.63b	17.03b	4.90a
	1.0	74.26a	7.59b	29.71a	5.71a
NAA	0.1	60.61c	8.19b	15.59b	3.19ab
	0.5	71.86ab	9.91b	15.98b	4.16ab
	1.0	70.76ab	7.83b	*15.79b	4.37ab
No auxin		11.31d	24.51a	03.06c	2.27b

Item	DF	MS (A	MS (B)	MS (C)	MS (D)
Replication	2	14.20	2.25	1.20	2.8
Treatment	6	49.32***	5.79	3.91**	1.28
Error	12	5.02	1.40	0.50	0.76

1. Means within a column followed by the same letters (P=0.05) are not significantly different according to Duncan's Multiple Range Test.

, * - significant at 1 % and 0.1% respectively.

* Formation of callus was noticed.

Table 22. Effect of different auxins and MS salt strength on *in vitro* rooting of mulberry genotypes. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Strength of MS	Growth regulators (mg/l)	Genotypes	% of shoot formed root (A)	Average root number (B)	Average root length (cm)(C)
1/4	IAA 1.0	BSRM-19	51	21.96	3.78
	1.0	BSRM-18	36	17.09	3.11
	1.0	BSRM- 3	32	15.33	2.41
	Mean		**38.36a**[1]	**8.12a**	**3.1a**
	NAA 1.0	BSRM-19	42	10.67*	2.88
	1.0	BSRM-18	45	8.01*	3.03
	1.0	BSRM -3	48	14.24	3.67
	Mean		**42.13a**	**10.98b**	**2.99a**
	IBA 1.0	BSRM-19	62	19.89	4.66
	1.0	BSRM-18	49	23.63	4.11
	1.0	BSRM -3	40	11.21	2.22
	Mean		**45.16a**	**18.24a**	**3.66a**
	IBA 0.5+NAA 0.5	BSRM-19	74	33.78	4.83
		BSRM-18	29	7.88	2.04
		BSRM -3	33	9.00	3.88
	Mean		**42.33a**	**16.88a**	**3.58a**
Half	IAA 1.0	BSRM-19	47	11.36	2.55
	1.0	BSRM-18	49	11.09	3.11
	1.0	BSRM -3	24	7.11	2.89
	Mean		**39.0c**	**11.52c**	**2.85a**
	NAA 1.0	BSRM-19	53	18.67	3.44
	1.0	BSRM-18	67	24.37	3.11
	1.0	BSRM -3	72	21.44	2.79
	Mean		**53.23ab**	**21.49b**	**3.11a**
	IBA 1.0	BSRM-19	79	40.71	3.88
	1.0	BSRM-18	66	31.09	4.66
	1.0	BSRM -3	69	25.33	2.43
	Mean		**57.73a**	**32.37a**	**3.65a**

Table 22 (continued)

	IBA 0.5+NAA 0.5	BSRM-19	58	19.99	2.96
		BSRM-18	52	9.88	3.46
		BSRM -3	68	21.71	2.55
	Mean		**50.43b**	**17.19bc**	**2.99a**
Full	IAA 1.0	BSRM-19	41	13.22	3.88
	1.0	BSRM-18	32	11.67	2.41
	1.0	BSRM -3	36	7.88	3.29
	Mean		**37.03a**	**10.92c**	**3.19a**
	NAA 1.0	BSRM-19	46	17.63	2.97
	1.0	BSRM-18	19	9.72*	1.92
	1.0	BSRM -3	38	10.61	3.01
	Mean		**35.53a**	**12.65bc**	**2.63a**
	IBA 1.0	BSRM-19	57	26.44	4.62
	1.0	BSRM-18	39	19.66	3.54
	1.0	BSRM -3	32	22.10	2.39
	Mean		**40.66a**	**22.73a**	**3.51a**
	IBA 0.5+NAA 0.5	BSRM-19	49	23.99	2.84
		BSRM-18	27	6.82	2.09
		BSRM -3	36	11.00	3.11
	Mean		**37.53a**	**19.27ab**	**2.69a**

Item	DF	MS (A)	MS (B)	MS (C)
Replication	2	50.15	40.66	1.11*
Hormone (H)	3	136.65***	210.94***	0.85*
Strength (S)	2	474.09***	87.27*	0.32
Genotype (G)	2	196.59***	178.71***	1.31*
H x S	6	43.99*	70.99**	0.19
H x G	6	52.55*	35.68	0.88*
S x G	4	46.52	1.19	0.75
H x S x G	12	29.97	86.98***	1.31***
Error	70	18.80	21.56	0.30

1. Means within a column followed by the same letters (P=0.05) are not significantly different according to Duncan's Multiple Range Test.
*, **, *** - significant at 5%, 1% and 0.1% respectively.

Table 23. Survival percentage of *in vitro* derived plantlets in the field.

Genus	Species	Genotypes	% of survival after 6 months	% of survival after 1 year
Morus	*alba*	BSRM -1	59	47
		BSRM -3	84	70
		BSRM -8	57	52
		BSRM -17	72	63
		BSRM -18	95	80
		BSRM -19	91	82

Table 24. Morphological comparison of 6 genotypes of mulberry. Figures in the parenthesis for *in vitro* grown plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Geno-types	Plant height (cm) A	Leaf number/ branch B	Single leaf area (cm) C	Weight of 100 leaves (g) D	Internodal distance(cm) (cm) E
BSRM -1	87.7(99.6)	26.9(42.7)	70.3(71.4)	144.3(201.1)	3.41(3.12)
BSRM -3	85.2(97.1)	25.3(44.2)	58.7(57.3)	130.4(189.2)	3.22(3.01)
BSRM -8	70.1(67.2)	34.3(26.2)	31.1(29.5)	69.9(62.8)	2.77(3.12)
BSRM-17	76.3(87.4)	23.9(36.5)	58.8(59.9)	110.4(203.1)	2.91(2.01)
BSRM-18	94.6(81.3)	26.3(19.6)	67.5(63.1)	143.2(119.9)	3.57(4.97)
BSRM-19	92.4(94.7)	28.7(39.9)	55.9(58.7)	119.6(211.1)	3.31(2.89)

Item	DF	MS(A)	MS(B)	MS(C)	MS(D)	MS(E)
Replication	2	20.56	54.38	2.9*	2033.51	0.3
Genotype (G)	5	198.79***	42.05	392.03***	3142.90	0.71***
Origin (O)	1	52.08*	159.14**	0.48	6019.61*	0.01
G x O	5	52.73**	68.32*	3.32**	1213.42	0.32
Error	22	10.19	18.56	0.56	1354.51	0.02

*, **, *** - significant at 5%, 1% respectively.

Table 25. Nutritional comparison of 6 mulberry genotypes. Figures in the parenthesis for *in vitro* grown plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Genotypes	Nitrogen (%) A	Potassium (%) B	Phosphorus (%) C	Calcium (%) D	Magnesium (%) E
BSRM-1	2.58(2.59)	0.15(1.15)	0.16(1.15)	0.62(0.63)	0.12(0.13)
BSRM-3	2.13(3.01)	0.11(0.10)	0.19(2.19)	0.59(0.62)	0.9(0.13)
BSRM-8	2.01(2.00)	0.9(0.10)	0.10(2.11)	0.71(0.76)	0.11(0.10)
BSRM-17	3.61(3.96)	0.51(1.50)	0.16(0.21)	0.51(1.50)	0.21(0.19)
BSRM-18	2.32(2.31)	0.39(1,43)	0.13(0.12)	0.72(1.72)	0.19(0.18)
BSRM-19	3.03(2.31)	0.15(2.16)	0.15(0.17)	0.61(2.56)	0.15(0.12)

Items	DF	MS(A)	MS(B)	MS(C)	MS(D)	MS(E)
Replication	2	0.32NS	14.2	4.8	2.56	0.48
Genotype(G)	5	2.58***	4.49	5.73	2.02	1.08*
Origin(O)	1	0.85	14.96	10.10	9.01	1.54
G x O	5	0.23	6.24	7.6	1.97	1.05*
Error	22	0.22	3.1	3.1	2.30	0.39

*, ***, significant at 5 % and 0.1% respectively.

Table 26. Comparative nutritional status (Moisture) at different stages of leaf maturity of BSRM-19 in different seasons raised through cutting and in vitro system. Figures in the parenthesis are for *in vitro* raised plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Leaf Maturity	Winter	Summer	Rainy
Tender	62.3(62.1)	53.4(53.3)	66.7(68.6)
Mid-mature	58.0(52.5)	52.0(52.5)	59.4(60.5)
Mature	57.1(57.2)	51.4(51.8)	58.9(59.3)

Items	DF	MS
Replication	2	151.64***
Seasons(S)	2	45.45***
Maturity (M)	2	0.98
Origin (O)	1	7.49***
S x M	4	0.53
S x O	2	0.03
M x O	2	0.11
S x M x O	4	0.32
Error	34	

*** significant at 0.1% level.

Table 27. Comparative nutritional status (Carbohydrate) at different stages of leaf maturity of BSRM-19 in different seasons raised through in vitro and cutting derived system. Figures in the parenthesis are for *in vitro* raised plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Leaf maturity	Winter	Summer	Rainy
Tender	22.1(22.0)	23.7(24.4)	22.5(20.5)
Mid mature	25.0(24.9)	26.5(26.1)	22.1(21.6)
Mature	27.9(27.9)	27.9(27.6)	23.7(23.3)

Item	DF	MS
Replication	2	18,88***
Seasons(S)	2	22.40***
Maturity(M)	2	22.25***
Origin(O)	1	0.54
S x M	4	2.05***
S x O	2	0.43
M x O	2	0.02
S x M x O	4	0.28
Error	34	0.18

*** - significant at 0.1%.

Table 28. Comparative nutritional status (Reducing sugar) at different stages of leaf maturity of BSRM-19 in different seasons raised through *in vitro* and cutting derived system. Figures in the parenthesis are for *in vitro* raised plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Leaf maturity	Winter	Summer	Rainy
Tender	10.0(10.1)	12.0(12.5)	8.1(9.3)
Mid-mature	10.6(10.8)	13.2(13.1)	7.7(8.7)
Mature	10.9(13,8)	12.9(13.8)	7.3(6.5)

Item	DF	MS
Replication	2	7.67
Seasons(S)	2	37.27**
Maturity(M)	2	0.22
Origin(O)	1	0.46
S x M	4	1.28
S x O	2	0.08
M x O	2	0.14
SxMxO	4	0.29
Error	34	4.30

** - Significant at 1% level

Table 29. Comparative nutritional status (Total sugar) at different stages of leaf maturity of BSRM-19 in different seasons raised through *in vitro* and cutting derived system. Figures in the parenthesis are for *in vitro* raised plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Leaf maturity	Winter	Summer	Rainy
Tender	10.8(10.8)	14.2(14.8)	8.3(10.0)
Mid-mature	12.1(12.7)	15.2(14.8)	8.3(9.5)
Mature	13.8(13.9)	15.6(16.0)	10.1(10.5)

Item	DF	MS
Replication	2	3.33
Seasons(S)	2	47.89***
Maturity(M)	2	5.22*
Origin(O)	1	1.17
S x M	4	0.70
S x O	2	0.39
M x O	2	0.09
S x M x O	4	0.15
Error	34	

*, *** significant at 5% and 0.1% respectively.

Table 30. Comparative nutritional status (Protein) at different stages of leaf maturity of BSRM-19 in different seasons raised through *in vitro* and cutting derived system. Figures in the parenthesis are for *in vitro* raised plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Leaf maturity	Winter	Summer	Rainy
Tender	28.3(28.9)	32.1(32.6)	26.6(27.3)
Mid-Mature	27.2(27.3)	29.0(29.4)	24.5(25.3)
Mature	24.4(24.7)	25.6(26.1)	20.8(22.2)

Item	DF	MS
Replication	2	2.19
Seasons(S)	2	32.9***
Maturity(M)	2	43.13***
Origin(O)	1	1.56
S x M	4	0.83
SxO	2	0.17
MxO	2	0.03
SxMxO	4	0.03
Error	34	1.18

*** significant at 0.1%

Table 31. Comparative nutritional status (Mineral) at different stages of leaf maturity of BSRM-19 in different seasons raised through *in vitro* and cutting derived system. Figures in the parenthesis are for *in vitro* raised plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Leaf maturity	Winter	Summer	Rainy
	15.6(15.6)	13.3(13.8)	17.8(18.0)
Mid-mature	18.0(18.1)	14.2(14.3)	22.0(22.1)
Mature	20.8(21.6)	17.7(16.6)	22.2(21.2)

Item	DF	MS
Replication	2	20.56
Seasons(s)	2	46.98*
Maturity(m).	2	28.29
Origin(o)	1	0.09**
SxM	4	0.69
SxO	2	0.11
MxO	2	0.18
SxMxO	4	0.25
Error	34	10.23

*,** - significant at 5% and 1% respectively.

Table 32. Comparative nutritional status (Protein) of leaves of different genotypes in different seasons. Figures in the parentheses are for *in vitro* derived plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Genotypes	Winter	Summer	Rainy
BSRM -3	23.3(23.4)	23.8(25.1)	22.3(22.2)
BSRM -8	25.9(26.0)	25.8(26.4)	23.4(24.2)
BSRM-18	27.2(27.3)	28.8(29.7)	24.0(25.2)
BSRM-19	27.7(28.3)	29.4(29.7)	24.6(26.1)

Item	DF	MS
Replication	2	5.39
Genotype(G)	3	21.72**
Season(S)	2	21.64**
Origin(O)	1	1.93
G x S	6	0.79
G x O	3	0.04***
S x O	2	0.21
G x S x O	6	0.18
Error	46	4.50

, * Significant at 1% and 0.1% respectively.

Table 33. Comparative nutritional status (Mineral) of leaves of different genotypes in different seasons. Figures in the parentheses are for *in vitro* derived plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Genotype	Winter	Summer	Rainy
BSRM -3	20.5(22.3)	16.4(16.6)	24.3(23.6)
BSRM -8	18.8(20.5)	17.6(16.7)	24.4(23.8)
BSRM-18	18.2(18.4)	15.6(17.8)	21.7(22.2)
BSRM-19	17.9(17.8)	16.5(17.2)	19.6(19.5)

Item	DF	MS
Replication	2	5.89
Genotype(G)	3	8.32*
Season(S)	2	62.67***
Origin(O)	1	1.0
G x S	6	2.99
G x O	3	0.25
S x O	2	0.66
G x S x O	6	0.61
Error	46	2.5

*, *** significant at 5% and 0.1% respectively

Table 34. Comparative nutritive value (Reducing sugar) of leaves of different genotypes in different seasons. Figures in the parentheses are for *in vitro* raised plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Genotypes	Winter	Summer	Rainy
BSRM -3	10.1(10.1)	9.8(10.1)	5.7(6.3)
BSRM -8	7.3(7.5)	8.1(10.1)	6.0(5.7)
BSRM-18	10.3(10.3)	12.0(12.1)	7.3(8.7)
BSRM-19	10.8(10.9)	10.9(10.1)	8.3(8.9)

Item	DF	MS
Replication	2	3.40
Genotype(G)	3	9.31**
Season(S)	2	23.73***
Origin(O)	1	0.67
G x S	6	1.16
G x O	3	0.12
S x O	2	0.13
G x S x O	6	0.41
Error	46	1.90

,* -significant at 1% and 0.1% respectively.

Table 35. Comparative nutritive value (Total sugar) of leaves of different genotypes in different seasons. Figures in the parentheses are for *in vitro* raised plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Genotype	Winter	Summer	Rainy
BSRM -3	8.9(10.5)	12.2(11.2)	8.3(9.3)
BSRM -8	10.7(10.5)	11.7(14.9)	8.1(10.1)
BSRM-18	12.6(15.0)	15.7(15.5)	8.7(10.8)
BSRM-19	12.8(13.1)	15.3(15.9)	10.0(10.5)

Item	DF	MS
Replication	2	4.8
Genotype(G)	3	12.97*
season(S)	2	41.86***
Origin(O)	1	6.31
G x S	6	1.29
GxO	3	0.56
SxO	2	0.28
GxSxO	6	1.05
Error	46	3.11

*, *** - significant at 5% and 0.1% respectively.

Table 36. Comparative nutritive status (Soluble carbohydrate) of leaves of different genotypes in different seasons. Figures in the parentheses are for *in vitro* raised plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Genotypes	Winter	summer	Rainy
BSRM -3	20.7(21.6)	24.6(24.4)	19.1(19.5)
BSRM -8	22.5(22.5)	24.7(24.6)	19.5(19.8)
BSRM-18	25.1(27.2)	29.6(29.3)	22.7(21.4)
BSRM-19	26.3(27.8)	28.7(28.7)	21.4(22.2)

Item	DF	MS
Replication	2	3.98
Genotype(G)	3	30.93***
Season(S)	2	75.57***
Origin(O)	1	0.70
G x S	6	1.45
G x O	3	0.15
S x O	2	0.93
G x S x O	6	0.35
Error	46	4.56

*** - significant at 0.1% .

Table 37. Comparative nutritive status (Starch) of leaves of different genotypes in different seasons. Figures in the parentheses are for *in vitro* raised plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Genotype	Winter	Summer	Rainy
BSRM -3	18.4(18.6)	21.0(21.4)	17.1(17.0)
BSRM -8	19.6(19.6)	21.3(19.1)	17.5(16.7)
BSRM-18	21.4(22.1)	24.4(24.5)	20.8(18.5)
BSRM-19	22.5(24.0)	23.7(23.2)	18.7(19.5)

Item	DF	MS
Replication	2	4.33
Genotype(G)	3	18.01*
Season(S)	2	34.28**
Origin(O)	1	0.20
G x S	6	1.21
G x O	3	0.74
S x O	2	0.92
G x S x O	6	0.50
Error	46	4.40

*, ** significant at 5% and 1% respectively.

Table 38. Comparative nutritive status (Moisture) of leaves of different genotypes in different seasons. Figures in the parentheses are for *in vitro* raised plants. Mean squares (MS) in the analysis of variance with degree of freedom (DF) are also shown.

Genotype	Winter	Summer	Rainy
BSRM -3	58.4(58,5)	54.3(54.7)	62.8(62.1)
BSRM- 8	58.9(58.2)	51.4(51.2)	60.2(60.5)
BSRM-18	59.1(61.7)	52.4(52.1)	59.7(59.5)
BSRM-19	58.4(58.5)	52.7(53.1)	62.2(61.3)

Item	DF	MS
Replication	2	40.40
Genotype(G)	3	3.08
Season(S)	2	149.26***
Origin(O)	1	0.03
G x S	6	2.87
G x O	3	0.27
S x O	2	0.41
G x S x O	6	13315.33***
Error	48	16.56

*** - significant at 0.1%.

Table 39. Effects of auxins and cytokinin singly and in combination on collogenesis and formation of somatic embryo like structure (SELS) from cotyledonary explant of BSRM-19.

Growth regulators(mg/l)		% of explant formed callus	Nature of callus		Formation of SELS
			Colour	Texture	
2,4-D	0.5	26	Cream	Compact	-
	1.0	31	Cream	Compact	-
	2.0	23	Cream	Compact	-
	3.0	21	Cream	Compact	-
NAA	0.5	26*	Light brown	Amorphous	-
	1.0	33**	Light brown	Amorphous	-
	2.0	32	Light brown	Amorphous	-
	3.0	19	Brown	Amorphous	-
2,4-D +BA	1.0 0.1	41	White cream	Friable	-
NAA +BA	1.0 0.1	24	Light brown	Friable	-
2,4-D +Kn	1.0 0.1	30	Cream	Amorphous	-
NAA +Kn	1.0 0.1	47	Light brown	Amorphous	-

'-' : denotes no response

* : root formed

Table 40. Effects of auxins and cytokinin singly and in combination on callogenesis and formation of somatic embryo like structure (SELS) from internodal explant of BSRM-19.

Growth regulators(mg/l)		% of explant formed callus	Nature of callus		Formation of SELS
			Colour	Texture	
2,4-D	0.5	62	Brown	Compact	-
	1.0	68	Brown	Compact	-
	2.0	64	Light brown	Compact	-
	3.0	47	Light brown	Compact	-
NAA	0.5	51*	Cream	Amorphous	-
	1.0	63*	White cream	Amorphous	-
	2.0	57**	Yellowish cream	Amorphous	
	3.0	43	Cream	Amorphous	-
2,4-D + BA	1.0 0.1	98	Greenish	Friable	++
NAA + BA	1.0 0.1	79	Light green	Friable	-
2,4-D + Kn	1.0 0.1	74	Light brown	Loose	+
NAA + Kn	1.0 0.1	78	Light brown	Loose	-

*, ** Indicates root & profuse root formation respectively.
'-' : indicates no SELS formed
'+' : indicates presence of 1 or 2 SELS.
'++' : Indicates presence of 3 - 4 SELS

Table 41. Effects of auxins and cytokinins singly and in combination on callogenesis and formation of somatic embryo like structure (SELS) from leaf explants of BSRM-19.

Growth-regulators	(mg/l)	% of explants formed callus	Nature of callus		Formation of SELS
			Colour	Texture	
2,4-D	0.5	59	Light brown	Semi compact	-
	1.0	52	Brown	Semi compact	-
	2.0	61	Yellowish brown	Compact	-
	3.0	39	Yellowish brown	Compact	-
NAA	0.5	42	Cream	Friable	-
	1.0	67*	Cream	Friable	-
	2.0	61**	Cream	Friable	-
	3.0	47	Cream	Friable	-
2,4-D + BA	1.0 0.1	62	Greenish	Amorphous	-
NAA + BA	1.0 0.1	61	Greenish	Friable	-
2,4-D + Kn	1.0 0.1	60	Greenish	Amorphous	-
NAA + Kn	1.0 0.1	57	Greenish	Friable	-

*,** indicates formation of root and profuse root respectively.
`-' : - no SELS formation.

Table 42. Response of internodal explants of different mulberry genotypes on callus induction and formation of somatic embryo like structure (SELS) in 1.0 mg/l 2,4-D + 0.1 mg/l BA.

Genotypes	% of explants formed callus	Nature of callus		Formation of SELS
		Colour	Texture	
BSRM-1	39	Light brown	Compact	-
BSRM-3	46	Light brown	Compact	-
BSRM-8	41	Cream	Compact	-
BSRM-17	63	Cream	Compact	-
BSRM-18	67	Light green	Friable	+

`-': indicates SELS not formed.
`+': indicates presence of 1 or 2 SELS.

4

DISCUSSION

4. DISCUSSION

During the last two decades plant tissue culture techniques have been used successfully in the propagation of many herbaceous plants (Conger, 1981). Considerable amount of work has also been done in the tree propagation (Mascarenhas *et al.*, 1988) but in comparison to herbaceous, ornamental and crop species most hardwood and softwood tree species of forest value have proven difficult to propagate. In view of their highly heterozygous nature and long duration, it is very important to develop methods of clonal propagation of superior trees. The traditional methods of vegetative propagation of woody perennial by rooting of cutting or grafting is difficult. Since seed propagation results in great variability, tissue culture seems to be the only method which offers some promise to the solution of the above problem.

Morus species shows great genetic diversity which suggests that selection and multiplication of elite tree by vegetative methods might lead to even higher yields. The reviews of Bonga (1977, 1981) and Durzan and Campbell (1974) amply discussed the significance of tissue culture work. In vegetative propagation programmes, mature trees are generally preferred over juvenile ones, even though mature trees are often much more difficult to propagate vegetatively than juvenile trees (Bonga, 1981). *In vitro* vegetative propagation of mature trees has, in few cases, been achieved with explants other than those taken from reproductive

structures. So far, nearly 180 species of woody plants have been studied *in vitro* with variable success in the production of shoots, roots, embryos and whole plantlets (Rao and Lee, 1986). More than 60 different media have been used for tissues of various woody species (Skirvin, 1980). The most commonly used is that of MS media (Murashige and Skoog 1962; Bonga 1980). In the present investigation of mulberry MS (Murashige and Skoog, 1962) was employed other than few experiments.

According to Cohen and Cooper (1982); Hutchinson (1981); Zaerr and Mapes (1982) many different kinds, concentrations and combinations of growth substances have been used for *in vitro* cloning of tree species and there is no single formula that works well for all species. This underlines the fact that, trees vary greatly both in their biochemical composition and in the response of their tissues. Therefore, different cytokinins, auxins, sugars, aminoacids etc., in different combinations and concentrations were tested to experience diverse response of explants in *in vitro* environment. In many tree species shoot tips, terminal buds and axillary buds show good growth as explant in the media. In certain timber trees including *Eugenia* and *Pterocarpus,* the buds respond very well, each producing as many 30-40 shoots in *in vitro* condition. These new shoots can be separated, rooted and transferred to soil (Rao and Lee, 1986). For mulberry (*Morus* sp) in the present research, nodal explants and shoot tips were used extensively for most of the experiments. Cotyledon and hypocotyl explants were not employed in all

experiments because they were proved to be unsatisfactory in *in vitro* condition in this investigation. Another reason for discarding these explants was, mulberry is highly heterozygous and emphasis was given to nodal segments and shoot apices to get true-to-type plants through *in vitro* culture. However, for embryogenesis different explants like leaf segments, cotyledon, nodal segments and shoot apices were employed. Plants that were raised by culturing nodal and shoot apex explants displayed different responses in *in vitro* condition.

The present investigation reveals that, out of three explants (cotyledon, shoot apex and nodal segment), nodal segments were found to be the best explant for multiple shoot proliferation followed by shoot apex explant. However, shoot apex exhibited better shoot elongation than nodal explants. Yadav *et al.,* (1990) has observed similar results in *Morus nigra.*

Cotyledonary explants were not satisfactory for *in vitro* proliferation because they displayed poor exhibition of multiple shoot proliferation in all media tested and in all genotypes examined. Oka and Ohyama (1981) were unable to obtain bud formation on the cotyledons of mulberry while Kim *et al.*, (1985) found that, in mulberry, excision of the cotyledons from the embryo was necessary to induce shoot formation. However, cotyledons have been the explant of choice for *in vitro* clonal propagation of many gymnosperms (Aitken *et al.*, 1981; David, 1982). Rao *et al.,* (1981 a) initiated cotyledon culture of two tropical fruits with very limited success.

Tissue culture methodology for plant regeneration from cotyledon explant is well established for herbaceous, ornamentals, fruits or vegetable crops especially for those belong to family Cucurbitaceae and Cruciferae (Rahman *et al.*, 1991, 1993b) However, to date, investigators have made relative slow progress on plant regeneration protocols for tree species (Mante *et al.*, 1989).

Highly significant differences among the media (MS, MMS, B5, WP, LS and N6) tested for *in vitro* proliferation were observed in this study. MS was found to be suitable followed by LS and WP media for *in vitro* shoot proliferation. B5 and N6 were not satisfactory. Different genotypes of mulberry used interacted with different media (G x M) significantly in many cases which indicated that the genotypes used responded differently in different basal media. Our findings correlate with the findings of Conger (1981) and Gautheret (1985) who reported that MS allowed 5 to 7 times active growth in wide range of plant species including, monocot and dicot.

In general, shoot regeneration capacity of mulberry genotypes was low in N6 formulations. From the results of the present study, it is apparent that, high salt content nutrient medium was required for optimum response of mulberry explants. N6 is low salt content nutrient medium. Concentration of Ca^{++}, Mg^{++}, NO_3^- etc. are much lower than that of MS or LS. Whereas, macro and micro nutrients are same in MS and LS and they differ only in vitamins content. LS contains thiamine only and MS has thiamine, niacin, pyridoxin-HCl and glycine. Therefore, it can be inferred

that, vitamins content is one of the important factor in inducing multiple shoot regeneration in mulberry. Jain *et al.*, (1990) also reported MS and LS as good basal media for *in vitro* performance of mulberry.

In vitro shoot proliferation efficiency of nodal explants of the 7 genotypes of mulberry differed significantly. Among the different genotypes tested, BSRM-19 exhibited highest (97.0%) percentage of shoot proliferation followed by BSRM-18 (89.3%) while BSRM-1 displayed 63.5% of shoot proliferation. Ohyama and Oka (1987) suggested detail study on *in vitro* propagation of different mulberry genotypes is necessary. *In vitro* propagation through axillary bud multiplication in different mulberry genotypes has also been reported by Jain *et al.*, (1990) who also observed that, overall response in tissue culture of mulberry is genotypically oriented, as various cultivars under the same *in vitro* culture conditions exhibit different responses. Genotypic variation in morphogenic competency was also observed by Hammat *et al.* (1989); Zaman *et al.*, (1992c); Lutova and Zabelina (1988); Wehner and Locy (1981); Zelcer *et al.*, (1984); Islam *et al.*, (1994b); Hossain *et al.*, (1995) and Kim *et al.*, (1988) in different crop plants. It can be concluded that, factors in addition to genes controlling the regeneration ability, influence genotype culture protocol (Kris and Bingham, 1988). Das and Mitra (1990) also observed genotypic effect on *in vitro* shoot proliferation of *Eucalyptus*.

A number of experiments were conducted to select the best cytokinin in

optimum concentration for multiple shoot proliferation from both nodal explants and shoot spices of mulberry. The preceeding observations indicated that, applications of cytokinins was very effective in multiple shoot proliferation from nodal segments and shoot apices of mulberry genotypes. Among the four cytokinins (BA, Kn, 2iP and Zn) tested, BA was found to be the best for *in vitro* multiple shoot proliferation followed by Kn, while 2iP and Zn were unsatisfactory. The superiority of BA over other cytokinins for multiple shoot proliferation in mulberry has also been reported by Yadav *et al.*, (1990). BA as a super cytokinin was also experienced by Hossain *et al.*, (1994); Rahman *et al.*, (1991); Karim *et al.*, (1993); Biswas *et al.*, (1993); Joarder *et al.*, (1993); Lundergan and Jenick (1980); Vieitez and Vieitez (1980) in different fruit plants and trees. Present investigation also showed that, when BA and Kn were added together (1.0 mg/l each) highest number (15-20) of shoots were produced per explant probably because of synergistic activities of BA and Kn. This kind of synergism was also reported by Zaman *et al.*, (1993 a) in *Morus alba.* cv. S1. It was observed that, BA alone (2.0 mg/l) was sufficient for multiple shoot (9-10 shoots /explant) proliferation from nodal explants of mulberry. Similar result was also reported by Zaman *et al.*, (1993b, 1994a) in *Morus alba* cv C776 using shoot tip explants of seedlings and also in nodal explants of *Morus alba* (Zaman *et al.*, 1989, 1991). Papadatou *et al.*, (1990) also claimed 2.0 mg/l BA as an optimum concentration for multiple shoot proliferation in guava. However, Jain *et al.*, (1990)

claimed 0.25-0.5 mg/l BA as an optimum dose for *in vitro* shoot proliferation of *Morus indica*. In *Eucalyptus*, Das and Mitra (1990) found highest number of shoots when NAA at 0.1 mg/l and BA at 1.0 mg/l was added to modified MS medium.

From the preceeding experiments it is observed that, as per as the multiple shoot proliferation is concerned nodal explants always found to be superior while shoot apex exhibited better elongation in *in vitro* condition. Yadav *et al.*, (1990) also reported the same observation in *Morus nigra* and Mhatre (1985) and Patel *et al.*, (1983) in *Morus indica*, where nodal explants gave birth highest number of microshoots. Nodal explants were also found to be the best explants for *in vitro* multiple shoot proliferation in many tree species. Das and Mitra (1990), Gill *et al.*, (1994) and Islam *et al.*, (1994a) claimed nodal segment as the best explants for *in vitro* multiple shoot proliferation in *Eucalyptus* species. Joysree (1992) observed highest shoot proliferation by culturing nodal segment of *Gmelina arborea* L. Yadav *et al.*, (1990) also confirmed the same result in *Syzygium cuminii* a tropical fruit tree.

However, shoot tip explants were found to hold second position for better shoot proliferation in mulberry which is also supported by Ohyama and Oka (1976) and Zaman *et al.*, (1993b) in *Morus alba* cv. C776. Shoot tip as a good source of explant has also been reported by Ara (1991) in *Sesbania grandiflora*, Papadatou *et al.*, (1990) in guava.

Seasonal changes in endogenous growth hormone type and concentration are

known to occur in trees (Alvim, 1976; Dunberg, 1976, Junttna, 1982). The progression of shoot growth during spring flushing is likely controlled, at best in part, by hormonal fluctuation. Internal changes in a tree's hormonal status, which lead to changes in shoot growth, may also dictate the type of growth of an explant undergoes when placed *in vitro*. *In vitro* development and organogenic potential of explants in various physiological states have been shown to be influenced by the levels of endogenous growth regulators (Cassells *et al.*, 1982; Brand and Lineberger, 1986). Bogdanovic (1986) reported that, even in controlled environmental conditions, organogenesis can be influenced by the seasons which is also supported by Gogala and Camloh (1988).

There is hardly any report in Bangladesh regarding seasonal effect on *in vitro* shoot proliferation of mulberry. Therefore, 4 separate experiments were conducted considering two types of explants (nodal segment and shoot apex), three genotypes (BSRM-19, BSRM-18 and BSRM-3), four basal media (MS, WP, LS and B5) and two cytokinins (BA and Kn) in different concentrations. From the preceeding experiments it has been observed that, different seasons had remarkable effect on *in vitro* shoot proliferation of mulberry. The opening and growth of buds were greatly affected by the seasons and the associated physiological condition of the maternal twig from which the explants were collected.

The results obtained from 4 different experiments conducted, the practical

experiences are discussed hereby. Explants that collected in different months (seasons) were cultured (same day of collection) to varify the *in vitro* seasonal effect. Highest percentage of shoot proliferation and highest number of shoots per explant from nodal segment were recorded during late winter to spring (February-April) followed by summer (May-June). However, with the advent of winter (November to January), the performances declined considerably. Hohtola (1988) observed best culture growth from April to June in Scot Pine and Chesick (1990) found highest shoot proliferation in Western larch in summer. Nekrasova (1964) observed that bud sprouted and elongated best in July which correlates with our findings. In the rainy season (July-August) and in autumn (September-October) the rate of shoot proliferation and the elongation were better than that of winter (November-January). Das and Mitra (1990) reported that explants collected during summer were the most responsive for multiple shoot proliferation. In our experiments, analysis of variance shows that, the item collection month (M), explant (E) and month-explant interaction (M x E) were highly significant (0.1% level). It was also observed that, genotype (G) and collection date of explant (C) interacted (G x C) significantly for multiple shoot proliferation. Different media tested in different seasons also varied significantly in exhibiting *in vitro* shoot proliferation of mulberry. Hence from the foregoing discussion it is evident that, there is a substantial effect of explant collecting period (season) on *in vitro* shoot proliferation of mulberry. Though our findings do not have

exact similarities with other tree plants mentioned above and with those that we could not mention might be due to physiological differences of different trees and climatic conditions (temperature, humidity, rainfall, etc.) of our country. From overall discussion it can be assumed that, the seasonal fluctuations in the stock plant affected the shoot proliferation considerably potential of the explants *in vitro* which is strongly supported by the work of Brand and Lineberger (1986).

In tropical and subtropical countries despite of taking much precautionary measures, microorganisms (bacteria and fungi) contaminates the culture media, infects the explants cultured and finally jeopardise the aim of experiments. The success of tissue culture largely depends on the uncontaminated shoot/callus proliferation rate. Shoot apices were collected from field grown mulberry genotypes of BSRM-3, BSRM-18 and BSRM-19 in different months (January-December, 93-94) and cultured. The idea for conducting this experiment was to find out two things : (a) to select the best period for explant collection with low contamination rate and (b) to select the comparatively resistant genotype for *in vitro* shoot proliferation. The basal part of the explant was readily (along with the media) and rapidly contaminated while, the apical portion of the proliferated shoots were infected very rarely which was also observed by Hohtola (1988). During winter, the rate of *in vitro* contamination was less than 10% in mulberry while during rainy season (July-August) some times the rate of contamination reached upto 50%. However, our

findings were contradictory to Hohtola (1988) who observed in Scot Pine that *in vitro* contamination was low from May to October and significantly higher from December to April. This contradictory findings might be due to their climatic condition of that country where the experiment was conducted or it might be due to the resistance /succeptible condition of Pine tree.

It was observed that, aminoacids like tyrosine, glutamine and aspergine in the media had remarkable effect on shoot regeneration potentiality of the explant. Dodds and Roberts (1985) suggested that, aminoacids can be added in the media for better shoot proliferation. Two different experiments were carried out in this direction. In one experiment, effect of different aminoacids (tyrosine, glutamine and aspergine) were tested on different genotypes (BSRM-3,BSRM-18 and BSRM-19) considering one explant (nodal segment). In another experiment, effects of different concentrations of tyrosine with different cytokinins (BA,Kn, 2iP and Zn) on *in vitro* culture of nodal and shoot apex of BSRM-19 were evaluated. From the results obtained it can be concluded that, aminoacids had the potentiality to enhance *in vitro* shoot proliferation frequency as well as the production of shoots. Statistically it has been proved that, genotype (G), aminoacid (A), concentrations (C), explants (E), different cytokinins and interaction among them were highly significant.

Among the aminoacids, tyrosine was found to be suitable at the concentration of 50 mg/l for nodal explants and 100 mg/l for shoot apex explant. Among the

genotypes tested, BSRM-19 and BA as a cytokinin suited well with the tyrosine. Zaman *et al.*, (1994b) also observed that addition of tyrosine along with BA improved shoot proliferation rate and number of shots in *in vitro* culture of *Morus alba* cv. C776. Islam *et al.*, (1993) also claimed tyrosine as an accelator additive for better *in vitro* proliferation of *Morus alba*. Addition of tyrosine in cytokinin supplemented media also improved shoot proliferation efficiency in *Sesbania cannabina* (Hossain *et al.*, 1990). Rahman *et al.* (1985) also observed better shoot proliferation by adding tyrosine in shoot proliferation media of *Corchorus capsularis*. Our results also show that, glutamine was not favorable for *in vitro* shoot proliferation of mulberry which is also supported by Rao *et al*,. (1992) where addition of glutamine in the media declined the regeneration frequency but the addition of either 100 mg/l aspergine or proline promoted the frequency of response in different varieties of sorghum. Hossain's work (1990) with *Aegle marmelos* (a perennial woody tree) also confirms our findings. He found that presence of tyrosine (100 mg/l), glutamine (50 mg/l) and caseinhydrolysate (300 mg/l) in the media sufficiently improved shoot regeneration potentiality of the explant. Besides, Arya *et al.*, (1981) reported promotive effect of tyrosine on shoot regeneration of *Aegle marmelos*. Though glutamine was found to be less suitable in our experiments, it has been used as routine additive in the embryo culture of many plants (Monnier, 1976).

Suitable micropropagation method was developed for *Morus laevigata*. Among the different species of *Morus, M. laevigata* appears to be a multipurpose tree because of its high quality timber, sweet edible fruits (2-2.5 inches length) and high nutritional quality (Krishna and Ramesh, 1989). Unlike most of the mulberry specics *M. laevigata* can not be propagated through conventional methods (cutting and grafting) because of its poor rooting ability (Rajon and Ravindran, 1989). On the other hand, Mahmood *et al.*, (1987) reported that silkworm larvae fed on *M. laevigata* leaves gained more weight and gave better cocoon yields than those fed on *M. alba.* Unlike other genotypes of *M. alba* this species preferred low concentrations of BA or Kn and at no circumstances rate of shoot proliferation was more than 70 %. In comparison to other genotypes used, this species produced less number of shoot from nodal segment. In the first week of culture, 2-3 changes of media were essential to remove possible amount of latex present in the explant, since the cells contain more latex than the genotypes of *M. alba.* The low concentrations of cytokinins required by the explants of *M. laevigata* can be attributed to the presence of higher levels of endogenous growth regulators which is also supported by the works of Cassells *et al.*, (1982) and Heide (1968). From different rooting experiments, it was observed that the response of *in vitro* derived microshoots of different genotypes of *M. alba* (BSRM-3, BSRM-8 and BSRM-19) and *M. laevigata* to root induction was different. The auxin type, strength of MS, pH levels, sucrose

concentration, age of microcutting, etc., were the most important factors which manipulated the *in vitro* rhizogenesis of mulberry.

Among the auxins, indole-3- butyric acid (IBA) was found to be the best for root initiation in mulberry followed by NAA. Investigation by Zaman *et al.*, (1993), Kim *et al.*, (1985) and Islam *et al.,* (1993), with different genotypes of mulberry observed that best auxin for rooting in mulberry was IBA. In other tree species, like *Syzygium cuminii* Yadav *et al.*, 1990) *Quercus suber* (Manzanera and Pardons, 1990), *Eucalyptus globosus* (Islam *et al.*, 1994), *Sesbania grandiflora* (Ara *et al.*, 1991) IBA was also found to be superior to NAA for root induction. In some cases addition of IBA and NAA together at low concentration induced satisfactory root induction. For example, in *Morus laevigata,* it was observed that, number of roots per shoot was highest when IBA and NAA were added together (each 0.1 mg/l) in the basal media which indicated the synergistic action of the two auxins. Use of double auxin for successful root induction has also been reported in woody trees (Roy *et al.*, 1990; Brave and Mehta, 1993; Ellyard, 1981; Datta and Datta, 1984) where an equimolar concentration of IBA + NAA gave birth better rooting than either of the auxin alone.

From the preceeding study it was noted that different levels of pH (4.0-7.0) and different concentrations of sucrose (10-50 g/l) had substantial effect on *in vitro* rooting of mulberry microshoots. Generally 5.5-6.0 pH range was favourable for *in*

vitro rooting of different genotypes of mulberry. These results are in agreement with the findings of Skirvin (1981) in other fruit trees. Our results indicate that mulberry prefers slightly acidic media conditions in *in vitro* rooting and more acidic and alkaline conditions are unfavorable for better root induction. However, Prunus, Cherries and Plum require different levels of pH for their successful rooting in *in vitro* (Nemeth,1986) indicating species-specific rooting response to the pH of the medium.

Different concentrations of sucrose conspicuously affected the initiation of root from *in vitro* proliferated microshoots. In the absence of sucrose, percentage of root induction was found to be drastically reduced which is similar to the findings of Zimmermann (1983) in apple. Highest number of roots per microshoot was induced in sucrose concentration of 30 g/l while highest length of root was achieved in comparatively higher concentration (40 g/l) which corresponds the work of Snir (1983) in sour cherry. The lowest sucrose concentration (10 g/l) was very negative for rooting of mulberry which has confirmed with the work of Manzanera and Pardons (1990) in *Quercus suber* L. Haissig (1974) and Thorpe (1982) have stated that rooting initiation is a process demanding much energy, which requires sucrose as a carbon source. Our results in mulberry corroborate with their findings.

In vitro root induction of mulberry was also influenced by the strength of media. Unlike shoot proliferation root initation was enhanced by half (1/2) strength

MS media. Number and length of roots were favoured by 1/2 MS which is also supported by the works of Sen *et al.*, (1992); Gill *et al.*,(1994); Zaman *et al.*, (1993c) in different tree species. However, there are instances where full strength MS was favorable. For example,Yadav *et al.*, (1990) and Manzanera and Pardons (1990) observed best root induction using full MS media in *Syzygium cuminii* and *Quercus suber* respectively.

Variation in rooting response was observed among the microcuttings taken from different subcultures. Microcuttings originated from initial culture had unsatisfactory rooting ability. On the other hand microcuttings harvested from later subcultures (especially 1st and 3rd subculture) displayed higher frequency of rooting. However, microcuttings belonged to 6th and onward subcultures exhibited poor rooting. Srikandarajah *et al.*, (1982) in apple cultivars and Rugini and Verma, (1982) in almond observed better rooting from microshoots originated by increasing the number of subcultures. Zimmerman and Broome (1981) also reported that, some apple cultivars which had been in culture for longer period tended to root better than those cultured for shorter time. Our findings in mulberry confirms this statement. The reculturing process may change the physiological state of the microcuttings which in turn promotes better rooting (Hu and Wang, 1983; Economou and Read, 1986).

Microclones of different genotypes of *Morus alba* irrespective to the explant

sources survived and flourished well in soil and natural environment. After 4-5 weeks of transplantation in field the plantlets showed vigorous and uniform growth. Morphological variations among the genotypes were visible in some cases. Increased vigor of microclones under field condition has also been reported for apple (Zimmerman, 1986), thornless blackberry (Swartz *et al.*, 1983). Successful micropropagation of tree species in a relatively new phenomenon (Mott, 1981; Thorpe *et al.*, 1990). Several woody species such as, populus, wild cherry, eucalyptus, red wood and radiata pine are now commercially propagated, while others such as, sandal wood, birch, teak and loblolly pine shows promise (Haissig *et al.*, 1987).

Morphogenic behavior and nutritional status of leaves of *in vitro* raised mulberry genotypes were evaluated and compared with those of cutting derived plants at the same age. Very scanty information is available regarding field performances of *in vitro* raised tree plants both in terms of morphology and nutritional aspects (Pandey and Singh, 1989). No literature is available on field performances (Morphogenic and Nutritional status) of *in vitro* raised mulberry plants. In the present investigation it was found that in some cases the *in vitro* derived mulberry plants displayed more vigorous growth (interms of plant height, leaf number per branch, leaf area, leaf weight and internodal distance) in comparison to those derived from conventional method (cutting). Similar results were reported

by Swart (1981) and Damino *et al.*, (1983) in strawberry.

Field performance of regenerated plantlets has been generally good, although some problems with respect to the flowering and fruit set have been observed with strawberry in Europe, oil plam in Malaysia. These problems seem to be related to the phytohormones regimes used. Not enough data are yet available on the long-term performance of micropropagated rootstocks and self-rooted scions of pome and stone fruits or roses, but initial performance is very favorable (Zimmerman, 1986; Mullins, 1987). In general, hard wood plantlets have performed well in the field, but early maturation of conifer plantlets, as well as initial plagiotropic growth in a few species have been reported (Thorpe *et al.*, 1990).

After 1 year of transplantion in soil *in vitro* derived plants of different genotypes displayed varied range (52-82%) of survivability which indicates genotypic specificity of mulberry. Survival percentage of different micropropagated tree species was different. For example, the survival percentage was 80% in *Eucalyptus* (Das and Mitra, 1990), 60% in *Syzygium cuminii* (Yadav *et al.*, 1990), 11.53% in *Quercus suber* (Manzanera and Pardos, 1990), 75% in *Morus nigra* (Yadav *et al.*, 1990), 58% in *Eucalyptus* (Gill *et al.*, 1994) and 75% in *Gmelina arborea* (Sen *et al.*, 1992).

Unlike morphogenic characters, nutritional characters of both *in vitro* derived and cutting derived (conventional method) plants of mulberry did not show any

significant difference. This is because the plantlets of different genotypes were directly regenerated from shoot apices or nodal segments. Our findings regarding nutritive value (*in vitro* derived and cutting derived plants) of different genotypes reveals that the *in vitro* derived plants were true-to-type. Nutritional value of different leaf at different maturity stages of the same genotype differed significantly both in case of *in vitro* and cutting derived plants but difference between the sources (*in vitro* and cutting) was non-significant. Variation in nutritional value at different leaf maturity stages has also been reported in cutting derived mulberry plants (Krishnaswami *et al.*,1970; and Qader *et al.,* 1991) which correlates with our findings.

Different seasons also had remarkable effect on accumulation of different nutrients in the leaves of different genotypes irrespective of their origin (*in vitro* or cutting). The nutritional value of different genotypes also varied, though leaves of *in vitro* derived plants possessed slight higher amount of nutrition in comparison to leaves of cutting derived plants and the difference of nutrition among the different origin of plants was negligible and non-significant. Pandey and Singh (1989) also reported little higher quantity of total sugar and reducing sugar in *in vitro* raised plants of papaya which is also supported by the work of Swartz (1981) in strawberry. Our finding in mulberry correlates with these observations. Krishnaswami *et al.*, (1970) and Qader *et al.,* (1991) also observed nutritional variation in leaves of

cutting derived mulberry genotypes. They also observed seasonal effect on accumulation of nutrients in leaves of cutting derived plants which partially has supported our findings.

The bio-chemical investigation of our study finally and conclusively reveals that, there is no significant differences between the nutritional value of leaves of *in vitro* and cutting derived plants. The nutritional variation that was recorded due to difference in leaf maturity (tender, mid-mature and mature), difference in genotype and difference in season. This suggests that, *in vitro* derived plants were true-to-type.

In mulberry (*Morus*. sp) callus formation has been surmounted (Oka and Ohyama, 1973; Oka and Ohyama, 1976; Seki *et al.*, 1974; Yamamoto and Kamata, 1971; Ghugale *et al.*, 1971) but the process of organogenesis is puzzling as shoot regeneration could not be obtained from callus (Patel *et al.*, 1983). A number of trials were performed to see the effect of auxins singly or in conjunction with cytokinins on callus induction and embryogenesis from different explants of different mulberry genotypes. Among the different explants used, (cotyledon,leaf segment and internode) calli induced only from internodal explants were found to produce somatic embryo like structures (SELS), whereas, cotyledon and leaf segments induced callus only.

Somatic embryo like structures (SELS) were formed on internodal segments of the genotypes BSRM-19 and BSRM-18. The other genotypes used were non-

responsive. High level of auxin (2,4-D) in combination with lower ones of cytokinin (BA) was found to be suitable for callus induction along with the formation of SELS. Oka and Ohyama (1976) reported that, for induction of callus in mulberry, BA together with auxin proved to be the best but Seki *et al.*, (1971) observed that, Kn along with auxin induced better callus induction in mulberry. However, neither of them claimed about the formation of any SELS in their investigations. Patel *et al.*, (1983) conducted various experiments using auxin and cytokinin to induce organogenesis in callus cultures of mulberry but failed to induce shoots in any of the combinations tested.

We have conducted a series of experiments for the germination of SELS, that we obtained from callus cultures of internodal segments of BSRM-19 and BSRM-18 on MS media supplemented with auxin and cytokinin. In no where, even a single SELS was found to germinate. However, Mhatre *et al.*, (1985) observed shoot buds from callus tissues of the leaf explants in *Morus indica* L. On the other hand Jain *et al.*, (1990) reported that, despite of intensive work, shoot bud formation and somatic embryogenesis from mulberry callus have not yet been achieved. Islam *et al.*, (1992) did not observe any shoot formation from callus cultures of *Morus laevigata* from different explants. To our best knowledge, there is hardly any report of true embryogenesis via callus formation in mulberry which is also supported by the works of Oka and Ohyama (1976); Seki *et al.*, (1971); Patel *et al.*, (1983), Jain *et al.*,

(1990) and Islam *et al.*, (1992). In chickpea (Iqbal *et al.*, 1989; Islam and Riazuddin, 1995) it has been found that, with the passage of time the nucleic acid and peroxidase content of callus deteriorated and no organogenesis was achieved from callus cultures. As the organ formation is genetically controlled, the different aged calli can be tested whether any deterioration of nucleic acid and peroxidase content is encountered with the passage of time. Thus, more thorough and concentrated efforts need to be focussed on some of the basic problems concerning the various factors inducing differentiation before any meaningful work can be done on *in vitro* embryogenesis and other genetic variability for the improvement of mulberry tree.

SUMMARY

5. SUMMARY

The objectives and purposes of the present investigation were to standardize a suitable and stable micropropagation method from different explants of mulberry (*Morus* sp.) simultaneously to study the embryogenesis.

Nutritional standard of leaves of *in vitro* derived plants were evaluated through biochemical tests.

The results of the entire investigation reveals that, micropropagation of *Morus alba* L. and *Morus laevigata* from nodal segments and shoot apices is quite possible.

The shoot proliferation ability of different explants was different. Nodal explant was found to be most suitable and magnificent for multiple shoot proliferation followed by shoot apex explant. Cotyledonary explants exhibited poor performance.

Effects of different basal media were significantly noticed in the present study. Explants preferred MS medium followed by LS for better *in vitro* proliferation. B5 and N6 were unsatisfactory while WP was found to be intermediate.

Genotypic specificity on *in vitro* shoot proliferation was also studied. Explants (node and shoot apex) of BSRM-19 and BSRM-18 gave large number of multiple shoot in suitable media composition while explants from rest of the

genotypes tested produced fewer number of shoots. Performance of different cytokinins on *in vitro* shoot proliferation was not alike. BA (2.0 mg/l) was found to be most suitable cytokinin followed by Kn while, 2iP and Zn were not suitable. However, additive action of cytokinins (BA 1.0 + Kn 1.0 mg/l) was also common. Highest number of shoots (16.22) was produced due to additive action of two cytokinins.

Explant collection period (months) significantly influenced the *in vitro* cloning of different genotypes of *Morus* sp. Late winter and spring (February-March and April) were the best period for explant collection from field-grown plants. Pre-winter and winter (November-December and early January) periods were unsatisfactory for better *in vitro* shoot proliferation. However, in these periods rate of *in vitro* contamination was low (less than 10%) while in rainy seasons (July-August) percentage of contamination was higher (about 50 %). *Aspergillus, Penicillium* and *Bacillus* bacteria were found to be responsible for *in vitro* contamination. Among the different aminoacids tested, tyrosine (50 mg/l for nodal segments and 100 mg/l for shoot apex) was found to enhance the *in vitro* cloning.

Vegetative propagation of *Morus laevigata* is very difficult by conventional (cutting) methods due to its poor rooting ability. Micropropagation method for *M.*

laevigata was also well established by culturing nodal explants. Unlike other genotypes of *M. alba*, explants of *M. laevigata* preferred low concentration (0.5-1.0 mg/l) of BA. Similarly root initiation of this species also varied with other genotypes of *M. alba*. Microshoots of *M. laevigata* rooted well by synergistic action of IBA and NAA (0.1 mg/l each). Explants with genotypes of *M.alba* for rooting indicated that most of the genotypes preferred low concentrations of IBA (0.1-0.5 mg/l). Microshoots of BSRM-19, BSRM-18 rooted better than that of other genotypes. Addition of NAA along with IBA sometimes produced little amount of callus at the base of the microcuttings.

In vitro rooting of mulberry was remarkably influenced by pH levels of culture media and pH ranges from 5.5-6.0 were found to be favourable. Different concentrations of sucrose also influenced *in vitro* rooting and it was found that 25 g/l sucrose was optimum for best root induction.

Microcuttings taken from initial culture and from that of 4th and onward subculture exhibited poor rooting ability, while microcutting harvested from 1st, 2nd and 3rd subculture induced better rooting.

Rooted microcuttings were initially transplanted in polybags containing non-sterile garden soil and compost (1:1). After few days of indoor acclimatization they were finally transplanted in soil where they resumed growth and survived well.

In vitro plantlets were found to be morphogenically healthy in comparison to

cutting derived plants at the same age.

After one year of transplantation nutritional status of leaves of *in vitro* grown plants were studied and compared with the nutritional value of leaves of cutting derived plants which revealed that, nutritionally they (cutting and *in vitro* derived plants) were not significantly different and consequently the *in vitro* derived plants were of true-to-type in nature.

For somatic embryogenesis calli were induced from different explants. Internodal segments were found to be suitable for best callogenesis. Cotyledonary and leaf explants gave unsatisfactory results in respect to callus formation. A few somatic embryo like structures (SELS) were observed on the calli derived from internodal segment of BSRM-19 and BSRM-18. However, these somatic embryo like structures did not germinate even after subculture on variety of media tested.

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