DEVELOPMENT OF *IN VITRO* REGENERATION SYSTEM AND ITS UTILIZATION FOR GENETIC TRANSFORMATION IN MULBERRY *(MORUS* spp.)

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that Ms. D. S. VIJAYA CHITRA has carried out the research work embodied in the present thesis entitled "Development of *in vitro* regeneration system and its utilization for genetic transformation in mulberry *(Moms* spp.)" for the degree of Doctor of Philosophy under my supervision in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad.

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DECLARATION

I hereby declare **that** the work presented in this thesis entitled **"Development of *in vitro* regeneration system and its utilization for genetic transformation in mulberry *(Morus* spp.)"** has been carried out by me under the supervision of Dr. G. Padmaja in the Department of Plant Sciences, School of Life Sciences, University of Hyderabad and this work has not been submitted for any degree or diploma of any other University or Institute.

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To my father who has always Seen my source of inspiration and strength...

To my mother whose ambition for me and whose sacrifices have made this day possible...

Their faith in me has always Boosted my morale

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Vijaya Chitra

CONTENTS

ABBREVIATIONS

ATP	Adenosine triphosphate
BAP	6-benzyl amino purine
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CDPK	Calcium dependent protein kinase
CaMV 35S	Cauliflower mosaic virus 35S promoter
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide tri-phosphate
DTT	Dithiotheretol
2,4-D	2, 4-Dichlorophenoxyacetic acid
2-D	Two-dimensional
EDTA	Ethylenediamine tetraacetic acid
gm	gram
GUS	(3 -glucuronidase
y ^{32}P	Gamma P-32
HC1	Hydrochloric acid
$HgCl_2$	Mercuric chloride
H3PO4	Phosphoric acid
hr	hour
IAA	Indole-3-acetic acid
IBA	Indole-butyric acid
IEF	Isoelectric focusing
IPA	Indole-3-propionic acid
kDa	Kilodaltons
KH_2PO_4	Monopotassium phosphate
K2HPO4	Dipotassium phosphate
KN	Kinetin
M	Molarity
mM	Millimolar
MW	Molecular weight
MS	Murashige and Skoog's medium
$MgCl_2$	Magnesium chloride
mm	millimeter
min	minute
N	Normality
NAA	α-Naphthaleneacetic acid
NaF	Sodium fluoride
NaOH	Sodium hydroxide
nos nopal	ine synthase
npt II	neomycin phosphotransferase II
OD	Optical Density
LB agar	Luria-Bertani agar

LB broth	Luria-Bertani broth
LS medium	Linsmaier and Skoog's medium
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
pI	Isoelectric point
PMSF	Phenyl methyl sulphonyl fluoride
PPFD	Photosynthetic photon flux density
PVP Polyvinyl	pyrrol idone
rpm	rotations per minute
SDS	Sodium dodecylsulphate
SE .	Standard error
TDZ	1 -phenyl-3-(1,2,3-thiadiazol-5-yl)Urea (thidiazuron)
TE Buffer	Tris-EDTA buffer
TEMED	N, N, N',N'-tetramethylethylenediamine
TIBA	Triodobenzoic acid
Tris	Tris(hydroxymethyl) aminomethane
µg	microgram
ul	microlitre
uc	microcurie
V/V	Volume/Volume
W/V	Weight/Volume
X-gluc	5-Bromo-4-chloro-3 indolyl β-D-glucuronic acid
Z	Zeatin

Introduction

Mulberry is an indispensable crop for the sericulture industry as it serves as the exclusive source of feed for silkworms *(Bombyx mori* L.). Conventionally, mulberry is propagated through cuttings, seed and grafting. Mulberry cultivation by cuttings is widely used and popular method for raising saplings. Only 30-40% of the stem cuttings survive the time period between pruning, transportation and final transplantation. Moreover, successful rooting from stem cuttings depends on the favourable environmental conditions and is genotype dependent (Ohyama and Oka, 1987; Jain *et al.,* 1990). Many elite varieties have poor rooting ability and propagation through cuttings is restricted to only certain months of the year (Narayan *et al.,* 1989). Since cross-pollination is the rule in mulberry (Das, 1983; Hossain *et al,* 1992), propagation through seeds does not conserve true-to-typeness because of its heterozygous nature. Methods of conventional vegetative propagation through grafting are not economically viable since it involves skilled manpower, expensive nursery facilities and a minimum time period of 4-5 years to obtain plants ready for harvest (Bhau, 1999). Also the success through grafting depends on internal factors like compatibility, nutrient and water content of the scion, activity of the cambium, as well as external factors like atmospheric temperature and soil moisture.

The problems faced by the plant breeders in propagating this plant by conventional methods can be overcome by using the applications of tissue culture technology. *In vitro* plant propagation through culture of meristems and axillary buds has been used for plant multiplication in a -number of timber species, woody fruit and ornamental crops. Micropropagation provides a valuable tool for rapid multiplication of mulberry plants, as large number of genetically identical plants can be produced in a relatively short time and space. Axillary buds are widely used for micropropagation as

they have entire rudimentary vegetative shoot and can be induced to develop into plants easily, which are similar to the parental type. Meristems are also extensively used for rapid multiplication of shoots, since the constituent cells are genetically identical to the donor plants (Skirvin, 1981). With recent developments in the field of molecular biology and gene manipulations, the meristem tip culture has also been adopted as a tool for gene transfer in higher plants (Ulian *et al.,* 1988; Gould *et ed.,* 1991). *In vitro* plant regeneration from the apical/axillary shoot buds and nodal explants has been reported in a number of species of mulberry (Mhatre *et al.,* 1985; Ohyama and Oka, 1987; Sharma and Thorpe 1990; Chattopadhayay *et al.,* 1990; Hossain *et al.,* 1992; Tewari *et al.,* 1995; Pattnaik and Chand, 1997; Chitra and Padmaja, 1999). However, most of these protocols are largely genotype specific and may not be applicable to valuable genotypes (Bhojwani, 1992). Thus the present study was undertaken with an aim to evaluate the regeneration potential of four elite cultivars and to establish an efficient *in vitro* system for their rapid propagation by culture of nodal explants. In addition, our study was extended to compare the influence of season on axillary bud sprouting and regeneration from nodal cuttings in field and *in vitro* conditions.

The application of biotechnological tools for genetic improvement of mulberry attains greater significance, which in turn depends upon the availability of efficient regeneration system. For woody species like mulberry, however *de novo* organogenesis from differentiated tissues is often regarded difficult and is still limited to a few species. Regeneration in mulberry has been attempted from the axillary buds, hypocotyls, cotyledon, leaf and stem explants (Mhatre *et al.,* 1985; Oka and Ohyama, 1986; Chattopadhyay *et al.,* 1990; Jain and Datta, 1992). Only few authors have reported the *in vitro* regeneration of plantlets from the adventititious buds formed on the leaf explants

derived from aseptically grown shoots (Oka and Ohyama, 1981; Mhatre *et al.,* 1985), cultured embryos (Kim *et al.,* 1985) and from the axillary buds (Yamanouchi *et al.,* 1999; Vijayan *et al.,* 2000) in some of the species of *Morus.* Most of the regeneration work from leaf explants dealt with temperate varieties that are non-adaptive in tropical environments and also there is limited information available on regeneration from leaf explants of tropical varieties (Vijayan *et al.,* 2000). Thus in the present study, an attempt has been made to develop a highly efficient method of plant regeneration from the leaves of economically important cultivars of mulberry.

An important requirement for plants propagated *in vitro* is that the plants should be genetically identical to the source plants since genetic variation was frequently observed in plants regenerated from the tissue culture (Larkin and Scowcroft, 1981). The real applicability of micropropagated plants would ultimately depend on the comparative field performance with those of cutting grown plants. Thus the present investigation was extended to assess the field performance of micropropagated plants in comparison to cutting derived plants for growth characteristics and vegetative morphology for 3 consecutive years.

A better knowledge of understanding of biochemical and molecular aspects of differentiation will have useful applications in tissue culture methods and more importantly in mulberry biotechnology. Proteins are valuable indicators of differentiation and have been used in taxonomy, as genetic markers and could be useful to identify specific stages of development during the *in vitro* studies (Yuffa *et al*., 1994). There are relatively few studies, which revealed the biochemical changes during the process of differentiation and this type of characterization is necessary for the study of differential gene expression during the developmental program that accompanies plant regeneration

from plant tissue cultures. For studying protein changes associated with *in vitro* organogenesis, an experimental system is required in which the developmental events associated with organogenesis can be defined (Christianson and Warnick, 1988). In the present study, protein differences associated with leaf tissues during different stages of callogenesis and shoot organogenesis have been studied.

Protein phosphorylation is ubiquitous in biology (Gilroy and Trewavas, 1990). Protein phosphorylation and dephosphorylation are considered important regulatory mechanisms by which the activity of key enzymes and receptor molecules is altered in response to a wide variety of external stimuli (Cohen, 1982). In plants, the responses of cells or tissues to external stimuli, such as light (Raymond and Douglas, 1990), phytohormones (Raz and Fluchr, 1993; Mizoguchi *et al.,* 1994), polyamines (Chang and Kang, 1999) and environmental stress (Kyo and Harada, 1990; Yupsanis *et al.,* 1994; Suzuki and Shinshi, 1995; Reddy and Prasad, 1995) are mediated in part by the expression of genes whose products contribute to a given physiological effect. Various genes involved in protein phosphorylation during the development of living cells have been isolated and characterized (Stafstrom *et al.,* 1993; Wilson *et al.,* 1993; Kieber *et al*., 1993; Mizoguchi *et al*., 1993, 1994; Lynn and Walker-Simmons, 1995). No attempt has been made to investigate the changes in protein phosphorylation during the process of differentiation in mulberry. Hence, protein phosphorylation patterns during different stages of callus proliferation and shoot organogenesis from the leaves of mulberry cultivars have been studied.

Mulberry cultivation is fraught with many problems in the form of biotic and abiotic stresses. Among the various biotic stresses, fungal diseases cause a major damage to this crop (Philip *et al*., 1996). Mulberry is improved qualitatively and quantitatively

by conventional genetic approaches. However, the perennial and highly heterozygous nature of the plant coupled with prolonged juvenile period limits the speed of improvement using conventional methods (Ravindran and Lakshmi Sita, 1994). Further, the dioecious nature of the taxon and the genetic linkage of desirable and weak traits limit the success of genetic improvement. Genetic engineering offers a suitable alternative as it facilitates the introduction of desirable genes from different sources for developing resistance to various abiotic and biotic stresses. However, this approach demands the development of genetic transformation technology for integration of desirable genes. Among the various genetic transformation techniques, *Agrobacterium* mediated transformation is preferred because of its simplicity, efficiency and relatively neat packaging, and stable integration of transferred DNA into the plant genome (Gasser and Fraley, 1989). Despite the importance of mulberry in sericulture, there is only one report available on genetic transformation. Machii (1990) succeeded in transferring kanamycin resistant gene and β-glucuronidase (GUS) gene *via Agrobacterium* transformation of leaf discs and confirmed the expression of Kan R and GUS genes in regenerated plantlets. In the present study, an attempt has been made to genetically transform mulberry through *Agrobacterium* transformation of shoot meristems.

Keeping the above background in view, the present investigation was undertaken with the following objectives:

- To establish an efficient protocol for micropropagation of four elite cultivars of mulberry using nodal explants.
- To study the seasonal influence on axillary bud sprouting in field and *in vitro* conditions.

- To develop an efficient method for plant regeneration from leaves of four elite cultivars of mulberry.
- To study the field performances of the micropropagated plants in comparison to the plants derived through cuttings for three consecutive years.
- To analyze the changes in protein patterns during callogenesis and shoot organogenesis from the leaves using SDS-PAGE and two-dimensional gel analysis.
- To study the changes in protein phosphorylation associated with different stages of callogenesis and shoot organogenesis from leaves.
- To establish a transformation system for mulberry using *Agrobacterium* mediated transformation of shoot meristems.

Review of literature

Mulberry plant is the major factor determining productivity and the profitability in the sericulture industry. The leaves of mulberry are a major economic component in sericulture industry since the quality and the quantity of leaf produced per unit area has a direct bearing on cocoon harvest. Thus, maximization of mulberry leaf yield per unit area will lead to the realization of two most important objectives namely increased cocoon production per hectare and reduced cost of production (Krishnaswami, 1990). Mulberry cultivation is an applied science, which involves a detailed study of morphological, physiological and ecological features of mulberry. The study encompasses cultivation techniques suited for the production of best mulberry leaves suited for the rearing of silkworms and to obtain rich harvest of such leaves from a constant cultivation area in a reasonably inexpensive manner.

Sericulture provides subsistence to approximately 6 million people in India (Anuradha and Pullaiah, 1992). Textile industry is heavily dependent on natural silk, as the world market requires about 25 million Kg of silk every year. Over 90% of Indian silk is mori silk, since mulberry foliage constitutes the chief feed for the silkworm (Datta, 1994). The precise origin of sericulture in India is not very clear. However, the historians believe that sericulture industry was brought to India from China through Khotan in 140 B. C. Indian scholars believe that sericulture was practiced in the foot hills of Himalayas much earlier to that and originated in the Ganga and Brahmaputra river basins. Modern day sericulture was started during 1875, during the regime of Tippu Sultan ruler of Mysore kingdom, who brought mulberry from Bengal to the south and planted it in Kollegal and adjoining areas, while the silkworm *Bombyx mori* races were brought by him from China (Mundkar and Muniraju, 2002).

In India, most states have taken up sericulture as an important agro-industry with excellent results. The total acreage of mulberry in India is around 282,244 ha (Table 1). The genus *Morus* consists of trees and shrubs and is distributed in the temperate and sub-tropical regions of the northern hemisphere (Rao and Bapat, 1993). There are about 68 species of the genus *Morus,* the majority of them occur in Asia, especially in China (24 species) and Japan (19 species). Continental America is also rich in its *Morus* species. The genus is poorly represented in Africa, Europe and Middle East and is not present in Australia. In India there are many species of *Morus,* of which *Morus alba, M. indica, M. serrata* and *M. leavigata* grow wild in the Himalayas. Several varieties have been introduced belonging to *M. multicaulis*, *M. nigra, M. sinensis* and *M. phillippinensis.* Though mulberry cultivation is practiced in various climates, the major area is in tropical zone covering Karnataka, Andhra Pradesh and Tamil Nadu states, constituting approximately 90% of total area. In the sub-tropical zone, West Bengal, Himachal Pradesh and north-eastern states have major areas under mulberry cultivation.

The most important factor in the management of sericulture is the improvement of mulberry cultivation for achieving higher leaf yields. Mulberry leaves are essential for the survival of silkworms, since silkworms are monophagus insects, which grow only by feeding on mulberry leaves. It will also not be possible for the silkworms to complete the growth in the absence of mulberry. Thus the cultivation of mulberry is the most important factor in the production of silkworm eggs, rearing of silkworm cocoons and on the whole in the entire operation of sericulture. The presence of volatile alcohols, (3, y-hexanol in the leaves of mulberry serve as the attractant for the silkworm strains and cultures. Besides being an important host plant, mulberry is also one of the important multipurpose trees and referred to as kalpaviruksha (Dandin and Ramesh, 1987) as all the

parts of the plant have many uses. Some species of mulberry are grown for their edible fruits and timber (Patel *et al.*, 1983).

The diploid (2n) chromosome number of the *Morns alba* L. is 28, but highly polyploid number, up to 308 is also found in this plant (Ho-Rak Kim *et al.*, 1985). Among the polyploids, the triploids have many desirable traits, including better shoot and leaf growth, higher nutritional quality and resistance to cold and diseases. But, the production and multiplication of the triploids are time-consuming (Das, 1983).

Mulberry plant regenerates itself naturally by seeds or propagated through cuttings or graftings. Propagation through seeds is undesirable as enormous heterozygosity prevails in mulberry plant owing to its cross-pollination (Das, 1983). The mass clonal propagation of mulberry through cuttings is a potentially valuable method to accelerate the improvement programmes of this important woody species (Yadav *et al.*, 1990). However, most of the protocols are genotype specific and may not be applicable to valuable genotypes (Bhojwani, 1992). Moreover, seasonal response in rooting of cuttings and low survival frequency of rooted cuttings limits the application of conventional modes of vegetative propagation for large-scale multiplication of selected strains and cultivars (Tewari *et al.*, 1990). Propagation through grafting is generally not followed due to the various internal factors such as moisture and physiological state of the scion (Rajan and Ravindran, 1989). In vegetatively propagated plants like mulberry it will take many years to evolve a desirable clone from economic and commercial point of view by conventional hybridization methods (Rao *et al.*, 1989).

In vitro techniques particularly those concerning regeneration of the whole plants from adult tissues, permitting the alteration of a few characters through exploitation of somaclonal variation or application of gene transfer, can accelerate the recovery of

improved genotypes (Caboni *et al,* 1999). Though conventional breeding has helped in developing some elite cultivars, its successful application depends largely on a reliable plant regeneration system. Tissue culture techniques such as micropropagation provide an alternative to the routine vegetative propagation of woody species with desirable traits (Biondi and Thorpe, 1982; Thorpe, 1983). Clonal selection and propagation of tree species by tissue and organ culture techniques have considerable potential in breeding and improvement (Mhatre *et al.,* 1985). Tissue culture methods are used by nearly 600 companies throughout the world to produce more than 500 million units annually from almost 50,000 varieties of plants (Vasil, 1994). Plant regeneration from variety of explants has been successfully achieved in many species. In general, three modes of *in vitro* plant regeneration, namely axillary shoot proliferation, organogenesis and embryogenesis have been recognized (Murashige, 1977; George and Sherrington, 1984). Although the rate of plant regeneration is usually higher through organogenesis or embryogenesis, the meristem and shoot tip culture, which allows shoot proliferation through axillary branching, is still considered a method of choice for *in vitro* mass propagation because the method is less prone to the risk of genetic instability (Vasil and Thorpe, 1994).

Micropropagation has many advantages over conventional methods of plant propagation with application in horticulture, agronomy and forestry (Debergh and Zimmermann, 1991; Jeon *et al,* 1995; Hartmann *et al,* 1997). The advantage of micropropagation over conventional seed propagation is that it is possible to rapidly propagate the desirable genotypes (Jones *et al.,* 1982) and also result in production of both non-chimeric and true-to-type plants (Vasil, 1994). The origin of micropropagation can be traced to the early and the pioneering studies of Morel (1960) on the development

of virus free plants from cultured shoot meristems, and the elucidation of the role of cytokinins in shoot morphogenesis in plant tissue cultures by Skoog and Miller (1957). During the past four decades, these observations have prompted many scientists to exploit *in vitro* techniques for the rapid clonal propagation of a wide variety of herbaceous dicotyledonous species as well as many species of evergreen deciduous trees (Zimmerman *et al,* 1986; Vasil 1986, 1991; Debergh and Zimmerman, 1991; Ahuja, 1993; Vasil and Thorpe, 1994). Micropropagation involves the abolition of apical dominance resulting in the derepression and multiplication of the axillary buds.

In mulberry, a highly heterozygous plant, *in vitro* propagation using axillary buds or through culture of tissues having resident meristem is a powerful option to multiply species that are difficult to propagate *via* conventional means (Skirvin, 1981). For successful micropropagation, the buds (axillary buds) or shoot tip cultures (meristems) are preferred as buds have entire rudimentary vegetative shoot and offers no risk of obtaining cuttings different from mother plant than does the classical propagation in the green house (Anuradha and Pullaiah, 1992). Although micropropagation as a means of regeneration is quite popular in mulberry, varietal variations are immense (Bhatnagar *et al,* 2001). Tewary *et al.* (1996) observed significant variation in the sprouting frequencies in 10 genotypes of mulberry in response to cytokinin and auxin. Oka and Ohyama (1975) performed experiments in order to know the suitability of type of material for *in vitro* propagation of two varieties of mulberry. They inferred that young greenish buds with long or short stems grew into leafy shoots whereas in another variety brownish buds did not show any growth. The growth and proliferation of nodal explants was greatly influenced by the time of explant collection in three *Morus* species (Pattnaik *et al,* 1996). In *Morus cathayana* and *M. ihou,* high frequency bud break coupled with

maximum number of shoots was recorded with nodal explants collected between July and October. In *Morus serrata,* the explants collected between November and February yielded the best result. In *Morus australis,* most explants collected during November to February produced inflorescences during shoot elongation. Barve and Mehta (1994) reported high frequency of sprouting from the axillary buds of *Commiphora wightii* collected in the months of April to June then collected between July to August.

Nutritional and hormonal factors play a very crucial role in the growth and development of plants *in vitro* (George, 1996). Interspecific as well as intraspecific variation in culture response of *Morus* has already been reported by earlier workers (Tewari *et al,* 1995, Pattnaik *et al,* 1996, Pattnaik and Chand, 1997). Anuradha and Pullaiah (1992) achieved a high frequency of axillary bud sprouting from nodal explants on medium supplemented with low concentration of 2,4-D. Jain *et al.* (1990) observed sprouting in Shiminochi and Minozuwa varieties of mulberry in the presence of 2,4-D. Gamborg *et al.* (1976) stated that 2,4-D was a powerful suppressant of organogenesis and it was not used in experiments involving root and shoot initiation. The presence of cytokinin was essential for inducing high frequency bud break in some species of *Morus.* Yadav *et al.* (1990) demonstrated that BAP is a better cytokinin than K for growth and multiplication of shoots of *M. nigra.* In contrast, Tewari *et al.* (1999) reported that TDZ at 0.1 mg/l was found to be more effective than BAP for bud break and shoot proliferation in *M. indica* cvs. RFS-175 and K-2, whereas 0.5 mg/1 TDZ was better than BAP in *M. indica* cv. S1. Incorporation of TDZ not only significantly reduced the days required for bud break but also increased the percentage of bud breaks and the number of shoots per explant in *M. indica.* In *Morus laevigata,* the maximum production of shoots from the explants and the greatest length of shoots were obtained using 1.0 mg/1 BAP.

Regeneration of plants *via de novo* shoot formation is necessary for application of gene transfer technology and for screening plants for somaclonal variation. Plant regeneration *via* adventitious shoot formation has been reported from hypocotyls, cotyledons (Kim *et al.,* 1985), stem segments (Narayan *et al.,* 1989; Jain and Datta, 1992) and leaf explants (Oka and Ohyama, 1981; Mhatre *et al.,* 1985; Machii, 1992). Oka and Ohyama (1981) reported that leaf explants of mulberry derived either from subcultured shoots or primary leaves of seedlings had regenerative capacity. They observed that the entire leaf and the basal half of the leaf produced buds in the transitional zone but not at any other region. The apical half of a leaf that was free of the transitional zone did not exhibit any capacity to initiate buds. This result suggested the existence of some specific potency of producing buds in the transitional zone. Kim *et al.* (1985) reported adventitious shoot formation from cotyledonary, leaf, hypocotyls and shoot tip explants on medium containing high levels of BAP with or without a low concentration of NAA. High frequency of buds were induced from the leaves with petioles in *Morus indica* (Mhatre *et al.,* 1985) in contrast to *Morus alba* (Oka and Ohyama, 1981) where sessile leaves yielded more number of buds. Cytokinins are known to induce axillary as well as adventitous shoot formation from meristematic explants (George, 1993). TDZ has been employed for the induction of callus, shoot regeneration, somatic embryogenesis in cultured explants and on intact seedlings, protoplast culture and *in vivo* formation of outgrowths from roots and at the crown, in many herbs, shrubs and trees (Huetteman and Preece, 1993; Lu, 1993; Murthy *et al,* 1998). Thidiazaron is known to mimic the effects of both cytokinins and auxins on growth and differentiation of cultured explants. TDZ was found to be the best for multiple shoot proliferation from embryos axes while BAP promoted the maximum number of shoots from shoot apices in *Ricinus communis* L. The

differential response of cytokinins in both the explant types could be due to differences in uptake, recognition by the cells, or in the mechanisms of action of the cytokinins (Sujatha and Reddy, 1998). Yamanouchi *et al.* (1999) observed the highest frequency of adventitious bud formation from immature mulberry leaves on medium containing 1μM TDZ and 1 uM abscisic acid. Vijayan *et al.* (2000) observed a distinct genotypic effect for shoot differentiation from the leaf explants of tropical mulberry varieties. Genotypes S-799 and Sujanpur-5 having thinner cuticle and leaf blade showed a very high frequency of shoot differentiation compared to the other genotypes. From these results it was suggested that cuticle plays a role in the regeneration ability of leaves in mulberry and a heavier leaf cuticle may act as a barrier to the expansion of epidermal cells as evidenced from the poor leaf expansion and nodulation noticed in the cuticular leaves. Kapur *et al.* (2001) cultured leaf explants of two elite cultivars of mulberry obtained from field-grown plants, *in vitro* raised seedlings and *in vitro* maintained axillary shoot cultures on various media for regeneration. The regeneration percentage was maximum from the *in vitro* leaf explants on MS with 1.1 mg/l TDZ followed by the *in vitro* raised explants. Bhatnagar *et al.* (2001) studied the effect of various growth regulators especially of TDZ on seed germination and subsequent differentiation capabilities of hypocotyls, cotyledon, leaf, internode, root and petiole explants of *M. indica* cultivars K-2 and DD. TDZ at a concentration of 5.0 uM gave the highest percentage of adventitious bud formation in both the hypocotyls and cotyledon explants obtained from the seedlings germinated on 0.5 uM TDZ. The leaf explants produced adventitious buds after 30 days of culture on 2.5 uM TDZ. Elongation of the shoots from the regenerated shoot buds was achieved by subculture on MS medium containing 0.5 mg/1 BAP and 0.5 mg/1 GA_3 and 2.0 mg/1 $AgNo_3$.

Shoot multiplication is an important factor for suitability of tissue culture method for mass propagation of tree species. Mhatre *et al.* (1985) reported induction of multiple shoots on BAP supplemented medium in contrast to induction of a single plantlet per axillary bud on MS with KN or MS with Z supplemented medium in *Morus indica* L. Culture medium containing both auxins and cytokinins produced the best response of multiple shoot formation in *Morus alba* L. (Chattopadhyay *et al.*, 1990). A single axillary bud of *Morus alba* produced more than 20 plants pointing to the possibility of rapid clonal multiplication for commercial exploitation (Chattopadhyay *et al.*, 1990). Pattnaik *et al.* (1995) reported high frequency of sprouting and multiple shoot formation from the nodal segments of three indigenous varieties namely Kanva-2, S-1635 and S-36 and two Japanese varieties namely Goshoerami and Kenmochi on MS medium with 0.1 mg/l BAP whereas higher concentration had suppressive effect on the morphogenetic potential of the shoot buds. Tewari *et al.* (1999) also observed a strong monopodial growth habit in *Morus* cultures when low concentrations of BAP were used and multiple shoots were induced by higher concentration. However, these shoots failed to elongate at the later stage of culture in *Morus indica.* The inhibitory effect of BAP on shoot proliferation at concentrations higher than 1.0 mg/1 was noticed in *Morus* species (Ohyama and Oka, 1987). In *Morus laevigata,* multiple shoots were induced in the presence of BAP at 5.0 mg/1 but these shoots failed to elongate thereby resulting in rosette shoot clumps (Pattnaik *et al,* 1996). Kim *et al.* (1985) achieved elongation of the shoots induced from hypocotyls segments of mulberry by gradual reduction rather than abrupt removal. Mhatre *et al.* (1985) reported that presoaking of explants in cytokinins prior to culture was beneficial for shoot bud induction in axillary bud and leaf explants whereas stem segments proved recalcitrant. They inferred that pre-treatment confers a

physiological status on the explant that is favourable for shoot bud initiation. Yadav *et al.* (1990) reported that BAP was better than K for growth and multiplication of shoots of *M. nigra.* The shoots multiplied at a rate of 50 to 80 shoots every 4 weeks in the presence of 1.0 mg/l BAP. Hossain *et al.* (1992) reported induction of multiple shoots from nodal explants of 10-year-old tree of *M. laevigata* on MS medium with different concentrations (0.5-5.0 mg/1) of BAP. Shoot proliferation increased with the increase of BAP up to 2.5 mg/1 but further increase of the BAP level suppressed shoot proliferation. It was also reported that the shoot multiplication rate increased with the increasing number of subcultures but then declined after 7-8 subcultures.

The type of carbon source supplemented in the medium has been found to affect the plant regeneration with fructose being more effective than sucrose for *in vitro* plant production from bud culture of *Morus alba* L. (Oka and Ohyama, 1982). Tewari *et al.* (1999) observed an increase in the shoot number in each passage with increase in callus from the base when transferred to fresh medium in the V-1 and S-34 cultivars *of Morus.*

The advantage of micropropagation over other type of propagation is that shoot apex is priori present and does not have to be induced, only root induction being required (Anuradha and Pullaiah, 1992). The stimulatory effect of auxins in the root formation depends partly on the type of auxin employed. Oka and Ohyama (1981) achieved root induction from shoots regenerated from leaf explants of *M. alba* on medium containing 0.1 mg/1 IBA. Narayan *et al.* (1989) successfully rooted 95% of mulberry shoots within 3 weeks by supplementing MS medium with 0.5 mg/1 NAA. Anuradha and Pullaiah (1992) observed vigorous rooting of 35% in Mysore local and 45% in Kanva-2 varieties of *Morus alba* L. on a medium fortified with 1.0 mg/1 NAA. They considered NAA to be more efficient rooting agent than the other auxins like 1AA and 2,4-D. Rao and Bapat

(1993) observed abundant rooting from primary axillary bud cultures of *Morus **indica*** L. on MS medium with IAA, which has sparse on NAA and 2,4-D. On the contrary, IBA was an effective auxin for induction of rooting from the regenerated shoots in *Morus alba* L. (Kathiravan *et al.*, 1997; Bhau and Whaklu, 2000) and *Morus bombycis* Koidz (Jain and Datta, 1992).

The information on regeneration from callus cultures in the genus *Morus* has lagged behind. Media manipulation and explant choice are still among the key factors for the successful plant regeneration from different cultivars (Ohyama and Oka, 1987). The morphogenetic potential is dependent upon a number of factors, but the reduced regeneration potential due to callus ageing may be because of the accumulation of inhibitory substances (Halperin, 1986) or may be due to decreased metabolism, transport and interactions between growth regulators (Hansen *et al*., 1987; Van staden and Mooney, 1987; Palni *et al.*, 1988). Jain and Dutta (1992) presented a method for *de novo* induction and development of multiple shoot buds from callus of internodal segments and the regeneration of complete plants of *Morus bombycis* cultivar Schimanochi. Callus was induced from different explants *viz.* stem segments (Oka and Ohyama, 1973), leaf explants (Ogurtsov *et al.*, 1986; Tewari *et al.*, 1989) and hypocotyls segments (Ohnishi and Kobayashi, 1991a and b)) of mulberry genotypes on 2,4-D supplemented medium while proliferation of the callus was found to be better on a medium containing KN, IAA or NAA (Ohnishi *et al*., 1986). Oshigane (1989, 1990) reported plant regeneration from callus obtained from different strains of *M. bombycis*, *M. alba, M. multicaulis* on MS medium supplemented with auxins and cytokinins. Narayan *et al.* (1989) and Rao and Raghunath (1983) obtained shoot regeneration from callus of internodal segments on MS medium supplemented with BAP (2.0-3.0 mg/l),

while Jain and Datta (1992) reported shoot bud induction on LS medium supplemented with BAP in *M. bombycis*. The LS medium was found to be superior over MS medium for adventitious bud formation in the presence of NAA and KN from hypocotyls derived callus (Oshigane, 1989, 1990). Rhizogenesis was reported from hypocotyl derived callus cultures of mulberry on medium containing auxins *viz.*, IAA, NAA (Seki *et ai,* 1971), and IB A, IP A (Ghugle *et ai,* 1971). Ohnishi and Kobayashi (1991c) achieved rooting from suspension cells of callus cultures induced from hypocotyls of mulberry. Islam *et al.* (1992) observed rhizogenesis from callus derived from internodal segments and leaf explants of *M. laevigata* on MS medium supplemented with NAA (Islam *et al.,* 1992).

In vitro plantlets with profuse rooting are best adaptable for the field conditions (Ohyama, 1970). Jain *et al.* (1990) transferred a minimum of 50 plantlets of different genotypes of mulberry to the soil with a survival rate of 83-92%. The regenerants of *Morus laevigata* transferred to the field after acclimatization survived with frequency of 80% (Hossain *et al.,* 1992). Mhatre *et al.* (1985) reported 90% of survival of the tissue cultured plants upon transfer to soil. Anuradha and Pullaiah (1992) also successfully transferred 50% plantlets of *Morus alba,* var. Kanva-2 to the pots for acclimatization.

Genetic variation has been frequently observed in plants regenerated from tissue culture (Larkin and Scowcroft, 1981). Environmental conditions imposed during tissue culture typically include an artificial support medium containing minerals, plant growth regulators and carbohydrates as a source of carbon for growing the explants, low light regimes and high relative humidity. These particular conditions during *in vitro* culture induce anatomical, morphological and physiological changes in the explants (Kozai. 1991; Preece and Sutter, 1991). Morphological changes undergone by micropropagated

plantlets during acclimatization have been reported in 'Pixy' plum (Brainerd *et al.*, 1981), red raspberry (Donnelly and Vidaver, 1984) and *Liquid stryraciflua* L. (Lee *et al.*, 1985).

Very less information is available regarding the field performance of micropropagated tree plants both in terms of morphology and nutritional aspects (Pandey and Singh, 1989). The applicability of micropropagated plants would ultimately depend on the comparative field performance with those of cutting raised plants (Zaman *et al.*, 1997). Jain and Dutta (1992) demonstrated that it is possible to produce a large number of stable true-to-type plants of *Morus* by comparing the *in vitro* grown plants with vegetatively grown saplings for their morphological characters such as leaf shape (lobation), petiole length, area of leaf lamina and internodal length. The field performance of plants raised *in vitro* showed certain characteristics which could be useful for obtaining more yield of leaves per plant, such as more branching, short internodes and variation in phyllotaxy in mulberry (Rao and Bapat, 1993).

The use of the *in vitro* technology has made significant contribution to our understanding of the processes of organized development namely *de novo* organogenesis and somatic embryogenesis (Thorpe, 1993). Though regeneration has been reported in a number of plant species in cultures (Flick *et al.*, 1983), the regulatory mechanism(s) underlying morphogenesis still remains one of the most mysterious and challenging problems of biology (Sunkukar *et al.*, 1986). Proteins are valuable indicators of differentiation and have been used in taxonomy, as genetic markers and for identifying specific stages of plant development. Variation in protein patterns in differentiating and non-differentiating tissues has been reported in *Oryza saliva* L. (Chen and Luthe, 1987) in barley and sugarcane (Ramagopal, 1989, 1994) and in *Nicotiana tabacum* (Garcia *et al.*, 1992). Protein changes have been used as markers to delineate stages in axillary bud

development in pea (Stafstrom and Sussex, 1988) and to identify the time of bud regeneration in detached pine cotyledons (Villalabos *et al.,* 1984). Aitken-Christie *et al.* (1985) found that cotyledon explants of radiata pine that were predisposed to form shoots in culture, contained depleted protein reserves and unhydrolyzed storage lipids. The ability of conifer cotyledons to form shoots in the presence of BAP was distinguished based on their protein profiles (Elles and Judd, 1987). Hahne *et al.* (1988) studied the changes in protein composition accompanying embryogenesis in cell suspension cultures of *Dactylis glomerata* L. and identified several proteins that were specific for embryos or callus under various culture conditions. Leshem and Sussex (1990) employed SDS-PAGE to analyze changes in the protein profiles of melon *{Cucumis melo* L.) seedlings or of detached cotyledons cultured on media that induced the formation of either roots or shoots in order to identify any biochemical events that could serve as markers for organ regeneration. They observed that when polypeptides having molecular weights of 20-25 kDa were expressed for more than 3 days and cotyledons were cultured on an auxin containing medium, roots were regenerated. When these polypeptides were present for 3 days only or less and cotyledons were cultured on a cytokinin containing medium, shoots were regenerated. Kalea and Bhatla (1999) investigated the polypeptides patterns in hypocotyls and cotyledon explants of *Helianthus annuus* L. during induction of callus and rooting in response to NAA treatment. They reported that two proteins of MW 20 and 30 kDa were suppressed in the explants raised on auxin medium for 10 days whereas a new polypeptide of MW 23 kDa appeared in the tissue extracts of 10 day-old hypocotyls explants, accompanying callus and root initiation.

Analysis of proteins of cultured cotyledons of *Pseudotsuge menziesii* (Mirb.) Franco (Hasegawa *et al.,* 1979; Yasuda *et al.,* 1980) and *Pinus ponderosa* Laws. (Ellis

and Judd, 1987) by SDS-PAGE demonstrated that protein differences exist between cotyledons that are or are not competent for *in vitro* shoot regeneration. Wezniak and Partridge (1988) reported that a specific protein (27 kDa) was found to be associated with sorghum callus growth and was more abundant in callus in which regeneration potential was lost. Rajyalakshmi *et al.* (1991) analyzed soluble protein content as well as polypeptide profiles from morphogenic and non-morphogenic calli in wheat {*Triticum aestivum* L.). Several polypeptides were accumulated in the range around 20 kDa only in morphogenic calli whereas 23 kDa polypeptide was exclusively present in the non-morphogenic calli. Dodeman and Ducreux (1996) reported that protein pattern in cell suspension cultures was closer to that of somatic plantlets than to earlier stages of embryogenesis in carrot. Furthermore, the cell suspension pattern was closer to that of roots, which are auxin inducible, than to the other organ of the plantlets suggesting a direct control of auxins on protein gene expression. Blanco *et al.* (1997) analyzed the protein changes associated with plant regeneration in embryogenic calli of sugarcane (*Saccharum* sp.). The results pointed out the association of soluble protein content and callus regenerative ability and suggested the presence of a marker protein (between 55-70 kDa) for embryogenic callus regenerative ability in CP 5243 cultivar.

Protein phosphorylation is ubiquitous in biology (Cohen, 1982; Gilroy and Trewavas, 1990; Hunter, 1987; Ranjeva and Boudet, 1987). Phosphorylation is a form of post translational modification of proteins that provides a mechanism by which organisms can respond rapidly to changes in their internal and external environments over time scales that would preclude a transcriptional response (Reddy and Prasad, 1995). The post-translational phosphorylation of serine, threonine or tyrosine residues of cellular protein has an important role in mediating the action of various hormones and growth

factors (Weinstein, 1983). Protein phosphorylation in plants has been demonstrated in response to light, auxin and heat shock treatment (Scharf and Nover, 1982; Morre *et al.*, 1984; Budde and Randall, 1990). Protein phosphorylation and protein kinases have also been implicated in phytoalexin elicitation and synthesis (Kurosaki *et al.*, 1987; Dietrich *et al.*, 1990), in the action of phytotoxins of phytopathogens (Bidwai and Takemoto, 1987), plant pathogenesis (Crum *et al.*, 1988, Vera and Conejero, 1990), pollen embryogenesis (Kyo and Harada, 1990) and in the systemic induction of protein inhibitors (Farmer *et al.*, 1989).

Calcium (Ca^{+}) is a universal second messenger that regulates a variey of cellular and physiological processes in eukaryotic cells (Poovaiah and Reddy, 1993). In analogy to animal system, stimulus-induced elevations in cytosolic Ca^{2+} are believed to be perceived by a group of calcium binding proteins including Ca^{2+} dependent protein kinases and calmodulin (Roberts and Harman, 1992). $Ca^{}$ and calmodulin-dependent protein kinases have been described in a variety of plants. These activities were associated with plasma membranes (Blowers *el al.*, 1988), chloroplasts (Bennett *et al.*, 1980), soluble fractions (Veluthumbi and Poovaiah, 1984; Bogre *et al.*, 1988) and chromatin (Davis and Polya, 1983). Hetherington and Trewavas (1982) showed a stimulation by calmodulin of endogenous protein phosphorylation in a membrane fraction from pea shoots. Polya and Davies (1982) reported on the partial purification of a histone protein kinase from wheat germ, which could be stimulated by calmodulin.

The pioneer work of Murray and Key (1978) has shown the predominant effect of 2,4-D in the increase of the phosphorylation of nuclear proteins from soybean. There was a striking increase in the phosphorylation of a 48 kD protein after 24 hr treatment. Characteristic changes in the amount of a major phosphoproteins (CDPK 52-54 kDa)

were detected in alfalfa protoplasts grown in the presence of 2,4-D (Dudits *et al.,* 1993). As the number of the dividing cells increased in culture, the Ca^{2+} dependent phosphorylation of the 52-54 kDa protein doublet also showed elevated signals. After partial purification of these proteins it turned out that the 52-54 kDa protein was a Ca^{2+} dependent protein kinase (CDPK) with autophosphorylation capability. Tan and Kamada (2000) examined the patterns of protein phosphorylation in embryogenic cells (EC) and non-embryogenic cells (NC) that had lost the ability to form somatic embryos. Two-dimensional polyacrylamide gel electrophoresis and subsequent autoradiography revealed the presence of 31 phosphoproteins in EC but not in NC. Kyo and Harada (1990) examined the electrophoretic patterns of the entire complement of phosphorylated proteins from cultured pollen grains of *Nicotiana rustica* and observed remarkable differences between the patterns during normal development and embryogenic de-differentiation. Two dimensional gel electrophoresis and autoradiography revealed a pattern of protein phosphorylated *in vivo*, which consisted of six spots and was specific to embryogenic pollen grains. This characteristic pattern of phosphorylation was observed neither with pollen grains engaged in normal development (maturation) nor with non-embryogenic pollen derived from pollen grains younger or older than those at the mid-bicellular stage. Cordewener *et al.* (2000) investigated the changes in protein synthesis and phosphorylation during microspore embryogenesis in *Brassica napus* by two-dimensional (2-D) gel electrophoresis. Comparison of 2-D patterns of phosphorylated protein revealed minor differences between embryogenic and non-embryogenic cultures, except for the level of phosphorylation of hsp 70. Chang and Kung (1999) reported that the phosphorylation level of a 30 kDa nuclear protein was decreased by the growth-promoting hormones, 1AA and ethylene, while it was increased by a growth inhibitor,

spermine. These results suggested that there is a correlation between phosphorylation/dephosphorylation of the 30 kDa nuclear protein and the control of cell growth in *Ranunculus* petioles.

Mulberry is affected by several diseases caused by fungi, bacteria, mycoplasma, viruses and nematodes (Minamizawa, 1997). These diseases affect the growth of mulberry and cause considerable damage to the plant and loss in leaf yield. Feeding of diseased leaves has also been found to affect adversely the growth and development of the silkworms, cocoon yield and silk quality. Among the various biotic stresses, fungal diseases cause a major damage to the crop. Notable among them is the leaf spot, which is commonly caused by a pathogen *Cercospora moricola* (Cooke) Sacc. (Ullal and Narasimhanna, 1994) and reduces the leaf yield by 10-20% (Philip *et al,* 1996). In vegetatively propagated plant like mulberry it takes many years to evolve a desirable clone by conventional hybridization (Rao *et al,* 1989). In addition due to the cross-pollinating and perennial nature of crop, improvement of specific characters through conventional breeding is slow and cumbersome. Transformation approaches offer an attractive alternative to the conventional techniques for transfer of desirable genes without affecting the genetic makeup of the plant. Success in the generation of transgenic plants depends on efficient DNA delivery system, a method of regeneration and selection of fertile plants from plants derived from a single transformed cell (Chang *et al,* 1996). Of all the transformation techniques, *Agrobacterium tumefaciens* provides a reliable and well-documented means for introducing foreign DNA into plant cells. *A. tumefaciens* mediated delivery of foreign genes into numerous plant species has been extensively described since Horch *et al.* (1985) first demonstrated the utility of the system with subsequent documentation by other workers (Wordragen and Dons, 1992;

Fisk and Dandekar, 1993; Ishida *et al,* 1996; Hiei *et al,* 1994). The leaf disc transformation method established by Horsch *et al.* (1985) has been used for plant species that are susceptible to *Agrobacterium tumefaciens* infection and can be regenerated from leaf explants. Although shoot regeneration from leaf disks has been possible in some species (Nehra *et al.,* 1989), such systems are highly genotype specific limiting their use in genetic transformation of only few genotypes (Nehra *et al.,* 1990). Das *et al.* (2002) reported on the development of a very efficient regeneration and transformation protocol *via* somatic embryogenesis from leaf disc culture in four important genotypes of grapes. The leaf discs were subcultured on a double-layered medium after 3 days of coculture to overcome the problem of necrosis or cell death. Putative transformed calli growing on kanamycin were found to be positive for GUS and *npt* II genes.

Trujillo *et al.* (2001) optimized a transformation system for leaf explants of two of the Andean potato cultivars using *Agrobacterium tumefaciens* strain LBA 4404 containing pBI 121. Calli and shoot formation occurred after 5-8 weeks on a selection medium containing 3 mg/l Z and 1 mg/l IAA. Of the kanamycin resistant plantlets obtained, 51 % of diacol capiro cultivar and 13 % of parda pastusa cultivar were confirmed to be transformants by histochemical assays, polymerase chain reaction analyses and Southern blotting techniques. Zhan *et al.* (1997) developed a new protocol for increasing the efficiency of production of transgenic aspen plants. The new protocol was based on the leaf disc method but extended its power by inducing transformed roots on medium containing IB A (1.5 mg/1) and high concentration of kanamycin and then regenerating plants from transformed roots on medium containing zeatin (0.6 mg/1) and kanamycin (100 mg/1). This protocol greatly improved recovery of transformed shoots,

almost eliminated the occurrence of escapes and reduced the occurrence of chimeric shoots.

Since in most cases the totipotent meristematic cells are capable of developing into a fertile plant without an intermediate callus phase and the system is generally applicable to arrange of cultivars within a single species, the use of meristem and shoot tip culture has been recently proposed as an alternative to leaf disk system for *Agrobacterium* mediated genetic transformation (Ulian *et al.*, 1988). The use of the shoot apex as the explant for transformation has been reported in *Zea mays* (Gould *et al.* 1991) and cotton (Gould and Magallanes-Codeno, 1998). Machii (1990) has reported the genetic transformation of mulberry by incorporating foreign genes into mulberry system using *Agrobacterium tumefaciens* LBA 4404 as vector and succeeded in transferring kanamycin resistant gene and P-glucuronidase (GUS) gene through Ti plasmid PBI 121 to mulberry leaf discs and confirmed the expression of Kan r and GUS gene in regenerated plantlets.

Table-1: Area under mulberry cultivation in different states

State	Area (ha)
Andhra Pradesh	38,084
Assam	2813
Jammu & Kashmir	4717
Karnataka	166,000
Kerala	1164
Madhya Pradesh	2043
Manipur	25, 957
Tamil Nadu	9491
Uttar Pradesh	5665
West Bengal	21,358
Other	4934
Total	**282,244**

Materials and Methods

Mulberry cultivars M-5, S-36, S-13 (*Morus indica* L.) and China White (*Morus alba* L.) were procured **from Department** of Sericulture, P2, L. R. Seed Farm, Kammadanam, Mahboobnagar, Andhra Pradesh, India. Protein molecular weight marker for SDS-PAGE (Phosporylase b 97.4 kDa, Bovine Serum Albumin 66 kDa, Ovalbumin 43 kDa, Carbonic anhydrase 29 kDa, Soybean Trypsin Inhibitor 20 kDa and Lysozyme 14.3 kDa) and DNA molecular weight marker (Lambda DNA/*EcoRI* + *Hind III*) for PCR were purchased from Bangalore Genei, India. Growth regulators such as 2,4-D, BAP, NAA, IAA, IBA, TDZ, KN and Z were purchased from Sigma Chemical Company, St. Louise, U.S.A. Calmodulin, X-Gluc and antibiotics such as Carbenicillin, Cefotaxime, Kanamycin monosulfate and Rifampicin were purchased from Sigma Chemical Company, U. S. A. [γ-^{32}P] ATP (Specific activity >100 TB q/m mol) was purchased from BRIT (Board for Radioactive and Isotope Technology), Hyderabad. X-ray films and intensifying screens were purchased from Kodak, USA. Nonidiet P-40 and Freund's adjuvants (complete and incomplete) were purchased from Sigma Chemical Company. All other Chemicals /Reagents used were of extra pure and analytical grade manufactured from India by different firms.

Establishment of mulberry nodal cuttings in field:

Nodal cuttings of 20-22 cm containing at least 4-5 axillary buds were collected from two-year-old field grown plants of *Morus indica* L. cultivars M-5, S-36, S-13 and *Morns alba* L. cv. China White at different stages of growth *viz.*, developing, assimilation, storing and winter resting stage. The characteristic features of the above cultivars are given in Table 2. The leaves of China White cultivar were dark green and larger in size (Fig. 2) but had difficulty in rooting through cuttings compared to other cultivars. The nodal cuttings excised from different cultivars were immediately planted

Table 2: Characteristic features of mulberry cultivars used in the present study

Species	Cultivar	Sex	Attributes
Morus indica L.	M-5	Predominantly female	Resistant to powdery mildew, leaf spot and bacterial blight. High rooting ability and wide adaptability.
Morns indica L.	S-36	Female	Tolerant to leaf spot and powdery mildew, moderately susceptible to leaf rust and tukra infestation. Most suitable to young age silkworm rearing. Moderate rooting ability.
***Morus** indica* L.	S-13	Male	Resistant to leaf spot and powdery mildew, moderately resistant to leaf rust and tukra infestation. Deep rooting system, profuse branching with short **internodes** and superior leaf quality. Moderate rooting ability.
Morus alba L.	China White	Female	Large leaf size with thick, dark green foliage. Weight of single leaf very high. Low rooting ability.

Fig. 1: Different cultivars of mulberry established from nodal cuttings in the field.

M-5

S-36

S-13

China White

Fig: 1

Fig. 2: Comparison of the leaf sizes of four cultivars of mulberry.

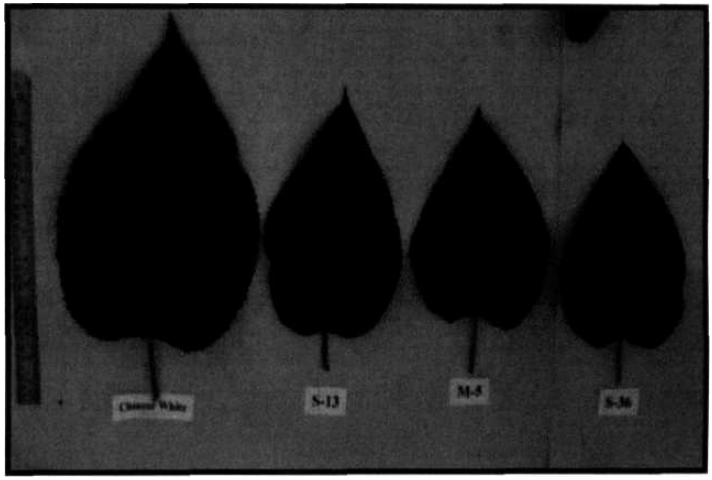


Fig:2

in the pots containing soil and manure in a ratio of 3:1 and kept out in the field for studying the influence of different seasons on axillary bud sprouting and regeneration from nodal cuttings. The environmental conditions in different seasons are mentioned **in** the Table 3. The pots were watered on alternate days in all the seasons. The appearance of the shoot meristems from the axillary buds was taken into consideration for calculating the sprouting frequency. The data on axillary bud sprouting was scored after 30 days and plant establishment frequency (PEF) from nodal cuttings was determined after 60 days. Twenty nodal cuttings were used for each experiment and all the experiments were repeated thrice.

Induction of axillary **bud** sprouting *in vitro:*

For *in vitro* studies, nodal explants of 3-4 cm in length were collected from the same plants as described above. The nodal explants were kept under running tap water **for** *Vi* hr and surface sterilized in 70% alcohol for 1 min followed by 0.1% mercuric chloride ($HgCl_2$) for 15 min under sterile conditions. The explants were then rinsed 4-5 times in sterile distilled water with a duration of 5 min each. The sterilized explants were placed on MS (Murashige and Skoog, 1962) medium containing 3% sucrose and 0.8% agar. The culture medium was supplemented individually with 2,4-D, KN and BAP in varied concentrations for inducing sprouting and shoot differentiation. All the experiments were repeated thrice at different times and ten explants were used for each treatment.

Induction of shoot organogenesis from leaves:

Leaves with or without petiole derived from sprouted axillary buds, *in vitro* multiplied shoots or the shoots that have rooted were used for induction of shoot organogenesis. They were taken from different positions of the shoots and cultured with

either abaxial or adaxial side in contact with the medium. The culture medium was supplemented with BAP, TDZ, Z and KN individually in varied concentrations and also in combination with auxins such as NAA, IAA and IBA. To determine the influence of container type on shoot organogenesis from leaf explants, the cultures were established in culture bottles (11.5 X 5.5 cm), plastic petridishes (diameter 90 mm) and test-tubes (15 X 2.5 cm) containing 30 ml, 30 ml and 15 ml of medium, respectively. The petridishes were sealed with parafilm. Studies were also conducted to observe the effect of presoaking of leaves in MS liquid medium with BAP (0.5-2.0 mg/1) for different durations (24-72 hr) on induction of shoot organogenesis after culture on MS medium with 2.0 mg/1 BAP. The influence of sugars such as sucrose, fructose, maltose and glucose at 3% in the presence of 4.0 mg/1 TDZ on induction of shoot organogenesis from leaves of four cultivars was studied. The appearance of adventitious buds from the leaf was taken into consideration for calculating the shoot organogenesis from the leaves.

Induction of **callus** from the leaf explants:

Leaves of 1.0-4.0 cm in length were cultured on MS medium supplemented with 2.0 mg/1 2,4-D for induction of callus from the explants.

Multiplication of shoots:

Shoot tips of 2-3 cm derived from sprouted axillary buds, leaf regenerated shoots or multiplied shoots were cultured on MS medium supplemented individually with cytokinins such as BAP, TDZ, KN and Z at 0.5-2.0 mg/1 for induction of multiple shoots. The effect of sucrose, maltose, glucose and fructose at 3% on induction of multiple shoots of 4 cultivars was studied by culturing shoot tips on MS medium with 0.5 mg/1 BAP. The multiplication potential of shoots was studied during repeated subculture of shoot tips on MS medium supplemented with 0.5 mg/1 BAP for 10 months at 30 days

intervals. The appearance of the shoot buds from the base of the explant and also from the axils of leaves was taken into consideration for calculating the multiplication frequencies. The average number of shoots induced per explant was recorded after 30 days of culture.

Root induction from shoots:

Healthy shoots derived from the sprouted axillary buds, leaves or from the multiple shoots were transferred to MS medium supplemented individually with 2,4-D, IAA, IBA and NAA at 0.1-2.0 mg/l for root induction. The observations on the duration for root induction, nature of roots induced and the frequency of root induction were recorded.

For all the experiments on induction of axillary bud sprouting, shoot multiplication and root induction from shoots, the cultures were maintained at 25± 2° C under a 16 hr photoperiod with a photosynthetic photon flux density (PPFD) of 83.6 μE m" s^{-1} provided by white fluorescent tubes.

Acclimatization of regenerated plants:

Regenerated plants having well developed roots were removed from culture bottles and washed free of agar. They were transferred to plastic pots containing soil and organic manure (3:1) and kept in a net house under shade for 15-20 days. In the first week of transfer, the plantlets were covered with polythene covers to maintain humidity. After 15-20 days of acclimatization, the plantlets were transferred to earthen pots and planted out in field. The percentage survival was recorded after 6 weeks of transfer to field.

Comparative study of field performance of micropropagated plants and cutting derived plants:

Plants derived from nodal cuttings and micropropagated plants (rooted after three subcultures on shoot proliferation medium) were grown in field plots in rows at a spacing of 60 cm with blocks of 24 plants in each plot. The field was mulched with field manure (soil and organic manure in 3:1 ratio) after planting and irrigated every 3 days. The cutting propagated and micropropagated plants were then picked up randomly from the rows and sacrificed ten each for 3 consecutive years to study the various morphological parameters such as height of the plant, thickness of the main shoot, total number of branches per plant, total number of leaves per plant, length of the internodes, weight of 100 leaves and leaf yield per plant.

SAMPLES FOR SDS-PAGE:

Samples for studying the protein profiles during callus proliferation from the leaves:

Leaves of 1-4 cm derived from the *in vitro* multiplied shoots were cultured on MS medium with 2.0 mg/l 2,4-D for induction of callus. Protein profiles were analyzed from the samples collected at explant stage (1^{st} sample), swelling of the explant (2^{nd} sample), slight initiation of callus (3^{rd} sample) and complete proliferation of callus (4^{th} sample).

Samples for studying the protein profiles during shoot organogenesis from leaves:

For studying the changes in protein profiles associated with shoot organogenesis, leaves of 1-4 cm derived from the ***in*** *vitro* multiplied shoots were cultured on MS medium with 2.0 mg/1 BAP and the samples were collected at explant stage (1^{st} sample), swelling of the explant (2^{nd} sample), induction of shoot buds (3^{rd} sample) and induction of shoots of 0.5-1.0 cm (4^{th} sample). The basal region of the leaves eliminating the apical

portion (1st sample) or the basal region with the differentiated shoots was used for sampling.

SAMPLES FOR TWO-DIMENSIONAL GEL ELECTROPHORESIS:

Protein was extracted from the leaves cultured on MS medium with 2 .0 mg/l 2,4-D or medium with 2.0 mg/1 BAP for detecting the proteins expressed during callus proliferation and shoot organogenesis, respectively. The samples used consisted of leaf explants, callus proliferated from leaf explants and induction of shoots of 0.5-1.0 cm from leaf explants.

SAMPLES FOR PROTEIN PHOSPHORYLATION:

Total protein was extracted from the leaf samples collected at different stages (as described in SDS-PAGE analysis) after culture on MS medium with 2.0 mg/1 2,4-D that stimulated callus proliferation or from those cultured on MS medium with 2.0 mg/1 BAP that triggered shoot organogenesis. The protein was subjected to *in vitro* phosphorylation for analyzing the changes in protein phosphorylation during de-differentiation and shoot differentiation.

PROTEIN EXTRACTION:

All the samples mentioned above were weighed 100 mg each and ground in a pre-chilled motor and pestle in 1ml of 50 mM Tris HC1 buffer (pH 5.7) containing 5 mM $MgCl_2$, 2mM K_2HPO_4, 1mM EDTA, 5 mM DTT, 2 mM KH_2PO_4, 5 mM DTT, 2% PVP, 20% glycerol, 10mM NaF, 10mM β-mercaptoethanol and 2mM PMSF. After homogenization, the samples were centrifuged at 4°C centigrade for 20 min at 12,000 rpm. The supernatant was taken and soluble protein content was estimated by Lowry's (1951) method with minor modifications as given below.

Protein estimation by Lowry's method:

Solution A consisted of 4% Sodium carbonate in 0.2 N Sodium potassium tartrate, Solution B consisted of 1% Cupric sulphate, and Solution C was 2% Sodium potassium tartrate and Solution D was 1N Folin's reagent (commercial). The working solution was obtained by mixing solutions A, B, C in a ratio of 23:1:1 and this solution was used within 24 hr of preparation. One ml of the working solution was added to one ml of protein sample, mixed well and allowed to stand for 10 minute. Then 0.2 ml of solution D was added rapidly while vortexing the sample. After 30 minutes, absorbance of the sample was recorded at 750 run. Bovine serum albumin (BSA Fraction V) was used as a standard protein (5-50 µg/ml).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed following the method of Laemmli (1970) with minor modifications. The separation of proteins was performed in 5% stacking gel and 10% resolving gel. Both the resolving and stacking gel contained 2.4% bisacrylamide as a cross linker and 0.1% SDS. The final buffer concentrations were 0.45 M Tris HC1 pH (8.9) in resolving gel and 0.2 M Tris HC1 (pH 6.7) in stacking gel. Ammonium persulphate and N,N,N,N-tetra methylethylene diamine (TEMED) were used as polymerizing reagents in final concentration of 0.05% and 0.1%, respectively. The electrode buffer comprised of 0.0247 M Tris HC1 and 0.19 M glycine and 0.1% SDS in one liter of distilled water (pH 8.3). The samples were mixed with sample buffer consisting of 0.5 M Tris HC1 (pH 6.8) and boiled at 90°C for 3 min. The samples (10-20 µl) having 50 µg protein were loaded in slab gel wells of the gel of 8 x 8 x 0.1cm dimension which was polymerized in plain glass plates and was fixed to Broviga (India) vertical slab gel apparatus. Gels were run at room temperature at

a voltage of 75 and 100 DC (direct current) for stacking and resolving gel, respectively. Electrophoresis was carried out until the bromophenol blue dye marker reached about 3-4 mm from the bottom of the gel. Then the gels were removed, and stained overnight with 0.25% w/v Commassie Brilliant Blue R 250 in methanol: glacial acetic acid: water (50:7:43) v/v. Medium range molecular weight marker (Bangalore Genei Pvt. Ltd.) was used for calibration.

TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS:

Proteins were separated by isoelectro focusing in 0.3 x 10 cm long tubes, according to the method of O'Farrel (1975). The second dimension slab gel for molecular weight was performed by SDS-PAGE as described previously and the gels were silver stained according to the method of Blum *et al.* (1987).

(a) Isoelectric Focusing (First dimension):

Polyacrylamide used was 30% (1.8% bis acrylamide as cross linker) in tube gels (0.35 x 8 cm) in the presence of 9.5 M urea, 10% Nonidet P-40 and 2% ampholines (pH 4-6 range, 1.6% and pH 3.5-10 range, 0.4%) for the first dimension. The gels were pre-run at 200 volts for 15 min, 300 volts for 30 min and 400 volts for 30 min with 0.03 M Sodium hydroxide (NaOH) as cathodic solution and 0.01 M Phosphoric acid (H_3PO_4) as anodic solution. After pre-run anodic and cathodic solutions were replaced with fresh solutions. Approximately 50µg of protein in the sample buffer (9.5M urea, 2% ampholines, 10% w/v Nonidet P-40 and 5% β- mercaptoethanol) was loaded on the gels. The protein samples were overlaid with 10 ul of 8 M urea followed by 0.03 M NaOH. One tube gel was run without protein sample for measuring the pH. Electrophoresis was carried out at 600 volts for 12 hr, 700 volts for 1 hr and finally for 1hr at 800 volts. The gels were extruded out of the tube and equilibrated in the equilibration buffer (0.0625

Tris HC1, pH 6.7 with 10% glycerol, 0.005M DTT and 2.3% SDS) for 1 hr, thereafter the gels were removed immediately and used for second dimension or for pH measurement. The gels were stored at -70°C until further use.

(b) Second Dimension:

The second dimension was performed in a discontinuous SDS-PAGE as previously described and silver stained. The IEF gels were placed on stacking gel and sealed with 1% agarose made in equilibration buffer. The standard markers were loaded in corner side of the gels. The gels were removed and silver stained.

(c) Measurement of pH:

The isoelectric focused gels were cut into 1 cm pieces and placed in individual test tubes containing 1.0 ml of distilled water. The test tubes were vortexed and kept overnight at room temperature. The pH was measured with pH meter.

(d) Silver Staining:

The proteins were detected in the gel by silver staining procedure according to Blum *et al*. (1987). The gels were fixed for more than 1hr in a fixative containing 50% methanol, 7.5% acetic acid and 0.5 ml of 37% formaldehyde per litre. The gels were washed with 50% ethanol for 3 times in 20 min interval. The washed gels were pretreated with 0.002% Sodium thiosulphate solution for exactly one minute and rinsed 3 times quickly with distilled water. The pretreated gels were impregnated for 20 min in 0.2% silver nitrate solution containing 0.02775% formaldehyde. The gels were rinsed 2 times with distilled water and developed for the proteins with the solution containing 6% sodium carbonate, 0.0185% formaldehyde and 0.0004% Sodium thiosulphate. The gels were placed in fixative solution for 10 min for the development of protein spots in appropriate intensity and subsequently stored in 50% methanol at 4°C.

IN VITRO PHOSPHORYLATION STUDIES:

Protein phosphorylation was carried out in 3 steps following the procedure of Mann *et al.* (1991). Phosphorylation of the protein sample, separation of the protein samples by SDS-PAGE as described previously and exposure of the gels to X-ray films,

(a) Phosphorylation of the sample:

(i) Ca^{2+} independent phosphorylation:

Calcium independent phosphorylation of endogenous substrate proteins extracted from the leaves during different stages of callus proliferation and shoot differentiation was carried out following standard methods (Babu *et al.*, 1994; Ali *et al.*, 1998). Equal amount of protein (100 µg) was taken from different samples of callus proliferation and shoot organogenesis. The reaction was carried out in 50 µl of 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ and 1mM DTT and 100 µM EGTA to chelate Calcium. The reaction was initiated by the addition of 4 µCi [*y*- ' P]ATP to the reaction mixture containing the sample and incubated at room temperature for 1 min. The reaction was terminated by Laemmli sample buffer (0.125 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 2% β-mercaptoethanol and 0.01% bromo phenol blue) and the mixture was heat denatured for 2 min in boiling water. The proteins were separated by SDS-PAGE on 10 % polyacrylamide gel as described by Laemmli (1970).

(ii) Calcium dependent phosphorylation:

The Calcium dependent phosphorylation was performed exactly as described above, excepting that the EGTA in the reaction mixture was replaced with 0.4 mM Calcium chloride.

(iii) Calmodulin dependent phosphorylation:

The Calmodulin dependent phosphorylation was also performed as presented above. The reaction was carried out in 50 ul of 50 mM Tris–HCl (pH 7.4), 10 mM Magnesium acetate, 1mM DTT, 0.4 mM $CaCl_2$, 2 μM calmodulin and 100 ug of protein for the different samples of callus proliferation and shoot organogenesis from the leaves.

(b) SDS-PAGE:

SDS-PAGE was performed as described previously and stained with commassie Brilliant Blue R 250 in 0.25% w/v methanol, glacial acetic acid and water in ratio.s of (50:7:43), and dried.

(c) Exposure of Gels to X-ray film:

The dried gels were exposed to the Kodak X-ray films by keeping them in the cassettes with intensifying screens (Kodak) and stored at -70°C for 2 days. The exposed films were developed and fixed with commercially available developer and fixer. Autoradiograms obtained were compared with the dried gels for determination of accurate molecular weights of the proteins.

***AGROBACTERIUM* MEDIATED GENETIC TRANSFORMATION:**

Shoot meristems of 0.5 cm in length were excised from healthy shoots of S-36 cultivar at the end of 3rd stage of shoot proliferation (MS medium with 0.5 mg/1 BAP) and used in transformation experiments. The work dealing with *Agrobacterium* mediated transformation was done in strict observance of national safety regulations.

***Agrobacterium* construct:**

A. tumefaciens strain GV2260 harbouring binary vector p'GUSINT" having *gus* gene with intron, driven by CaMV 35S promoter and *npt* II gene driven by *nos* promoter was used as vector system in genetic transformation of mulberry. The *Agrobacterium*

strain was maintained on LB agar medium containing 100 mg/1 rifampicin, 100 mg/1 carbenicillin and 50 mg/1 kanamycin. A single colony of *Agrobacterium* was cultured in 25 ml of LB broth medium with the above antibiotics at 28°C on a rotary shaker (200 rpm) for about 18 h until an optical density (OD) 600 of approximately 0.5 was reached. Aliquots of the culture were centrifuged at 3000 rpm for 5 min. The pellets were suspended in MS medium containing 3% sucrose in 1:10 dilution and used for infecting the explants.

Tissue **culture selection and plant regeneration:**

The medium used for inducing shoot proliferation and root induction from shoots was MS medium with 3% sucrose and 0.8% agar. Initially, the shoot meristems and leaves excised from healthy shoots were cultured on MS medium with 0.5 mg/1 BAP supplemented with a range of concentration of kanamycin (10-100 mg/1) to evaluate its effect on shoot bud differentiation. In addition, the sensitivity of the shoots to root induction was tested by culturing healthy shoots on MS medium with 0.1 mg/1 IBA or 0.1 mg/1 2.4-D and 10-100 mg/1 kanamycin.

Healthy shoot meristems were infected with the *Agrobacterium* by exposing the cut end of the shoot meristem to bacterial suspension for 10 min, blotted on sterile filter paper and transferred to MS medium with 0.5 mg/1 BAP. After 2 days of co-cultivation on MS medium with 0.5 mg/1 BAP at 22°C in the dark, the explants were placed on MS medium with 0.5 mg/1 BAP and 250 mg/1 cefotaxime for 2 days for eliminating the *Agrobacterium.* The shoot meristems were then transferred to MS medium with 0.5 mg/1 BAP, 250 mg/1 cefotaxime and 100 mg/1 kanamycin as the selection agent. The shoots meristems that have responded for bud differentiation were subsequently transferred to MS medium with 0.5 mg/1 BAP and 100.0 mg/1 kanamycin. The muliple shoots that

were induced were subcultured on the same medium for five times to avoid the possible escapes. Healthy shoots (2-3 cm) derived from the cultures at the end of 5th subculture on selection medium were transferred to MS medium with 0.1 mg/l IBA or 0.1 mg/l 2,4-D with 50 mg/1 kanamycin for root induction. The shoots that developed healthy roots were transferred to plastic pots containing soil and organic manure and the humidity was maintained by covering with polythene cover for 10-15 days. Subsequently, the plants were transferred to earthen pots and maintained in the greenhouse.

Extraction of genomic DNA from the leaves of the untransformed and putative transformed plants:

Genomic DNA was isolated from leaf tissues by the procedure based on CTAB (Cetyl trimethyl ammonium bromide) method of Saghai-Maroof *et al*. (1984). Leaves were collected from the putative transformants and untransformed plants (controls) established in the field. One gram of leaf tissue was quick frozen in liquid nitrogen and ground to fine powder in a mortar and pestle. The fine powder was thoroughly extracted with 5 ml of warm CTAB extraction buffer (1M Tris (pH 7.5), 5 M NaCl, 0.5 M EDTA (pH 8.0), 14 M β-mercaptoethanol) containing 1% CTAB and incubated at 65° C for 60-90 min. About 5 ml of Chloroform/isoamyl alcohol (24:1) was added to this solution and rocked gently on a shaker for 5 min and centrifuged at 5000 rpm for 10 min at room temperature.

To the supernatant, an equal volume of isopropanol was added and the precipitated DNA was hooked or centrifuged at 5000 rpm for 10 min. The pellet recovered was dissolved in 1ml of TE buffer and equal volume of phenol:chloroform was added to the DNA sample. The aqueous phase was transferred to a fresh tube and precipitated with 1/10 volume of 3M sodium acetate and 2.5 volumes of ice-cold absolute

ethanol. The precipitated DNA was pelleted by spinning at 5000 rpm for 10 min, dried and suspended in appropriate volume of TE. The concentration of DNA was determined spectro-photometrically and its quality through gel analysis.

GUS **analysis:**

GUS gene expression in the leaves and callus cultures of the untransformed and putative transformed plants was detected using the method of Jefferson (1987). Tissues were incubated for 12 hr at 37 ^{0} C in 50 mM Sodium phosphate buffer (pH 7.0) containing 1mM X-gluc. Following overnight incubation the tissues were rinsed in 70 % ethanol and the development of blue colour was monitored.

Polymerase Chain reaction (PCR):

PCR was performed on a perkin thermal cycler, with DNA extracted from putative transformants and untransformed control plants. Each reaction (50 µl) contained 0.2 µg DNA, primers, 1.25 units of Taq polymerase (GIBCO BRL Life Technologies. Burlington, Ontario), 1.5 mM $MgCl_2$, 10 mM dNTP mix and primers, covered with mineral oil. The samples were heated to 94^{0}C for 3 min, followed by 35 cycles of 94^{0}C (1 min), 55^{0}C for 1 min and 72°C for 1 min, with a final 10 min extension at 72^{0}C. The amplified products were electrophoresed on 1% agarose gel and visualized by ethidium bromide staining.

The primer sequences for *npt 11* gene are:

npt II Left

5' GAG GCT ATT CGG CTA TGA CTG 3'

npt II Right

5' ATC GGG AGC GGC GAT ACC GTA 3'

Results

Seasonal effect on axillary bud sprouting in field conditions:

The influence of season on axillary bud sprouting and plant establishment frequency from the nodal cuttings placed in soil was studied in field conditions. A high frequency of sprouting as well as a high plant establishment rate from cuttings was observed in summer compared to the rainy and winter seasons in all four cultivars. Adventitious roots were formed from the base of the nodal cuttings during shoot development in all four cultivars (Fig. 3). Axillary buds of M-5, S-36, S-13 cultivars sprouted in 10-12 days and plant establishment frequency from nodal cuttings varied from 63.3-93.3% in different seasons (Fig. 4). In China White cultivar, asynchronous sprouting was observed in all seasons and at the end of 60 days only 23.3% of the nodal cuttings established into plants in summer indicating the difficult-to-root nature of the cultivar (Table 3).

Sprouting of **the axillary buds *in vitro:***

Nodal explants of 3-4 cm bearing axillary buds were cultured on MS medium supplemented individually with 2,4-D, KN and BAP for inducing sprouting. Nodal explants having greenish axillary buds enveloped by 2-3 whorls of scales on medium tender shoots responded efficiently for sprouting. Axillary buds without scales from very tender shoots turned brown upon sterilization treatment and buds from very old shoots with more than 2-3 whorls of scales did not sprout even after 30 days of culture. Contamination in the cultures was observed to be season dependent. The maximum contamination of 40.0% occurred during winter followed by the rainy and summer seasons with frequencies of 25.0% and 15.0%, respectively.

The sprouting frequency from axillary buds of four cultivars varied from 10.0-83.3% in summer followed by rainy season (Fig. 5 and Table 4). A low frequency of

Table 3: **Seasonal effect on axillary bud sprouting and plant establishment rate from the cuttings of different mulberry cultivars in the field.**

Seasons (Months)	Cultivar							
	M-5		S-36		S-13		China White	
	Sprouting (%)	PE*[a] (%)	Sprouting (%)	PE* (%)	Sprouting (%)	PE * (%)	Sprouting (%)	PE* (%)
Summer[b] (February - May)	96.7± 3.3 a	93.3± 2.2 a	93.3± 2.5 a	90.0±0.1a	90.0± 0.0 a	90.0± 0.0 a	70.0± 0.7 a	23.3± 2.3 a
Rainy[c] (June-September)	86.7± 3.3 a	86.7± 1.9 a	86.7± 1.7 a	86.7± 2.1a	83.3± 2.1a	80.0±1.2 b	66.7± 2.2 a	20.0± 0.8 a
Winter[d] (October - January)	70.0± 5.8 b	70.0± 2.2 b	66.7± 1.7 b	66.7±1.7 b	66.7± 1.7 b	63.3± 1.9 c	53.3± 0.9 b	16.7± 1.8 a

[a] *PE – Plant Establishment Frequency

[b] Mean temperature (°C): 23.9 - 31.4; sun shine (h) 8.2 - 10.1; relative humidity (%) I: 57.0 – 82.0, II: 19.0 – 38.0.

[c] Mean temperature (°C): 26.1 – 28.4; sun shine (h) 4.3 – 6.8; relative humidity (%) I: 80.0 – 88.0, II: 52.0 – 69.0.

[d] Mean temperature (°C): 19.1 – 26.0; sun shine (h) 7.9 – 9.9; relative humidity (%) I: 81.0 – 87.0, II: 33.0 – 51.0.

Mean followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Fig. 3: Comparison of rooting from the nodal cuttings of four cultivars after 45 days of transfer to soil.

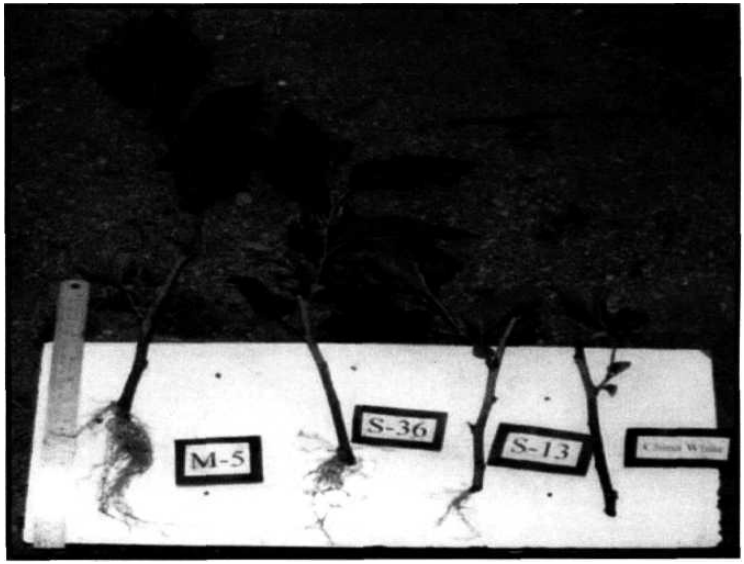


Fig:3

Fig. 4: Establishment of plants from nodal cuttings of four cultivars after 45 days of transfer to soil.

Fig:4

Table 4: Seasonal effect on *in vitro* axillary bud sprouting of different cultivars of mulberry

Treatments (mg/l)	Sprouting frequency (%) *											
	M-5			S-13			S-36			China While		
	S	R	W	S	R	W	S	R	W	S	R	W
Basal	36.7 + 3.3	33.3 + 3.3	23.3 + 8.8	50.0 + 5.8	43.3 + 6.7	30.0 + 5.8	56.7 4 6.7	46.7 + 6.7	40.0 + 5.8	30.0 + 5.8	23.3 + 3.3	16.7 + 8.8
0.3 2.4-D	80.0 + 5.8	76.7 + 3.3	56.7 ± 3.3	73.3 + **3.3**	63.3 + 3.3	40.0 + 5.8	83.3 + 3.3	76.7 + 3.3	60.0 + 5.8	50.0 + 0.0	43.3 + 3.3	33.3 + 6.7
1.0 **2,4-D**	63.3 + 3.3	56.7 + 3.3	50.0 ±5.7	66.7 + **6.7**	46.7 + 3.3	23.3 + **3.3**	56.7 + 3.3	46.7 + 3.3	33.3 + 8.8	30.0 + 5.8	23.3 + 6.7	6.7 + 3.3
2.0 2,4-D	56.7 + **6.7**	46.7 + 8.8	36.7 **+ 6.8**	53.3 + **3.3**	33.3 ± 6.7	6.7 + 3.3	36.7 + **3.3**	26.7 + 3.3	6.7 + 3.3	23.3 + 3.3	0.0	**0.0**
0.3 KN	26.7 + 6.7	20.0 + 5.8	10.0 + 5.8	73.3 + 3.3	60.0 + 5.8	43.3 + 3.3	46.7 + **3.3**	33.3 + 3.3	30.0 + 5.8	53.3 + 3.3	33.3 + 8.8	23.3 + 3.3
1.0 KN	13.3 + 3.3	10.0 + 5.8	6.7 + 3.3	60.0 + 5.8	53.3 + 8.8	26.7 + 6.7	43.3 + **6.7**	26.7 + 6.7	16.7 + 3.3	70.0 + 5.8	56.7 + 6.7	43.3 + 3.3
2.0 KN	10.0 + 5.8	6.7 ±3.3	0.0	56.7 + 3.3	36.7 + 6.7	16.7 + 3.3	13.3 + 3.3	6.7 + 3.3	0.0	76.7 **+ 3.3**	60.0 + 5.8	46.7 + 3.3
0.3 BAP	23.3 + 3.3	10.0 + 5.8	**0.0**	56.7 + 3.3	50.0 + 5.8	46.7 + 6.7	40.0 + 5.8	33.3 + 6.7	23.3 + 6.7	40.0 + 5.8	26.7 + **3.3**	16.7 + 8.8
1.0 BAP	30.0 + 5.8	26.7 + 3.3	16.7 + 3.3	43.3 + **3.3**	36.7 + **3.3**	26.7 + **3.3**	30.0 + 5.8	20.0 + 5.8	16.7 + 8.8	26.7 + 3.3	16.7 + 8.8	10.0 + 5.8
2.0 BAP	40.0 +5.8	30.0 + 5.8	20.0 + 5.8	40.0 + 0.0	33.3 +3.3	20.0 + 5.8	16.7 + 3.3	0.0	0.0	16.7 +3.3	10.0 + 5.8	**6. /** **+** 3.3

* The values represent the mean (± SE) of three independent experiments. Ten explants were used for each experiment.

S - Summer (February- May)
R - Rainy (June-September)
W- Winter (October-January)

Fig. 5: Induction of sprouting from the axillary buds of four cultivars of mulberry.

M-5

S-36

S-13

China White

Fig:5

sprouting (0.0-60.0%) was observed in winter indicating the strong influence of season on axillary bud sprouting. A high frequency of axillary bud sprouting (56.7-83.3%) from the nodal explants of M-5 and S-36 cultivars was induced on medium supplemented with 0.3 mg/l of 2,4-D and sprouting occurred in 5-7 days. When the concentration of 2,4-D was increased in the MS medium, the sprouting frequency decreased (0.0-66.7%) in all the four cultivars of mulberry. Medium supplemented individually with KN (0.3-2.0 mg/1) and BAP (0.3-2.0 mg/1) induced axillary bud sprouting at frequencies ranging from 0.0-46.7% and 0.0-40.0% in M-5 and S-36 cultivars, respectively and the duration of sprouting was longer (18-20 days) compared to 2,4-D. Axillary buds of the S-13 cultivar collected in summer exhibited sprouting with the same frequency of 73.3% on medium supplemented individually with 2,4-D (0.3 mg/1) and KN (0.3 mg/1). However, axillary buds cultured on 2,4-D medium sprouted in 9-10 days whereas those cultured on medium with KN sprouted in 22-24 days. In the China White cultivar, MS medium with 2.0 mg/1 KN favoured a high frequency of sprouting (46.7-76.7%) compared to 2,4-D supplemented medium with the buds sprouting at the same duration of 12-14 days.

Development of shoots as well as inflorescences was observed from sprouted axillary buds of all four cultivars irrespective of the season during which nodal explants were collected. The female inflorescences that were induced in M-5 and S-36 cultivars, when allowed to grow along with the shoot, the growth of the shoot remained arrested (Fig. 6a). Subsequently, when the shoot along with the inflorescence was transferred to rooting medium containing 1.0 mg/1 2,4-D, the female inflorescence ripened with no seed set resulting into parthenocarpic fruits (Fig. 6b). When the female inflorecences were removed at an early stage of growth, the shoots resumed their growth with complete vigour. The removal of the male inflorescence was however, not required in S-13

Fig. 6a: Induction of shoot along with female inflorescence from the sprouted axillary bud of S-36 cultivar on MS medium with 0.3 mg/l **2,4-D.**

Fig. 6b: A complete plantlet of S-36 cultivar with ripened fruit on MS medium with 10 mg/1 2,4-D.

Fig : 6a

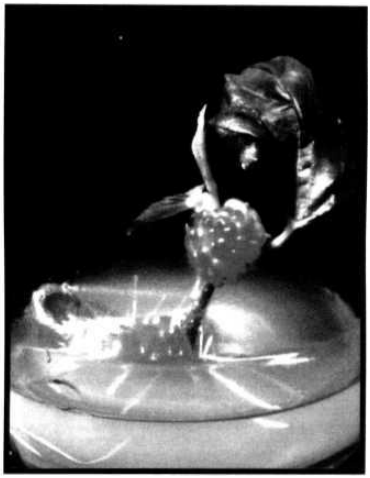


Fig : 6b

cultivar **as the** flowers withered away immediately after the pollen was shed from the anthers without affecting the growth of the shoot.

Induction of direct shoot organogenesis from the leaves:

Plant regeneration *via* direct shoot organogenesis has been achieved from the cultured leaf explants of all four cultivars. The age and the orientation of the leaves remarkably influenced the response of leaves for direct organogenesis. The leaves from very young (smaller than 1.0 cm) and very old leaves (more than 4 cm) did not respond for shoot regeneration even after 60 days of culture on the media tested. Hence, leaves of an intermediate size (1-4 cm length) were used for further studies on shoot organogenesis. The leaves present at the apical region and very old leaves situated at the lower position of the shoots failed to exhibit any response, whereas the leaves present at the middle portion of the shoot responded efficiently for shoot organogenesis. Shoot bud induction was observed from leaves cultured with the abaxial surface in contact with the medium. The type of culture vessel that was used for culture of leaves influenced the organogenic response. The response of shoot organogenesis was high when leaf explants were cultured in the test tubes (2.5 x 15.0 cm) whereas those cultured in culture bottles (11.5 x 5.5 cm) and petridishes (diameter 90 mm) responded with a low frequency.

Presoaking of the leaves for 24-72 hr in MS liquid medium containing BAP (0.5-2.0 mg/l) had no significant effect on induction of shoot buds in all four cultivars. The source from which the leaves were derived did not have any affect on shoot organogenesis as the leaves derived from the various sources such as the sprouted axillary buds, multiple shoots and from the shoots that have developed roots responded similarly for shoot organogenesis. The leaves without petiole exhibited a high frequency response for shoot bud induction in contrast to the leaves with the petiole, which failed to

differentiate into shoots even when left on the medium for 60 days. There was no induction of shoot buds from the leaves that were cultured on MS medium without growth regulators and only increased in size at the end of 30 days of culture.

High frequency of shoot organogenesis (77.6-89.2%) was achieved from the leaves that were cultured for a limited period of 8-10 days on 4.0 mg/1 TDZ supplemented medium and subsequently transferred to MS medium with 2.0 mg/1 BAP (Fig. 7). Low levels of TDZ (2.0-3.0 mg/1) promoted shoot bud differentiation at low frequencies of 6.8-33.4% in different cultivars whereas medium with 4.0 mg/1 TDZ favoured high frequency of shoot organogenesis. Distinct, 8-9 visible meristems were produced from a single leaf besides, there were number of smaller, continuously proliferating shoot buds on medium with 4.0 mg/1 TDZ. But, from a single leaf only 2-3 buds developed into shoots of 0.5-1.5 cm at the end of 30 days and growth of the rest of the adventitious shoot buds remained arrested. These shoot buds resumed growth only upon transfer to 2.0 mg/1 BAP supplemented medium and 16-18 shoots developed in all four cultivars after 30 days of transfer. The frequency of adventitious shoot bud induction as well as the number of shoot buds induced per explant decreased in all four cultivars with the increase in concentration of TDZ (Table 5).

Direct shoot organogenesis was achieved with a frequency ranging from 0.0-46.7% in different cultivars from the leaves that were cultured directly on medium with BAP (2.0-5.0 mg/1) without preculturing on TDZ supplemented medium. Adventitious shoot buds of 5-6 in number differentiated from the basal portion of the leaf after 30 days of culture on medium with 2.0 mg/1 BAP. The leaves cultured on medium with 3.0 mg/1 and 4.0 mg/1 BAP responded for shoot organogenesis with a frequency of 13.6-32.5% and 0.0-18.5%, respectively in different cultivars. A further increase in the concentration

Table 5: Effect of BAP and TDZ on direct shoot organogenesis from the leaves of four cultivars of mulberry

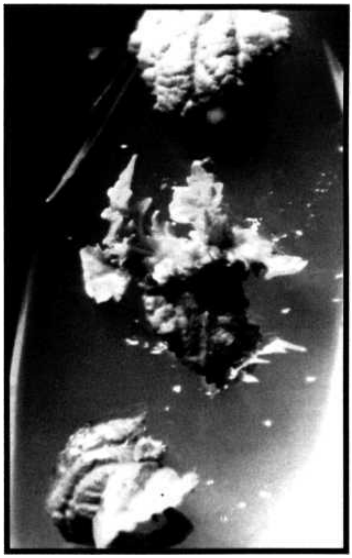
Type of cytokinin	Concentration (mg/l)	% of shoot regeneration			
		Cultivar			
		M-5	S-36	S-13	China White
BAP	2.0	46.7 + 2.2 a	44.4 + 4.4 a	43.7 + 1.9 a	30.1 + 1.5 a
	3.0	32.5 + 3.7 ab	28.4 + 3.0 b	22.7+ 1.6 bc	13.6+ 1.8 b
	4.0	18.5+ 1.0c	8.3 +1.4 c	14.3 +2.8 cd	0.0
	5.0	8.7 + 1.9 d	6.1 + 0.8 cd	11.0 +1.2 d	0.0
TDZ	2.0	13.3 + 3.4 cde	19.3 + 1.9 e	11.4 + 0.8 de	6.8 + 3.5 bc
	3.0	27.4 + 4.7 ab	33.4 + 3.4 b	21.0 + 2.8 bdf	17.3 +2.8 bc
	4.0	84.9 + 3.7 f	89.2 + 3.7 f	80.6 + 5.2 g	77.6 + 3.5 d
	5.0	64.7 + 3.4 g	67.7 + 3.4 g	48.1 + 3.2 a	43.9 + 3.7 e

Means followed by the same letter in a column arc not significantly different ($p < 0.05$) by Newman-Keul`s multiple range test.

Fig. 7: Direct shoot regeneration from leaf explants of different mulberry cultivars exposed to 4.0 mg/i TDZ for 8-10 days followed by subculture to medium with 2.0 mg/l BAP.

M-5

S-36



S-13

China White

Fig:7

of BAP (5.0 mg/1) decreased the frequency of organogenesis by 0.0-11.0% in all four cultivars with the thickening of the proximal and marginal portion of the leaves without any induction of shoot buds.

The leaves cultured on MS medium supplemented individually with KN (2.0-5.0 mg/1) and Z (2.0-5.0 mg/1) did not exhibit any shoot organogenesis. Combination of BAP (10.0 mg/1) and IBA (1.0 mg/1) induced shoot buds in 26-28 days at a low frequency of 10.0-13.5% from leaf explants of M-5, S-13 and S-36 cultivars, whereas in the China White cultivar, no response was observed even after 60 days of culture. The addition of fructose, glucose or maltose instead of sucrose did not have any effect on leaf organogenesis and the leaves turned pale cream in colour within 30 days of culture on glucose supplemented medium.

Multiplication of the shoots:

The response of shoot proliferation from the shoot tips derived from the axillary buds and leaf regenerated shoots was studied by culturing on MS medium with BAP, TDZ, KN and Z individually at 0.5-2.0 mg/1 and 3% sucrose. Multiple shoots were induced from the shoot tips differentiated from the axillary buds (Fig. 8) and leaves (Fig. 9) at a high frequency of 80.0-90.3% and 78.7-93.3%, respectively on MS medium with 0.5 mg/1 BAP (Table 6 and 7). In all four cultivars, multiple shoots were induced in 9-10 days on MS medium with 0.5 mg/1 BAP with induction of shoots of 2.4-3.6 cm in 30 days from shoot tips derived from the axillary buds and leaves. Medium supplemented with 1.0 mg/1 BAP triggered induction of 2.4-5.2 shoots with frequencies ranging from 67.8-73.4% and 70.5-82.6% from shoot tips derived from axillary bud and leaf regenerated shoots, respectively in different cultivars (Fig. 10a and 10 b). The shoot tips derived from both axillary buds and leaves when cultured on 2.0 mg/1 BAP also

Table 6: *Effect of different cytokinins on multiple shoot induction from the shoots differentiated from the axillary buds*

Type of cytokinin (0.5 mg/l)	Cultivar											
	M-5			S-36			S-13			China White		
	FOS*	NOS*	LOS*	FOS*	NOS*	LOS*	FOS*	NOS*	LOS*	FOS*	NOS*	LOS*
BAP	87.8 ± 3.4a	7.4±0.2a	3.2 ±0.2a	90.3 ±0.7a	7.5 ±0.2a	3.4 ±0.3a	84.2 ±3.8a	6.6 ±0.2a	3.5 ±0.2a	80.0 ±0.9a	6.5 ±0.2a	2.6 ±0.3a
TDZ	57.0 ± 3.6b	1.6 ±0.2b	1.5 ±0.1b	66.8 ±6.0b	1.7 ±0.1b	1.4 ±0.3b	50.9 ±6.6b	1.5 ±0.1b	1.4 ±0.1b	47.5 ±3.4b	1.3 ±0.2b	1.5 ±0.1b
KN	63.6 ±4.0bc	3.3 ±0.2c	1.4 ±0.1b	67.2 ±2.8bc	3.6 ±0.2c	1.6 ±0.2b	60.1±0.9bc	3.4 ±0.3c	1.4 ±0.2b	60.4 ±0.7c	3.5 ±0.2c	1.3 ±0.1b
Z	40.2 ±1.0d	2.8 ±0.2c	1.1 ±0.1b	43.7 ±3.8d	2.5 ±0.2d	1.3 ±0.1b	37.1±3.7d	2.6 ±0.2d	1.3 ±0.1b	27.8 ±2.7d	2.4 ±0.2d	1.1 ±0.2b

Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

FOS* Frequency of multiple shoot induction (%)

NOS* Number of shoots

LOS* Length of the shoots (cm)

Fig. 8: Multiple shoot induction from the axillary bud derived shoots of different cultivars of mulberry after 30 days of culture on MS medium with 0.5 mg/1 BAP.

M-5

S-36

S-13

China White

Fig:8

Table 7: Effect of different cytokinins on multiple shoot induction from the shoots regenerated from leaves.

Type of cytokinin (0.5 mg/l)	Cultivar											
	M-5			S-36			S-13			China White		
	FOS*	NOS*	LOS*	FOS*	NOS*	LOS*	FOS*	NOS*	LOS*	FOS*	NOS*	LOS*
BAP	93.3±1.9a	10.3±0.4a	3.6±0.05a	82.9±1.6a	10.6 ± 0.2a	3.4±0.1a	78.7±1.8a	10.5±0.4a	3.0±0.1a	81.1±1.8a	9.4±0.3a	2.4±0.1a
TDZ	76.0±1.3b	1.6±0.1b	1.6± 0.1b	73.6±3.0b	1.7 ± 0.1b	1.6±0.1b	72.1±0.8b	1.5±0.1b	1.5±0.1b	72.0±1.7b	1.5±0.1b	1.3±0.1b
KN	60.1±1.8c	3.6±0.2c	1.7 ± 0.2b	69.5±2.0bc	3.5 ± 0.1c	1.7±0.1b	64.2±1.3c	3.6±0.1c	1.5±0.1b	67.4±1.0b	3.5±0.1c	1.3 ± 0.2b
Z	56.3±1.6c	2.4±0.1d	1.2 ± 0.1c	65.1±2.0b	2.6 ± 0.1d	1.3 ± 0.1c	57.1±1.8	2.7±0.2d	1.2±0.1b	55.1±2.0c	2.3±0.1d	1.0 ± 0.1b

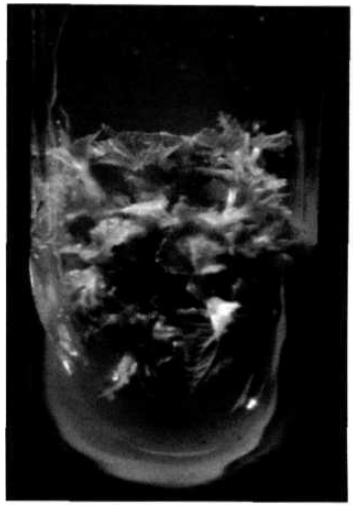
Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

FOS* Frequency of multiple shoot induction (%)

NOS* Number of shoots

LOS* Length of the shoots (cm)

Fig. 9: Multiple shoot induction from the shoots regenerated from leaves of different cultivars of mulberry after 20 days of culture on MS medium with 0.5 mg/1 BAP.



M-5

S-36

S-13

China White

Fig:9

Fig. 10a: Effect of different levels of cytokinins (0.5-2.0 mg/1) on frequency of multiple shoot induction from the shoots differentiated from the axillary buds.

Fig. **10b:** Effect of different levels of cytokinins (0.5-2.0 mg/1) on frequency of multiple shoot induction from the shoots differentiated from the leaves.

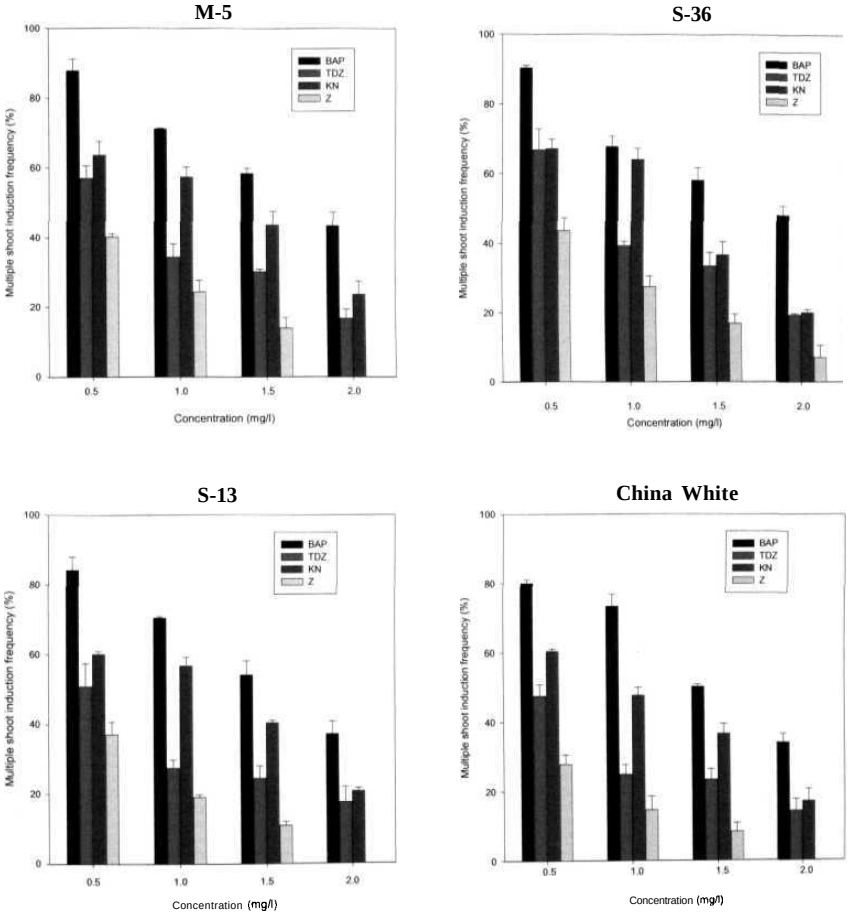

M-5
S-36
S-13
China White
BAP
TDZ
KN
Z
Multiple shoot induction frequency (%)
Concentration (mg/l)
0
20
40
60
80
100
0.5
1.0
1.5
2.0

Fig: 10a

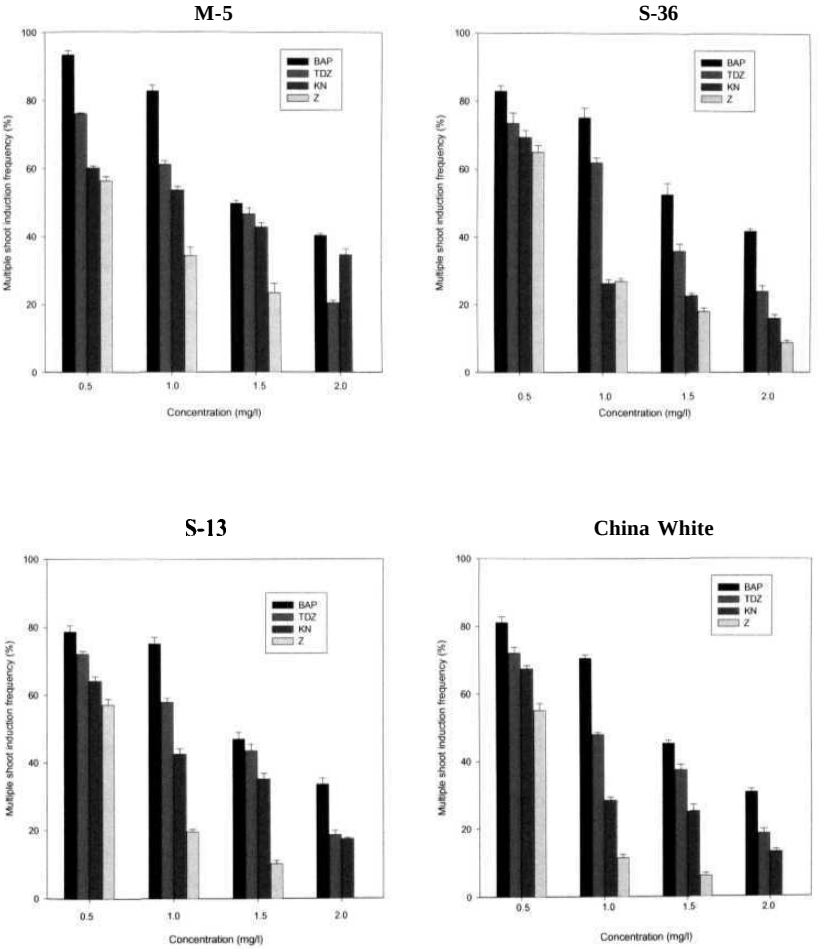

M-5
S-36
S-13
China White
BAP
TDZ
KN
Z
Multiple shoot induction frequency (%)
Concentration (mg/l)
0
20
40
60
80
100
0.5
1.0
1.5
2.0

Fig : 10b

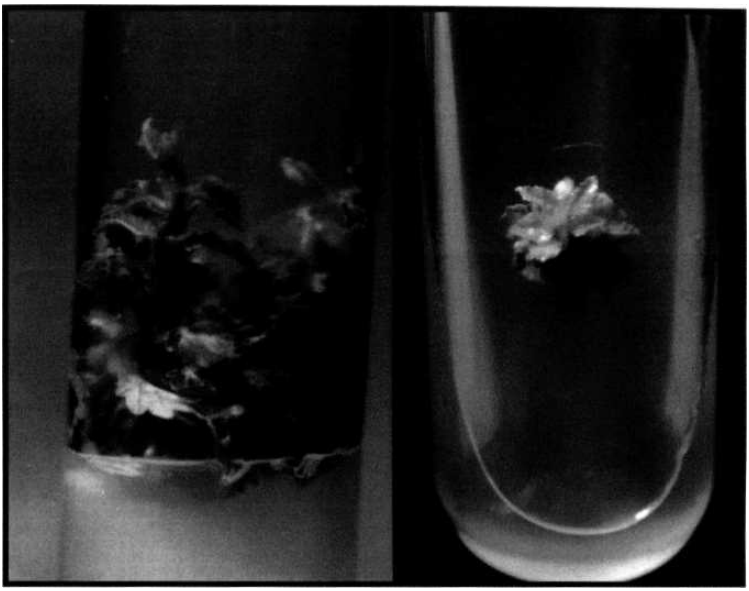
developed shoot buds from the base but the shoots failed to grow and appeared as clumps (Fig. 11), which did not resume growth even when left for 60 days on the same medium. Transfer of shoot clumps to medium with low levels of BAP (0.5 mg/I) facilitated further growth of the shoots with induction of shoots of 1.5-2.0 cm after 30 days of culture.

Thidiazuron at 0.5 mg/l supported induction of 1.3-1.7 shoots from the base of the shoot tips derived from sprouted axillary buds and leaf regenerated shoots of all four cultivars with a frequency of 47.5-66.8% and 72.0-76.0% and length of 1.4-1.5 cm and 1.3-1.6 cm, respectively. The leaves of the multiple shoots increased in size and turned dark green with the thickening of the veins at the end of 30 days of culture. The shoot buds that have appeared from the base became fused, and developed into shoots that were fasciated with thickening of the leaves as the growth proceeded. Shoots cultured on medium with TDZ (2.0 mg/1) developed callus from the base that was cream coloured and had sectors of green patches (Fig. 11). Callus with green sectors was placed on medium with 0.5-2.0 mg/1 BAP for promoting shoot differentiation. However, no shoot bud differentiation was observed from the callus even after 30 days of culture. Following the above observation, internodal segments without shoot meristems were cultured on medium with 2.0 mg/1 TDZ for observing their response for callus induction and subsequent plant regeneration. However, the internodal explants turned brown and did not show any sign of callus initiation. This observation suggested that the presence of meristematic region is essential for induction of callus from the internodal explants. Shoot tips cultured on medium with KN and Z responded differently with no induction of shoot buds from the base of the culture. However, axillary shoots were induced at a frequency of 27.8-67.2% and 55.1-69.5% from the shoot lips derived from axillary bud and leaf regenerated shoots, respectively in different cultivars. The main shoot increased

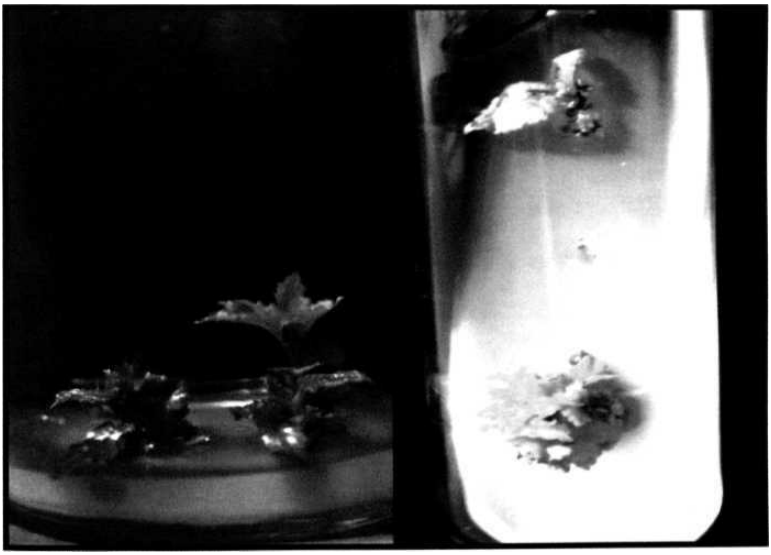
Fig. 11: Comparison of multiple shoot induction from axillary bud derived shoots of S-36 cultivar on medium supplemented with different concentrations of cytokinin.

A&B) 0.5mg/l BAP and 2.0 mg/l BAP

C&D) 0.5mg/l **TDZ** and **2.0** mg/1 **TDZ**



A B



C D

Fig: 11

in length with induction of axillary shoots of 1.0-1.7 cm at the end of 30 days of culture (Fig. 12). Shoot tips cultured on medium with high levels (1.0-2.0 mg/l) of KN and Z developed dark brown callus with a retardation of shoot growth.

Attempts were also made to study the effect of various sugars such as sucrose, fructose, maltose and glucose at 3% on shoot proliferation rates from shoot tips derived from axillary buds (Table 8). The frequency of multiple shoot induction did not vary significantly in the presence of sucrose (66.7-81.7%) and fructose (75.0-88.3%) and decreased on medium containing maltose (31.7-53.3%) in different cultivars. However, the shoot proliferation rate increased at a rate of 6.5 per culture in all 4 cultivars by the incorporation of fructose (3%) instead of sucrose (3%) in the multiplication medium containing 0.5 mg/1 BAP and the growth of the shoots was vigorous (Fig. 13). The average length of shoots was 5.2 cm in the presence of fructose whereas on medium containing sucrose, 3.8 cm long shoots were induced. Shoots cultured on MS medium with glucose (3%) turned pale green and withered away whereas those cultured on maltose (3%) supplemented medium developed shoot buds from the axils of the leaves and there was no further growth of the buds. However, these buds resumed growth upon transfer to medium supplemented with either sucrose or fructose and shoot multiplication was achieved. Studies were also conducted to observe the multiplication rates of shoots derived from sprouted axillary buds upon each subculture (Table 9). The rate of shoot multiplication increased upon each subculture on medium with 0.5 mg/1 BAP. Dark brown coloured callus developed from the base of the shoots from 5^{th} subculture onwards in all four cultivars (Fig. 14). The development of the callus from the base had no effect on the shoot multiplication rates of cultivars. Repeated subculturing of shoots triggered induction of more number of shoots from the base and the mean number of shoots

Fig. 12: Multiple shoot induction from axillary bud derived shoots of S-36 cultivar after 30 days of culture on MS medium with 0.5 mg/l of different cytokinins.

BAP KN

TDZ

Fig: 12

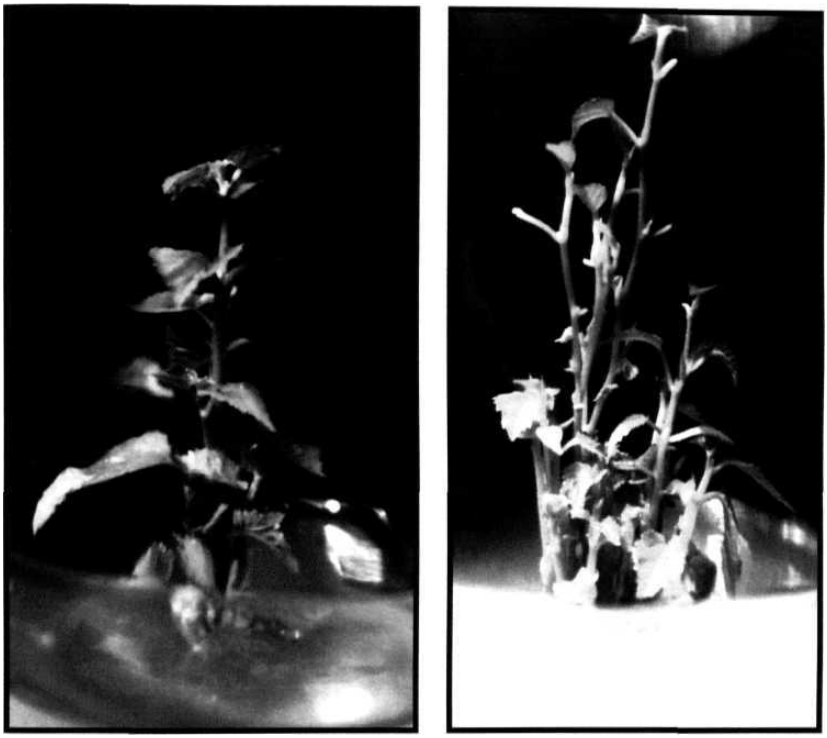
Table 8: Effect of different sugars on multiple shoot induction in 4 cultivars of mulberry

Type of sugar (3%)	Frequency of multiple shoot induction (%)			
	M-5	S-36	S-13	China White
Sucrose	81.7 + 4.4 a	90.0 + 2.9 a	73.3 + 1.7 a	66.7 + 6.0 a
Fructose	88.3 + 3.3 a	**91.7 + 3.3 a**	83.3 + 4.4 a	75.0 + 5.8 a
Glucose	16.7 + 1.7b	**11.7 + 3.3 b**	0.0	0.0
Maltose	53.3 + 6.0 c	53.3 + 4.4 c	**45.0 + 2.9 b**	31.7 + 3.3 b

Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Fig. 13: Multiple shoot induction from axillary bud derived shoots of S-36 **cultivar** on MS medium with sucrose/fructose

a) 3 % sucrose
b) 3 % fructose



a **b**

Fig : 13

Table 9: Mean number of shoots induced during series of subcultures in different cultivars of mulberry

	Mean shoot number			
Subculture cycles	M-5	S-36	S-13	China White
S_1	7.5 + 0.17a	7.5 + 0.18a	6.6 + 0.17a	7.2 + 0.18a
S_2	9.4 + 0.12b	9.5 +0.15b	7.6 + 0.17ab	9.3 + 0.18b
S_3	10.7 + 0.15c	10.8 + 0.15c	9.3 + 0.10bc	10.1 + 0.09c
S_4	11.6 + **0.06d**	11.8 + 0.19d	10.2 + 0.12dc	11.2 + 0.06d
S_5	12.7 + **0.19e**	12.7 + 0.06e	11.6 + **0.15ed**	12.2 + 0.15e
S_6	14.7+"0.07f	14.8 + 0.12f	13.7 + 0.25ef	14.2 + 0.12f
S_7	**15.5** + 0.13g	15.8 ± 0.15g	14.7 + 0.12gf	15.2 + 0.12g
S_8	17.1 + 0.09h	17.1 +0.21h	15.8 + 0.09gh	16.7 + 0.13h
S_9	**18.9** + **0.24i**	**18.9** + **0.15i**	16.5 + 0.12ih	17.4 + 0.03i
S_{10}	19.8 + 0.03j	20.0 + 0.19]	17.7 + 0.06i	**19.5** ± 0.18j

Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Fig. 14: Multiple shoot formation with development of callus from the base of shoots of S-36 cultivar on medium with 0.5 mg/l BAP at 5^{th} subculture.

Fig : 14

produced from a single shoot tip culture of **different cultivars varied from** 17.7-20.0 **at** 10^{th} **subculture.**

Root induction from the shoots:

The effect of auxins such as 2,4-D, NAA, IAA and IBA on root induction from shoots of four mulberry cultivars was studied. The source from which the shoots were derived had a marked effect on root induction. Medium supplemented with 1.0 mg/l 2,4-D favoured a high frequency of root induction (86.7-100.0%) from the shoots derived from the sprouted axillary buds and the roots were induced in 9-10 days **in** all four cultivars (Table 10). Differences were noticed in the nature of roots induced depending on the auxin used in the medium (Fig. 15). Thin slender roots were induced in 17-18 days on IBA and IAA supplemented medium in all four cultivars and numerous medium thick roots were induced in 13-14 days on medium supplemented with NAA. Roots induced on 2,4-D medium were thicker, stronger, fewer in number than those induced on IAA and NAA. However, no difference in the functionality of the roots was observed during acclimatization. Repeatedly subcultured shoots rooted well on MS medium supplemented with low concentration of auxins.

Shoots regenerated from the leaves responded differently with respect to frequency as well as the nature of roots induced in comparison to axillary bud regenerated shoots (Table 11). Low levels of auxins (0.1 mg/1) favoured high frequency of rooting from leaf regenerated shoots whereas axillary bud regenerated shoots rooted efficiently when auxins were provided at 1.0 mg/1. The frequency as well as the nature of roots induced from the leaf regenerated shoots varied with the type and concentration of auxin. Healthy, thick roots that enabled the plants to survive better in the field were obtained on medium with 0.1 mg/1 IBA (Fig. 16). Roots were induced in 12-14 days with

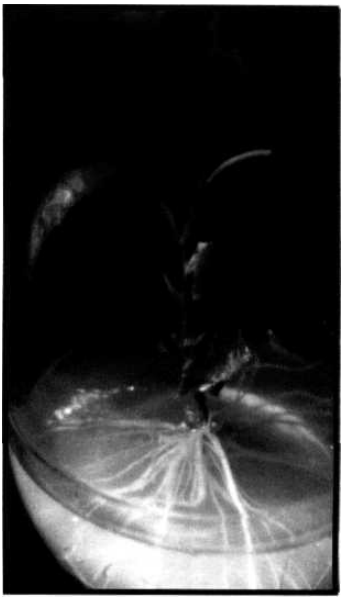
Table 10: ***Effect of auxins on root induction from axillary bud derived shoots of four cultivars of mulberry***

Type of auxin (1.0 mg/l)	% of rooting				Nature of the roots induced
	Cultivar				
	M-5	S-36	S-13	China White	
NAA	0.0	76.7 ± 3.3 a	73.3 ± 1.8 a	70.0 ± 5.8 a	Infinite, medium thick roots
IAA	53.3 ± 1.7 a	66.7 ± 3.3 ab	56.7 ± 2.8 b	60.0 ± 0.0 a	Thin roots
2,4-D	86.7 ± 2.4 b	100.0 ± 0.0 c	96.7 ± 2.3 c	96.7 ± 3.3 b	Thick, strong roots
IBA	66.7 ± 5.8 a	60.0 ± 5.8 b	46.7 ± 3.3 b	36.7 ± 3.3 c	Long slender roots

Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Fig. 15: Root induction from the axillary bud derived shoots of M-5 cultivar after 30 days of culture on MS medium with different auxins.

2,4-D



IAA

IBA

NAA

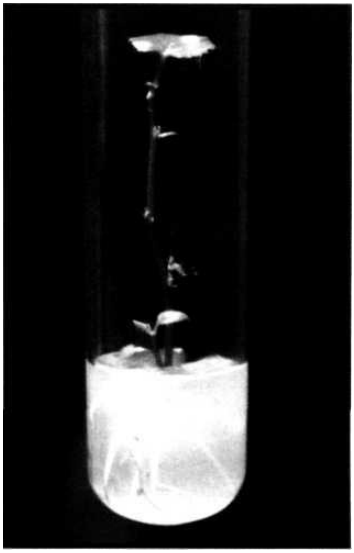
Fig : 15

Table 11: Effect of different auxins on induction of rooting from the leaf regenerated shoots of mulberry

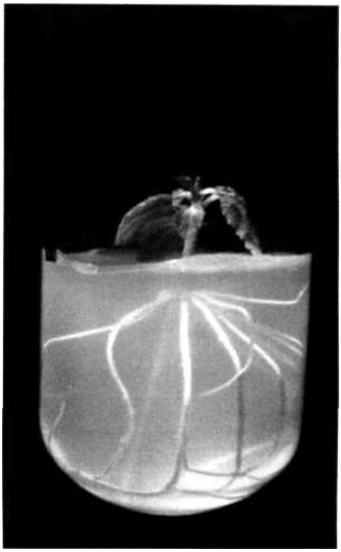
Type of auxin	Concentration (mg/l)	% of rooting			
		Cultivar			
		M-5	S-36	S-13	China White
IAA	0.1	62.0+ 1.1 a	67.1 + 1.9 a	45.7 + 1.5 a	41.7 + 2.5a
	1.0	53.6 + 2.6 b	57.0 + 2.4 b	28.9 + 0.2 b	19.2 + 0.4 b
IBA	0.1	86.6 + 2.1 c	85.2 + 2.3 c	80.2 + 0.4 c	76.0+ 1.5 c
	1.0	64.3 + 2.4 a	63.4 + 2.4 ab	43.8 + 2.5 a	44.2 + 2.5 a
NAA	0.1	30.4 + 1.1d	33.6 + 2.8 d	24.2+ 1.0 b	13.5 + 0.8 d
	1.0	17.0 + 2.4 e	20.9 + 0.3 e	10.4 + 0.7 d	7.1 + 0.2 e
2,4-D	0.1	72.6 + 1.9 f	78.7+ 1.2 c	68.0 + 2.5 e	60.3 + 1.1 f
	1.0	53.4 + 2.0 b	57.5 + 3.2 b	43.4 + 1.9 a	38.8+ 1.1 a

Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Fig. 16: Root induction from the leaf regenerated shoots of different cultivars on MS medium supplemented with 0.1 mg/l IBA.



M-5



S-36

S-13

China White

Fig: 16

a frequency of 76.0-86.6% in different cultivars at 0.1 mg/1 IBA whereas higher levels of IBA (1.0 mg/1) **resulted** in **abundant** callus development from the base of the culture (Fig. 17). Incorporation of 0.1 mg/1 2,4-D triggered root induction with a frequency of 60.3-78.7% in different cultivars. Slender, thin roots were induced from the shoots cultured on medium supplemented with NAA (0.1 mg/1) and IAA (0.1 mg/1) at a frequency of 13.5-33.6% and 41.7-67.1%, respectively in different cultivars.

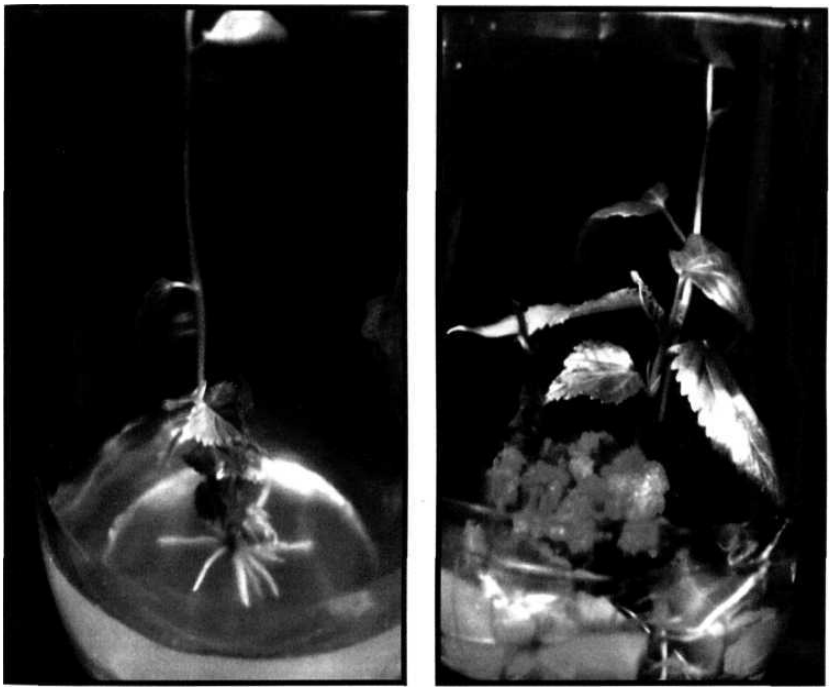
Shoots cultured on medium with 1.5 mg/1 IBA developed cream coloured friable callus from the base within four weeks of culture (Fig. 18a)). However, the internodal explants without meristems upon culture on MS medium with 1.5 mg/1 IBA, underwent rhizogenesis directly from the explants without any callus formation. Transfer of friable callus onto medium supplemented with BAP (0.5-1.0 mg/1) resulted in rhizogenesis along with development of nodular structures that appeared as globular staged somatic embryos (Fig. 18b). However, further development of the somatic embryos was not observed even when left on the same medium for 60 days. At higher concentration of BAP (2.0 mg/1), the callus became completely brown within a week of culture. Shoot tips cultured on medium supplemented individually with 1.5 mg/1 2,4-D, IAA and NAA developed hard callus from the base of the explant, which showed no response for regeneration when transferred to medium with BAP (0.5 to 2.0 mg/1).

Establishment of micropropagated plants in field:

Regenerated plants of all four cultivars with well developed roots and 4-5 healthy leaves were transferred to pots containing soil and organic manure (3:1 ratio) and the humidity was maintained by covering with a plastic cover. The plants were acclimatized for 15-20 days and subsequently transferred to field. The survival frequency of the regenerated plants was more in rainy than in winter and summer seasons. The plants

Fig. 17: Comparison of root induction from the leaf regenerated shoots of S-36 cultivar on MS medium supplemented with 1BA.

a) 0.1 mg/l 1BA
b) 1.0 mg/l 1BA



a b

Fig:17

Fig. 18a: Development of callus from the base of the shoot of S-36 cultivar on MS medium with 1.5 mg/l IB A.

Fig. 18b: Development of globular shaped embryos along with rhizogenesis in the callus developed from the base of the shoot upon transfer to medium with 0.5 mg/1 BAP.

Fig : 18a

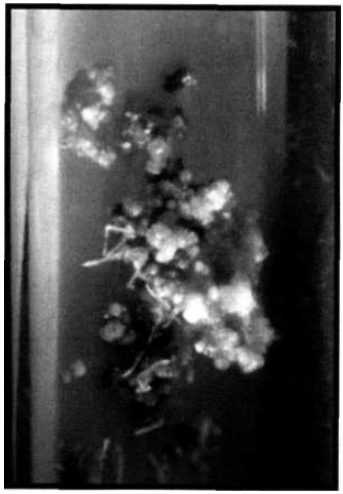


Fig : 18b

regenerated from axillary buds (Fig. 19) established at a high frequency of 66.8-95.5% in comparison to plants regenerated from leaves (Fig. 20), which established at a frequency of 56.5-80.3% in different cultivars (Table 12).

Field performance of micropropagated plants:

The growth characteristics and vegetative morphology of micropropagated plants were compared with the plants raised through cuttings for tluee consecutive years (Table 13). In all four cultivars, *in vitro* raised plants were healthier than cutting raised plants. The height of the cutting propagated plants of different cultivars varied from 364.0-514.3 cm in comparison to micropropagated plants which exhibited a height of 192.7-396.3 cm in the first year. The leaf area of the cutting raised plants was more in comparison to the micropropagated plants. The thickness of the main shoot of the cutting derived plants was more (7.3-14.2 cm) than the micropropagated plants (5.1-9.4 cm) in all four cultivars. But, the number of branches/plant was more for micropropagated plants (7.7-14.0) and an enormous increase in the number of branches/plant was noticed in micropropagated plants at the end of 3rd year (25.3-26.7). In contrast, the cutting derived plants exhibited a marginal increase in the number of branches/plant every year. The internodal distance of the micropropagated plants was lesser (2.4-4.5 cm) than that of the cutting derived plants (3.2-6.2 cm). The weight of the single leaf of the cutting raised plants was more compared to micropropagated plants in all four cultivars. However, this did not alter the yield of micropropagated plants as they had more number of branches/plant (Fig. 21-24).

***Table 12:* Establishment frequency of plants upon transfer to pot in different seasons**

Season	Establishment frequency (%)							
	M-5		S-36		S-13		China White	
	AP*	LP*	AP*	LP*	AP*	LP*	AP*	LP
Summer	80.2 ±0.8 a	67.1 ±1.6 a	76.2 ±1.1 a	64.5 ±1.9 a	75.0 ±2.0 a	60.6 ±1.6 a	66.8 ±1.8 a	56.5 ±1.7 a
Rainy	92.4 ±1.3 b	80.3 ±0.9 b	95.5 ±1.3 b	74.9 ±2.3 b	85.9 ±2.6 b	71.5 ±1.7 b	82.4 ±1.7 b	70.7 ±1.1 b
Winter	83.6 ±1.8 a	75.4 ±1.6 c	86.3 ±1.9c	71.1 ±1.5 b	81.3 ±1.4 ab	66.4 ±1.2 b	73.9 ±2.5 c	66.4 ±2.4 b

Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

AP*- Plants regenerated from the axillary shoots

LP*- Plants regenerated from the leaves

Summer- February-May

Rainy- June-September

Winter- October- January

Fig. 19: Establishment of micropropagated plants derived from axillary buds of different cultivars in field.

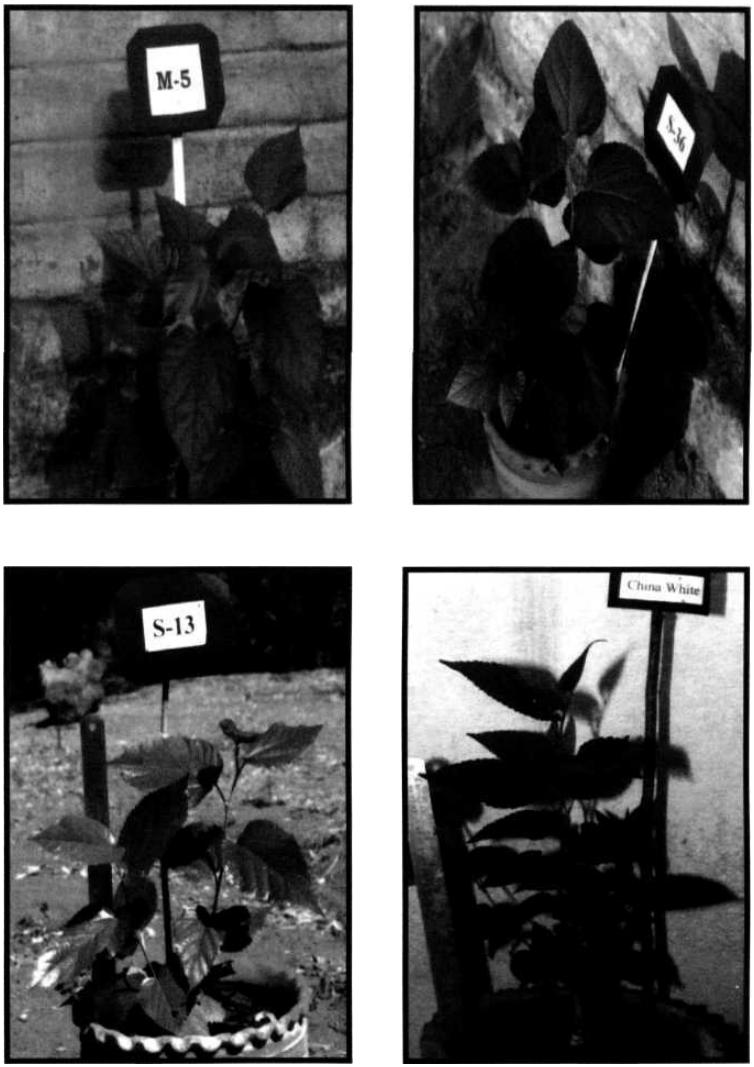


Fig:19

Fig. 20: Plantlets regenerated from leaves after 15 days of transfer to pots containing garden soil and organic manure (3:1).

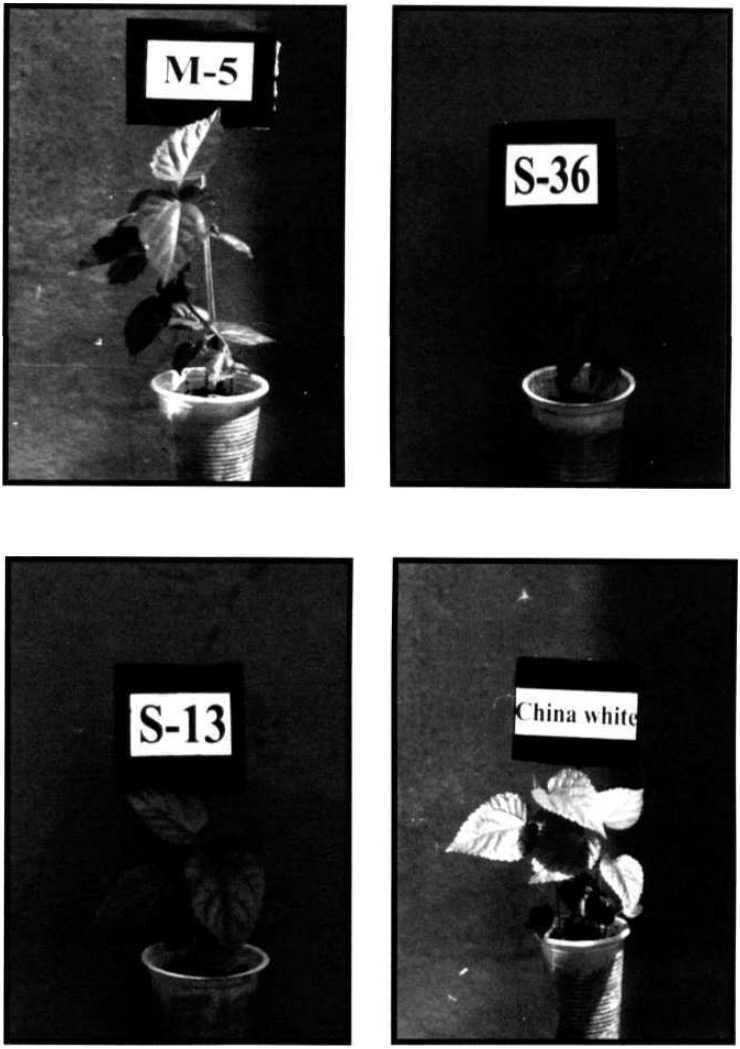


Fig : 20

Table 13: *Field performance of micropropagated plants in comparison to cutting derived plants for three consecutive years*

Morphological Characters	Year	M-5		S-36		S-13		China white	
		CP*	MP*	CP*	MP*	CP*	MP*	CP*	MP*
Length of the plant (cm)	1st	438.0 ± 1.7	386.3± 4.3	506.3 ± 3.7	396.3 ± 0.9	514.3 ± 3.2	356.7 ± 2.4	364.0 ±3.2	192.7 ± 2.0
	2nd	490.3 ± 1.5	412 ± 3.0	581.3 ± 2.4	406.0 ± 1.7	570 ± 1.6	446.0 ± 4.7	420.3 ± 5.8	228.0 ± 6.6
	3rd	525.0 ± 3.8	503 ± 2.4	613.3 ± 3.4	463.7 ± 2.1	609 ± 4.9	533.3 ± 6.7	504.0 ± 3.6	317.0 ± 3.6
Thickness of the shoot (cm)	1st	9.6 ± 0.1	7.3 ± 0.3	14.2 ± 0.2	9.4 ± 0.3	10.1 ± 0.1	5.1 ± 0.1	7.3 ± 0.2	5.4 ± 0.3
	2nd	10.7 ± 0.1	9.4 ± 0.3	15.3 ± 0.3	10.2 ± 0.2	11.3 ± 0.3	6.6 ± 0.2	8.7 ± 0.2	7.3 ± 0.2
	3rd	11.5 ± 0.3	11.4 ± 0.3	15.9 ± 0.1	11.0 ± 0.03	12.4 ± 0.3	7.5 ± 0.3	9.2 ± 0.5	9.3 ± 0.2
No of branches	1st	11.0 ± 0.6	14.0 ± 0.6	9.0 ± 0.6	12.3 ± 0.9	5.3 ± 0.3	7.7 ± 0.3	9.7± 0.7	12.7 ± 0.9
	2nd	13.0 ± 0.6	16.7 ± 0.9	11.3 ± 0.9	16.0 ± 1.5	7.3 ± 0.9	16.0 ± 0.6	11.0 ± 0.6	21.0 ± 1.2
	3rd	14.0 ± 0.6	25.3 ± 1.2	12.7 ± 0.7	26.3 ± 1.2	8.7 ± 0.9	25.7 ± 0.9	14.0 ± 0.6	26.7 ± 0.9
Total no of the leaves/plant	1st	542.0 ± 12.5	842.3 ± 26.4	906.3 ± 9.0	970.0 ± 14.3	417.3 ± 6.3	539.7 ± 20.2	225.3 ± 12.2	335.3 ± 7.3
	2nd	737.3 ± 24.4	1028.7± 25.5	140.3 ± 26.5	1642.0 ± 18.6	839.0± 19.4	954.2 ± 27.6	737.7 ± 18.9	847.7 ± 21.4
	3rd	1066.0± 35.1	1983.0 ± 14.2	530.0 ± 21.4	2012.0 ± 17.6	1217.7 ± 14.2	1768.6 ± 29.9	1239.0 ± 19.7	1364.7 ± 23.6
Length of the internode (cm)	1st	5.1 ± 0.1	3.3 ± 0.2	6.2 ± 0.1	4.5 ± 0.1	3.2 ± 0.1	2.4 ± 0.1	3.8 ± 0.1	3.9 ± 0.05
	2nd	5.2 ± 0.03	3.9 ± 0.1	6.5 ± 0.2	4.8 ± 0.1	3.8 ± 0.1	3.0 ± 0.07	4.2 ± 0.08	4.3 ± 0.05
	3rd	5.3 ± 0.1	4.1 ± 0.1	6.8 ± 0.1	4.9 ± 0.02	4.0 ± 0.04	3.6 ± 0.1	4.5 ± 0.02	4.6 ± 0.09
Weight of 100 leaves (gm)	1st	651.7 ± 1.2	271.0 ± 1.2	635.3 ± 2.0	652 ± 1.2	507 ± 2.7	702.0 ± 1.6	1053.3 ± 1.8	955.3 ± 2.7
	2nd	684.0 ± 2.7	414.0 ± 2.1	764.7 ± 2.0	772.0 ± 1.2	591 ± 1.2	864.7 ± 2.3	1193.7 ±1.8	1205.3 ± 3.2
	3rd	724.0 ± 2.1	583.0 ± 1.2	843.3 ± 2.0	862.7 ± 1.5	676.0 ± 2.0	914.0 ± 2.7	1223.0 ± 2.1	1354.3 ± 2.4
Yield/plant	1st	3660.7 ± 0.8	2277 ± 11.2	5812.3 ± 13.0	6355.7 ± 4.7	2133.5 ± 2.1	3858.7 ± 11.9	2392.3 ± 2.7	3233.3 ± 2.7
	2nd	5428.1 ± 1.4	4311.3 ± 5.8	8393.7 ± 4.2	12051.7 ± 8.2	5073.3 ± 3.3	8586.3 ± 6.1	9164.7 ± 24.2	10650.7 ± 9.8
	3rd	7824.1 ± 3.8	11374 ± 6.2	12254.2 ± 17.7	17294.3 ± 23.9	8401.9 ± 26.8	16466.3 ± 26.4	15239.9 ± 9.1	17591 ± 35.0

CP*- Cutting derived plant
MP*- Micropropagated plants

Fig. 21: Comparison of field performance of one-year old plants of M-5 cultivar established from nodal cuttings with micropropagated plants.

Cutting raised plants

Micropropagated plants

Fig : 21

Fig. 22: Comparison of field performance of one-year old plants of S-36 cultivar established from nodal cuttings with micropropagated plants.

Cutting-raised plant

Micropropagated plant

Fig : 22

Fig. 23: Comparison of field performance of one-year old plants of S-13 cultivar established from nodal cuttings with micropropagated plants.

Cutting-raised plants

Micropropagated plants

Fig : 23

Fig. 24: Comparison of field performance of one-year old plants of China White cultivar established from nodal cuttings with micropropagated plants.

Cutting-raised plant

Micropropagated plant

Fig : 24

SDS-PAGE analysis during callus proliferation and shoot organogenesis from leaf explants:

In an effort to identify a developmental marker specific to shoot organogenesis, protein profiles of different stages of shoot organogenesis were compared with different stages of callus proliferation from leaf explants of M-5 and S-36 cultivars. Leaf explants cultured on MS medium with 2.0 mg/l 2,4-D produced callus whereas those cultured on 2.0 mg/1 BAP underwent shoot organogenesis. SDS-PAGE analysis was made from the protein extracted from the leaves at different stages of callus proliferation and shoot organogenesis. The first stage was represented by the explant stage (0 day), 2nd stage of shoot organogenesis and callus proliferation was collected when the leaves exhibited swelling and enlargement in size and 3r stage was collected at induction **of** shoot buds for shoot organogenesis and slight initiation of callus for callus proliferation. The fourth stage of shoot organogenesis was collected when the shoots have attained the height of 0.5-1.0 cm and the final stage of callus proliferation was collected when the basal half has completely developed into callus. Analysis of protein profiles during callus proliferation and shoot organogenesis in M-5 cultivar revealed high expression of 49 kDa protein in leaf explants which decreased in intensity following culture. Proteins of 103, 94, 82 and 76 kDa were not detectable in leaf explants but were seen as clear bands during shoot organogenesis. These proteins appeared at high intensity in early stages of callus induction but decreased in intensity when there was complete proliferation of the callus. There was an increase in intensity of 39 kDa protein during the induction of shoot organogenesis whereas the intensity of this protein was very low in the leaf explants. This protein increased in amounts during the initial stages of callus proliferation and was present in lower amounts in leaf derived callus. A protein of 25 kDa was specifically

expressed during callus proliferation and was not detectable in leaf explants or during induction of shoot organogenesis. A significant number of proteins with MW 68, 56, 53 and 42 were common to callus proliferation and shoot organogenesis and appeared at more or less at the same level during all stages of culture (Fig. 25a and b).

A protein of M W 49 kDa was present in abundance in leaf explants and decreased in intensity during induction of shoot organogenesis in S-36 cultivar. Proteins of 103 kDa and 94 kDawere not detectable in leaf explants and expressed at a low intensity during all the stages of callus proliferation and shoot organogenesis. Differences in staining intensities were noticed with respect to 55 and 53 kDa proteins which were expressed at high levels in the 4^{th} stage of shoot organogenesis whereas the intensity of these proteins was low in the corresponding stages of callus proliferation. Proteins of MW 125 and 68 kDa were expressed at the same intensity at all stages of callus proliferation and leaf organogenesis. The intensity **of** 82 and 76 kDa proteins increased at the 2^{nd} stage of shoot organogenesis and callus proliferation and continued to express at the same level during the subsequent stages of culture. On the contrary, a protein of 39 kDa was expressed in low levels in leaf explants and increased in intensity during induction of shoot organogenesis. However, this protein increased in intensity during the initial stages of callus induction and appeared faintly when the leaf explants completely developed into callus (Fig. 26a and b).

Two- dimensional analysis of protein during organogenesis and callus induction:

SDS-PAGE analysis during callus proliferation and shoot organogenesis from leaf explants revealed major differences in the expression of 39 kDa protein which expressed in relatively higher levels during final stages of shoot organogenesis whereas the relative amount of this protein was low in callus derived from leaf explants. To more precisely

Fig. 25a: SDS-PAGE gel of protein extracts prepared from leaf explants of M-5 cultivar at different stages of callus proliferation and stained with coomassie blue.

Lane 1 : Molecular weight marker
Lane 2 : Leaf explant (0 day)
Lane 3 : Swelling of the explants
Lane 4 : Initiation of callus from leaf explants
Lane 5 : Callus derived from leaf explants

Arrows point to the bands of interest

Fig. 25b: SDS-PAGE gel of protein extracts prepared from leaf explants of M-5 cultivar at different stages of shoot organogenesis and stained with coomassie blue

Lane 1: Molecular weight marker
Lane 2 : Leaf explant (0 day)
Lane 3 : Swelling of the explants
Lane 4 : Induction of shoot buds from leaf explants
Lane 5 : Development of shoots (0.5-1.0 cm) from the explants

Arrows point to the bands of interest

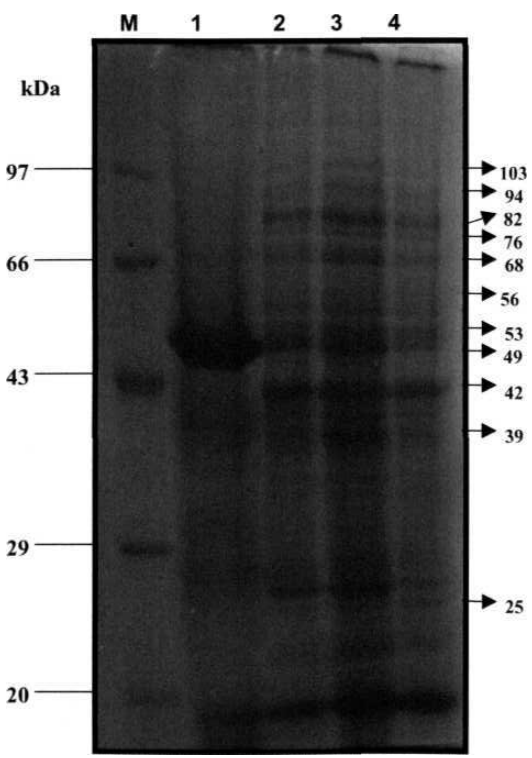


Fig : 25a

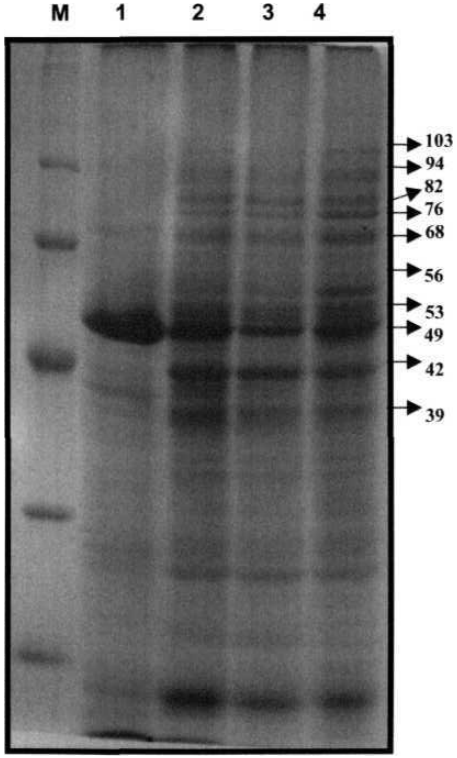


Fig : 25b

Fig. 26a: SDS-PAGE gel of protein extracts prepared from leaf explants of S-36 cultivar at different stages of callus proliferation and stained with coomassie blue

Lane 1 : Molecular weight marker
Lane 2 : Leaf explant (0 day)
Lane 3 : Swelling of the explants
Lane 4 : Initiation of callus from leaf explants
Lane 5 : Callus derived from leaf explants

Arrows point to the bands of interest

Fig. **26b:** SDS-PAGE gel of protein extracts prepared from leaf explants of S-36 cultivar at different stages of shoot organogenesis and stained with coomassie blue

Lane 1 : Molecular weight marker
Lane 2 : Leaf explant (0 day)
Lane 3 : Swelling of the explants
Lane 4 : Induction of shoot buds from leaf explants
Lane 5 : Development of shoots (0.5-1.0 cm) from the explants

Arrows point to the bands of interest

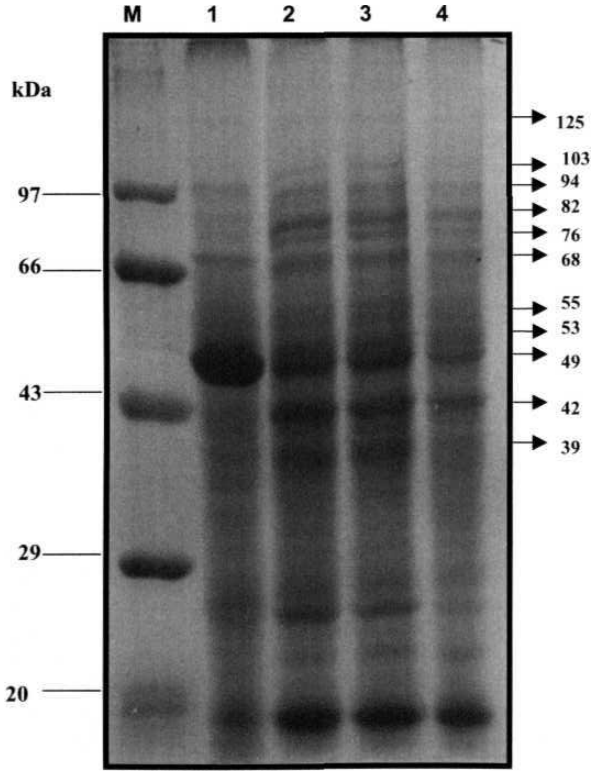


Fig : 26a

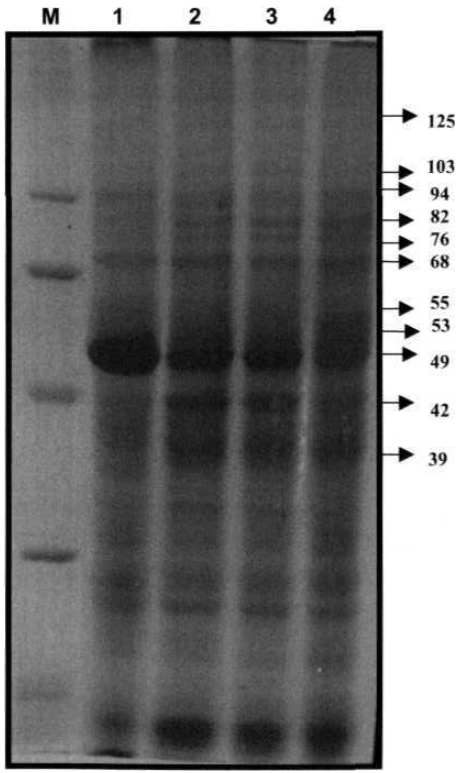


Fig : 26b

identify differences in the specific proteins during organogenesis and callus proliferation from leaf explants, the protein extracts of these samples were analysed by **two-**dimensional gel electrophoresis followed by highly sensitive silver staining. During the final stages of shoot organogenesis, 39 kDa protein appeared intensely and was expressed in four isoforms with pI values ranging from 4.2 to 5.8 in both the cultivars. However, 39 kDa protein was expressed in two isoforms with pI values of 4.2 and 5.8 were observed in leaf derived callus of M-5 cultivar in contrast to S-36 cultivar where only one isoform with pI value of 4.2 was detectable. A protein of MW 62 kDa was more expressed in the leaf explants of S-36 cultivar than in M-5 cultivar with pI values ranging from 4.3-6.5 and 4.2-5.4, respectively. This protein was expressed during shoot organogenesis with pI values of 4.6 and 5.0 in M-5 cultivar and pI value of 4.3 in S-36 cultivar whereas callus of both the cultivars showed very faint expression. SDS-PAGE analysis revealed the presence of 49 kDa protein at a high intensity in leaf explants and decreased in intensity during the process of de-differentiation and shoot differentiation. 2-D analysis revealed that protein of 49 kDa protein was expressed with pI values ranging from 3.0-4.8 in leaf explants of M-5 cultivar whereas this protein separated into isoforms with pI values ranging from 4.8-5.6 during shoot organogenesis and pI values ranging from 4.5-5.8 during callus proliferation. In S-36 cultivar, 49 kDa protein was expressed with isoforms ranging from 3.0-4.5 in the leaf explants, whereas isoforms with pI values ranging from 4.5-5.3 were observed in the final stages of shoot organogenesis and callus proliferation. The expression of 32 kDa protein was more intense in S-36 cultivar in comparison to M-5 cultivar with pI values ranging from 3.5-5.4 and 3.2-4.2, respectively in the leaf explants. During the final stages of shoot organogenesis, 32 kDa protein was expressed with a pI value of 4.2 in M-5 and S-36 cultivars whereas this

protein was expressed with pI values of 4.5-6.2 and 4.5-5.3 in the callus of M-5 and S-36 cultivars, respectively. Most of the proteins in the leaf explants were found to be towards the acidic side in both the cultivars whereas in the final stages of callus proliferation and shoot organogenesis, the proteins were distributed from acidic to neutral side (Figs. 27 and 28).

Changes in protein phosphorylation during callus proliferation and shoot organogenesis in M-5 cultivar:

The phosphorylation pattern varied with respect to intensity during callus proliferation and shoot organogenesis from leaf explants. Analysis of protein phosphorylation during various stages of callus proliferation revealed differences in the intensity of phosphorylation of proteins of MW 68 kDa, 56 kDa and 46 kDa. The intensity of phosphorylation of these proteins increased at the 2^n stage of callus induction in the absence of Ca^{2+} compared to the presence of Ca^{2+} and calmodulin in the phosphorylation reaction buffer. External addition of calmodulin resulted in the de-phosphorylation of 68, 56 and 46 kDa proteins in the leaf derived callus. Irrespective of Ca^{2+} in the buffer, 56 kDa protein was intensely phosphorylated whereas 46 kDa protein was dephosphorylated in the final stages of callus proliferation (Fig. 29a and b; 30a and b).

Protein of 56 kDa was more intensely phosphorylated in the absence/presence of Ca^{2+} at the 2nd stage of shoot organogenesis whereas the presence of Ca^{2+} and calmodulin increased the intensity of phosphorylation of 68, 56 and 46 kDa proteins. At the 3rd stage of induction of shoot buds from the leaf explants, 68 and 46 kDa proteins were highly phosphorylated in the presence of Ca^{+} and calmodulin in comparison to presence or absence of Ca^{2+} in the phosphorylation reaction buffer. During the final stages of shoot

Fig. 27: Two dimensional electrophoretic analysis of the protein extracted from leaf explants of M-5 cultivar during shoot organogenesis and callus proliferation.

(i) : Leaf explants

A - 62 kDa protein

a - 49 kDa protein

b - 32 kDa protein

(ii): Development of shoots from leaf explant

A - 62 kDa protein

a - 49 kDa protein

b - 32 kDa nrotein

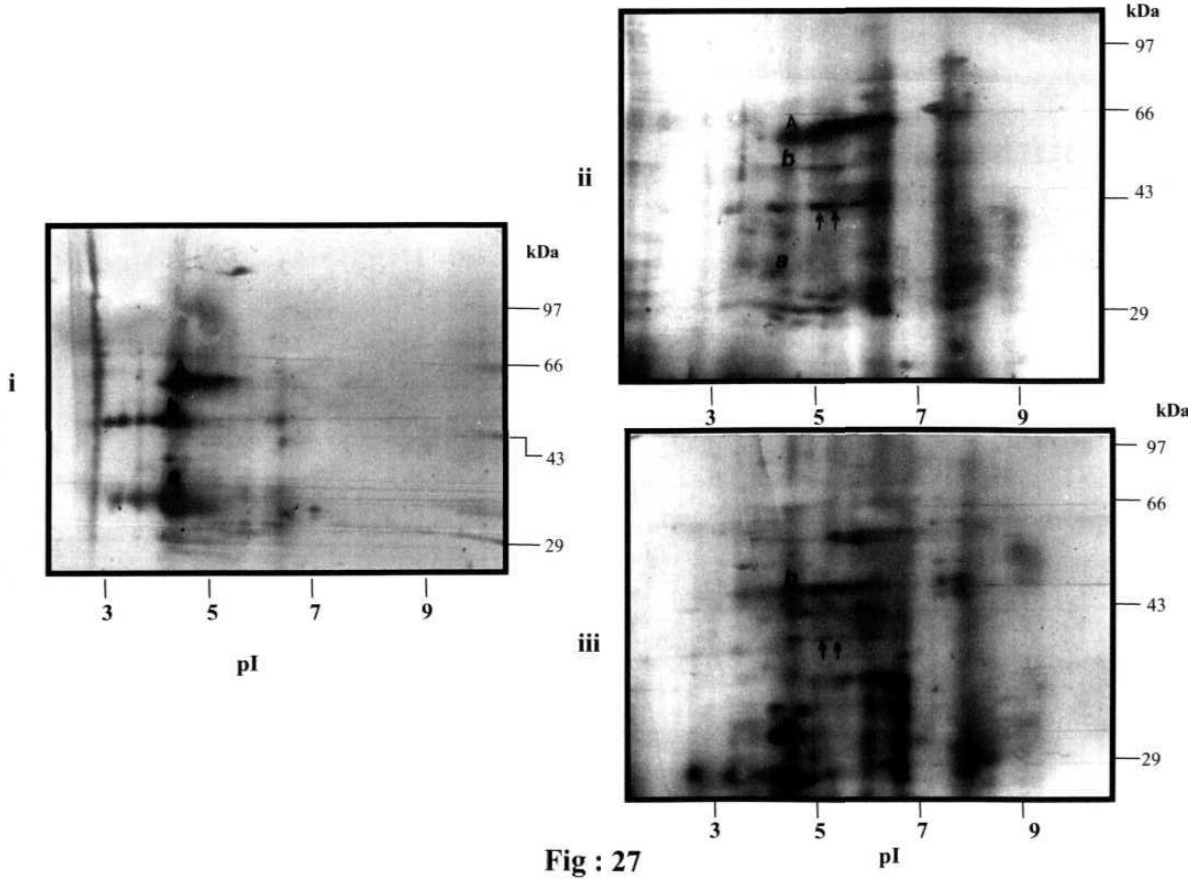
↑↑ - 39 kDa protein

(iii) : Callus derived from leaf explants

a - 49 kDa protein

b - 32 kDa protein

↑↑ - 39 kDa protein


i
kDa
97
66
43
29
3
5
7
9
pI
ii
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97
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43
29
A
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B
3
5
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9
iii
kDa
97
66
43
29
3
5
7
9
pI

Fig : 27

Fig. 28: Two dimensional electrophoretic analysis of the protein extracted from leaf explants of S-36 cultivar during shoot organogenesis and callus proliferation.

(i): Leaf explants

A - 62kDa protein

a - 49 kDa protein

b - 32 kDa protein

(ii) : Development of shoots from leaf explant

A - 62kDa protein

a - 49 kDa protein

b - 32 kDa protein

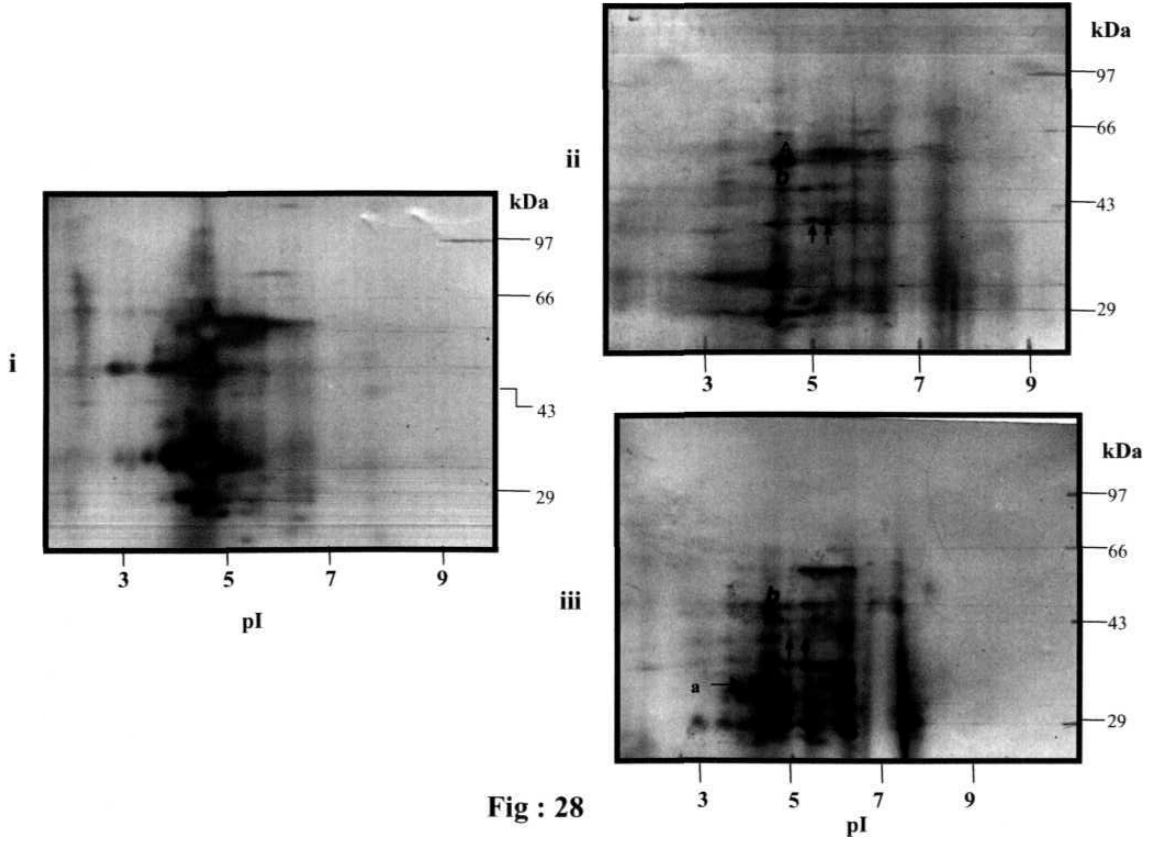
↑↑ - 39 kDa protein

(iii) : Callus derived from leaf **explants**

a - 49 kDa protein

b - 32 kDa nrotein

↑↑ - 39 kDa protein


i
kDa
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iii
kDa
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43
29
a
3
5
7
9
pI

Fig : 28

Fig. 29a: Changes in protein phosphorylation pattern during callus proliferation from leaf explants of M-5 cultivar on MS medium with 2.0 mg/l 2,4-D.

Lane 1 Leaf explant (0 day)
Lane 2 Swelling of the explants
Lane 3 Initiation of callus from leaf explants
Lane 4 Callus derived from leaf explants

Total protein was extracted from the leaves during different stages of callus proliferation, and the extracts were labeled by addition of 4 μCi (γ-^{32}P) ATP in the presence and absence of Ca^{2+} in the phosphorylation reaction mixture at room temperature, separated by SDS-PAGE and exposed to X-ray films for 2 days.

Arrows indicate the position in the gel of the major labeled proteins.

Fig. 29b: SDS-PAGE analysis of total proteins during different stages of callus proliferation from leaf explants of M-5 cultivar after culture on MS medium with 2.0 mg/l 2,4-D.

Lane M: Molecular weight marker
Lane 1,2, 3 & 4: as above.

Arrows point to the bands of interest

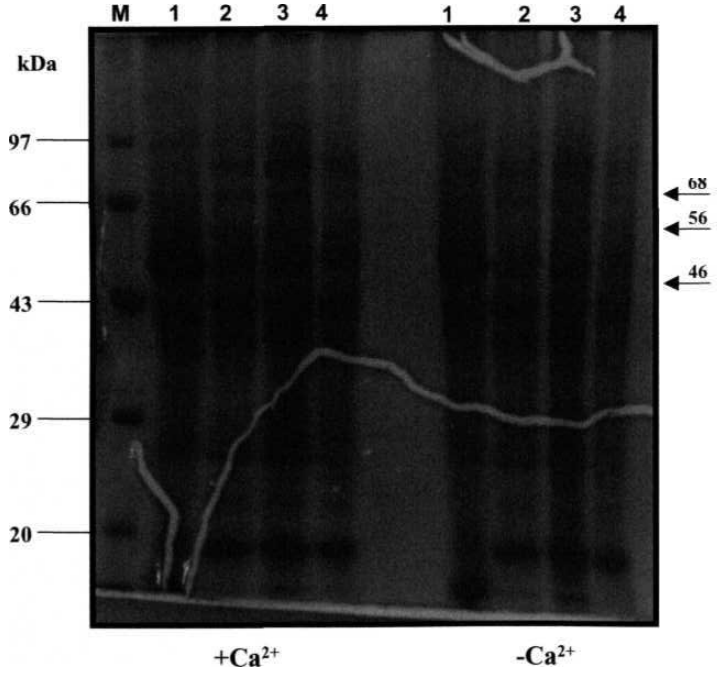


Fig : 29a

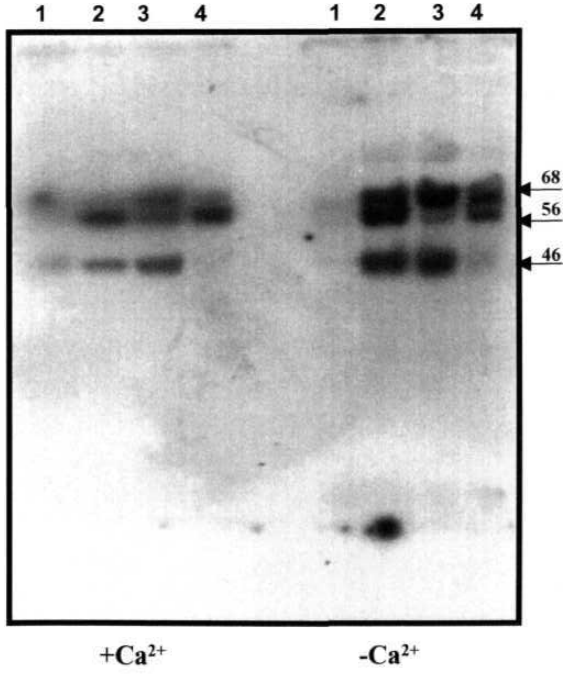


Fig : 29b

Fig. 30a: Changes in protein phosphorylation pattern during callus proliferation from leaf explants of M-5 cultivar on MS medium with 2.0 mg/l 2,4-D.

Lane 1: Molecular weight marker
Lane 2 : Leaf explant (0 day)
Lane 3 : Swelling of the explants
Lane 4 : Initiation of callus from leaf explants
Lane 5 : Callus derived from leaf explants

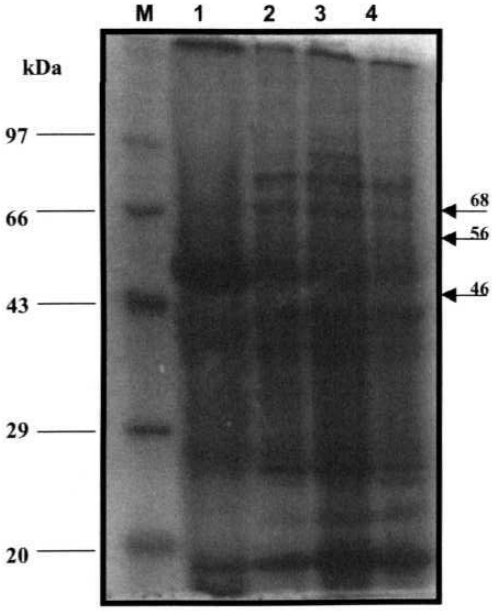
Total protein was extracted from the leaves during different stages of callus proliferation, and the extracts were labeled by addition of 4 μCi (γ-^{32}P) ATP in the presence of Ca^{2+} and calmodulin in the phosphorylation reaction mixture at room temperature, separated by SDS-PAGE and exposed to X-ray films for 2 days.

Arrows indicate the position in the gel of the major labeled proteins.

Fig. 30b: SDS-PAGE analysis of total proteins during different stages of callus proliferation from leaf explants of M-5 cultivar after culture on MS medium with 2.0 mg/l 2,4-D.

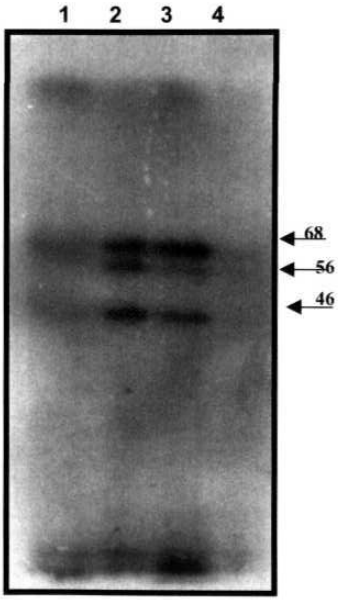
Lane M: Molecular weight marker
Lane 1,2, 3 & 4: as above.

Arrows point to the bands of interest



Calmodulin

Fig : 30a



Calmodulin

Fig : 30b

organogenesis, phosphoprotein of 68 kDa was more intensely phoshphorylated compared to 56 and 46 kDa proteins irrespective of Ca^{2+} and calmodulin (Fig. 31a and b; 32a and b).

Changes in protein phosphorylation during callus proliferation and shoot organogenesis in S-36 cultivar:

Analysis of the phosphorylated proteins of leaf extracts on SDS-PAGE revealed major bands of 68 and 46 kDa and a minor band of 56 kDa. A clear difference in the pattern of phosphorylation was observed in the presence and absence of Ca^{2+} in the phosphorylation buffer. Protein of 56 kDa was phosphorylated at a low intensity in leaf explants whereas the intensity of phosphorylation increased at the 2^{nd} and 3^{rd} stage of callus proliferation followed by a decrease in the intensity of phosphorylation in the callus. There was no significant difference observed in the intensity of the phosphorylation of 68, 56 and 46 kDa proteins in the absence/presence of Ca^{2+} and calmodulin in the leaf derived callus whereas the level of phosphorylation of these proteins increased in the presence of Ca^{2+} and calmodulin (Fig. 33a and b; 34a and b).

Phosphoproteins of 68, 56 and 46 kDa were detectable as faint bands in the presence of Ca^{2+} whereas the intensity of phosphorylation increased in the absence of Ca^{2+} and presence of Ca^{2+} and calmodulin at the 2^{nd} stage of shoot organogenesis. There was an intense phosphorylation of 68, 56 and 46 kDa proteins during induction of shoot buds (3^{rd} stage) from the leaf explants irrespective of Ca^{2+} and calmodulin in the extraction buffer. During the final stages of shoot organogenesis, 68 kDa protein was highly phosphorylated whereas 56 kDa and 46 kDa proteins were faintly phosphorylated irrespective of Ca^{2+} and calmodulin (Fig. 35a and b; 36a and b).

Fig. 31a: Changes in protein phosphorylation pattern during shoot organogenesis from leaf explants of M-5 cultivar on MS medium with 2.0 mg/l BAP

Lane 1 : Leaf explant (0 day)
Lane 2 : Swelling of explants
Lane 3 : Induction of shoot buds from leaf explants
Lane 4 : Development of shoots (0.5-1.0 cm) from the explants

Total protein was extracted from the leaves during different stages of shoot organogenesis, and the extracts were labeled by addition of 4 μCi (γ-^{32}P) ATP in the presence and absence of Ca^{+} in the phosphorylation reaction mixture at room temperature, separated by SDS-PAGE and exposed to X-ray films for 2 days.

Arrows indicate the position in the gel of the major labeled proteins.

Fig. 31b: SDS-PAGE analysis of total proteins during different stages of shoot organogenesis from leaf explants of M-5 cultivar after culture on MS medium with 2.0 mg/1 BAP

Lane M: Molecular weight marker
Lane 1,2, 3 & 4: as above.

Arrows point to the bands of interest

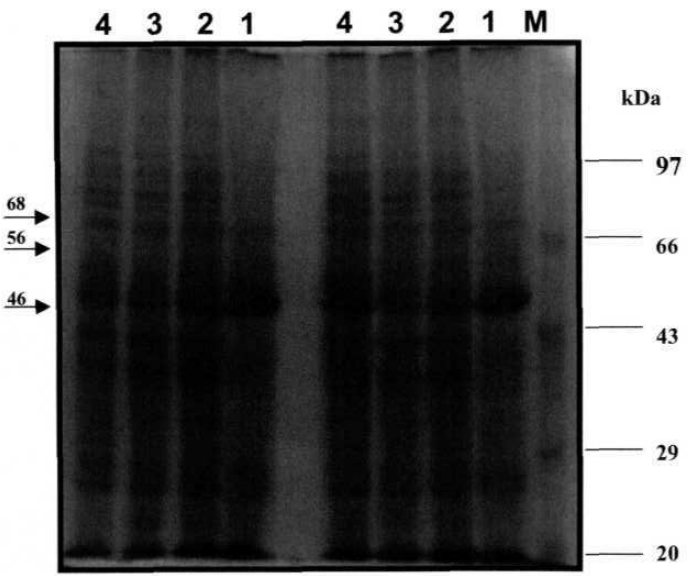


Fig : 31a

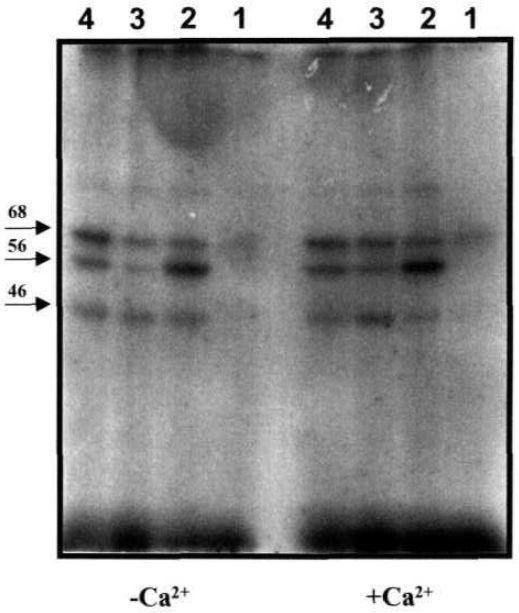


Fig : 31b

Fig. 32a: Changes in protein phosphorylation pattern during shoot organogenesis from leaf explants of M-5 cultivar on MS medium with 2.0 mg/l BAP

Lane 1 : Leaf explant (0 day)
Lane 2 : Swelling of the explants
Lane 3 : Induction of shoot buds from leaf explants
Lane 4 : Development of shoots (0.5-1.0 cm) from the leaf explants

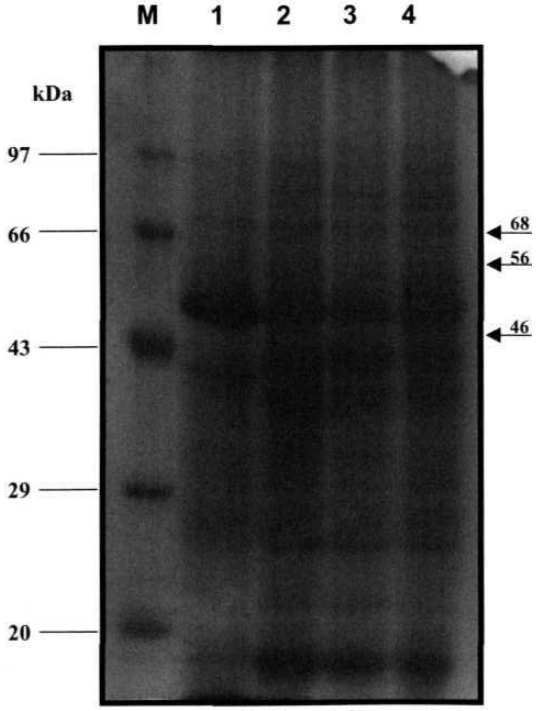
Total protein was extracted from the leaves during different stages of callus proliferation, and the extracts were labeled by addition of 4 μCi (γ-^{32}P) ATP in the presence Ca^{2+} and calmodulin in the phosphorylation reaction mixture at room temperature, separated by SDS-PAGE and exposed to X-ray films for 2 days.

Arrows indicate the position in the gel of the major labeled proteins.

Fig. 32b: SDS-PAGE analysis of total proteins during different stages of shoot organogenesis from leaf explants of M-5 cultivar after culture on MS medium with 2.0 mg/1 BAP

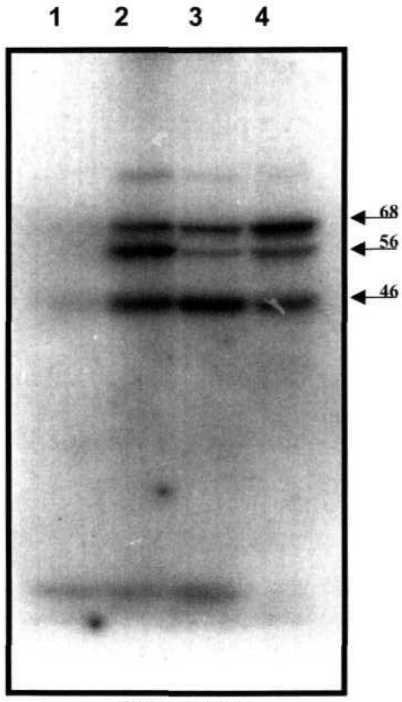
Lane M: Molecular weight marker
Lane 1,2, 3 & 4: as above.

Arrows point to the bands of interest



Calmodulin

Fig 32a



Calmodulin

Fig : 32b

Fig. 33a: Changes in protein phosphorylation pattern during callus proliferation from leaf explants of S-36 cultivar on MS medium with 2.0 mg/l 2,4-D.

Lane 1: Molecular weight marker
Lane 2 : Leaf explant (0 day)
Lane 3 : Swelling of the explants
Lane 4 : Initiation of callus from leaf explants
Lane 5 : Callus derived from leaf explants

Arrows point to the bands of interest

Total protein was extracted from the leaves during different stages of callus proliferation, and the extracts were labeled by addition of 4 μCi (γ-^{32}P) ATP in the presence and absence of Ca^{2+} in the phosphorylation reaction mixture at room temperature, separated by SDS-PAGE and exposed to X-ray films for 2 days.

Arrows indicate the position in the gel of the major labeled proteins.

Fig. 33b: SDS-PAGE analysis of total proteins during different stages of callus proliferation from leaf explants of M-5 cultivar after culture on MS medium with 2.0 mg/l 2.4-D.

Lane M: Molecular weight marker
Lane 1,2, 3 & 4: as above.

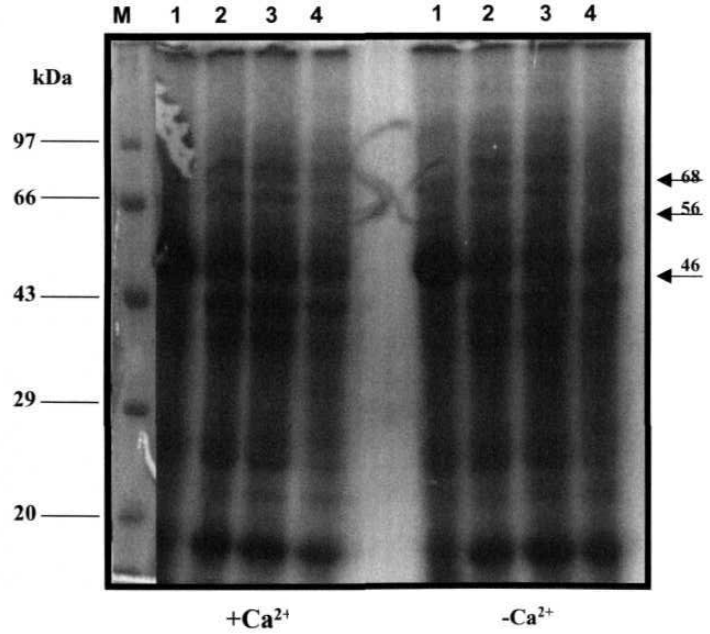


Fig : 33a

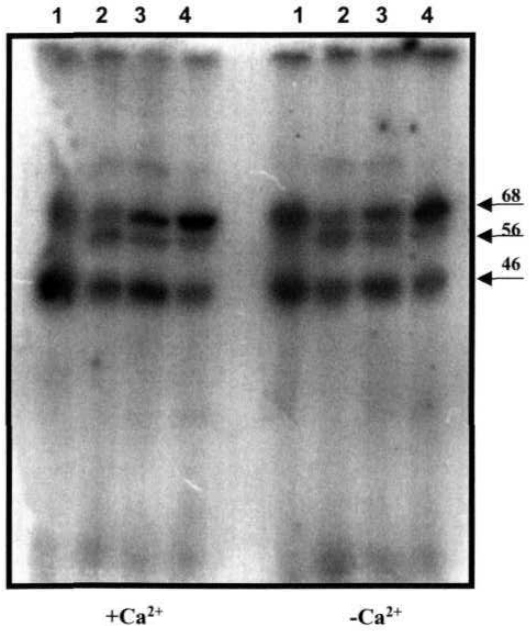


Fig : 33b

Fig. 34a: Changes in protein phosphorylation pattern during callus proliferation from leaf explants of S-36 cultivar on MS medium with 2.0 mg/l 2,4-D.

Lane 1 : Leaf explant (0 day)
Lane 2 : Swelling of the explants
Lane 3 : Initiation of callus from leaf explants
Lane 4 : Callus derived from leaf explants

Arrows point to the bands of interest

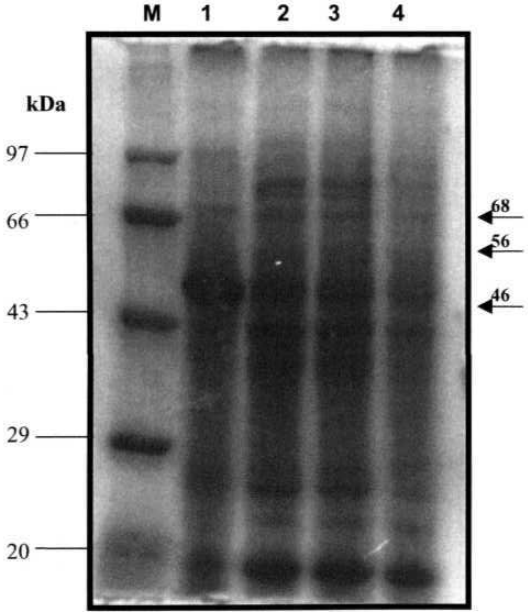
Total protein was extracted from the leaves during different stages of callus proliferation, and the extracts were labeled by addition of 4 μCi (γ-^{32}P) ATP in the presence of Ca^{2+} and calmodulin in the phosphorylation reaction mixture at room temperature, separated by SDS-PAGE and exposed to X-ray films for 2 days.

Arrows indicate the position in the gel of the major labeled proteins.

Fig. 34b: SDS-PAGE analysis of total proteins during different stages of callus proliferation from leaf explants of M-5 cultivar after culture on MS medium with 2.0 mg/1 2,4-D.

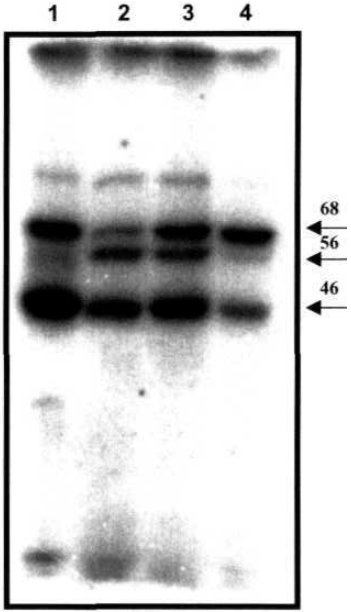
Lane M: Molecular weight marker
Lane 1,2, 3 & 4: as above.

Arrows point to the bands of interest



Calmodulin

Fig : 34a



Calmodulin

Fig : 34b

Fig. 35a: Changes in protein phosphorylation pattern during shoot organogenesis from leaf explants of S-36 cultivar on MS medium with 2.0 mg/1 BAP

Lane 1 : Leaf explant (0 day)
Lane 2 : Swelling of the explants
Lane 3 : Induction of shoot buds from leaf explants
Lane 4 : Development of shoots (0.5-1.0 cm) from the leaf explants

Total protein was extracted from the leaves during different stages of shoot organogenesis, and the extracts were labeled by addition of 4 μCi (γ-^{32}P) ATP in the presence and absence of Ca^{+} in the phosphorylation reaction mixture at room temperature, separated by SDS-PAGE and exposed to X-ray films for 2 days.

Arrows indicate the position in the gel of the major labeled proteins.

Fig. 35b: SDS-PAGE analysis of total proteins during different stages of shoot organogenesis from leaf explants of S-36 cultivar after culture on MS medium with 2.0 mg/1 BAP

Lane M: Molecular weight marker
Lane 1,2, 3 & 4: as above

Arrows point to the bands of interest

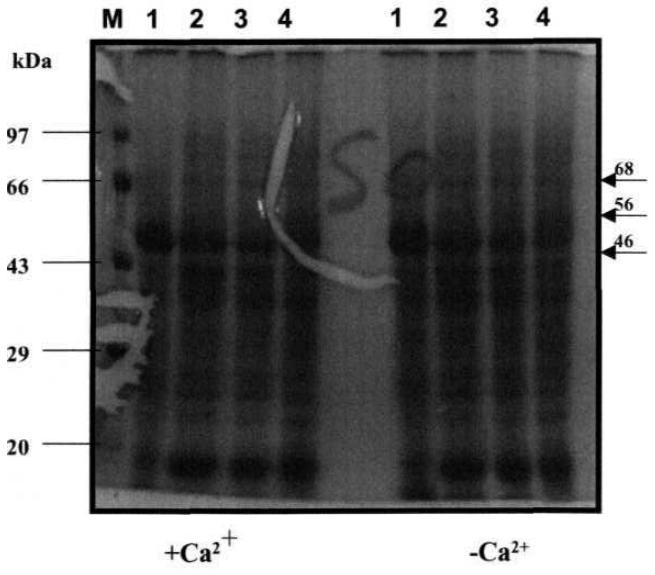


Fig : 35a

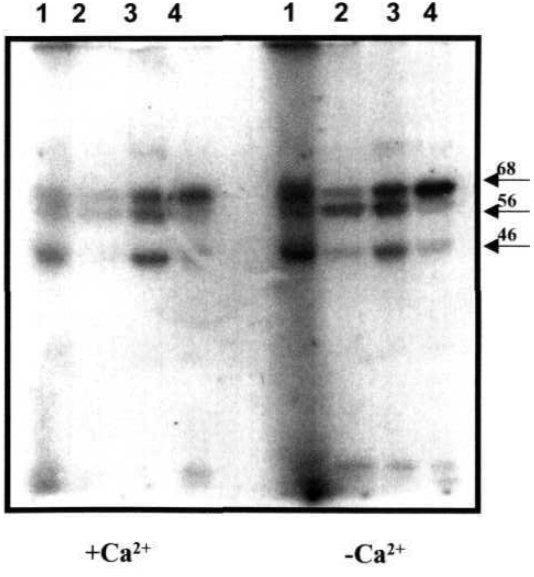


Fig : 35b

Fig. 36a: Changes in protein phosphorylation pattern during shoot organogenesis from leaf explants of S-36 cultivar on MS medium with 2.0 mg/l BAP.

Lane 1 : Leaf explant (0 day)
Lane 2 : Swelling of the explant
Lane 3 : Induction of shoot buds from leaf explants
Lane 4 : Development of shoots (0.5-1.0 cm) from the leaf explants

Arrows point to the bands of interest

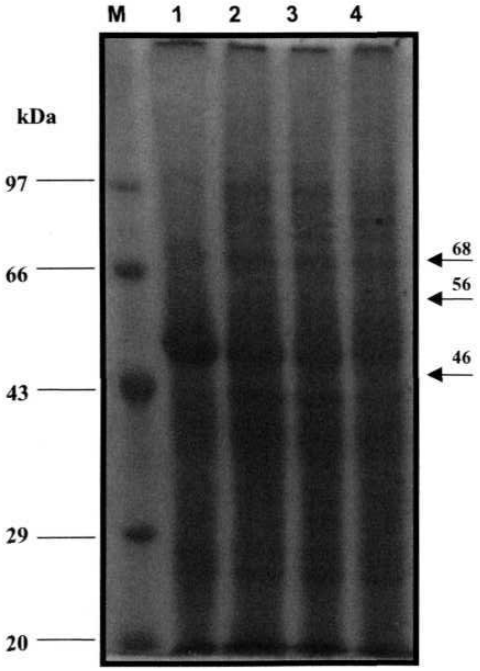
Total protein was extracted from the leaves during different stages of shoot organogenesis, and the extracts were labeled by addition of 4 μCi (γ-^{32}P) ATP in the presence of Ca^{+} and calmodulin in the phosphorylation reaction mixture at room temperature, separated by SDS-PAGE and exposed to X-ray films for 2 days.

Arrows indicate the position in the gel of the major labeled proteins.

Fig. 36b: SDS-PAGE analysis of total proteins during different stages of shoot organogenesis from leaf explants of S-36 cultivar after culture on MS medium with 2.0 mg/1 BAP.

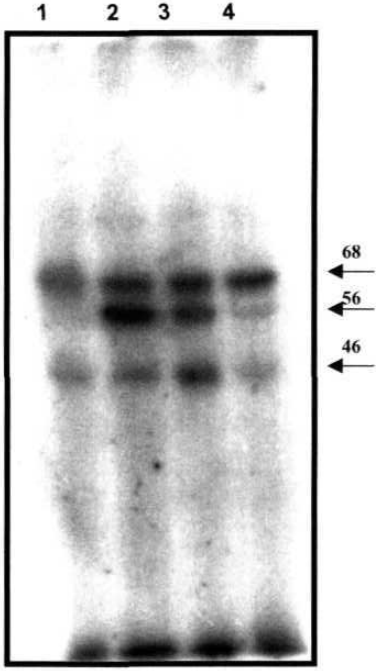
Lane M: Molecular weight marker
Lane 1,2, 3 & 4: as above

Arrows point to the bands of interest



Calmodulin

Fig : 36a



Calmodulin

Fig : 36b

***Agrobacterium* mediated genetic transformation of S-36 cultivar:**

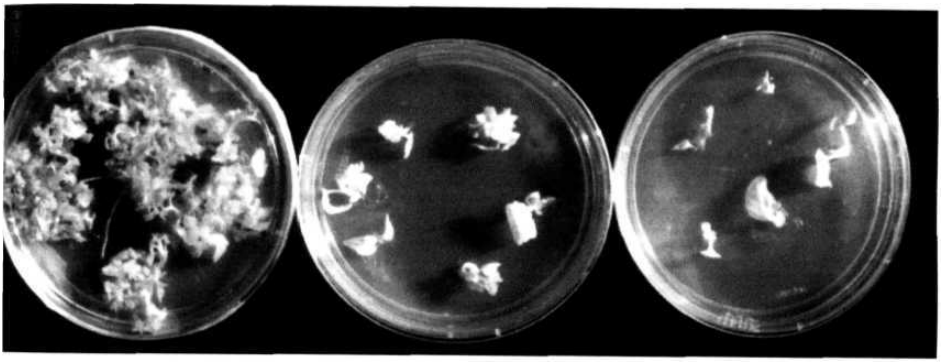
Shoots and leaves derived from the *in vitro* multiplied shoots (MS medium with 0.5 mg/1 BAP) were used for the genetic transformation studies. Shoot meristems of 0.5 cm and leaves of 1.0-4.0 cm and shoots of 2-3 cm length were cultured on medium containing 10-100 mg/1 kanamycin to determine the concentration that is lethal for shoot organogenesis, multiple shoot induction and root induction from the regenerated shoots. Leaves cultured on medium with 4.0 mg/1 TDZ and kanamycin showed no response for shoot organogenesis even when kanamycin was used at a low concentration of 10.0 mg/1 in the medium. The response of shoot multiplication decreased (66.2-6.2%) as the concentration of kanamycin increased (10.0-70.0 mg/1) with complete inhibition of shoot proliferation at 100 mg/1 kanamycin (Table 14). As shoot organogenesis was completely suppressed at a low concentration of kanamycin, further studies on genetic transformation were carried out using shoot meristem explants (Fig. 37). The rooting phase was very sensitive to kanamycin compared to shoot multiplication. The percentage of shoots that rooted decreased (53.8-4.8%) with increase in the concentration of kanamycin (10.0-70.0 mg/1) on IBA and 2,4-D supplemented medium. Kanamycin had a marked effect on root development with induction of 1-2 roots of 1.0-1.5 cm after 30 days of culture in comparison to control shoots which rooted profusely on IBA or 2,4-D supplemented medium with induction of roots of 6.7-8.2 cm after 30 days of culture. Initially, a single colony of *Agrobacterium* strain harbouring p'GUSINT' was cultured in LB broth medium overnight on a orbital shaker at 200 rpm and a temperature of 28" C until the turbidity reached OD of 0.5 at 600 nm. Shoot tips were infected with *Agrobacterium* strain for 5 min and then were placed on MS medium with 0.5 mg/1 BAP. After 48 hr of co-cultivation, the shoot tip explants were incubated on MS medium with 0.5 mg/1 BAP

Table 14: Effect of kanamycin on **survival** of various **explants**

Explant	1 ype of hormone (mg/l)	Concentration of kanamycin (mg/1)	% explants survived	Type of response
Meristem	0.5 BAP	0.0	90.0+ 1.5	Multiple shoot induction
		10.0	66.2+ 1.5	
		30.0	42.7 + 2.1	
		50.0	13.6 + 2.0	
		70.0	6.2 + 1.7	
		100.0	0.0	
Leaf	4.0 TDZ	0.0	83.0+ 1.3	Regeneration of the shoots from the leaf
		10.0	0.0	
		30.0	0.0	
		50.0	0.0	
		70.0	0.0	
		100.0	0.0	
Shoot	0.1 IBA	0.0	89.7+ 1.5	Rooting from the shoots
		10.0	53.8+ 1.8	
		30.0	22.7 ± 1.5	
		50.0	11.6 + 1.5	
		70.0	6.1 +0.8	
		100.0	0.0	
	0.1 2,4-D	0.0	72.7+ 1.2	Rooting from the shoots
		10.0	51.7+ 1.5	
		30.0	20.2 + 1.5	
		50.0	10.6 ± 1.2	
		70.0	4.9 + 0.7	
		100.0	0.0	

Means followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman-Keul's multiple range test.

Fig. 37: Kanamycin sensitivity of shoot tip explants of S-36 cultivar to shoot proliferation on MS medium with 0.5 mg/1 BAP.



10 mg/I **50 mg/1** **100 mg/1**

Fig : 37

and 250 mg/l cefotaxime for 2 days for eliminating the *Agrobacterium*. Subsequently, the shoot tips were placed on selection medium containing MS medium with 0.5 mg/1 BAP and 100 mg/1 kanamycin and scored for the putative transformed shoots. An average of 27.0% of shoot tips developed shoot buds from the base and the shoots reached a length of 0.5-1.0 cm at the end of 30 days of culture on the selective medium (Table 15 and Fig. 38). In contrast, the non-transformed shoot tips (controls) did not show any shoot bud induction and ultimately died after 30 days of culture on kanamycin supplemented medium. Shoots induced from co-cultivated shoot tips were subjected to repeated selection on MS medium supplemented with 0.5 mg/1 BAP and 100 mg/1 kanamycin. Shoots that attained a height of 2-3 cm at the end of 5th subculture on selection medium were separated and transferred to rooting medium containing 0.1 mg/1 IBA and 0.1 mg/1 2,4-D individually with 50 mg/1 kanamycin (Fig. 39 a and b). Selection for transformants using kanamycin in the rooting medium was very effective as a high frequency of shoots that rooted (40.5-46.8%) on IBA or 2,4-D containing medium showed GUS expression. Leaves excised from the putative transformed shoots/plants were placed on medium with 2.0 mg/1 2,4-D for callus induction for histochemical GUS assay. An average of 66.7% of the regenerated plants showed GUS expression in contrast to those selected on shoot multiplication medium where only 43.2% of shoots showed GUS expression in the leaves and callus. Leaves and callus induced from the controls did not show GUS activity (Fig. 40 a and b).

Genomic DNA was isolated from the leaves that were collected from the putative transformants and non-transformed plants established in the field (Fig. 41). The putative transformed plants were subjected to PCR analysis to confirm the presence of the *npt* II gene using specific primers for *npt* II gene. A DNA fragment of 600 bp corresponding to

Table 15: Selection of the putative transformants on MS medium supplemented with kanamycin

Explant	I ype of hormone (mg/l)	Kanamycin (mg/1)	% response	Type of response
Meristems	0.5 BAP	50.0	**13.6 + 2.0** (Control)	Multiple shoot induction
		100.0	0.0 (Control)	
		50.0	39.3 + 2.6	
		100.0	27.0+ 1.3	
Shoot	0.1 IB A	50.0	11.6 + 1.5 (Control)	Root induction from the shoots
		100.0	0.0 (Control)	
		50.0	46.8 + 2.3	
		100.0	0.0	
Shoot	0.1 2,4-D	50.0	10.6+ 1.3 (Control)	
		100 0	0.0 (Control)	
		50.0	40.5 + 0.9	
		100.0	0.0	

Means followed by the same letter **in** a column are not significantly different ($p < 0.05$) **by** Newman-Keul's multiple range test.

Fig. 38: Response of shoot proliferation from non-transformed and transformed shoot tips on selection medium containing 0.5 mg/1 BAP and 100 mg/1 kanamycin.

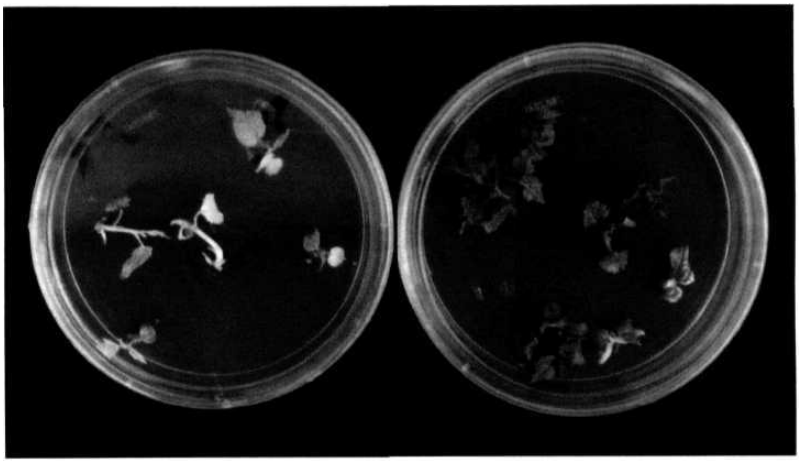


Fig : 38

Fig. 39a: Comparison of root induction from the non-transformed and transformed shoots of S-36 cultivar on MS medium with 0.1 mg/l IBA and 50 mg/l kanamycin after 30 days of culture.

Fig. 39b: Comparison of root induction from the non-transformed and transformed shoots of S-36 cultivar on MS medium with 0.1 mg/1 2,4-D and 50 mg/1 kanamycin after 30 days of culture.

Fig : 39a

Fig: 39b

Fig. 40a: X-gluc reaction of leaves excised from non-transformed and transformed plantlets of S-36 cultivar: only transformed leaves stained blue.

Fig. 40b: GUS gene expression in callus derived from non-transformed and transformed leaves of S-36 cultivar, only calli from transformed leaves stained blue.

Fig. 40a

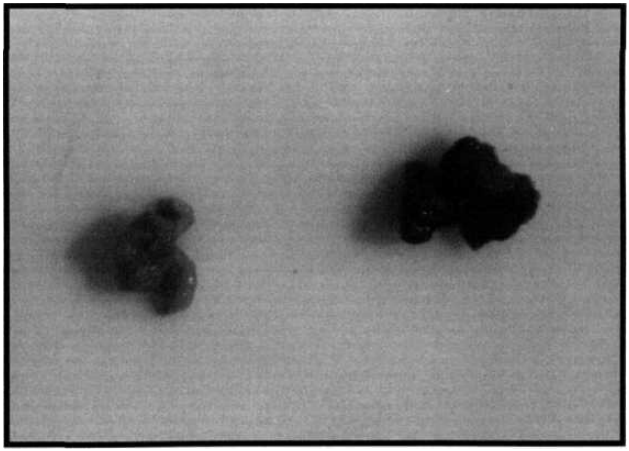


Fig. 40b

Fig. 41: Transgenic plants of S-36 cultivar established in greenhouse after 30 days after transfer.

Fig : 41

the expected size was amplified in 7 of the 8 transgenic plants with slight difference in the amplification whereas no amplification was observed in non-transformed plants (Fig. 42).

Fig. 42: PCR analysis for detecting the *npt* II in transformants of S-36 cultivar.

M : Molecular weight marker (100 bp DNA ladder)
N : Leaf explants of non-transformed plants
T_1 - T_7 : Leaf explants from individual transformed plants

Arrow indicates the 600 bp fragment of the ***npt*** 11 gene

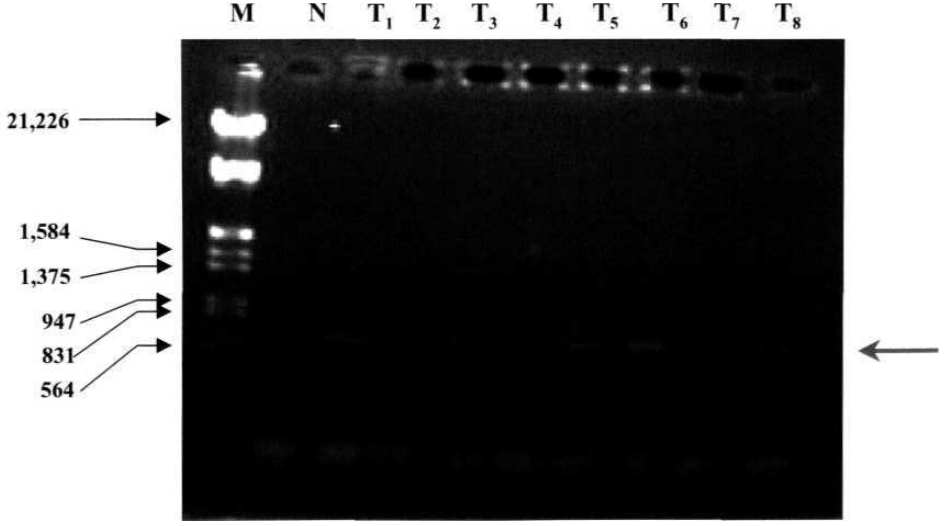


Fig : 42

Discussion

In vitro plant propagation through culture of axillary buds and shoot meristems has been successfully used for rapid multiplication of a number of timber species, woody fruits and ornamental crops (George, 1993). Clonal multiplication of superior phenotypes and valuable breeding stocks offers immense scope in tree improvement programmes. Micropropagation methods are specifically applicable to species for which clonal propagation is required. Mulberry is a highly heterozygous plant and propagation through axillary buds would ensure genetic uniformity and stability among the regenerants. Nodes with axillary buds are the most convenient, accessible and the efficient explants for micropropagation in mulberry. It offers several advantages which include the ease of obtaining explant material, a strong tendency for profuse multiple bud induction, the relative ease with which the shoots could be induced to root and the high percentage of *ex vitro* plant survival after acclimatization. In the present study, nodal explants having greenish axillary buds covered with 2-3 whorls of scales responded efficiently for sprouting whereas buds enveloped by more than 3 whorls of scales did not sprout. Oka and Ohyama (1975), and Anuradha and Pullaiah (1992) have reported that nodal explants of mulberry having greenish axillary buds showed the best response for sprouting and shoot differentiation. The nodal explants collected from old shoots did not exhibit sprouting, whereas explants collected from medium-aged lateral shoots sprouted within 5-7 days of culture. In comparison, the actively growing young lateral shoots with tender axillary buds failed to sprout, as these buds did not survive the sterilization treatments. Pattnaik and Chand (1997) have reported high frequency of bud break from the nodal segments collected from actively growing young lateral shoots compared to the old shoots of 2-3 months in *Morus cathayana* Hemsl, *M. ihou* Koiz and *M. serrata* Roxb.

A comparative study of seasonal effects on axillary bud sprouting and plant establishment rate from the nodal cuttings was undertaken *in vitro* and field conditions. In field conditions, plants established at high frequency (23.3-93.3%) from nodal cuttings in summer in comparison to the rainy and winter seasons in all four cultivars. In the China White cultivar, axillary buds sprouted asynchronously and only 16.7-23.3% of nodal cuttings established into plants in different seasons in field conditions indicating the difficult-to-root nature of the cultivar. Zaman *et al.* (1997) have reported that propagation of mulberry through cuttings is restricted to a single season (September-October) in Bangladesh. The time of the year at which the explants were collected from seedlings stock plants had an influence on axillary shoot growth. This was related to the differences in the physiological condition of the stock plants grown under natural environmental conditions. The best period for shoot culture initiation was April to June and this period was more favorable for the explants to produce significantly more number of shoots and the shoots were also longer than during other times of the year. Seasonal variation in axillary shoot proliferation has been reported for *Artocarpus heterophyllus* (Amin and Jaiswal, 1993) and *Eucalyptus tereticornis* (Das and Mitra, 1990). In the present study, *in vitro* bud break as well as the frequency of aseptic cultures obtained was strongly influenced by the season as well as the age of the lateral shoots from which the nodal explants were collected. The contamination rate was more in nodal explants that were excised from the old lateral branches compared to the medium-aged lateral branches. Similar observation was made earlier in mulberry by Pattnaik and Chand (1997). Maximum contamination of cultures was observed in winter season followed by rainy and summer seasons in all four cultivars of mulberry (Chitra and Padmaja, 2001). Quraishi *et al.* (1996) reported that high contamination was observed in nodal explants of

Cleistanthus collinus collected during July to September whereas minimum contamination occurred during January to June. In the present study, axillary buds of all four cultivars sprouted at a higher frequency in summer (10.0-83.3%) than in rainy (0.0-76.7%) and winter seasons (0.0-60.0%). Pattnaik and Chand (1997) observed that the growth and proliferation of the nodal explants was greatly influenced by the time of explant collection in *Morus cathayana*, *M. ihou* and *M. serrata*. Civinova and Sladsky (1990) suggested that the differential responses of cultured winter and spring buds of temperate species such as oak, aspen and black locust could be linked to the dynamics of endogenous substances in the tree. Quraishi *et al.* (1996) reported that April to June is the best period for shoot culture initiation in *Cleistanthus collinus* and the nodal segments produced significantly more shoots than during other times of the year. It was suggested that the differences in the physiological condition of the stock plants grown under natural environmental conditions might be the reason for differential growth responses *in vitro*.

Different cultivars may require different growth regulators for obtaining optimal response for sprouting and shoot differentiation. In M-5 and S-36 cultivars, high frequency of sprouting (6.7-83.3%) was induced on 2,4-D supplemented medium than on KN supplemented medium. In summer, axillary buds of S-13 cultivar sprouted with the same frequency of 73.3% on medium supplemented individually with 2,4-D (0.3 mg/l) and KN (0.3 mg/1) whereas in China White cultivar, high frequency of sprouting (76.7%) was induced on medium supplemented with KN (2.0 mg/1). Anuradha and Pullaiah (1992) achieved high frequency of axillary bud sprouting from nodal explants of *Morus alba* L. on medium supplemented with low levels of 2,4-D. In contrast, Mhatre *et al.* (1985) reported that BAP was more effective than KN and Z in inducing axillary bud sprouting and shoot differentiation in mulberry. Vasil and Thorpe (1994) stated that the

morphogenetic competence of different genotypes was under the control of physiological or epigenetic factors like exogenous growth regulators and culture conditions rather than true genetic factors.

Efficient plant regeneration protocol is a prerequisite for the application of *in vitro* genetic manipulation techniques, such as variant selection and transformation for the improvement of economically more desirable characters. Leaves are the best explants for adventitious shoot bud regeneration because they have the advantage of ease in manipulation in genetic transformation studies and large number of explants can be obtained from a single plant (Kapur *et al,* 2001). In the present study, the leaves derived either from the *in vitro* induced axillary buds or from the multiple shoots were used for the regeneration studies. The age and the orientation of the leaves were the determining factors affecting induction of direct shoot organogenesis of the leaves. The best response of shoot organogenesis was observed from the leaves of intermediate age (1.0-4.0 cm) that were cultured with the abaxial side in contact with the medium. The regeneration ability of *Morus spp.* was greatly dependent on the combination of growth regulators, explant type and the mulberry genotype (Jain and Datta, 1992; Kathiravan *et al.,* 1995; Sahoo *et al.*, 1997, Vijayan *et al.,* 2000). Yamanouchi *et al. (*1999) reported stable regeneration of plantlets from the immature leaves isolated from winter buds of field-grown mulberry. It is inferred from the studies that the size of the leaf plays an important role in endogenous differentiation potential of leaves in induction of adventitious shoot bud formation. Mhatre *et al. (*1985) concluded from their studies that the upright position of the leaf explants is favorable for maximum induction of shoot buds in *Morus indica* L.

The positive and negative influence of culture vessels on organogenesis has been reported earlier (Wright *et al.,* 1987; McClelland and Smith, 1990). It has been suggested

that organogenesis is influenced by the amount of ethylene and carbon dioxide present in the culture vessel. In the present study, the leaf explants cultured in glass tubes have regenerated at high frequency compared to leaves cultured in the culture bottles and plastic petridishes. In this case, probably the higher amount of ethylene in the culture tubes has helped in the organogenetic response of the leaf explants as it regulates cell division during the induction phase while carbon dioxide may be required to modify the biosynthesis and action of ethylene (Kumar *et al,* 1987). It has also been shown that culture vessels with closures allowing free gas exchange (cotton plugs) are more conducive for regeneration rather than hermetically sealed culture vessels like culture bottles with polypropylene caps and plastic petridishes sealed with parafilm (Proft *et al,* 1985; Aartrijk *et al.*, 1985).

A two-stage culture procedure consisting of culture of leaves on 4.0 mg/l TDZ for a limited period until the induction of adventitious shoot buds followed by a secondary medium consisting of BAP (2.0 mg/1) to promote shoot development was developed. Use of primary and secondary media has been successful with apple (Fasolo *et al,* 1989), pear (Singh and Bhatia, 1988), *Populus* (Russel and Mccown, 1986), and *Rhododendron* (Preece and Imel, 1991). Cytokinins are known to induce axillary as well as adventitious shoot formation from meristematic explants (George, 1993). TDZ is the most potent of the diphenyl ureas that have been evaluated for plant tissue culture (Mok *et al,* 1982) and is less susceptible to plant degrading enzymes than the endogenous cytokinins and also active at low concentrations than the amino purine cytokinins (Mok *et al,* 1987). In the present study, TDZ when used singly in MS medium at 4.0 mg/1 triggered induction of shoot buds from the leaf explants in 8-10 days at frequencies ranging from 77.6-89.2% in different cultivars. Direct shoot organogenesis was also observed from leaves cultured

on medium containing 2.0-5.0 mg/l BAP with frequencies ranging from 0.0-46.7% in different cultivars, and induction of less number (10-11) of shoot buds compared to TDZ. The shoot buds proliferated from the cut apical edges of the leaf explants and might have originated *de novo* as well as from the pre-existing meristems. Though many shoot buds were induced on TDZ supplemented medium, only 2-3 shoot buds increased to a height of 0.5-1.5 cm in 30 days and the rest of the buds resumed growth only upon transfer to BAP (2.0 mg/1) supplemented medium. Similar observation was made in *Pinus strobes* L. by Pijut *et al.* (1991). In the present study, medium supplemented with high levels of TDZ (5.0 mg/1) suppressed shoot elongation in all cultivars. The leaves cultured on medium with 10.0 mg/1 BAP and 1.0 mg/1 IBA responded for shoot differentiation at a very low frequency of 10.0-13.5%. TDZ is resistant to all cytokinin oxidases and induces accumulation of endogenous cytokinins (Kaminek, 1992) that might have inihibited shoot elongation. Yamanouchi *et al.* (1999) achieved high frequency of shoot regeneration from the immature leaves isolated from apical buds and winter buds on medium with 1 uM TDZ compared to 10 uM BAP in 8 cultivars of mulberry. Variation in the activity of different cytokinins can be explained by their differential uptake rate reported in different genomes (Blakesley, 1991), varied translocation rates to meristematic regions and metabolic processes, in which the cytokinin may be degraded or conjugated with sugars or amino acids to form biologically inert compounds as reported by Tran Tranh Van and Trinh (1990), and Kaminek (1992).

A good multiplication rate is the most important factor in rendering a propagation protocol cost-effective. *In vitro* propagation techniques using meristem culture have been developed for several species of *Morus* (Ohyama and Oka, 1987; Ivanicka, 1987, Jain *et al.*, 1990). In the present study, the effect of four cytokinins (BAP, TDZ, KN and Z) on

multiple shoot induction was studied using shoot tips derived from the axillary buds and leaves. Shoots differentiated from the axillary buds and shoots regenerated from the leaves exhibited differences in terms of multiplication with induction of more number of shoots from the leaf regenerated shoots on BAP supplemented medium. Shoot multiplication was maximum in all four cultivars when cytokinins were used at 0.5 ing/1 in MS medium. Of the different cytokinins tested, BAP at 0.5 mg/l was found to be the best for shoot multiplication of axillary bud and leaf regenerated shoots with induction of 6.5-7.5 and 9.5-10.6 shoots, respectively in all four cultivars at the end of 30 days. Shoots were induced with a high frequency (78.7-93.3%) on medium containing 0.5 mg/1 BAP with 4-5 shoots from the base of the shoots and 2-3 shoots from the axils of the leaves whereas on medium with TDZ (0.5 mg/1), shoots and leaves became thick, with induction of 1.3-1.7 shoots from the base of single tip culture. Shoots induced on TDZ supplemented medium were fused and appeared fasciated. The length of the shoots (1.3-1.6 cm) was also less than the shoots induced on BAP supplemented medium (2.4-3.6 cm). There was no induction of shoots from the base of the shoot tips that were cultured on KN and Z supplemented medium whereas 2.3-3.6 multiple shoots were seen from the axils of the leaves after 25-30 days of culture. Palacios *et al*. (2002) reported that the addition of KN caused basal callusing rather than shoots, from the cut stem bases in contact with the medium and reduced the efficiency of shoot production in *Lonicera tatarica*.

In the present study, higher concentrations of cytokinins supported profuse callusing and reduction in shoot multiplication in mulberry. Increase in the concentration of BAP and TDZ to 2.0 mg/1 in the medium decreased the shoot number with retardation of shoot growth. Shoot tips cultured on medium with 2.0 mg/1 TDZ developed callus

from the base that had sectors of green patches. However, shoot differentiation from the callus could not be induced even after subculture on medium with BAP. Huetman and Preece (1993) reported high proliferation rates at very low TDZ concentrations in woody plant species, which had low proliferation on BAP supplemented medium. They further inferred from their studies that inhibition of shoot elongation by TDZ may be consistent with its high cytokinin activity and should not be considered a toxic effect. They have also opined that TDZ should be used carefully as it resulted in hyperhydricity or morphological abnormalities (eg. shoot fasciation) among the regenerated shoots when the concentrations were too high. The formation of stunted or the fasciation of the shoots on TDZ supplemented medium has been reported in several plant species such as apple (van Nieuwkerk *et al.,* 1986) and *Rhododendron* (Preece and Imel, 1991). Hossain *et al.* (1992) observed an increase in shoot proliferation in *Morus laevigata* with the increase in BAP concentration upto 2.5 mg/l and further increase in BAP level suppressed shoot proliferation rate.

The type of sugar is a major factor as are the nature, concentrations and ratios of various endogenous and exogenous auxins and cytokinins. In the present study, shoots differentiated from the sprouted axillary buds showed an increase in proliferation at a rate of 6.5 shoots per culture in all cultivars when fructose (3%) was used in place of sucrose (3%). Shoot tips cultured on glucose supplemented medium turned pale green whereas those cultured on maltose supplemented medium showed induction of buds with no further growth. However, these buds resumed growth and developed into shoots upon transfer to sucrose or fructose supplemented medium. Oka and Ohyama (1982) suggested fructose was a better source of carbon than sucrose for *in vitro* plant induction from bud culture of *Morus alba* L.

Repeated subculturing of the shoot tips differentiated from the axillary buds enhanced the shoot multiplication rate and a 10-12 fold increase in shoot number was observed at the end of 10th subculture in all four cultivars. With the increase in the culture passage, more shoots were induced from the base of the shoots and callus development from the base was observed from 5th subculture onwards. The increase in shoot proliferation rates during series of subcultures may possibly be due to *in vitro* adaptation. Tewari *et al.* (1995) observed an increase in the shoot number in each passage with increase in callus from the base when transferred to fresh medium in the V-1 and S-34 cultivars of *Mortis*. Repeated subculturing of nodes and leaves of *Aegle marmelos* (L.) from shoot cultures helped to achieve continuous production of callus-free, healthy shoots for at least five subculture cycles (Ajithkumar and Seeni, 1998). A similar phenomenon was also observed in *Morus australis* (Pattnaik *et al.*, 1996).

The production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plants in soil (Ohyama, 1970). The stimulatory effect of auxins in the root formation from the shoots depends partly on the type of auxin employed in *Morus alba* L. (Anuradha and Pullaiah, 1992). In the present study, roots were induced with varying frequencies and the number of roots induced as well as the nature of roots induced in the presence of different auxins varied. Medium supplemented with high levels of auxins (1.0 mg/1) was found to be effective for shoots differentiated from the axillary buds whereas low levels of auxins favoured rooting from the repeatedly subcultured shoots and leaf regenerated shoots of different cultivars. Of all the auxins tested, 2,4-D at 1.0 mg/1 was found to be a better rooting agent for the shoots differentiated from the axillary buds whereas IBA at 0.1 mg/1 was found to be the best for the leaf regenerated shoots of all four cultivars. Infinite number of roots was induced on

NAA supplemented medium whereas few thin roots were induced on IAA supplemented medium, which were difficult to handle during transfer of the plantlets to soil. Higher levels of IBA in the rooting medium resulted in the development of callus from the base of the regenerated shoots. The differential response of rooting observed in axillary bud and leaf regenerated shoots in presence of different auxins might be due to the differences in the endogenous hormonal levels in the tissues, that could have contributed to various degrees of sensitivity of tissues towards exogenous auxins. Similarly, Kim *et al.* (1985) observed substantial amount of callus formation at the base of the leaf regenerated shoots on 1.0-5.0 mg/1 IBA supplemented medium whereas in the presence of 0.1 mg/1 IBA, few but healthier and more vigorously growing roots were formed directly at the base of the shoots. The presence of callus between the root and shoot resulted in poor vasculature connection, which made field survival of the plantlets difficult. Hossain *et al.* (1992) observed root formation from the shoots excised from the nodal explants on 1.0 mg IBA or NAA in *Morus laevigata* Wall. Bhau and Wakhlu (2001) reported that IBA was the most effective auxin (0.5 mg/1) for root induction from the shoots regenerated from the callus of *Morus alba* L. whereas higher levels of auxins encouraged callus formation from the cut ends of the explants.

In the present study, shoot tips cultured on medium with 1.5 mg/1 IBA developed abundant cream colored friable callus from the base of the cultures. The callus upon further subculture became nodular with rounded and prominent structures that appeared as globular stage somatic embryos, which did not undergo further development. Internodal segments without shoot meristems when cultured on medium with 1.5 mg/1 IBA did not develop any callus and underwent rhizogenesis directly from the tissue, indicating a high endogenous levels of auxins in such tissues that is known to play an

important role in organogenesis (Narayanswamy, 1977; Halperin, 1986). Jain and Dutta (1992) also opined that high endogenous auxin levels may be inhibitory for the induction of caulogenesis from mulberry tissues and reported the *de novo* induction and development of multiple shoot buds from callus of internodal segments and regeneration of complete plants of *Morns bombycis* cultivar, Shimanochi.

The number of plants, which are successfully transferred from tissue culture vessels to soil conditions, can only effectively measure the success of any micropropagation system. Maintenance of high humidity is necessary for some time after transplantation, for the continued turgidity of the tissue-cultured leaves, until the freshly acclimatized leaves develop (Donnely and Vidaver, 1984). The importance of physiological changes during acclimatization and its effects on leaf gas exchange, growth and plant survival needs to be studied particularly plant performance, since plantlet performance can vary according to plant species and environmental conditions during acclimatization (Luna *et al*., 2001). In the present study, plants regenerated from axillary bud and leaf cultures exhibited differences in survival frequency upon transfer to soil. Plants originating from axillary buds survived with high frequencies (66.8-95.5%) compared to those derived from leaf explants (56.5-80.3%). This differences in survival frequency may be possibly due to the differences in the nature of roots induced, with induction of healthy and prolific root development observed in axillary bud cultures compared to few and slender roots observed in leaf regenerated in the presence of different auxins.

An important requirement for plants produced *in vitro* is that the plants must be genetically identical to the source plants. It was observed that, in all the cultivars *in vitro* raised plants were much vigorous and exhibited more number of branches than the

cutting raised plants which may be due to their previous exposure to cytokinins in the culture medium. Maximum height was recorded in cutting derived plants compared to micropropagated plants in all four cultivars of mulberry for three consecutive years. But Murashige (1978) and Zaman *et al.* (1997) observed a reversed trend of more height of micropropagated plants than the conventionally propagated plants for cuttings. In the present study, the thickness of the shoot was more (7.3-14.2 cm) for the cutting derived plants than in micropropagated plants (5.4-9.4 cm). The leaf number of micropropagated plants was more (335.3-970.0) compared to field propagated plants (225.3-906.3), but leaf area of micropropagated plants was less in comparison to cutting derived plants. Even though the weight of the single leaf was more in cutting propagated plants, the yield of micropropagated plants was high due to the presence of more number of branches. The internodal distance was less in micropropagated plants and hence, the leaves were closely present on the shoots. Pandey and Singh (1989) also found the same type of morphogenic expressions while comparing the *in vitro* raised and seedling raised plants of *Carica papaya*. Jain and Datta (1992) compared the performance of the *in vitro* grown plants of *Morus bombycis* cultivar, Shimanochi with vegetatively grown saplings for various morphological characters like leaf shape, petiole length, area of leaf lamina and internodal distance. The study revealed that *in vitro* grown plants did not exhibit any significant quantitative variation as compared to the conventionally grown plants, indicating the varietal multiplication to be of true-to-type. Morrison *et al.* (2000) reported- that micropropagated plants of *Vaccinium augustifolium* that did not result from subcultures had stem characteristics more similar to stem cuttings than to seedlings, with less branching than seedlings and fewer vegetative buds for burying. They inferred that subculturing on cytokinin-rich media apparently induces the juvenile branching

characteristics that provide micropropagated plants with the desirable morphological and growth habits of seedlings with the benefits associated with asexual propagation. Increased vigour of microclones under field condition has also been reported for apple (Zimmerman, 1986), thornless blackberry (Swartz *et al.*, 1983). The plants regenerated *via* micropropagation are derived from characteristic organized meristems or meristematic cells. These cells are by nature genetically stable and less prone to mutational changes (Vasil, 1994). Thus, the results obtained in the present study clearly demonstrated that micropropagation gave rise to superior clonal populations with respect to number of branches/plant and leaf yield/plant that will be suitable for the mass production of plants.

Growth and differentiation involve the metabolism of various proteins at different stages. Identification of such proteins associated with somatic embryogenesis and organogenesis is important to elucidate the biochemical and molecular mechanisms underlying the process. Many reports have appeared on the quantitative and qualitative differences in protein profiles of embryogenic and non embryogenic calli in a variety of plants (Sung and Okimoto 1981, Choi and Sung, 1984, de Veries *et al.*, 1988 in case of carrot; Chen and Luthe, 1987 for rice; Stirn and Jacobson, 1987 for pea and Hahne *et al.*, 1988 for orchard grass). However, no considerable effort has been made to study the biochemical aspects particularly the protein profiles with respect to organogenesis or embryogenesis in mulberry. In the present study, the protein profiles during different stages of callus proliferation and shoot organogenesis from leaf explants were studied to identify markers specific for de-differentiation and shoot differentiation. In the leaf cultures of mulberry, the cells either proliferate as callus or differentiate into shoots. These cells undergo the process of de-differentiation when the auxin 2,4-D (2.0 mg/1) is

used in the medium whereas in the presence of BAP (2.0 mg/l), direct shoot regeneration is observed. In order to determine whether the induction of callus or shoot regeneration induced from leaf explants by manipulation of growth regulators in the culture media is correlated with the biochemical events, equal amount of proteins were extracted from the above cultures and separated on SDS-PAGE. A comparison of the protein profiles of leaves during different stages of callus proliferation and shoot induction showed similarities as well as differences in the expression of few proteins. A 49 kDa protein was predominant in leaf explants of M-5 and S-36 cultivars and decreased in intensity during the process of callus proliferation and shoot organogenesis. This possibly represents the product of gene concerned with leaf physiology. A protein of 39 kDa was expressed in low levels in leaf explants and increased in intensity during induction of shoot organogenesis in both the cultivars. However, this protein increased in intensity during the initial stages of callus induction and appeared faintly when the leaf explants completely developed into callus. In contrast, in M-5 cultivar, a protein of 25 kDa specifically expressed during callus proliferation and was not detectable during shoot organogenesis, and might be associated with unorganized growth. However, protein characteristic exclusively to callus proliferation could not be detected in S-36 cultivar. Protein changes have previously been used as markers to delineate stages in axillary bud development in pea (Stafstrom and Sussex, 1988), and to identify the time of bud regeneration in detached pine cotyledons (Villalobos *et al.*, 1984). A significant number of the new proteins were common to callus proliferation and shoot organogenesis in M-5 cultivar and were expressed at relatively constant level throughout the culture period. They possibly represent the products of basic metabolic "house-keeping' genes that are components of normal metabolic functions such as cell division and stress responses.

Sung and Okimoto (1983) showed that undifferentiated cells produce 'callus-specific' proteins, and that these 'callus-specific' and 'embryonic' proteins are co-ordinately regulated. The ability of conifer cotyledons to form shoots in the presence of benzyladenine was distinguished based on their protein profiles (Ellis and Judd, 1987). Chen and Luthe (1987) reported that 45 kDa polypeptide consistenly increased in non-embryogenic (NE) rice *(Oryza sativa* L.) calli when it was subcultured on regeneration medium for 9-12 days. There was a little change in the abundance of the 45 kD polypeptide group in embryogenic calli which suggested that it may be correlated with unorganized growth in NE calli. Guru *et al*. (1999) studied the protein pattern of differentiating and non-differentiating calli using SDS-PAGE in two varieties of chickpea *viz.,* BG 267 (kabuli) and BG 362 (desi). The study demonstrated the influence of genotype on the proteins related to differentiation and the role of these proteins in morphogenesis in chickpea. A protein band of molecular weight around 50 kDa was identified in the differentiating calli of the kabuli variety, which was absent in the non-differentiating calli.

One-dimensional system reveals only quantitative differences as multiple bands whereas two-dimensional analysis shows the qualitative contributions of different isoforms to each protein species. The 2-D analysis performed during callus proliferation and shoot organogenesis from leaves revealed the separation of 49 kDa into various isoforms with pl values ranging from 3.0-4.8 in M-5 cultivar and 3.0-4.5 in S-36 cultivar. With respect to shoot organogenesis, 39 kDa protein is of particular interest as it is more abundant in the final stages of shoot organogenesis in M-5 and S-36 cultivars and thus can be correlated with shoot differentiation. A few proteins showed differential expression during shoot organogenesis and callus proliferation from the leaves of M-5

and S-36 cultivars. In M-5 ana s-36 cultivars, 39 kDa protein separated into font-spots during shoot organogenesis with pI values ranging from 4.2-5.8. However 39 kDa protein was expressed in two isoforms with pI values of 4.2 and 5.8 in callus of M-5 cultivar in contrast to S-36 cultivar where only one isoform with pi value of 402 was detectable. This observation shows that isoforms of 39 kDa protein vary in expression during callus proliferation and shoot organogenesis in M-5 and S-36 cultivars. Protein with MW 62 kDa was more expressed in the leaf explants of S-36 cultivar than in M-5 cultivar with pI values ranging from 4.3-6.5 and pI values of 4.2-5.4, respectively. This protein was expressed during organogenesis with pI values of 4.6 and 5.0 in M-5 cultivar and pI value of 4.3 in S-36 cultivar whereas callus of both the cultivars showed very faint expression. This complexity may be the result of post-translational protein modification or of the existence of multiple genes encoding similar but non-identical gene-products, or a combination of the two (Hakman *et al*., 1990). The function of these proteins is presently unknown, however, they can serve as developmental markers for understanding the process of shoot differentiation in mulberry. Hahne *et al*. (1985) reported that callus specific proteins were not detected in the leaf (explanting stage), apparently because it is a tissue type without direct equivalent in whole plants and thus not unexpectedly contains unique proteins. Coleman and Ernst (1991) studied the protein differences among internodal stem explants of *Populus deltoids* that were determined for either shoot regeneration or callus growth by SDS-PAGE and two-dimensional PAGE. Three 32 kDa proteins with pI values of approximately 6.5-7.5 were detected only in callus inducing (cim) and shoot inducing (sim) treatments for which high levels of adventitious shoot regeneration was observed. A 35 kDa protein of approximately pI 7.0 was observed only in the cim/sim treatments which resulted in callus determined growth.

Phosphorylation is a form of post-translational modification of proteins that provides a mechanism by which organism can respond rapidly to changes in their internal and external environments over time scales that would preclude a transcriptional response. Factors that are known to alter phosphorylation are light, growth hormones, polyamines and environmental stresses (Raymond and Douglas, 1990; Mizoguchi *et al*., 1994; Reddy and Prasad, 1995; Chang and Kang, 1999). Protein kinase activity in plants has been shown to occur in both particulate and soluble fractions obtained from plant homogenates (Keates and Trewavas, 1974; Lin and Key, 1976). In the present study, the presence of protein kinase activity was demonstrated during different stages of callus proliferation and shoot organogenesis from leaf explants of M-5 and S-36 cultivars. Further studies are needed to elucidate the biochemical and regulatory properties of protein kinase activity associated with extracts obtained from leaf explants during induction of callus and shoot organogenesis. The incorporation of ^{32}P into endogenous proteins present in the different samples containing the protein kinase was determined and exogenous substrates were not applied. The substrate proteins were separated after phosphorylation by SDS-PAGE and autoradiographed. This procedure allows only the determination of the net phosphorylation and does not discriminate between protein kinase and phosphatase activities.

Calcium (Ca^{2+}) is a universal second messenger that regulates a variety of cellular and physiological processes in eukaryotic cells (Poovaiah and Reddy, 1993). The roles of calcium are mediated by a group of calcium-binding proteins including Ca^{2+}-dependent protein kinases (CDPKs) and calmodulin (CAM, Roberts and Harmon, 1992). In the present study, changes in protein phosphorylation in the absence/presence of Ca^{+} and calmodulin during callus proliferation and shoot organogenesis from leaf explants of

M-5 and S-36 cuitivars were investigated. SDS-PAGE analysis revealed differences in the expression of 39 kDa protein, which appeared in high intensity in the final stage of shoot organogenesis whereas it appeared as a faint band in the leaf-derived callus of M-5 and S-36 cuitivars. Evidences of phosphorylation experiments indicated variation with respect to intensity of phosphorylation of 68, 56 and 46 kDa proteins in the absence/presence of Ca^{2+} and calmodulin. Proteins of 68 and 56 kDa appeared as clear bands with a slight variation in the intensity whereas 46 kDa protein could not be visualized as clear band during callus proliferation and shoot organogenesis on SDS-PAGE gel. This means that changes in the intensity of phosphorylation of these proteins are not accompanied by significant changes in the quantity of these proteins. Phosphoproteins of MW 68, 56 and 46 kDa appeared as faint bands in the leaf explants of M-5 cultivar and the intensity of these phosphoproteins increased during shoot organogenesis with a slight variation in the level of phosphorylation in the absence/presence of Ca^{2+} and calmodulin. In contrast, these proteins exhibited varying degrees of phosphorylation during initial stages of callus proliferation with a complete dephosphorylation of 46 kDa protein in the leaf derived callus irrespective of absence/presence of Ca^{2+} and calmodulin. This result suggests that there is a correlation between phosphorylation and dephosphorylation of the 46 kDa protein and unorganized cell growth in leaf explants of M-5 cultivar. In S-36 cultivar, phosphoprotein of 68 kDa and 46 kDa appeared as major bands and 56 kDa protein as a minor band in contrast to M-5 cultivar where all the three phosphoproteins appeared as faint bands. Although these phosphorylated bands were common for callus proliferation and shoot organogenesis, the relative intensity of the bands differed between callus proliferation and shoot organogenesis. Phosphorylation of 46 kDa protein was reduced in the final stages of

shoot organogenesis whereas intense phosphorylation was observed in the leaf derived callus of S-36 cultivar. In contrast, the leaf derived callus of M-5 cultivar was characterized by the absence of 46 kDa phosphoprotein. This observation shows the varietal differences in the pattern of phosphorylation during the process of de-differentiation and shoot differentiation in leaf explants of mulberry. Vilardell *et al.* (1990) reported that RAB-17 (responsive to ABA) were highly phosphorylated in mature maize embryos, while these protein accumulated in ABA-treated callus without considerable phosphorylation. Tan and Kannada (2000) identified one specific phosphoprotein, ECPP-44 (Embryogenic Callus Phosphoprotein) that was found to be induced in all treatments that resulted in embryogenic competence. It was suggested that ECPP-44 might be related to the acquisition of embryogenic competence. Cordewener *el al.* (2000) investigated the changes in protein synthesis and phosphorylation during microspore embryogenesis in *Brassica napus* by two-dimensional (2-D) gel electrophoresis. Comparison of 2-D patterns of phosphorylated proteins revealed minor differences between embryogenic and non-embryogenic cultures, except for the level of phosphorylation of hsp 70.

In the present study, addition of calmodulin resulted in a decrease in the phosphorylation of 68, 56 and 46 kDa proteins during callus proliferation whereas enhanced phosphorylation was observed when shoots of 0.5-1.0 cm differentiated from leaf explants of M-5 cultivar. Wolff *el al.* (1981) suggested that calmodulin may influence the degree of protein phosphorylation through regulation of both phosphatases and kinases. Veluthambi and Poovaiah (1984) presented evidence for the role of Ca^{2+} and calmodulin in regulating the phosphorylation of membrane and soluble proteins from corn coleoptiles. Some reports identifying plant calmodulin-dependent protein kinases

were based in part on the observation that micromolar levels of calmodulin stimulate protein kinase activity 1.5-6-fold (Polya *et al.,* 1983; Salimath and Marme, 1983; Paliyath and Poovaiah, 1985; Veluthambi and Poovaiah, 1986). Blowers *et al.* (1985) showed that autophosphorylation of a protein kinase, which had been isolated from pea membranes by electroblotting, was stimulated by the addition of calcium and calmodulin.

The transfer of foreign genes into plants has opened new ways to study regulation of development and also provided new approaches to achieve certain breeding objectives of economically important plants (Bent, 1996; Kazun *et al.,* 1997). Among the various genetic transformation methods available, *Agrobacterium* mediated transformation has been extensively applied to many plant species because this method offers several advantages such as the differential integration of transgenes, potentially low copy number and preferential integration into transcriptionally active regions of the chromosome over the direct gene delivery method like particle bombardment (Koncz *et al.,* 1989; Hiei *et al.,* 2000). The critical point for the optimization of a transformation procedure is the efficient production of plants from cultured explants. Successful transformation using *Agrobacterium* depends not only on the efficiency of plant regeneration system but also on the sensitivity of the cultured tissues to antibiotic. In the present study, the sensitivity of the tissues at different stages of plant regeneration, *i.e.* shoot organogenesis from leaf explants, multiplication of shoots and root induction from the shoots was determined by supplementing kanamycin at 10.0-100.0 mg/l in the respective medium. The concentration of kanamycin in the selective medium had a significant effect on the survival of explants. No survival was recorded from the leaf explants even at 10.0 mg/1 kanamycin, whereas multiple shoots were induced with frequencies of 13.6-66.2% from the shoot tips cultured on medium with 10.0-50.0 mg/1 kanamycin. Shoot induction and

root induction was completely suppressed from the shoots when kanamycin was used at 100.0 mg/l in the respective medium. The visible effect of kanamycin at 50.0 mg/l on root induction was delayed root induction along with reduction in the number and the growth of the roots. It is well known that the roots are very sensitive to antibiotics used in plant transformation experiments (Bennici, 1974; David, 1984). The efficiency of transformation with *Agrobacterium* depends on the efficiency of selection that allows growth of all the transformed cells and the frequency of regeneration that occurs among the transformed cell population (Dandekar *et al*., 1993). In the present study, as the leaf explants turned necrotic even at a low concentration of kanamycin, shoot tips were used as initial explants for transformation experiments. About 27% co-cultivated shoot tips yielded resistant shoots on medium containing BAP and kanamycin (100.0 mg/1). Regenerated shoots from the putative transformed explants were propagated on the same medium for five passages to eliminate the possibility of chimeras Subsequently, the putative transformed shoots were transferred to rooting medium containing kanamycin for root induction. Leaves and calli of kanamycin resistant plants were evaluated using the GUS histochemical assay. An average of 66.7% of the regenerated plants showed GUS expression in contrast to those selected at the shoot multiplication stage where only 43.2% of shoots showed GUS expression in the leaves and callus. These results suggested that there are escapes on kanamycin selection medium and that selection for transformants was more effective with the use of kanamycin at the rooting stage. Regeneration of escapes could be explained by the loss of foreign gene expression or by the ineffective kanamycin selection where non-transformed cells are protected from the selective agent by the surrounding transformed cells (Zhan *et al*., 1997). Although there is a possibility of escapes using shoot tips, repeated proliferation of shoots on selection

medium followed by the use of kanamycin in the rooting medium will minimize the possibility of escapes. The only earlier report on genetic transformation of mulberry was by Machii (1990) who succeeded in transferring kanamycin resistant gene and β-glucuronidase (GUS) gene through Ti plasmid PB1 121 to mulberry leaf discs and confirmed the expression of Kan R and GUS gene in regenerated plants. The use of the shoot apex as the explant for *Agrobacterium* mediated tranformation has been reported in petunia (Ulian *et al*., 1988), pea (Hussey *et al*., 1989), sunflower (Schrammeijer *et al*., 1990), *Zea mays* (Gould *et al*., 1991) and cotton (Gould Gould and Magallanes-Codeno, 1998). Xie and Hong (2002) described a protocol for *Agrobacterium* mediated transformation of *Acacia mangium* using rejuvenated shoots as the explants. Stem segments of rejunevated shoots were co-cultivated with *Agrobacterium tumefaciens* strain LBA 4404 harbouring binary vector pBI 121 and the selection for transgenic shoots was performed through five consecutive steps on MS medium supplemented with 1.0 mg/l TDZ, 0.25 mg/l IAA and different concentrations of geneticin and timentin. Thirty-four percent of the stem segments produced resistant multiple adventitious shoot buds, of which 30 % expressed the β- glucuronidase gene.

In the present study, examination of the GUS positive transgenic plants from eight separate tranformation events by PCR confirmed that 7 of the 8 transgenic plants contained the expected DNA fragment of 600 bp with a slight difference in the amplification. No amplified fragment was observed in the non-transformed plants.

Summary

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Conclusions

The study has focused on establishment of an efficient *in vitro* regeneration system for axillary bud and leaf explants and its utilization for genetic transformation of mulberry. The significant findings that emerged from the study are as follows:

- High frequency of sprouting from axillary buds was observed in summer compared to the rainy and winter seasons in all four cultivars in field and *in vitro* conditions.
- In China White cultivar, sprouting was found to be asynchronous and at the end of 60 days only 23.3% of the nodal cuttings established into plants in summer in field conditions.
- High frequency of axillary bud sprouting (56.7-83.3%) in M-5 and S-36 cultivars was observed on medium supplemented with 0.3 mg/1 2,4-D whereas in S-13 cultivar, the axillary buds sprouted with the same frequency of 73.3% on medium supplemented individually with 2,4-D (0.3 mg/1) and KN (0.3 mg/1). In China White cultivar, medium fortified with 2.0 mg/1 KN favoured high frequency of axillary bud sprouting (76.7%) from nodal explants.
- A brief culture of leaves for 8-10 days on medium with 4.0 mg/1 TDZ followed by transfer to 2.0 mg/1 BAP supplemented medium triggered high frequency shoot organogenesis (77.6-89.2%) in all four cultivars.
- Shoots differentiated from axillary buds and leaf explants responded efficiently for shoot multiplication in the presence of 0.5 mg/1 BAP with induction of more number of shoots from leaf regenerated shoots (9.4-10.6) in comparison' to axillary bud regenerated shoots (6.5-7.5).

- Addition of fructose instead of sucrose in the multiplication medium increased the shoot number at a rate of 6.5 per culture and also the growth of the shoots was vigorous in all four cultivars.
- Repeated subculture of shoots enhanced the shoot multiplication rates with production of 17.7-20.0 shoots/culture at 10^{th} subculture in different cultivars.
- Shoots differentiated from the axillary buds rooted at a high frequency (86.7-100.0%) on medium with 1.0 mg/l 2,4-D with induction of healthy roots after 9-10 days of culture in different cultivars.
- A differential response was observed from the shoots regenerated from the leaf explants and repeatedly subcultured shoots which rooted at a high frequency on medium supplemented with low level of IBA (0.1 mg/1) whereas increase in the level of IBA (1.0 mg/1) resulted in induction of callus from the base of the shoots.
- The field performance of the micropropagated plants in comparison to cutting derived plants demonstrated the superiority of the micropropagated plants with regard to number of branches/plant and leaf yield/plant.
- SDS-PAGE analysis of proteins during induction of shoot organogenesis from leaves showed that 49 kDa protein was highly expressed in leaves and decreased in intensity following culture. An increase in the intensity of 39 kDa protein was noticed as shoot organogenesis progressed in M-5 and S-36 cultivars.
- Two-dimensional gel analysis on the protein extracted from the leaves revealed the separation of 49 kDa protein into various isoforms with pI values ranging form 3.0-4.8 in M-5 cultivar and 3.0-4.5 in S-36 cultivars. Protein of 39 kDa separated into four spots during shoot organogenesis with pI values ranging from 4.2-5.8 in M-5 and S-36 cultivars. This protein was expressed in two isoforms

with pI values of 4.2 and 5.8 in callus of M-5 cultivar in contrast to leaf derived callus of S-36 cultivar where only one isoform with pI value of 4.2 was observed.

- Protein phosphorylation studies during callus proliferation and shoot organogenesis form leaves of M-5 and S-36 cultivars revealed the presence of 68, 56 and 46 kDa phosphoproteins in varying intensities in the presence/absence of Ca^{+2} and calmodulin. The most noticeable change was observed with respect to 46 kDa protein which was dephosphorylated in the leaf derived callus of M-5 cultivar whereas intense phosphorylation was observed during shoot organogenesis. In contrast, in S-36 cultivar, 46 kDa protein was intensely phosphorylated in the leaf derived callus whereas faint phosphorylation was observed during induction of shoots (0.5-1.0 cm) from leaf explants.
- Shoot meristems derived from *in vitro* proliferated shoots were co-cultivated with *Agrobacterium* strain harbouring binary vector having ***gus*** intron and *npt* II gene and the selection **for** the transformed shoots was performed on medium with 0.5 mg/l BAP and 100 mg/l kanamycin. An average of 27.0% of shoot tips yielded resistant shoots which when tested for GUS expression at the end of 5^{th} subculture revealed that 43.2% of shoots were GUS positive.
- Selection of transformants using kanamycin in rooting medium was very effective as 66.7% of the regenerated plants showed GUS expression in contrast to untransformed shoots which did not show GUS expression. PCR analysis using primers for the *npt* II gene showed the expected band of 600 kb in 7 out of 8 putative transformants whereas no amplified fragment was observed in the untransformed (control) plants.

In summary, reproducible protocols for the rapid *in vitro* multiplication of four elite cultivars of mulberry has been developed. A two-step approach involving short incubation of leaves for 8-10 days on TDZ supplemented medium followed by transfer to medium with BAP for obtaining high frequency shoot organogenesis from leaves of four elite cultivars has been developed. Evaluation of field performance of micropropagated plants in comparison to cutting derived plants demonstrated the superiority of the micropropagated plants with regard to number of branches/plant and leaf yield/plant. Studies on changes in protein profiles during callogenesis and shoot organogenesis showed the differential expression of 39 kDa protein during de-differentiation and shoot differentiation from leaf explants of M-5 and S-36 cultivars and thus this protein can be used as a marker for understanding the shoot regeneration process. Analysis of protein phosphorylation revealed that proteins of MW 68, 56 and 46 kDa were phosphorylated in varying intensities in the absence/presence of Ca^{2+} and calmodulin during callogenesis and shoot organogenesis from leaf explants of M-5 and S-36 cultivars. It would be of interest to identify the target proteins and characterize the associated protein kinase(s). Once the proteins associated with shoot organogenesis are identified, further studies on amino acid composition, sequencing and comparison with databases will help in understanding their specific role in the process of shoot differentiation. Furthermore, a procedure for *Agrobacterium* mediated transformation of mulberry using shoot meristems is developed which can be exploited for transferring biotic as well as abiotic resistant genes for its improvement.

References

Aartrijk, J. V., Bloom-Barnhoorn, G. H. and Bruisma, J. (1985) Adventitious bud formation from bud-scale explants of *Lilium speciosum* Thumb. *In vitro:* effects of aminoethxyvinyl glycine, 1-aminocyclopropane-1-carboxylic acid and ethylene. J. Plant Physiol. 117:401-410.

Ahuja, M. R. (1993) Micropropagation of woody plants. Kluwer academic publishers, Dordrecht.

Aitkin-Christie, J., Singh, A. P., Horgan, K. J. and Thorpe, T. A. (1985) Bot. Gaz. 146: 196-203.

AjithKumar, D. and Seeni, S. (1998) Rapid clonal multiplication through *in vitro* axillary shoot proliferation of *Aegle marmelos* (L.) corr., a medicinal tree. Plant Cell Rep. 17:422-426.

Ali, S. **M., Sarah,** B., **Stevan,** P. R. **and** Rose, P. (1988) Phosphorylation of synaptic proteins in chick forebrain: changes with development and passive avoidance training. J. Neurochem. 50: 1579-1587.

Amin, M. N. and Jaiswal, V. S. (1993) *In vitro* response of apical bud explants from mature tree of jack fruit (*Atrocarpus heterophyllus*). Plant Cell Tiss. Org. Cult. 33: 59-65.

Anuradha, M. and Pullaiah, T. (1992) Micropropagation of mulberry *{Morns alba* L.). Annali Di Botanica, 50: 35-42.

Babu, P. P., Ratnakumari, L. and Vemuri, M. C. (1994) Differential changes in cell morphology, macromolecular composition and membrane protein profiles in neurons and astrocytes in chronic ethanol treated rats. Mol. Cell. Biochem. 130: 29-40.

Barve, D. M. and Mehta, A. R. (1994) *In vitro* clonal propagation of mature trees of *Commiphora wightii*. In: (ed.) Tandon, P., Advances in plant tissue culture in India. Proc. of **XIII** Plant tissue culture conference 232-240.

Bennett, J. (1977) Phosphorylation of chloroplast membrane polypeptides. Nature, 269: 344-346.

Bennici, A. (1974) Cytological analysis of root, shoots and plants regenerated from suspension and solid *in vitro* culture of haploid *Pelargonium.* Z. Pflanzenzuchtg, 72: 199-205.

Bent, A. F. (1996) Plant disease resistance genes: Function meets structure. Plant Cell, 8: 1757-1771.

Bhatnagar, S., Anita, K. and Khurana, P. (2001) TDZ-Mediated differentiated in commercially valuable Indian Mulberry, *Morus indica* cultivars K-2 and DD. Plant Biotechnol. 18: 61-65.

Bhau, B. S. (1999) Tissue culture studies of some difficult-to-root temperate varieties of *Morus multicaulis* (PERR.). Ph.D.Thesis, Jammu, India.

Bhau, B. S. and Wakhlu, A. K. (2001) Effect of genotype, explant type and growth regulators on organogenesis in *Morus alba.* Plant Cell Tiss. Org. Cult. 66: 25-29.

Bhojwani, S.S. (1992) Plant tissue culture and its relevance to mulberry breeding. Brainstorming meeting on genetics and biotechnology of silkworm and mulberry. CSRTI, Mysore, India. 1-11.

Bidwai, A. P. and Takemoto, J. Y. (1987) Bacterial phytotoxin, syringomycin, induces a protein kinase-mediated phosphorylation of red beet plasma membrane polypeptides. Proc. Natl. Acad. Sci. USA. 84: 6755-6759.

Biondi, S. and Thorpe, T. A. (1982) Clonal propagation of forest tree species. In: (ed.) Rao, A., Proc. of COSTED symposium on "Tissue culture of economically important plants". COSTED and Asian Network of Biological Sciences, Singapore. 197-204.

Blakscy, D. (1991) Uptake and metabolism of 6-benzyladenine in shoot proliferation of *Musa* and *Rhododendron.* Plant Cell Tiss. Org. Cult. 25: 69-74.

Blanco, M. D. L. A., Nieves, N., Snchez, M., Borroto, C. G., Castillo, R., Gonzalez, J. L., Escalona, M., Baez, E. and Hernandcz, Z. (1997) Protein associated with plant regeneration in embryogenesis calli of sugarcane (*Saccharum* sp.). Plant Cell Tiss. Org. Cult. 51: 153-158.

Blowers, D. P., Hetherington, A. and Trewavas, A. (1985) Isolation of plasma membrane-bound calcium/calmodulin-regulated protein kinase from pea using western blotting. Planta, **166:** 208-215.

Blowers, D. P., Wendy, F. B. and Anthony, J. T. (1984) Rapid changes in plasma membrane protein phosphorylation during initiation of cell wall digestion. Plant Physiol. 86: 505-509.

Blum, H., Beier, H. and Gross, H. J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis, 8: 93-99.

Bogre, L., Olah, Z. and Dudits, D. (1988) Calcium dependent protein kinase from alfalfa *(Medicago varia):* partial purification and autophosphorylation. Plant Sci. 58: 135-144.

Brainerd, K. E., Fuchigami, L. H., Kurat Kowski, D and Clark, C. S. (1981) Leaf anatomy and water stress of aseptically cultured 'Pixy' plum grown under different environments. Hortic. Sci. 16: 173-175.

Budde, R. J. A. and Randall, D. D. (1990) Light as a signal influencing the phosphorylation status of plant proteins. Plant Physiol. 94: 1501-1504.

Caboni, E., Tonelli, M. G., Lauri, P., Angeli, S. D. and Damiano, C. (1999) *In vitro* shoot regeneration from leaves of wild pear. Plant Cell Tiss. Org. Cult. 59: 1-7.

Chang, C, **Ben,** A. **M., Kathleen, B. E. and Guiltinan, M. J.** (1996) *In vitro* plantlet regeneration from cotyledon, hypocotyls and root explants of hybrid seed geranium. Plant Cell. Tiss. Org. **Cult.** 45: 61-66.

Chang, S. C. and Kang, B. G. (1999) Effects of spermine and plant hormones on nuclear protein phosphorylation in *Ranunculus* petioles. J. Plant Physiol. 154: 463-470.

Chattopadhyay, S., Chattopadhyay, S. and Datta, S. **K.** (1990) Quick *in vitro* production of mulberry *(Morus alba* L.) plantlets for commercial purpose. Ind. J. Exp. Biol. 28(6): 522-525.

Chen, L. J. and Luthe, D. S. (1987) Analysis of proteins from embryogenic and non-embryogenic rice *(Oryza sativa* L.) calli. Plant Sci. 48: 181-188.

Chitra, D. S. **and Padmaja, G.** (1999) Clonal propagation of mulberry *(Morus indica* L. cultivar M-5) through *in vitro* culture of nodal explants. Scientia Hortic. 80: 289-298.

Chitra, D. S. V. and Padmaja, G. (2002) Seasonal influence on axillary bud sprouting and micropropagation of elite cultivars of mulberry. Scientia Hortic. 92: 55-68.

Choi, J. H. and Sung, Z. R. (1984) Two-dimensional gel analysis of carrot somatic embryonic proteins. Plant Mol. Biol. Rep. 2: 19-25.

Christianson, M. L. and Warnick, D. A. (1988) Organogenesis *in vitro* as a developmental process. Hortic. Sci. 23: 515-519.

Civinova, B. and Sladsky, Z. (1990) Stimulation of the regeneration capacity of tree shoot segment explants *in vitro.* Biol. Plant. 32: 407-413.

Cohen, P. (1982) The role of protein phosphorylation in neutral and hormonal control of cellular activity. Nature, 296: 613-620.

Coleman, D. G. and Ernst, S. G. (1991) Protein differences among *Populus deltoids* internodal stem explants determined for shoot regeneration or callus growth. Plant Sci. 75: 83-92.

Crum, C. J., Hu, J., Hiddinga, J. and. Roth, D. (1988) Tobacco **mosaic virus** stimulates the phosphorylation of a plant protein associated **with double-stranded** RNA-dependent protein kinase activity. J. Biol. Chem. 263: 13440-13443.

Dandekar, A. M., McGranahan, G. H. and James, D. J. (1993) Transgenics woody plants. In: (eds.) Schain-dow, Wu. R., Transgenic plants. Academic press New York. 2: 129-151.

Dandin, S. B. and Ramesh, S. R. (1987) A kalppavruksha called mulberry. Indian Silk 87: 49-53.

Das, B. C. (1983) Mulberry taxonomy, cytogenetics and breeding. National Seminar on Silk Research and Development, March 10-13, Bangalore, India.

Das, D. K., Reddy, M. K., Upadhyaya, K. C. and Sopory, S. K. (2002) An efficient leaf disc culture method for the regeneration *via* somatic embryogenesis and transformation of grape (*Vitis vinifera* L.). Plant Cell Rep. 20: 999-1005.

Das, T. and Mitra, G. C. (1990) Micropropagation *of Eucalyptus tereticornis.* Plant Cell Tiss. Org. Cult. 22: 95-103.

Datta, R. K. (1994) Production and demand of silk in India, In: Global silk scenario-2000, Proc. of the International Conference on Sericulture, Oxford IBM. 44-54.

David, C, Chilton, M. D. and Tempe, J. (1984) Conservation of T-DNA in plants regenerated from hairy root cultures. Biotechnol. 2: 73-76.

Davies, J. R. and Polya, G. M. (1983) Purification and properties of high specific activity protein kinase from wheat germ. Plant Physiol. 71: 489-495.

De Vries, S. C, Booij, H., Janssens, R., Vogels, R., Saris, L., Lo Schiavo, F., Terzi, M. and Van Kammen, A. (1988) Carrot somatic embryogenesis depends on the phytochrome/controlled presence of correctly glycosylated extracellular proteins. Genes Dev. 2:462-476.

Debergh, P. C. and Zimmerman, R. H. (1991) Micropropagation-Technology **and** Application. Kluwer Academic Publishers, Dordrecht.

Dietrich, A., Mayer, J. E. and Hahlbrock, K. (1990) Fungal elicitor triggers rapid, transient, and specific protein phosphorylation in parsley cell suspension cultures. J. Biol. Chem. 265: 6360-6368.

Dodeman, V. L. and Ducreux, G. (1996) Isozyme patterns in zygotic and somatic embryogenesis of carrot. Plant Cell Rep. 16: 101-105.

Donnelly, D. J. and Vidaver, W. E. (1984) Pigment content and gas exchange of red raspberry ***in*** *vitro* and *ex vitro.* J. Am. Hortic. Sci. 109: 177-181.

Dudits, D., Bogre, L. Dedeoglu, D., Magyar, Z., Kapros, T., Felfoldi, F. and Gyorgye, J. (1993) Key components of cell cycle control during auxin-induced cell division. In: (ed.) Ormrod J. C., Kluwer Academic Publishers, Dordrecht, 111-131.

Ellis, D. D. and Judd, R. C. (1987) SDS-PAGE analysis of bud-forming cotyledons of *Pinus ponderosa.* Plant Cell. Tiss. Org. Cult. 11: 57-65.

Farmer, E. E., Pearc, G. and Ryan, C. **A.** (1989) *In vitro* phosphorylation of plant plasma membrane proteins in response to the proteinase inhibitor-inducing factor. Proc. Natl. Acad. Sci. USA. 86: 1536-1542.

Fasolo, F., Zimmerman, R. H. and Fordham, I. (1989) Adventitious shoot formation on excised leaves of *in vitro* grown shoots of apple cultivars. Plant Cell Tiss. Org. Cult. 16: 75-87.

Fisk, H. J. and Dandekar, A. M. (1993) The introduction and expression of transgenes in plants. Scientia Hortic. 55: 5-36.

Flick, C. E., Evans, D. A. and Sharp, W. R. (1983) Organogenesis. In: (eds.) Evans, D. A., Sharp, W. R., Ammirato, V. P. and Yamada, Y. Macmillan, New York, 13-81.

Gamborg, O. L., Murashige, T., Thorpe, T. A. and Vasil, I. K. (1976) Plant tissue culture media. *In Vitro.* 12: 473-478.

Garcia, R. B., Walling, L. L. and Murashige, T. (1992) Analysis of polypeptides associated with shoot formation in tobacco callus cultures. Am. J. Bot. 79: 481-487.

Gaśser, C. S. **and Fraley, R. T.** (1989) Genetically engineering plants for crop improvement. Science, 244: 1293-1299.

George, E. **F.** (1993) Plant propagation by tissue culture. Part I: The techonology. Second edition, Exegetics Ltd. England. 470-471.

George, E. F. (1996) Plant propagation by tissue culture. Exogenics, Edington UK. **574.**

George, E. F. and Sherrington, P. D. (1984) Plant propagation by tissue culture-Handbook and directory of commercial laboratories. Eastern Press, Reading.

Ghugale, D. D., Kulkarni, D. and Narsimhan, R. (1971) Clonal propagation of *Morus alba.* Ind. J. Exp. Biol. 9: 381-384.

Gilroy, S. **and Trewavas, A.** (1990) Signal sensing and signal transduction across the plasma membranes. In: (eds.) Larsson, C. and Moller, 1. M. The Plant Plasma Membrane, Structure, Function and Molecular Biology. Springer-Verlag, Berlin, Heidelberg. 203-232.

Gould, J. and Magallanes-Codeno, M. (1998) Adaptation of shoot apex ***Agrobacterium*** inoculation and culture to cotton transformation. Plant Mol. Biol. Rep. 16: 284-

Gould, J., Devey, M., Hasegawa, O., Ulian, E. C, Peterson, G. and Smith, R. H. (1991) Transformation of *Zea mays L.* using *Agrobacterium tumefacians* and shoot apex. Plant Physiol. 95: 426-434.

Guru, S. K., Chandra, R., Khetrapal, S., Raj, A. and Polisetty, R. (1999) Protein pattern in differentiating explants of chick pea *(Cicer arietinum* L.). Ind. J. Plant Physiol. 4:147-151.

Hahne, G., Mayer, J. E. and Lorz, H. (1988) Embryogenic and callus specific proteins in somatic embryogenesis of the grass, *Dactylis glomerata* L. Plant Sci. 55: **267-**79.

Hakman, I., Stabel, P., Engstrom, P. and Eriksson, T. (1990) Storage protein acculmulation during zygotic and somatic embryo development in ***Picea*** *abies* (Norway spruce). Physiol. Plant. 80: 441-445.

Halperin, W. (1986) Attainment and retention of morphogenetic capacity ***in*** *vitro.* In: (ed.) Vasil, I. K. Cell culture and somatic cell genetics of plants, Plant regeneration and genetic variability. Academic Press, Inc. Orlando, Florida. 3: 3-47

Hansen, C. E., **Meins,** F. **and Aebi,** R. (1987) Hormonal regulation of zeatin-riboside accumulation by tobacco cells. Planta, 172: 520-525.

Hartmann, H. T., Kester, D. E., Davies, F. T. **and Geneve, R. L.** (1997) Plant propagation principles and practices. Sixth edition. Prentice Hall Inc., Upper Saddle River, N. J.

Hasegawa, P. M., **Yasuda,** T. **and Cheng,** T. (1979) Effect of auxin and cytokinin on newly synthesized proteins of cultured Douglas-fir cotyledons. Physiol. Plant. 46: 211-217.

Hetherington, A. and Trewavas, A. (1982) Calcium dependent protein kinase in pea shoot membranes. FEBS Lett. 145:67-71.

Hiei, Y., Komari, T., Ishida, Y. and Saito, H. (2000) Development *of Agrobacterium* mediated transformation method for monocotyledonous plants. Breed. Res. 2: 205-213.

Hiei Y. Ohata, S., Komari, T. and Kumashiro, T. (1994) Efficient transformation of rice *(Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of T- DNA. Plant J. 6: 271-282.

Horsh, R. B., Fry, J. E., Hoffman, N. L., Eicholtz, D., Rogers, S. G. and Fraley, R. T. (1985) A simple and general method for transferring genes into plants Science 227: 1229-1231.

Hossain, M., Rahman, S. M., Zaman, A., Joader, O. I. and Islam, R. (1992) Micropropagation of *Morus laevigata* Wall from mature trees. Plant Cell Rep 11 • 522-524.

Huetteman, C. A. and John Preeece, E. (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tiss. Org. Cult. 33: 105-119.

Hunter, T. (1987) A thousand and one protein kinases. Cell, 50:823-829.

Hussey, G., Johnson, R. D. and Warren, S. (1989) Transformation of meristematic cells in the shoot apex of cultured pea shoots by *Agrobacterium tumefaciens* and *A. rhizogenes*. Protoplasma, 148: 101-105.

Huttner, W. B. and Greengard, P. (1979) Multiple phosphorylation sites in protein and their differential regulation by cyclic AMP and calcium. Proc. Nalt. Acad. Sci. USA 76: 5402-5406.

Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T. and Kusahiro, T. (1996) High efficiency of transformation of maize *(Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nat. Biotech. 14: 745-750.

Islam, R., Zaman, A., Hossain, M., Barmar, A. C, **Joarder, O. I. and Hossain,** A. B. (1992) Effect of growth regulators on *in vitro* callogenesis in *Morus laevigata* Wall. Bull. Sericult. Res. 3:55-58.

Ivanicka, J. (1987) *In vitro* micropropagation of mulberry *Morus **nigra*** L. Scientia Hortic. 32: 33-40.

Jain, A. K. and Datta, R. K. (1992) Shoot organogenesis and plant regeneration in mulberry *(Morus bombycis* Koidz) : factors influencing morphogenetic potential in callus cultures. Plant Cell Tiss. Org. Cult. 29(1): 43-50.

Jain, A. K., Dandin, S. B. and Sengupta, K. (1990) *In vitro* propagation through axillary bud multiplication in different mulberry genotypes. Plant Cell Rep. 8: 737-740.

Jefferson, R. A. (1987) Assaying chimeric genes in plant: the GUS gene fusion system. Plant Mol. Biol. Rep. 5: 387-405.

Jeon, B. R., Fujiwara, K. and Kozai, T. (1995) Environmental control and photoautotropic micropropagation. In: (ed.) Janik, J. Hortic. Rev. 17: 123-170.

Jones, L. H., Barfield, D., Barret, J., Flook, A., Pollock, K. and Robinson, P. (1982) Cytology of oil palm culture and regenerated plants. In : (ed.) Fujiwara, A. Plant Tiss. Cult. Tokyo.

Kalea, G. and Bhatla, S. **C.** (1999) Expression of soluble polypeptides during **auxin-**modulated rooting in sunflower (*Helianthus annus* **L.).** Ind. **J.** Plant **Physiol 4:36-39.**

Kaminek, M. (1992) Progress in cytokinin research. TIBTECH. 10: 159-162.

Kapur, A. Bhatnagar, S. and Khurana, P. (2001) Efficient regeneration from mature leaf explants on Indian mulberry ***via*** organogenesis. Serocologia, 41: 1-8.

Kathiravan, K., Ganapathi, A. **and Shajahan,** A. (1997) Adventitous shoot formation and plant regeneration from callus cultures of mulberry (*Morus alba* L.). Sericologia, 37: 727-733.

Kathiravan, K., Shajahan, A. and Ganapathi, A. (1995) Regeneration of plantlets from hypocotyls derived callus of *Morus* alba L. Israel J. Plant Sci. 43: 259-262.

Kazun, K., Curtis, M. D., Goulter, K. C. **and Manners, J. M.** (1997) *Agrobacterium tumefaciens* mediated **transformation** of double haploid canola ***(Brassica napus)*** lines. Aust. J. Plant Physiol. 24: 97-102.

Keates, R. A. B. **and Trewavas,** A. J. (1973) Protein kinase activity associated with isolated ribosomes from peas and *Lemna.* Plant Physiol. 54: 95-99.

Kieber, J., Rothenberg, M., Roman, G., Feldmann, K. A. **and Ecker, J. R.** (1993) CTRL, **a** negative regulator of the ethylene response pathway in *Arabidopsis.* encodes a member of the Raf family of protein kinase. Cell, 72: 427-441.

Kim, H. R., Patel, K. R. and Thorpe, T. A. (1985) Regeneration of mulberry plantlets through tissue culture. Bot. Gaz. 146: 335-346.

Koncz, C, Martin, N., Mayerhafer, R., Koncz-Kulman, Z., Korber, H., Redei, G. P. and Schell, J. (1989) High frequency T-DNA mediated gene tagging in plants. Proc. Natl. Acad. Sci. USA. 86: 8467-8471.

Kozai, T. (1991) Acclimatization of micropropagated plants. In: Bajaj, Y. P. S. (ed.) Biotechnology in Agriculture and Forestry, Vol. 17. High-Tech. Micropropagation. Springer-Verlag, Berlin, Heildelberg. 127-141.

Krishnaswami, S. (1990) Mulberry cultivation in South India. Central Silk Board. Bangalore. pp. 1-9.

Kumar, P. P., Reid, D. M. and Thorpe, T. A. (1987) The role of **ethylene and** carbon dioxide in differentiation of shoot buds in excised cotyledons of *Pinus radiata in vitro*. Physiol. Plant. 69: 244-252.

Kurosaki, F., Tsurusawa, Y. and Nishi, A. (1987) The elicitation of phytoauxins by Ca^{2+} and cyclic AMP in carrot cell. Photochemistry, 26: 1919-1923.

Kyo, M. and. Harada, H. (1990) Specific phosphoproteins in the initial period of tobacco pollen embryogenesis. Planta, 182: 58-63.

Laemmli, U. **K.,** (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature, 277: 680-685.

Larkin, P. J. and Scowcroft, W. R. (1981) Somaclonal variation a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 67: 443-455.

Lee, N., Wetzstein, Y. and Sommer, H. E. (1985) Effects of quantum flux density on photosynthesis and chloroplast ultrastructure in tissue-cultured plantlets and seedlings of *Liquidambar stryraciflua L.* towards acclimatization improvement and field survival. Plant Physiol. 78: 637-641.

Leshem, B. and **Sussex,** I. **M.** (1990) Polypeptides of cultured melon cotyledons serve as markers of root and shoot organogenesis. J. Plant Physiol. 137: 155-159.

Lin, P. P. C. **and** Key, J. L. (1976) Lysine-rich histone H1 kinase from soybean hypocotyls. Biochem. Biophys. Res. Commun. 73: 396-403.

Linsmaier, E. M. **and** Skoog, F. (1965) Organic growth factor requirements of tobacco tissue cultures, Physiol. Plant. 18: 100-127.

Lowry, O. H., Rosebrouch, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the folin phenol regent. J. Biol. Chem. 193: 265-275.

Lu, C. Y. (1993) The use of thidiazuron in tissue cultures. *In vitro* Cell Dev. Biol. 29: 92-96.

Luna, E. A. A., Davies Jr, F. T. and Egilla, J. N. (2001) Physiological changes and growth of micropropagated chile anchopepper plantlets during acclimatization and post- acclimatization. Plant Cell, Tiss. Org. Cult. 66:17-24.

Lynn, D. H. and Walker-Simmons, M. K. (1995) The wheat abscisic acid-responsive protein kinase m RNA PKABA-1 is up-regulated by dehydration, cold temperature and osmotic stress. Plant Physiol. 108:1203-1210.

Machii, H. (1990) Leaf disc transformation of mulberry plant ***(Mortis** alba* L.) by *Agrobacterium* Ti plasmid. J. Seric. Sci. Jpn. 59: 105.

Machii, H. (1992) Organogenesis from immature leaf cultures **in** mulberry, *Morus alba L.* **J.** Seric. Sci. **Jpn.** 61: 512-519.

Mann, N. H., Rappka, R., and Herdman, M. (1991) Regulation of protein phosphorylation in the cyanobacterium *Anabaena* strain, PCC 7120 I Gen Microbiol. 137:331-339.

McClelland, M. T. and Smith, M. A. **L.** (1990) Vessel type, closure and explant orientation influence in vitro performance of five woody species. Hortic. Sci. 25: 797-800.

Meane, L. M. and Debergh, P. C. (1983) Rooting of tissue cultured plants under in vivo conditions. Acta Hortic. 131:201-208.

Mhatre, M., Bapat, V. A. **and Rao, P.** S. (1985) Regeneration of plants from the culture of leaves and axillary buds in mulberry (*Morus indica* L.). Plant Cell Rep. 4: 78-80.

Miller, C. O. (1961) Kinetin and related compounds in plant growth. Ann. Rev. Plant Physiol. 12: 395-408.

Minamizawa, S. (1997) In: Moriculture - science of mulberry cultivation. Oxford and IBH publishing Co. Pvt. Ltd., New Delhi.

Mizoguchi, T., Gotoh, Y., Nishida, E., Yamaguchi-Shinozaki, K., Hayashida, N., Iwasaki, T., Kamada, H. and Shinozaki, K. (1994) Characterization of two cDNAs that encode MAP kinase homologues in *Arabidopsis thaliana* and analysis of the possible role of auxin in activating such kinase activities in cultured cells. Plant J. 5: 111-122.

Mizoguchi, T., Hayashida, N., Yamaguchi-Shinozaki, K., Kamada, H. and Shinozaki, K. (1993) ATMPKs: a gene family of plant MAP kinases in *Arabidopsis thaliana.* FEBS Lett. 336: 440-444.

Mok, M. C, **Mok, D. W.** S., **Armstrong, D. J., Shudo, K., Isogai, Y. and Okamoto, T.** (1982) Cytokinin activity of N-phenyl-N'–1,2,3-thiadiazol-5-ylurea (thidiazuron). Phytochemistry, 21: 1509-1511.

Mok, M. C, **Mok, D. W. S., Turner, J. E. and Mujer,** C. **V.** (1987) Biological and biochemical effects of cytokinin-active phenyl urea derivatives in tissue culture systems. Hortic. Sci. 22:1194-1196.

Morel, G. (1960) Producing virus-free cymbidium. Am. Orchid Soc. Bull. 29: 495-497.

Morre, D. J., Morre, J. T. and Varnold, R. L. (1984) Phosphorylation of membrane located proteins of soybean ***in*** *vitro* and response to auxin. Plant Physiol. 75: 265-268.

Morrison, S., John, M. S. and Litten, W. (2000) Morphology, growth and rhizome development of *Vaccinium angustifolium* Ait. Seedlings, rooted softwood cuttings and micropropagated plantlets. Hortic. Sci. 35: 738-741.

Mourgues, F., Chevreau, E., Lambert, C. and de Bondt, A. (1996) Efficient *Agrobacterium*-mediated transformation and recovery of transgenic plants from pear (*Pyrus communis* L.). Plant Cell Rep. 16: 245-249.

Mundkur, R. and Muniraju, E., (2002) Mulberry silkworm races of India. Plant Hortic. Tech. 3: 42-45.

Murashige, T (1978) The impact of plant tissue culture in agriculture. In: (ed.) Thorpe T. A. Frontiers of plant tissue culture. Univ. Calgary Press, Canada. 15-26.

Murashige, T. (1977) Plant cell and organ culture as horticultural practices. Acta Hortic. 78: 17.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Murray, M. G. **and** Key, **J. L.** (1978) 2, 4-dichlorophenoxyacetic acid-enhanced phosphorylation of soybean nuclear proteins. Plant Physiol. 61: 190-198.

Murthy, B. N. S., Murch, S. **J. and Saxena, P. K.** (1998) Thidiazuron: A potent regulator **of *in vitro*** plant morphogenesis. *In vitro* Cell Dev. Biol. 34: 267-275.

Narayan, P., Chakraborthy, S. **and Rao, G. S.** (1989) Regeneration of plantlets from the callus of stem segments of mature plants of *Morus alba* L. Proc. Indian Natl. Sci. Acad. 55: 469-472.

Narayanswamy, S. (1977) Regeneration of plants from tissue cultures. In: (eds.) Reinert, J. and Bajaj, Y. P. S. Applied and fundamental aspects of plant cell, tissue and organ culture. 179-206.

Nehra, N. S., Chibbar, R. N., Kartha, K. K., Datta, R. S. S., Crosby, W. L. and Stushnoff, C. (1990) Genetic transformation of strawberry by *Agrobacterium tumefaciens* using leaf disc regeneration system. Plant Cell Rep. 9: 293-298.

Nehra, N. S., Stushnoff, C. and Kartha, K. K. (1989) Direct shoot regeneration from strawberry leaf disks. J. Am. Soc. Hortic. Sci. 114: 1014-1018.

Ogurtsov, K. S., Atabekova, K. S. Khamirzaev, M. M. and Madyarov, S. H. R. (1986) Culturing the meristems of young mulberry leaves on an artificial nutrient medium. Sheik 1:3 (R).

Ohnishi, T. Yaasukura, F. and Tan, J. (1986) Preservation of mulberry callus by the addition of both abscissic acid (ABA) and p-amino benzoate (PABA) in culture medium. J. Sericult. Sci. Jpn. 55: 252-255.

Ohnishi, T. and Kobayashi, Y. (1991a) Separation of free cells from the hypocotyls of mulberry. J. Sericult. Sci. Jpn. 60: 117-124.

Ohnishi, T. and Kobayashi, Y. (1991b) Suspension culture of cells from the hypocotyls of mulberry. J. Sericult. Sci. Jpn. 60: 320-323.

Ohnishi, T. and Kobayashi, Y. (1991c) Root formation from suspension cells of mulberry hypocotyls. J, Sericult. Sci. Jpn. 60: 505-507.

Ohyama, K. (1970) Tissue culture in mulberry tree. Jpn. Agric. Res. Quart. 5: 30-34.

Ohyama, K. and Oka, S. (1987) Mulberry. In: (eds.) Bonga J. M. and Durzan D. J. Cell and Tissue Culture in Forestry, Martinus-Nijhoff, The Netherlands. 3: 272-284.

Oka, S. and Ohyama, K. (1973) Induction of callus and effects of constituents of medium on callus formation of mulberry trees. J. Seric. Sci. Jpn. 42: 317-324.

Oka, S. and Ohyama, K. (1975) Studies on ***in*** *vitro* of excised buds in mulberry tree. II. Effect of growth substances on the development of shoots from axillary bud. J. Sericult. Sci., Japan. 44: 444-450.

Oka, S., and Ohyama, K. (1981) *In vitro* initiation of adventitious buds and its modification by high concentration of benzyl adenine in leaf tissues of mulberry (*Morus alba* L.). Can. J. Bot. 59: 68-74.

Oka, S., and Ohyama, K. (1982) Sugar utilization in mulberry (*Morus alba* L.) bud culture. Proc. 5th Intl. Cong. Plant Tissue Cell Culture. 67-68.

Oka, S., and Ohyama, K. (1986) Biotechnology in agriculture and forestry, Trees I. In: (ed.) Bajaj, Y. P. S., Springer- Verlag, Berlin Heidelberg. Vol. I: 384-394.

Oshingane, K. (1989) On the redifferentiation of shoot callus to different culture in mulberry. J. Fac. Text. Sci. Tech. Schinshu Univ. E. Agri. Sericult. 107: 38-52.

Oshingane, K. (1990) On the redifferentiation of shoot callus to different culture in mulberry. J. Fac. Text. Sci. Tech. Schinshu Univ. E. Agri. Sericult. 0(11): 13-40.

Palacios, N., Christou, P. and Leech, M. J. (2002) Regeneration of *Lonicera talarica* plants ***via*** adventitious organogenesis from cultured stem explants. Plant Cell Rep. 20:808-813.

Paliyath, G. and Poovaiah, B. W. (1985) Calcium and calmodulin-promoted phosphorylation of membrane proteins during senescence in apples Plant Cell Physiol. 26: 977-986.

Palni, L. M. S., Burch, L. and Horgan, R. (1988) The effect of auxin concentration **on** cytokinin stability and metabolism. Planta, 174: 231-234.

Pandey, R. M. **and** Singh, S. P. (1989) Field performance of *in vitro* raised plants. Ind. J. Hortic. 33: 1-7.

Patel, G. K., Bapat, V. **A. and Rao, P.** S. (1983) *In vitro* culture of organ explants of *Morus irtdica:* Plant regeneration and fruit formation in axillary bud culture. Z. Pflanzenphysiol. 111: 465-468.

Pattnaik, S. **K. and Chand, P. K.** (1997) Rapid clonal propagation of three mulberries, *Morus cathayana* Hemsl., *M. ihou* Koiz. and *M. serrata* Roxb., through *in vitro* culture of apical shoot buds and nodal explants from mature trees. Plant Cell Rep. 16:503-508.

Pattnaik, S. **K., Sahoo, Y. and Chand, P. K.** (1995) Efficient plant retrieval from alginatc encapsulated vegetative buds of mature mulberry trees. Scientia Hortic. 90: 1-13.

Pattnaik, S. **K.,** Sahoo, Y. **and** Chand, P. K. (1996) Micropropagation of a fruit tree, *Morus australis* Poir Syn. *M. acidosa* Griff. Plant Cell Rep. 15: 841-845.

Philip, T., Sarkar, A. **and Govindaiah** (1996) Screening of some promising genotypes of mulberry for leaf spot and rust resistance. Ind. J. Seric. 35(2): 158-159.

Pickard, B. G. (1970) Comparison of calcium and lathanon ions in the *Avena* coleoptile growth test. Planta, 91:314-320.

Pijut, P. M., Michler, C. H. and Voelker, T. M. (1991) Effects of embryo explant orientation, thidiazuron, and agar on eastern white pine (*Pinus strobes* L.) adventitious shoot initiation. Proc. International Symp. on Applications of Biotechnology to Tree Cultures, Protection and utilization. Columbus OH. 126 (Abstract).

Polya, G. **M. and** Davies, J. R. (1982) Resolution and properties of a protein kinase catalyzing the phosphorylation of a wheat-germ cytokinin-binding protein. Plant Physiol. 71:482-488.

Polya, G. M., Davies, J. R. and Micucci, V. (1983) Properties of a calmodulin-activated Ca^{2+}-dependent protein kinase from wheat germ. FEBS Lett. 150: 167-171.

Poovaiah, B. W. and Reddy, A. S. N. (1993) Calcium and signal transduction in plants. Crit Rev Plant Sci. 12: 185-211.

Preece, J. E. and **Imel,** M.R. (1991) Plant regeneration from leaf explants of Rhododendron 'P. J. H. Hybrids'. Scientia Hortic. 48:159-170.

Preece, J. E. and Sutter, E. G. (1991) Acclimatization of micropropagated plants to the green house and field. In: (eds.) Debergh, P. C. and Zimmerman, R. H. Micropropagation technology and Application. Kluwer Academic Publishers, Dordrecht. 71-93.

Proft, M. P. D., Maene, L. J. and Debergh, P. C. (1985) Carbondioxide and ethylene evolution in the culture atmosphere *of* ***Magnolia*** cultured *in vitro.* Physiol. Plant. 65: 375-379.

Quraishi, A., **Koche, V.** and Mishra, S. K. (1996) *In vitro* **micropropagation** from nodal segments of *Cleistanthus collinus.* Plant Cell Tiss. Org. Cult. 45: 87-91.

Rajan, M. **V.** and Ravindran, V. (1989) In: Genetic resources of mulberry and utilization. Mysore, India, 81-86.

Rajyalakshmi, K., Grover, A., **Maheshwari,** N. Tyagi, A. K. and **Maheshwari,** S. C. (1991) High frequency regeneration of plantlets from the leaf-bases *via* somatic embryogenesis and comparison of polypeptide profiles from morphogenic and non-morphogenic calli in wheat (*Triticum aestivum*). Physiol. Plant. 82: 617-623.

Ramagopal, S. (1989) Barley proteins associated with tissue differentiation. J. Plant Physiol. 134: 395-405.

Ramgopal, S. (1994) Protein variation accompanies leaf differentiation in sugarcane (*Saccharum officinarum*) and is influenced by genotype. Plant Cell Rep. 6: 692-696.

Ranjeva, R. and **Boudet,** A. (1987) Phosphorylation of proteins in plants regulatory effects and potential involvement in stimulus/response coupling. Ann. Rev. Plant Physiol. 38: 73-93.

Rao, P. S. and Bapat, V. A. (1993) Micropropagation of sandalwood (*Santalum album* L.) and mulberry (*Morus indica* L.). In: (ed.) Ahuja, M. R. Micropropagation of Woody Plants. Kluwer Academic Publishers. The Netherlands. 317-345.

Rao, P. S., Bapat, **V.** A., Mhatre, **M.** and **Patel,** G. K. (1989) Application of plant cell, tissue and organ culture in mulberry improvement programme. Genetic resources of mulberry utilization. 125-130.

Rao, S. and Raghunath, **M.** K. (1993) Callus initiation, growth and plant regeneration in mulberry *(Morus alba* L.) cultivars. J. Swamy Bot. Club. 10: 17-21.

Ravindranan, R., and Lakshmi Sita, G. (1994) Micropropagation of difficult-to-root elite cultures and induction of embryogenesis in mulberry *{Morus indica* L.). In:

(ed.) Tandon, P. Advances in plant tissue culture in India. Proc. of **XIII Plant** tissue culture conference, 155-169.

Raymond, J. A. B. and Douglas, D. R. (1990) Light as signal influencing the phosphorylation status of plant proteins. Plant Physiol. 94: 1501-1504.

Raz, V. **and Fluhr, R.** (1993) Ethylene signal is transduced *via* protein phosphorylation events in plants. Plant Cell, 5: 523-530.

Reddy, G. N. and Prasad, M. N. V. (1995) Cadmium induced protein phosphorylation changes in rice *(Oryza saliva* L.) seedling. J. Plant Physiol. 145: 67-70.

Roberts, D. M. and Harmon, A. C. (1992) Calcium-modulated proteins: targets of intracellular calcium signals in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43: 375-414

Russel, J. A. and McCourn, B.H. (1986) Thidiazuron-stimulated shoot differentiation from protoplasts derived calli of *Populus.* VI International Cong. Plant Tiss. Cell Cult. Abstracts. 49.

Saghai-Maroof, M. A., Soliman, K. M., Jorgensen, R. A. and Allard, R. W. (1984) Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, Chromosomal location, and population dynamics. Proc Nalt Acad Sci USA. 81: 8014-8018

Sahoo, Y., Pattnaik, S. K. and Chand, P. K. (1997) *In vitro* clonal propagation of a medicinal plant *Ocimum basilicum* L. (sweet basil) by axillary shoot proliferation. *In vitro* Cell Dev. Biol. Plant. 33: 293-296.

Saito. H. (1992) *In vitro* propagation of mulberry through adventitious bud induction system, J. Seric. Sci. Jpn. 61: 46-51.

Salimath, B. P. and Marme, D. (1983) Protein phosphorylation and its regulation by Calcium and calmodulin in membrane fractions from zucchini hypocotyls. Planta, 158:560-568.

Scharf, K. D. and Nover, L. (1982) Heat shock-induced alterations of ribosomal protein phosphorylation in plant cell cultures. Cell, 30: 427-437.

Schrammeijer, B., Sijmons, P. C, van den Elzen, P. J. M. and Hockcma, A. (1990). Meristem transformation of sunflower *via Agrobacterium.* Plant Cell. Rep. **9:** 55-60.

Schulman, H. and Grengard, (1978) Stimulation of brain membrane protein phosphorylation by calcium and endogenous heat-stable protein. Nature, 271: 478-479.

Seki, H., Takeda, M., Trutrumi, K. and Ushiki, Y. (1971) Studies **on the** callus culture of the mulberry tree (1). The effect of concentrations of auxin and kinetin on the callus culture of the mulberry stem. J. Seric. Sci. Jpn. 40: 81-85.

Sharma, K. K. and Thorpe, T. A. (1990) *In vitro* propagation of mulberry (*Morus alba* L.) through nodal segments. Scientia Hortic. 42: 4: 307-320.

Singh, S. and Bhatia, S.K. (1988). Shoot proliferation of pear cultures on medium containing thidiazuron and benzylaminopurine. Hortic. Sci. 23: 803 (Abstract).

Skirvin, R. M. (1981) Fruit crops. In: (ed.) Conger, B. V. Cloning agricultural plants ***via in*** *vitro* techniques. CRC Press, Boca Raton, 51-139

Skoog, F. and Miller, C. **O.** (1957) Chemical regulation of growth and organ formation in plant tissues cultured ***in*** *vitro*. Symp. Soc. Exp. Biol. 11: 118-131.

Soll, J. (1988) Purification and characterization of a chloroplast outer-envelope bound, ATP-dependent protein kinase. Plant Physiol. 87; 898-903.

Stafstrom, J. P., Altschuler, M. and Anderson, D. H. (1993) Molecular cloning and expression of a MAP kinase homologue from pea. Plant Mol. Biol. 22: 83-90.

Stafstrom, J. P. and Sussex, 1. M. (1988) Patterns of protein synthesis in dormant and growing vegetative buds of pea. Planta, 176: 497-505.

Stewart, A.A., Ingebritsen, T. S., Manalan, A., Klee, C. B. and Cohen, P. (1982) Discovery of a Ca^{2+} and calmodulin-dependent protein phosphatase. FEBS Lett. 137:80-84

Stirn, S. and Jacobsen, H. J. (1987) Marker proteins for embryogenic differentiation patterns in pea callus. Plant Cell Rep. 6: 50-54.

Sujatha, M. and Reddy, T. P. (1998) Differential cytokinin effects on the stimulation of *in vitro* shoot proliferation from meristematic explants of castor (*Ricinus communis* L.). Plant Cell Rep. 17: 561-566.

Sung, Z. R. and Okimoto, R. (1981) Embryogenic protein in somatic embryogenesis of carrot. Proc. Natl. Acad. Sci. USA.78: 3683-3687.

Sung, Z. R. and Okimoto, R. (1983) Coordinate gene expression during somatic embryogenesis in carrots. Proc. Natl. Acad. Sci. USA. 80: 2661-665.

Suzuki, K. and Shinshi, H. (1995) Transient activation and tyrosine phosphorylation of **a** protein kinase in tobacco cells treated with a fungal elicitor. Plant Cell, 7: 639-647.

Swartz, H. J., Galletta, G. J. and Zimmerman, R. H. (1981) Field performance and phenotypic stability of tissue culture propagated strawberries. J. Amer Soc Hortic Sci. 106:667-673.

Swartz, H. J., Galletta, G. J. and Zimmerman, R. H. (1983) Field performance and phenotypic stability of tissue culture propagated thornless blackberries. J. Amer. Soc. Hortic. Sci. 108: 285-290.

Tan, S. K. **and Kamada, H.** (2000) Initial identification of a phosphoprotein that appears to be involved in the induction of somatic embryogenesis in carrot. Plant Cell Rep. 19: 739-747.

Tewari, A., Bhatnagar, S. and Khurana, P. (1999) *In vitro* response of commercially valuable cultivars of *Morus* species **to** thiadiazuron and activated charcoal. Plant **biotech.** 16:413-418.

Tewari, P. K. and Subba Rao, G. (1990) Multiple shoot formation through shoot apex culture of mulberry. Ind. J. For. 13: 109-111.

Tewari, P. K., Gupta, P. K. and Subba Rao, G. (1989) *In* ***vitro*** studies on the growth rate of callus of mulberry (*Morus alba* L.). Ind. J. For. 12: 34-35.

Tewari, P. K., Raghunath, M. and Sarkar, A. (1996) Genotypic differences in response to ***in*** *vitro* shoot development of mulberry *(Morns* spp.). Ind. J. Sericult. 35: 104-106.

Tewari, P. K., Sarkar, A., Kumar, V. and Chakraborti, S. (1995) Rapid *in vitro* multiplication of high yielding mulberry (*Morus* spp.) Genotypes V-1 and S-34. Ind. J. Seric. 34: 133-136.

Thorpe, T. A. (1980) Organogenesis ***in*** *vitro:* structural, physiological and biochemical aspects. Int. Rev. Cyt. Supp. 11: 71-111.

Thorpe, T. A. (1983) Biotechnological applications of tissue culture to forest tree improvement. Biotech. Adv. 1: 263-278.

Thorpe, T. A. (1993) *In vitro* organogenesis and somatic embryogenesis: Physiological and biochemical aspects. In: (eds.) Roubelakis, K. A., Angelakis and Tran Thanh Van, K. Markers of plant morphogenesis Plenum Press, New **York,**

Tran Thanh Van, K. and Trinh (1990) Organogenic differentiation. In: Bhojwani, S. S. (eds.), Plant tissue culture. Application and limitations. Elsevier, Amsterdam.

Trewavas, A. (1979) Nuclear phosphoproteins in germinating cereal embryos and their relationship to the control of mRNA synthesis and the onset of cell division. In: (eds.) Laidman, D. L. and Wyn Jones, R. G. Recent Advances in the biochemistry of cereals. Academic Press, New York. 175-208.

Trujillo, C., Rodriguez-Arango., Jaramillo, S., Hoyos, R., Orduz, S. and Arango, R. (2001) One step transformation, of two Andean **potato** cultivars (*Solanum tuberosum* L. sub sp. *Andigena*) Plant Cell Rep. 20: 637-641.

Ulian, E. C, Smith, R. H., Gould, J. H. and McKnight, T. D. (1988) Transformation of plants *via* the shoot apex. *In vitro* Cell Dev. Biol. 24: 951-954.

Ullal, S. R. **and** Narasimhanna, **M.** N. (1994) Diseases and pests of mulberry. In: Handbook of Practical Sericulture. Central Silk Board, Bangalore, pp. 42-52.

Van Staden, J. and Mooney, P. A. (1987) The effect of IAA on the metabolism of kinetin by *Catharanthus roseus* crown gall callus. J. Plant Physiol. 131: 297-303.

Vasil, I. K. (1986) Cell culture and somatic cell genetics of plants. Plant regeneration and genetic variability. Academic press, Orlando. Vol 3.

Vasil, I. **K.** (1991) Cell culture and somatic cell genetics of plants. Scale-up and Automation in plant propagation. Academic Press, San Diego. Vol. 8

Vasil, I. **K.** (1994) Automation of plant propagation. Plant Cell Tiss. Org. Cult. 39: 105-108.

Vasil, **I. K. and Thorpe,** T. A. (1994) Plant Cell Tissue Culture. Kluwer Academic Publishers, Dordrecht.

Veluthambi, K and Poovaiah, B. W. (1984a) Calcium promoted protein phosphorylation in plants. Science, 223: 167-169.

Veluthambi, K. and Poovaiah, B. W. (1984b) Polyamine-stimulated phosphorylation of proteins from corn (*Zea mays* I) coleoptiles. Biol. Chem. Biophys. Res **Commun.** 122: 1374-1380.

Veluthambi, K. and Poovaiah, B. W. (1986) *In vitro* and *in vivo* protein phosphorylation in *Avena sativa* L. coleoptiles. Effects of Ca^{2+}, calmodulin antagonists, and auxins. Plant Physiol. 81: 836-841.

Vera, P. **and Conejero, V.** (1990) *Citrus exocortis* viroid infection alters the ***in*** *vitro* pattern of protein phosphorylation of tomato leaf proteins. Mol. Plant Microbe Interact. 3: 28-32.

Vijayan, K., Chakraborti, S. P., and Roy, B. N. (2000) Plant regeneration from leaf explants of mulberry: Influence of sugar, genotype and 6-benzyladenine. Ind. J Exp. Biol. 38: 504-508.

Vilardell, J., Goday, A., Freire, M. A., Torrent, M., Martinez, M. C, Tornc, J. M. and Pages, M. (1990) Gene sequence, developmental expression, and protein phosphorylation of RAB-17 in maize. Plant Mol. Biol. 14: 423-432.

Villalabos, V. M., Leung, D. W. M. and Thorpe, T. A. (1984) Light-cytokinin interaction in shoot formation in cultured cotyledon explants of radiata pine Physiol. Plant. 61:497-504.

Wang, J. H. **and** Waissman, D. M. (1979) Calmodulin and its role in the second messenger system. Curr. Topics Cell Reg. 15: 47-107.

Wcinstein, I. B. (1983) Protein kinase, phospholipid and control of growth. Nature, 302: 750.

Wilson, C, **Eller,** N., **Gartner,** A., **Vicente, O. and Heberlebors,** E. (1993) Isolation and characterization of a tobacco cDNA clone encoding a putative MAP kinase. Plant Mol. Biol. 23: 543-551.

Wolff, D. J., Ross, **J.** M., Thompson, **P.** N., Brostrom, **M.** A. and **Brostrom, C. D.** (1981) Interaction of calmodulin with histones. Alteration of histone dephosphorylation. J. Biol. Chem. 256, 1846-1860.

Wordragen, M. F. **and Dons,** H. J. M. (1992) *Agrobacterium tumefacians* mediated transformation of recalcitrant crops. Plant Mol. Biol. Rep. 10: 12-36.

Wright, M. S., **Ward,** D. V., **Hinchee, M.** G. C. **and Kaufman,** R. J. (1987) Regeneration of soya bean (*Glycine max* L. Merr.) from cultured primary leaf tissue. Plant Cell Rep. 6: 83-89.'

Xie, D. and Hong, Y. (2002) *Agrobacterium* mediated genetic transformation of *Acacia mangium.* Plant Cell Rep. 20: 917-922.

Yadav, U., Madan, L. and **Jaiswal, V.** S. (1990) Micropropagation of *Morns nigra* L. from shoot tip and nodal explants of mature trees. Scicntia Hortic. 44: 61-67.

Yamanouchi, H., **Koyama,** A. and **Machii,** H. (1999) Effect of medium conditions on adventitious bud formation in immature mulberry leaves. JARQ. 33: 267-274.

Yasuda, T., **Hasegawa,** P. M. and Cheng, T. (1980) Analysis of newly synthesized proteins during differentiation of cultured Douglas-fir cotyledons. Physiol. Plant. 48: 83-87.

Yuffa, A. **M.,** Garcia, E. G. D. **and Nieto,** M. S. (1994) Comparitive study of protein electrophoretic patterns during embryogenesis in *Coffea arabica* cv Catimor. Plant Cell Rep. 13: 197-202.

Yupsanis, T. Moustakas, **M.** Eleftheriou, P. and **Damianidou,** K (1994) Protein phosphorylation-dephosphorylation in *Alfa alfa* seeds germinating under salt stress. J Plant Physiol. 143:234-240.

Zaman, A., Islam, R. and Joarder, O. I. (1997) Field performance and biochemical evaluation of micropropagated mulberry plants. Plant Cell Tiss. Org. Cult. **51: 61-**64.

Zhan, X., Kawai, S., Katayama, Y. and Morohoshi, N. (1997) A **new** approach based on the leaf disc method for *Agrobacterium* mediated transformation and regeneration of aspen. Plant Sci. 123: 105-112.

Zimmerman, R. H., Griesbach, R. J., Hammerschlag, F. A. and Lawson, R. H. (1986) Tissue culture as a plant production system for horticultural crops. Martinus Nijhoff Publishers, Dordrecht.

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Clonal propagation of mulberry (*Morus indica* L. cultivar M-5) through in vitro culture of nodal explants

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Clonal propagation of mulberry (*Morus indica* L. cultivar M-5) through in vitro culture of nodal explants

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Abstract

A high frequency of sprouting (80.0%) and shoot differentiation was observed in the primary cultures of nodal explants of *Morus indica* L. cultivar M-5 on MS medium supplemented with 2,4-D (0.3 mg/I). In vitro proliferated shoots were multiplied rapidly by culture of shoot tips on MS medium with BAP (0.5 and 1.0 mg/l) which produced the greatest multiple shoot formation. Multiplication was also achieved by culture of shoot tips on MS medium with BAP (4.0 mg/l) and GA_3 (0.05 mg/1) which facilitated the elongation of shoots followed by sprouting of axillary buds of in vitro grown shoots. A high frequency of rooting (86.7%) with development of healthy roots was observed from shoots cultured on medium with 2,4-D (1.0 mg/1). Plants with well developed roots were transferred to soil with a survival frequency of 80%.

Keywords: In vitro culture; Nodal explants; Micropropagation; *Morus indica* L.; Mulberry

1. Introduction

Mulberry *(Morus indica* L.), a woody perennial tree plays a very significant role in sericulture as its foliage constitutes the main diet for the silkworm

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; NAA, α-naphthaleneacetic acid; KN, kinetin; MS, Murashige and Skoog's medium; AC, activated charcoal

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(*Bombyx mori* L.). Conventionally, mulberry is propagated by cutting and grafting. However, propagation of mulberry through cuttings and grafting has certain limitations. Only 30-40% of stem cuttings survive the time between pruning, transportation and final transplantation (Bapat et al., 1987). Moreover, successful rooting from stem cuttings depends on the genotype, environmental factors and physiological state of the cuttings (Ohyama and Oka, 1987; Narayan et al., 1989). Tissue culture techniques such as micropropagation provide a fast and dependable method for production of a large quantity of uniform plantlets in a short time throughout the year. For successful micropropagation, axillary buds or shoot tip cultures are preferred as pre-existing meristems easily develop into shoots while maintaining clonal fidelity. The in vitro production of plantlets from axillary buds has been reported by various workers in different species of *Morus* (Jain et al., 1990; Sharma and Thorpe, 1990; Yadav et al., 1990; Rao and Bapat, 1993; Pattnaik et al., 1996; Pattnaik and Chand, 1997). In most of the earlier reports, BAP was found to be more effective than KN in inducing shoot development from axillary bud cultures of mulberry. Anuradha and Pullaiah (1992) achieved shoot differentiation from axillary bud cultures of *Morus alba* L. on MS medium supplemented with 2,4-D (0.5-1.5 mg/l). Subsequently rooting was induced with 35–45% frequency and they inferred that NAA was a more effective rooting agent than either IAA or 2,4-D. The present study differs from the earlier reports as a high frequency of shoot differentiation from nodal explants and root induction from regenerated shoots were achieved in the M-5 cultivar of mulberry on MS medium supplemented with 2,4-D.

2. Materials and methods

2.1. Induction of shoots from axillary buds of nodal explants

Nodal explants with axillary buds were collected during March-August from 5-year old mature tree of *Morus indica* L. cultivar M-5. The excised nodal explants (2-3 cm) were washed thoroughly under running tap water and then surface sterilized in 70% alcohol for 1 min followed by 0.1% mercuric chloride for 15 min. Finally, the explants were rinsed 4-5 times in sterile distilled water with a duration of 5 min each. The sterilized single nodal explants were cultured on MS (Murashige and Skoog, 1962) medium supplemented with 2,4-D, IAA, NAA, BAP and KN in varied concentrations and combinations for inducing sprouting and shoot differentiation. The cultures were maintained at 25 ± 2°C under a 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 83.6 $\mu E\ m^{-2}\ s^{-1}$ provided by white fluorescent tubes. All the experiments were repeated thrice at different times and each treatment had 10 replicate cultures. The appearance of the shoot meristem was taken into consideration for calculating the sprouting frequency.

2.2. Multiplication of shoots

Shoot tips (2-3 cm) obtained from in vitro differentiated shoots were transferred to MS medium supplemented with BAP (0.5–4.0 mg/l) either singly or in combination with GA_3 (0.05 mg/l) for induction of multiple shoots. The average number of shoots induced per explant as well as the length of the shoots was recorded after 4 weeks.

2.3. Rooting of shoots

Healthy shoots (3–4 cm) were transferred to MS medium containing different auxins such as 2,4-D (1.0 mg/l), IAA (1.0 mg/l), NAA (1.0 mg/l) or IBA (1.0 mg/l) with or without activated charcoal (0.2% w/v) for rooting. The effect of these auxins on induction of roots from shoots was examined after 30 days of culture.

2.4. Acclimatization of regenerated plants

Healthy plantlets with well developed roots were transferred to pots containing peatrite (a combination of perlite, vermiculite and established organic manure cocopeat) and soil (1:1) and maintained in the culture room (25 ± 2°C at 83.6 $\mu E\ m^{-2} s^{-1}$ PPFD) for 15-20 days. Humidity was maintained by covering with a plastic cover. The acclimatized plants were planted in the soil and transferred to field. The % survival was recorded after 6 weeks.

3. Results

3.1. Induction of sprouting and shoot differentiation from axillary buds of nodal explants

The frequency of contamination from primary cultures of nodal explants varied from 10% to 15%. Differences were observed in terms of viability of the nodal explants depending on the type of material selected for the study. Nodal explants that were slightly tender, medium in thickness (0.5-0.6 cm) and having greenish axillary buds responded efficiently for bud sprouting and shoot differentiation. Nodal explants that were very tender turned brown after disinfection treatments whereas hard and thick explants having brownish axillary buds did not show any sign of growth even after 30 days of culture. The survival percentage of nodal explants (0.5-0.6 cm thick) and their subsequent development into shoots varied from 10% to 80% on MS medium supplemented with various hormones (Table 1).

Table 1
Effect of hormones on sprouting of axillary buds from primary cultures of nodal explants and shoot differentiation of mulberry on MS medium

S. No.	Conc. of hormones (mg/l)	Sprouting (%)	Mean length of shoots (cm)[a]
1	Basal	36.7 c	1.6 a
2	1.5 BAP	33.3 c	3.9 c
3	2.0 BAP	40.0 c	4.1 bc
4	0.5 KN	13.3 b	1.4 a
5	1.0 KN	10.0 b	1.0 a
6	0.3 2,4-D	80.0 a	5.8 d
7	0.5 2,4-D	73.3 a	4.9 b
X	1.0 2,4-D	66.7 a	4.6 b
9	1.0 NAA+1.0 BAP	53.3 d	4.0 bc
10	1.0 IAA+2.0 KN	13.3 b	1.3 a

Mean followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman–Keul's multiple range test.
[a] Data scored after 30 days.

Axillary buds from the nodal explants cultured on MS medium without hormones sprouted with a frequency of 36.7% and the shoots grew to a height of 1.6 cm in 30 days (Table 1). Axillary bud sprouting from nodal explants occurred with a frequency of 33.3% and 40.0% on MS medium supplemented with 1.5 and 2.0 mg/1 BAP, respectively. The frequency of sprouting was very low on medium supplemented with KN (Table 1).

Efficient sprouting (80.0%) from axillary buds was observed on MS medium with 0.3 mg/l 2,4-D and shoots of 5.8 cm developed in 30 days (Fig. 1 and Table 1). The frequency of sprouting decreased with increase in the concentration of 2,4-D (Table 1). The combination of NAA and BAP at 1 mg/l each induced axillary bud sprouting with a frequency of 53.3% and shoots grew to a height of 4.0 cm at the end of 30 days. A significant decrease in sprouting from buds was noticed on MS medium supplemented with 1 mg/1 IAA and 2 mg/1 KN.

Axillary buds developed into shoots as well as inflorescences irrespective of the hormones used in the MS medium. Inflorescences were excised at an early stage of induction to accelerate the development of the shoots.

3.2. Induction of multiple shoots

Multiple shoots were induced with varying frequencies (37.5-79.2%) by culturing shoot tips of 2-3 cm on medium supplemented with 0.5-1.5 mg/1 BAP (Table 2). When the concentration of BAP was increased beyond 1.5 mg/1, there was no enhancement of multiple shoot induction. However, shoot tips cultured on MS medium with BAP (4.0 mg/l) and GA_3 (0.05 mg/1) showed a differential

Fig. 1. Shoot induced from the axillary bud of nodal explant cultured on MS medium with 0.3 mg/l 2,4-D after 30 days of culture.

Table 2
Effect of BAP and GA_3 on induction of multiple shoots of mulberry on MS medium

S. No.	Conc. of hormones (mg/l)	Multiple shoot induction (%)	Mean no. of multiple shoots induced per explant[a]	Mean length of shoots (cm)[a]
1	0.5 BAP	79.2 a	7.7 a	4.8 a
2	1.0 BAP	62.5 a	6.2 b	4.7 a
3	1.5 BAP	37.5 b	3.5 c	3.4 c
4	4.0 BAP+0.05 GA.,	66.7 a	8.5 a	1.8 b

Mean followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman–Keul's multiple range test.
[a] Data scored after 30 days.

response with elongation of shoots followed by sprouting of axillary buds which developed into shoots of 1.8 cm in 30 days (Fig. 2 and Table 2).

5.5. *Induction of rooting from shoots*

Multiple shoots were separated and healthy shoots were transferred to MS medium containing different auxins (Table 3). Thin slender roots were induced

Fig. 2. Shoot induction from axillary buds of in vitro elongated shoot on MS medium with 4 mg/1 BAP and 0.05 mg/1 GA, after 30 days of culture.

Table 3
Effect of auxins and activated charcoal (AC) on root induction from shoots of mulberry on MS medium

S. No.	Conc. of auxin (mg/1)	Rooting (%)	Average no. of roots per culture"	Average length of longest root (cm)[a]
1.	1.0 IAA	53.3 a	4.5 a	4.1 c
2.	1.0 IAA+0.2% AC	40.0 b	3.3 b	3.5 b
3.	1.0 IBA	66.7 a	10.3 c	3.3 b
4.	1.0 IBA+0.2% AC	56.7 a	5.9 d	3.0 b
5.	1.0 2,4-D	86.7 c	8.5 e	5.8 a
6.	1.0 2,4-D+0.2% AC	70.0 a	7.9 e	5.4 a

Mean followed by the same letter in a column are not significantly different ($p < 0.05$) by Newman–Keul's multiple range test.
[a] Data scored after 30 days.

from the base of the shoot with a frequency of 53.3% in the presence of IAA. Healthy thick roots were induced with a high frequency of 86.7% on medium supplemented with 2,4-D which increased in length to 5.8 cm in 30 days (Fig. 3). Rooting from shoots occurred with a frequency of 66.7% on medium with IBA

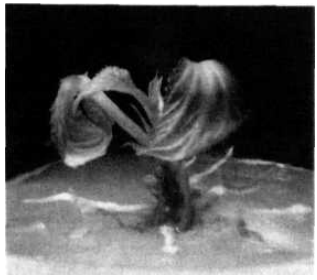

Fig. 3. Root induction from shoots of mulberry on MS medium with 1 mg/l 2,4-D after 20 days of culture.

(1 mg/l) and the roots were very thin when compared to those induced on 2,4-D medium. Activated charcoal (0.2% w/v) in the presence of all auxins tested decreased the frequency of root induction.

The plants with well developed roots were initially transferred to pots containing peatrite and soil (1:1) and the acclimatized plants were finally transferred to soil under field conditions. The plants transferred to the field survived with a frequency of 80% (Fig. 4).

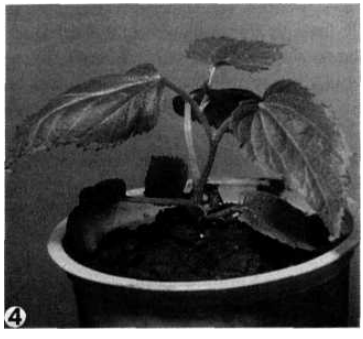

Fig. 4. Regenerated plant in the pot containing peatrite and soil (1 : 1) after 20 days of transfer.

4. Discussion

Nodal explants that were slightly tender and having greenish axillary buds responded efficiently for bud sprouting compared to hard nodal explants with brownish buds which showed no sign of growth. Oka and Ohyama (1975) reported that brownish buds of two cultivars (Kenmochi and Ichinose) of mulberry did not show any growth whereas young greenish buds of cultivar Kenmochi grew into leafy shoots.

In the present study, axillary buds from nodal explants cultured on MS medium without hormones exhibited a low frequency of sprouting and shoot growth was not vigorous. Various hormones like 2,4-D, IAA, NAA, BAP and KN were used either singly or in combinations in MS medium to see their effects on axillary bud sprouting and shoot differentiation. A high frequency of sprouting and vigorous shoot growth was observed on MS medium with 0.3 mg/l 2,4-D when compared to other concentrations tested. Although, 2,4-D is considered to suppress organogenesis (Gamborg et al., 1976) and is generally used in experiments involving callus induction, in the present study, low levels of 2,4-D (0.3 mg/l) triggered shoot differentiation with a high frequency (80%) compared to other hormones tested. Anuradha and Pullaiah (1992) reported that low concentration of 2,4-D (0.5 mg/1) stimulated sprouting whereas higher concentration (2.0 mg/1) resulted in rapid callus proliferation from axillary bud cultures of mulberry (*Morus alba* L.).

Culture medium containing BAP at concentrations of 1.5 and 2.0 mg/1 induced axillary bud sprouting with a frequency of 33.3% and 40.0%, respectively. The frequency of sprouting from axillary buds was low on medium supplemented with KN. Combination of BAP (1.0 mg/1) and NAA (1.0 mg/l) induced axillary bud sprouting at a higher frequency compared to BAP alone. Yadav et al. (1990) and Pattnaik and Chand (1997) observed that BAP was more effective than KN in inducing shoot development and multiple shoot induction from both apical shoot buds and nodal explants of the three species of mulberry.

Sprouted axillary buds in the present study developed into shoots as well as inflorescences. Induction of inflorescence from cultured axillary buds would be of significance in studies related to anther culture as it does not demand sterilization which has to be done when the inflorescences are obtained from field-grown plants. In *Morus australis*, most explants collected during November-February produced inflorescences during shoot elongation (Pattnaik et al., 1996).

Rapid multiplication of any crop plant can be achieved by inducing multiple shoots through in vitro culture. Multiple shoots were induced with a frequency of 79.2% in the presence of BAP at 0.5 mg/l and higher levels (1.5 mg/1) reduced the frequency. The inhibitory effect of BAP on shoot proliferation at concentrations higher than 1.0 mg/1 was noticed earlier in *Morus* species (Ohyama and Oka,

1987). In *Morus laevigata*, multiple shoots were induced in the presence of BAP at 5.0 mg/l but these shoots failed to elongate thereby resulting in rosette shoot clumps (Pattnaik et al., 1996). In the present study, when GA_3 (0.05 mg/l) was added along with BAP (4.0 mg/l), the shoots initially elongated followed by sprouting of axillary buds which developed into shoots of 1.8 cm in length. These shoots served as a source material for inducing multiple shoots or rooting and thus provided an alternative for rapid multiplication of mulberry. The promotive effect of GA_3 (0.3-0.4 mg/l) on axillary bud break when used along with BAP (1.0 mg/l) was reported earlier in *Morus* species (Pattnaik and Chand, 1997).

In the present study, auxins like IAA, IBA, 2,4-D and NAA were used singly to induce rooting from the shoots. Culture medium supplemented with 2,4-D induced rooting from shoots at a higher frequency (86.7%) than other auxins tested. Although relatively large number of roots were induced in the presence of IBA at 1.0 mg/l, these roots were thinner when compared to roots induced on 2,4-D medium which were thick and healthier. The average number of roots induced per shoot after 4 weeks of culture was highest with 0.25 mg/l IBA for *Morus nigra* (Yadav et al., 1990). Rao and Bapat (1993) observed abundant rooting from primary axillary bud cultures of *Morus indica* L. on MS medium with IAA which was sparse on NAA and 2,4-D. In contrast, Anuradha and Pullaiah (1992) reported that NAA was a more effective rooting agent than 2,4-D and IAA for *Morus alba* L. Narayan et al. (1989) successfully rooted 95% of mulberry shoots within 3 weeks by supplementing with 0.5 mg/l NAA. But our study with NAA as rooting agent yielded no response and 2,4-D was found to be a more effective rooting agent than IAA and IBA.

In the present study, complete plantlets of mulberry could be established al a high frequency in two steps from nodal explants by varying the levels of 2,4-D in MS medium. Further, multiple shoots proliferated when shoot tips excised from in vitro differentiated shoots were cultured on medium with BAP (0.5–1.0 mg/l). From our study it can be inferred that 2,4-D is a more effective shooting and rooting agent compared to BAP, KN, IAA and NAA for micropropagation of the M-5 cultivar of mulberry *(Morus indica* L.). These results provide an efficient in vitro method for the rapid propagation of the economically important M-5 cultivar of mulberry.

References

Anuradha, M., Pullaiah, T., 1992. Micropropagation of mulberry *(Morus alba L.)*. Annali Di Botanica. 15, 35–41.

Bapat, V.A., Mhatre, M. Rao, P.S.. 1987. Propagation of *Morus indica* L. (mulberry) by encapsulated shoot buds. Plant Cell Rep. 6, 393-395.

Gamborg, O.L., Murashige, T., Thorpe, T.A., Vasil, I.K., 1976. Plant tissue culture media. In Vitro 12, 473–478.

Jain, A.K., Dandin, S.B., Sengupta, K., 1990. In vitro propagation through axillary bud multiplication in different mulberry genotypes. Plant Cell Rep. 8, 737-740.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Narayan, P.. Chakraborty, S., Subba Rao, G., 1989. Regeneration of plantlets from the callus of stem segments of mature plants of *Mnrus alba L.* Proc. Indian Natl. Sci. Acad. 55, 469-472.

Oka, S., Ohyama, K., 1975. Studies on in vitro culture of excised bud in mulberry tree II. Effect of growth substances on the development of shoots from bud. J. Seric. Sci. Jpn. 44, 444-450.

Ohyama, K., Oka, S., 1987. Mulberry. In: Bonga, J.M., Durzan, D.J. (Eds.), Cell and Tissue Culture in Forestry, vol. 3. Nijhoff/Junk Publishers, Dordrecht, pp. 272-284.

Pattnaik, S.K., Sahoo, Y., Chand, P.K., 1996. Micropropagation of a fruit tree, *Morus australis* Poir. Syn. *M. acidusa* Griff.. Plant Cell Rep. 15, 841-845.

Pattnaik. S.K., Chand. P.K., 1997. Rapid clonal propagation of three mulberries, *Morus cathayana* Hemsl., *M. lhou* Koiz. and *M. serrata* Roxb., through in vitro culture of apical shoot buds and nodal explants from mature trees. Plant Cell Rep. 16, 503-508.

Rao, P.S., Bapat, V.A., 1993. Micropropagation of sandalwood (*Santalum album* L.) and mulberry (*Morus indica* L.). In: Ahuja, M.R. (Ed.), Micropropagation of Woody Plants. Kluwer Academic Publishers, The Netherlands, pp. 317–345.

Sharma, K.K., Thorpe, T.A., 1990. In vitro propagation of mulberry *(Morus alba* L.) through nodal segments. Scientia Hortic. 42(4), 307-320.

Yadav, V., Madan, L., Jaiswal, V.S., 1990. Micropropagation of *Morus nigra* L. from shoot tip and nodal explants of mature trees. Scientia Hortic. 44, 61-67.

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Seasonal influence on axillary bud sprouting and micropropagation of elite cultivars of mulberry

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Abstract

High frequency of sprouting from axillary huds was observed during summer in all four cultivars in field and in vitro conditions. In the China White cultivar, sprouting was asynchronous and at the end of 60 days only 23.3% of the nodal cuttings established into plants in summer in field conditions. Under in vitro conditions, a high frequency of sprouting was induced in M-5 and S-36 cultivars on medium supplemented with 1.36 μM 2,4-D. In the S-13 cultivar. the axillary huds collected during summer sprouted with the same frequency of 73.3% on medium supplemented individually with 2,4-D and KN at 1.36 and 1.39 uM concentrations, respectively, whereas in the China White cultivar, a high frequency of axillary bud sprouting was induced on KN (9.29 uM) supplemented medium. Shoot **multiplication** was achieved in all four cultivars by culture of shoot tips on MS medium with BAP (2.22 μM). Addition of fructose instead of sucrose in the multiplication medium increased the shool number and also the growth of the shoots was vigorous. In vitro differentiated shoots of all cultivars rooted with a high frequency on medium supplemented with 4.52 uM 2.4-D. Micropropagated plants were successfully established in soil in field conditions with a survival frequency of 85–90%.

Keywords: In vitro culture; Nodal explants; Seasonal effects; Micropropagation; *Morus indica* L.; *Morus alba* L.

Abbreviations: MS, Murashige and Skoog's medium; BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; KN, kinetin; NAA, α-naphthaleneacetic acid; Z, zeatin

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1. Introduction

Mulberry (*Morus* spp.) is a very important plant deciding the sericulture economics of the country since its foliage is used for rearing silkworms (*Bombyx mori* L.). Mulberry cultivation by cuttings is widely used and popular method for raising saplings. However, successful rooting from cuttings is dependent upon the favourable environmental conditions and genotype dependent (Ohyama and Oka, 1987; Jain et al., 1990). Many elite varieties have poor rooting ability and propagation through cuttings is restricted to only certain months of the year (Narayan et al., 1989). Mulberry can also be propagated through seeds but seed propagation is not practised due to the heterogeneous nature of the seedlings owing to its cross pollination (Das, 1983; Hossain et al., 1992). Micropropagation provides a valuable tool for rapid multiplication of plants, as large number of genetically identical plants can be produced in a relatively short time and space. Axillary buds are widely used for micropropagation as they have entire rudimentary vegetative shoot and can be induced to develop into plants easily which are similar to the parental type. Meristems are also extensively used for rapid multiplication of shoots, since the constituent cells are genetically identical to the donor plants (Skirvin, 1981). In vitro plant regeneration from the apical/axillary shoot buds and nodal explants has been reported in a number of species of mulberry (Mhatre et al., 1985; Ohyama and Oka, 1987; Hossain et al., 1992; Pattnaik and Chand, 1997; Chitra and Padmaja, 1999). The present study was undertaken with the aim to evaluate the regeneration potential of four elite cultivars and to establish an efficient in vitro system for their rapid propagation by culture of nodal explants. In addition, this study compares the influence of season on axillary bud sprouting and regeneration from nodal cuttings in field and in vitro conditions.

2. Materials and methods

2.1. Establishment of mulberry nodal cuttings in field

Nodal cuttings of 20–22 cm containing at least 4-5 axillary buds were collected from 2-year-old field grown plants of *Morus indica* L. cultivars M-5, S-36, S-13 and *Morus alba* L. cv. China While at different stages of growth, viz., developing, assimilation, storing and winter resting stage. The characteristic features of the above cultivars are given in Table 1. The nodal cuttings excised from different cultivars were immediately planted in the pots containing soil and manure in a ratio of 3:1 and kept out in the field for studying the influence of different seasons on axillary bud sprouting and regeneration from nodal cuttings. The environmental conditions in different seasons are mentioned in Table 2. The pots were

Table 1
Characteristic features of mulberry cultivars used in the present study

S. No.	Species	Cultivar	Sex	Attributes
1	*M. indica* L.	M-5	Predominantly female	Resistant to powdery mildew, leaf spot and bacterial blight. High rooting ability and wide adaptability
2	*M. indica* L.	S-36	Female	Tolerant to leaf spot and powdery mildew, moderately susceptible to leaf rust and tukra infestation. Most suitable to young age silkworm rearing. Moderate rooting ability
3	*M. indica* L.	S-13	Male	Resistant to leaf spot and powdery mildew, moderately resistant to leaf rust and tukra infestation. Deep rooting system, profuse branching with short internodes and superior leaf quality. Moderate rooting ability
4	*M. alba* L.	China White	Female	Large leaf size with thick, dark green foliage. Weight of single leaf very high. Low rooting ability

watered on alternate days in all the seasons. The appearance of the shoot meristems from the axillary buds was taken into consideration for calculating the sprouting frequency. The data on axillary bud sprouting was scored after 30 days and plant establishment frequency (PEF) from nodal cuttings was determined after 60 days. Twenty nodal cuttings were used for each experiment and all the experiments were repeated thrice.

Table 2
Seasonal effect on axillary bud sprouting and plant establishment rate from the cuttings of different mulberry cultivars in the field (means followed by the same letter in a column are not significantly different ($p < 0.05$) according to one way ANOVA followed by Newman–Keul's multiple range test)

Seasons	Cultivar							
	M-5		S-13		S-36		China White	
	Sprouting	PEP	Sprouting	PEF	Sprouting	PEF	Sprouting	PEF
Summer[b] (February-May)	96.7 a	93.3 a	90.0 a	90.0 a	93.3 a	90.0 a	70.0 a	23.3 a
Rainy[c] (June-September)	86.7 a	86.7 a	83.3 a	80,0 b	86.7 a	86.7 a	66.7 a	20.0 a
Winter[d] (October-January)	70.0 b	70.0 b	66.7 b	63.3 c	66.7 b	66.7 b	53.3 b	16.7 a

[a] Plant establishment frequency.
[b] Mean temperature (C): 23.9-31.4; sun shine (h) 8.2-10.1; relative humidity (*) I: 57.0–82.0, II: 19.0-38.0.
[c] Mean temperature (°C): 26.1-28.4; sun shine (h) 4.3–6.8; relative humidity (%) I: 80.0–88.0, II: 52.0–69.0.
[d] Mean temperature ("C): 19.1-26.0; sun shine (h) 7.9-9.9; relative humidity (%) I: 81.0-87.0, II: 33.0-51.0.

2.2. Induction of axillary bud sprouting in vitro

For in vitro studies, nodal explants of 3–4 cm in length were collected from the same plants as described above. The nodal explants were kept under running tap water for $\frac{1}{2}$ h and surface sterilized in 70% alcohol for 1 min followed by 0.1% mercuric chloride ($HgCl_2$) for 15 min under sterile conditions. The explants were then rinsed 4-5 times in sterile distilled water with a duration of 5 min each. The sterilized explants were cultured on MS (Murashige and Skoog, 1962) medium containing 3% sucrose and 0.8% agar (Hi-Media, Mumbai, India). The culture media were supplemented individually with 2,4-D, KN and BAP in varied concentrations for inducing sprouting and shoot differentiation. All the experiments were repeated thrice at different times and 10 explants were used for each treatment.

2.3. Multiplication of shoots

The effect of sugars such as sucrose, maltose, glucose and fructose on induction of multiple shoots of four cultivars was studied by culturing shoot tips (2-3 cm) derived from in vitro developed shoots on MS medium with 2.22 μM BAP. The multiplication potential of shoots during repeated subcultures was studied by subculturing shoot tips on MS medium supplemented with 2.22 μM BAP for 10 months at 30 days intervals. The average number of shoots induced per explant was recorded after 30 days of culture.

2.4. Root induction from shoots

Healthy shoots derived from the sprouted axillary buds or from the multiple shoots were transferred to MS medium supplemented individually with 2,4-D, 1AA, IBA and NAA at concentrations 4.52, 5.71, 4.92 and 5.37 uM, respectively, for inducing rooting. The frequency of rooting and the nature of roots induced were determined after 30 days of culture.

For all the experiments on induction of axillary bud sprouting, shoot multiplication and root induction from shoots, the cultures were maintained at 25 ± 2°C under a 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 83.6 $\mu E\ m^{-2}\ s^{-1}$ provided by white flourescent tubes.

2.5. Acclimatization of regenerated plants

Regenerated plants having well-developed roots were removed from culture bottles and washed free of agar. They were transferred to plastic pots containing soil and organic manure (3:1) and kept in a net house under shade for 15–20 days. In the first week of transfer, the plantlets were covered with polythene covers to

maintain humidity. After 15-20 days of acclimatization, the plantlets were transferred to earthen pots and planted out in field. The percentage survival was recorded after 6 weeks of transfer to field.

3. Results

3.1. Seasonal effect on axillary bud sprouting in fieldconditions

The influence of season on axillary bud sprouting and PEF from the nodal cuttings placed in soil was studied in field conditions. A high frequency of sprouting as well as a high plant establishment rate from cuttings was observed in summer compared to the rainy and winter seasons in all four cultivars. Adventitious roots were formed from the base of the nodal cuttings during shoot development in all four cultivars. Axillary buds of M-5, S-36, S-13 sprouted in 10–12 days and PEF from nodal cuttings varied from 63.3 to 93.3% in different seasons. In the China White cultivar, asynchronous sprouting was observed in all seasons and at the end of 60 days only 23.3% of the nodal cuttings established into plants in summer indicating the difficult-to-root nature of the cultivar (Table 2).

3.2. Sprouting of the axillary buds in vitro

Nodal explants of 3–4 cm bearing axillary buds were cultured on MS medium supplemented individually with 2,4-D, KN and BAP for inducing sprouting. Nodal explants having greenish axillary buds enveloped by 2-3 whorls of scales responded efficiently for sprouting. Axillary buds without scales turned brown upon sterilization treatment and buds with more than 2-3 whorls of scales did not sprout even after 30 days of culture. Contamination in the cultures was observed to be season dependent. The maximum contamination of 40.0% occurred during winter followed by the rainy and summer seasons with frequencies of 25.0 and 15.0%, respectively.

The sprouting frequency from axillary buds of four cultivars varied from 10.0 to 83.3% in summer followed by rainy season (0.0–76.7%). A low frequency of sprouting (0.0-60.0%) was observed in winter indicating the strong influence of season on axillary bud sprouting (Table 3). A high frequency of axillary bud sprouting (56.7-83.3%) from the nodal explants of M-5 and S-36 cultivars was induced on medium supplemented with 1.36 uM of 2,4-D and sprouting occurred in 5-7 days. Medium supplemented individually with KN (1.39-9.29 uM) and BAP (1.33-8.88 uM) induced axillary bud sprouting at frequencies ranging from 0.0 to 46.7% and 0.0 to 40.0%, respectively, in M-5 and S-36 cultivars and the duration of sprouting was longer (18-20 days) compared to 2,4-D. Axillary buds

Table 3
Seasonal effect on in vitro axillary bud sprouting of different cultivars of mulberry (S: summer (February–May); R: rainy (June–September); W: winter (October–January))

S. No.	Treatments (μM)	Sprouting frequency (%)[a]											
		M-5			S-13			S-36			China White		
		S	R	W	S	R	W	S	R	W	S	R	W
1	Basal	36.7 a ± 3.3	33.3 a ± 3.3	23.3 a ± 8.8	50.0 a ± 5.8	43.3 a ± 6.7	30.0 a ± 5.8	56.7 a ± 6.7	46.7 a ± 6.7	40.0 a ± 5.8	30.0 a ± 5.8	23.3 a ± 3.3	16.7 a ± 8.8
2	1.36 2,4-D	80.0 b ± 5.8	76.7 b ± 3.3	56.7 b ± 3.3	73.3 b ± 3.3	63.3 a ± 3.3	40.0 a ± 5.8	83.3 b ± 3.3	76.7 b ± 3.3	60.0 a ± 5.8	50.0 b ± 0.0	43.3 a ± 3.3	33.3 a ± 6.7
3	4.52 2,4-D	63.3 c ± 3.3	56.7 c ± 3.3	50.0 c ± 5.8	66.7 c ± 6.7	46.7 a ± 3.3	23.3 a ± 3.3	56.7 a ± 3.3	46.7 a ± 3.3	33.3 a ± 8.8	30.0 a ± 5.8	23.3 a ± 6.7	6.7 a ± 3.3
4	9.05 2,4-D	56.7 d ± 6.7	46.7 a ± 8.8	36.7 a ± 6.8	53.3 a ± 3.3	33.3 a ± 6.7	6.7 b ± 3.3	36.7 a ± 3.3	26.7 a ± 3.3	6.7 b ± 3.3	23.3 a ± 3.3	0.0	0.0
5	1.39 KN	26.7 a ± 6.7	20.0 a ± 5.8	10.0 a ± 5.8	73.3 d ± 3.3	60.0 a ± 5.8	43.3 a ± 3.3	46.7 a ± 3.3	33.3 a ± 3.3	30.0 a ± 5.8	53.3 c ± 3.3	33.3 a ± 8.8	23.3 a ± 3.3
6	4.65 KN	13.3 e ± 3.3	10.0 d ± 5.8	6.7 a ± 3.3	60.0 e ± 5.8	53.3 a ± 8.8	26.7 a ± 6.7	43.3 a ± 6.7	26.7 a ± 6.7	16.7 a ± 3.3	70.0 d ± 5.8	56.7 b ± 6.7	43.3 b ± 3.3
7	9.29 KN	10.0 f ± 5.8	6.7 e ± 3.3	0.0	56.7 a ± 3.3	36.7 a ± 6.7	16.7 a ± 3.3	13.3 c ± 3.3	6.7 c ± 3.3	0.0	76.7 e ± 3.3	60.0 c ± 5.8	46.7 c ± 3.3
8	1.33 BAP	23.3 a ± 3.3	10.0 f ± 5.8	0.0	56.7 a ± 3.3	50.0 a ± 5.8	46.7 a ± 6.7	40.0 a ± 5.8	33.3 a ± 6.7	23.3 a ± 6.7	40.0 a ± 5.8	26.7 a ± 3.3	16.7 a ± 8.8
9	4.44 BAP	30.0 a ± 5.8	26.7 a ± 3.3	16.7 a ± 3.3	43.3 a ± 3.3	36.7 a ± 3.3	26.7 a ± 3.3	30.0 d ± 5.8	20.0 d ± 5.8	16.7 a ± 8.8	26.7 a ± 3.3	16.7 a ± 8.8	10.0 a ± 5.8
10	8.88 BAP	40.0 a ± 5.8	30.0 a ± 5.8	20.0 a ± 5.8	40.0 f ± 0.0	33.3 a ± 3.3	20.0 a ± 5.8	16.7 e ± 3.3	0.0	0.0	16.7 a ± 3.3	10.0 a ± 5.8	6.7 a ± 3.3

[a] The values represent the mean (±S.E.) of three independent experiments. Ten explants were used for each experiment. Means followed by the same letter in a column are not significantly different ($p < 0.05$) from the response on basal medium according to one way ANOVA followed by Dunnett's multiple comparison test.

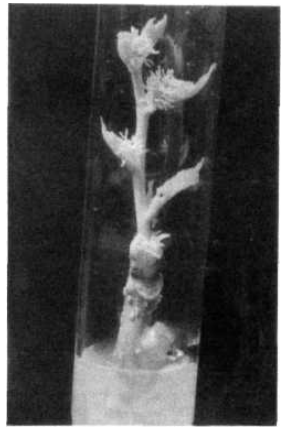

Fig. 1. Shoot development with induction of female catkins from the nodal explant of China White cultivar on MS medium with 9.29 μM KN.

of the S-13 cultivar collected in summer exhibited sprouting with the same frequency of 73.3% on medium supplemented individually with 2,4-D (1.36 μM) and KN (1.39 μM). However, axillary buds cultured on 2,4-D medium sprouted in 9–10 days whereas those cultured on KN medium sprouted in 22-24 days. In the China White cultivar, MS medium with 9.29 μM KN favoured a high frequency of sprouting (46.7-76.7%) compared to 2,4-D supplemented medium with the buds sprouting at the same duration of 12-14 days (Fig. 1). Development of shoots as well as of inflorescences was observed from sprouted axillary buds of all four cultivars irrespective of the season during which nodal explants were collected.

3.3. Multiplication of the shoots

Shoot tips excised from axillary buds were multiplied by culturing on MS medium containing 2.22 μM BAP and 3% sucrose. In all four cultivars, 6-8 shoots were induced in 9-10 days from shoot tips excised from axillary buds (Fig. 2). Attempts were also made to study the effect of various sugars such as sucrose, maltose, glucose and fructose on shoot proliferation rates from shoot tips derived from axillary buds. The shoot proliferation rate increased at a rate of 6.5 per culture by the incorporation of fructose (3%) instead of sucrose (3%) in the multiplication medium containing 2.22 μM BAP and the growth of the shoots was

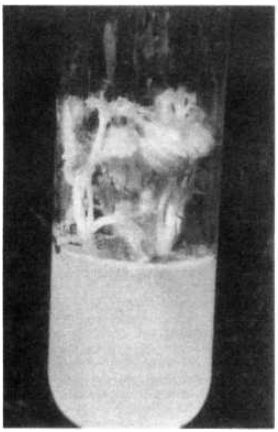

Fig. 2. Multiple shoot induction from in vitro raised shoot of China White cultivar after 30 days of culture on medium with 2.22 uM BAP.

vigorous. The average length of shoots was 5.2 cm in the presence of fructose whereas on sucrose supplemented medium, 3.8 cm long shoots were induced. Shoots cultured on MS medium with glucose (3%) turned pale green and withered away whereas those cultured on maltose (3%) supplemented medium showed no response for shoot multiplication with only induction of the buds from the axils of the leaves and there was no further growth of the buds. However, these buds resumed growth upon transfer to medium supplemented with either sucrose or fructose and shoot multiplication was achieved. Studies were also conducted to observe the multiplication rates of shoots upon each subculture. The rate of shoot multiplication increased upon each subculture on medium with 2.22 uM BAP (Table 4). Dark brown coloured callus developed from the base of the shoots from the fifth subculture onwards in all four cultivars (Fig. 3). The development of the callus from the base had no effect on the shoot multiplication rates of cultivars. Repeated subculturing of shoots triggered induction of more number of shoots from the base and the mean number of shoots produced from a single shoot tip culture of different cultivars varied from 17.7 to 20.0 at 10th subculture.

3.4. Root induction from the shoots

The effect of auxins such as 2,4-D, NAA, IAA and IBA on root induction from shoots of the four mulberry cultivars was studied. The frequency of root induction

Table 4
Mean number of shoots induced during series of subcultures in different cultivars of mulberry"

Subculture cycles	Mean shoot number			
	M-5	S-13	S-36	China White
S,	7.5 a	6.6 a	7.5 a	7.2 a
S_2	9.4 b	7.6 ab	9.5 b	9.3 b
S_3	10.7 c	9.3 bc	10.8 c	10.1 c
S_4	11.6 d	10.2 dc	11.8 d	11.2 d
S_5	12.7 e	11.6 ed	12.7 e	12.2 e
S_6	14.7 f	13.7 ef	14.8 f	14.2 f
S_7	15.5 g	14.7 gf	15.8 g	15.2 g
S_8	17.1 h	15.8 gh	17.1 h	16.7 h
S.,	18.9 i	16.5 ih	18.9 i	17.4 i
S_{10} 19.8	j 17.7	i 20.0	j 19.5	j

[a] Means followed by the same letter in a column arc not significantly different ($p < 0.05$) according to one way ANOVA followed by Newman-Keul'.s multiple range lest.

varied from 36.7 to 100.0% in the presence of different auxins. Medium supplemented with 4.52 uM 2,4-D favoured a high frequency of root induction (86.7-100%) and roots were induced in 9-10 days in all four cultivars (Table 5). Differences were noticed in the nature of roots induced depending on the auxin used in the medium. Thin slender roots were induced in 17–18 days on IBA and IAA supplemented medium in all four cultivars and numerous medium thick roots were induced in 13-14 days on medium supplemented with NAA. Roots induced on 2,4-D medium were thicker, stronger, fewer in number (Fig. 4) than

Fig. 3. Multiple shoot formation with development of callus from the base of shoot of S-36 cultivar on medium with 2.22 uM BAP at fifth subculture.

Table 5
Effect of auxins on root induction from in vitro raised shoots of four cultivars of mulberry[a]

Type of auxin (μM)	Rooting (%)				
	M-5	S-13	S-36	China White	Nature of the roots induced
5.37 NAA	0.0	73.3 a	76.7 a	70.0 a	Infinite, medium thick roots
5.71 IAA	53.3 a	56.7 b	66.7 ab	60.0 a	Thin roots
4.52 2,4-D	86.7 b	96.7 c	100.0 c	96.7 b	Thick, strong roots
4.92 1BA	66.7 a	46.7 b	60.0 b	36.7 c	Long slender roots

* Means followed by the same letter in a column are not significantly different ($p < 0.05$) according to one way ANOVA followed by Newman–Keul's multiple range test.

those induced on IAA and NAA. However, no difference in the functionality of the roots was observed during acclimatization.

3.5. Establishment of micropropagated plants in field

Regenerated plants of all four cultivars having well-developed roots were transferred to pots containing soil and organic manure and the humidity was

Fig. 4. Root induction from an in vitro raised shoot of China White cultivar after 30 days of culture on MS medium with 4.52 uM 2,4-D.

Fig. 5. Micropropagated plants established in field, 60 days after transfer.

maintained by covering with a plastic cover. After acclimatization for 15-20 days, the plants were finally transferred to field with a survival frequency of 85-90% (Fig. 5) and to date they are still growing with full vigour.

4. Discussion

The technique of micropropagation has been used for rapid clonal propagation of many fruit and forest trees (Bajaj, 1986; Hutchinson and Zimmerman, 1987). Mulberry is a highly heterozygous plant and propagation through axillary buds would ensure genetic uniformity and stability among the regenerants. In the present study, nodal explants having greenish axillary buds covered with 2-3 whorls of scales responded efficiently for sprouting whereas buds enveloped by more than three whorls of scales did not sprout. Anuradha and Pullaiah (1992) and Oka and Ohyama (1975) have reported that nodal explants of mulberry having greenish axillary buds showed the best response for sprouting and shoot differentiation.

A comparative study of seasonal effects on axillary bud sprouting and plant establishment rate from the cuttings was undertaken in vitro and field conditions. In field conditions, plants established at high frequency from nodal cuttings in summer in comparison to the rainy and winter seasons in all four cultivars. In the China White cultivar, axillary buds sprouted asynchronously and only 16.7–23.3% of nodal cuttings established into plants in different seasons in field conditions indicating the difficult-to-root nature of the cultivar. Zaman et al. (1997) have reported that propagation of mulberry through cuttings is restricted to a single season (September-October) in Bangladesh. In the present study, in vitro bud break as well as the frequency of aseptic cultures obtained was strongly influenced by the season. Axillary buds of all four cultivars sprouted at a higher

frequency in summer than in the rainy and winter seasons. Pattnaik and Chand (1997) observed that the growth and proliferation of the nodal explants was greatly influenced by the time of explant collection in *Morus cathayana*, *M. ihou* and *M. serrata*. Civinova and Sladsky (1990) suggested that the differential responses of cultured winter and spring buds of temperate species such as oak, aspen and black locust could be linked to the dynamics of endogenous substances in the tree. Quraishi et al. (1996) reported that April-June is the best period for shoot culture initiation in *Cleistanthus coUinus* and the nodal segments produced significantly more shoots than during other times of the year. It was suggested that the differences in the physiological condition of the stock plants grown under natural environmental conditions might be the reason for differential growth responses in vitro.

Different cultivars may require different hormones for obtaining optimal response for sprouting and shoot differentiation. In M-5 and S-36 cultivars, high frequency of sprouting was induced on 2,4-D supplemented medium than on KN supplemented medium. In summer, axillary buds of S-13 cultivar sprouted with same frequency of 73.3% on medium supplemented individually with 2,4-D (1.36 μM) and KN (1.39 μM) whereas in the China White cultivar, a high frequency of sprouting (76.7%) was induced on medium supplemented with KN (9.29 μM). In *M. alba* L., a high frequency of axillary bud sprouting from nodal explants was achieved on medium supplemented with low levels of 2,4-D (Anuradha and Pullaiah, 1992). In contrast, Mhatre et al. (1985) reported that BAP was more effective than KN and Z in inducing axillary bud sprouting and shoot differentiation in mulberry.

Shoot multiplication is an important factor determining the suitability of the tissue culture method for the mass propagation of tree species (Quraishi et al., 1996). In the present study, medium supplemented with 2.22 μM BAP was found suitable for shoot multiplication with induction of 6–8 shoots per culture in all cultivars at the end of 30 days. Shoot proliferation increased at a rate of 6.5 shoots per culture in all cultivars when fructose (3%) was used in place of sucrose (3%) as a carbohydrate source. Oka and Ohyama (1982) suggested fructose was a better source of carbon than sucrose for in vitro plant induction from bud culture of *M. alba* L. Shoot tips cultured on glucose supplemented medium turned pale green whereas those cultured on maltose supplemented medium showed induction of buds with no further growth. However, these buds resumed growth and developed into shoots upon transfer to sucrose or fructose supplemented medium. Repeated subculture of shoots enhanced the shoot multiplication rate and a 10-12-fold increase in shoot number was observed at the end of 10th subculture in all four cultivars. With the increase in the culture passage, more shoots were induced from the base of the shoots and callus development from the base was observed from fifth subculture onwards. The increase in shoot proliferation rates during series of subcultures may possibly be due to in vitro

adaptation. Tewary et al. (1995) observed an increase in the shoot number in each passage with increase in callus from the base when transferred to fresh medium in the V-1 and S-34 cultivars *of Morus*.

Production of plantlets with profuse rooting in vitro is important for successful establishment of regenerated plants in soil (Ohyama, 1970). Auxins such as NAA, IAA, IBA and 2,4-D were used singly to induce rooting from in vitro raised shoots of the cultivars. Roots were induced with varying frequencies and the number of roots induced as well as the thickness of roots induced in the presence of different auxins varied. Of all the auxins tested, 2,4-D was found to be a better rooting agent in all four cultivars. In contrast, Hossain et al. (1992) reported NAA and IBA to be an efficient rooting agent in *Morus laevigata* whereas Yadav et al. (1990) stated IBA to be the best auxin for induction of roots in *Morus nigra*.

In the present communication, reproducible protocols for the rapid multiplication of elite cultivars of mulberry are developed. A detailed study of seasonal effects on axillary bud sprouting in vitro and field conditions has been conducted and high frequency of sprouting was observed in summer as compared to the rainy and winter seasons in all four cultivars. We observed that 2,4-D is a better sprouting agent for M-5, S-13 and S-36 cultivars whereas KN was found to be superior in inducing high frequency sprouting in China White, a difficult-to-root cultivar of mulberry. Furthermore, fructose was found to be a more suitable source of carbon than sucrose for shoot multiplication of all four cultivars. Efficient root induction in all cultivars was achieved in the presence of 2,4-D and micropropagated plants were established in the field with a survival frequency of 85-90%. The methods described offer immense scope for the rapid in vitro propagation of mulberry for commercial use.

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References

Anuradha, M., Pullaiah, T.. 1992. Micropropagation of mulberry (*Morus alba* L.). Annali Di Botanica 15, 35-41.

Bajaj, Y.P.S., 1986. Biotechnology in Agriculture and Forestry, Vol. 11. Springer, Berlin.

Chitra, V.D.S.. Padmaja, G., 1999. Clonal propagation of mulberry *(Morus indica* L. cullivar M-5) through in vitro culture of nodal explants. Sci. Hort. 80, 289-298.

Civinova, B.. Sladsky, Z., 1990. Stimulation of the regeneration capacity of tree shoot segment explants in vitro. Biol. Plant. 32, 407-413.

Das, B.C., 1983. Mulberry taxonomy, cytogenetics and breeding. In: Proceedings of the National Seminar on Silk Research and Development, Bangalore, March 10-13, India.

Hossain, M.. Rahman, S.M., Zaman, A., Joarder, O.I., Islam, R., 1992. Micropropagation of *Morus laevigata* wall, from mature trees. Plant Cell Rep. 11, 522-524.

Hutchinson, J.F., Zimmerman, R.H., 1987. Tissue culture of temperate fruit and nut trees. Hort. Rev. 9, 273-349.

Jain, A.K., Dandin, S.B., Sengupta, K., 1990. In vitro propagation through axillary bud multiplication in different mulberry genotypes. Plant Cell Rep. 8, 737-740.

Mhatre, M., Bapat, V.A., Rao. P.S., 1985. Regeneration of plants from the culture of leaves and axillary buds in mulberry (*Morus indica* L.). Plant Cell Rep. 4, 78-80.

Murashige. T., Skoog, F., 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15, 473–497.

Narayan, P., Chakraborty, S., Subba Rao, G., 1989. Regeneration of plantlets from the callus of stem segments of mature plants of *Morus alba* L. Proc. Indian Natl. Sci. Acad. 55, 469-472.

Ohyama, K., 1970. Tissue culture in mulberry tree. Jpn. Agric. Res. Quart. 5, 30-34.

Ohyama, K., Oka, S., 1987. Mulberry. In: Bonga, J.M., Durzan, D.J. (Eds.), Cell and Tissue Culture in Forestry, Vol. 3. Nijhoff/Junk Publisher, Dordrecht, pp. 272-284.

Oka, S., Ohyama, K., 1975. Studies on in vitro culture of excised bud in mulberry tree. 11. Effect of growth substances on the development of shoots from bud. J. Seric. Sci. Jpn. 44, 444–450.

Oka, S.. Ohyama, K., 1982. Sugar utilization in mulberry (*Morus alba* L.) bud culture. In: Proceedings of the Fifth International Congress on Plant Tissue Cell Culture, pp. 67-68.

Pattnaik, S.K., Chand, P.K., 1997. Rapid clonal propagation of three mulberries, *Morus cathyana* Hemsl., *M. ihou* Koiz. and *M. serrata* Roxb., through in vitro culture of apical shoot buds and nodal explants from mature trees. Plant Cell Rep. 16, 503-508.

Quraishi, A., Koche, V., Mishra, S.K., 1996. In vitro micropropagation from nodal segments of *Cleistanthus collinus*. Plant Cell Tiss. Org. Cult. 45, 87-91.

Skirvin, R.M., 1981. Fruit crops. In: Conger, B.V. (Ed.), Cloning Agricultural Plants via In Vitro Techniques. CRC Press, Boca Raton. FL, pp. 51–139.

Tewary, P.K., Sarkar. A., Vineet, K.. Chakraborti, S., 1995. Rapid in vitro multiplication of high yielding mulberry (*Morus* spp.) genotypes V-1 and S-34. Indian J. Seric. 34, 133–136.

Yadav, U., Madan, L., Jaiswal, V.S., 1990. Micropropagation of *Morus nigra* L. from shoot lip and nodal explants of mature trees. Sci. Hort. 44, 61-67.

Zaman, A., Islam, R., Joarder, O.I., 1997. Field performance and biochemical evaluation of micropropagated mulberry plants. Plant Cell Tiss. Org. Cult. 51, 61-64.