STANDARDIZATION OF HARDENING PROTOCOL OF TISSUE CULTURED MULBERRY PLANTS (*Morus* sp.)

REDDY LAKSHMI, V.
PAK 5241

DEPARTMENT OF SERICULTURE
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE

STANDARDIZATION OF HARDENING PROTOCOL OF TISSUE CULTURED MULBERRY PLANTS (*Morus sp.*)

REDDY LAKSHMI, V.
PAK 5241

Thesis submitted to the

University of Agricultural Sciences, Bangalore

In partial fulfilment of the requirements

For the award of the degree of

MASTER OF SCIENCE

IN

SERICULTURE

BANGALORE **AUGUST, 2007**

Affectionately Dedicated
To My Beloved
Parents, Brothers
&
Friends

DEPARTMENT OF SERICULTURE
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE-560 065.

CERTIFICATE

This is to certify that the thesis entitled **STANDARDIZATION OF HARDENING PROTOCOL OF TISSUE CULTURED MULBERRY PLANTS (*Morus sp.*)** submitted by **Ms.REDDY LAKSHMI, V.,** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE** in **SERICULTURE** of the University of Agricultural Sciences, Bangalore, is a record of research work carried out by her during the period of his study in this University under my guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar titles.

Bangalore
August, 2007

(Dr.D.NUTHAN)
Major Advisor
Associate Director of Research (HQ)

Approved by:

Chairman : **Dr.D.NUTHAN**

Members :

1. Dr.K.P.CHINNASWAMY

2. Dr.D.DAYAL DOSS

3. Dr.FATHIMA SADUTALLA

4. Dr.VASUNDHARA

ACKNOWLEDGEMENT

"No scientific endeavour is a result of an individuals efforts and so comes the time to look back on the path traversed during this endeavour and to remember the faces and spirits behind the action with sense of gratitude."

Neither the space nor words are big enough to express my loyal and venerable thanks for giving me such wonderful person as my teacher during the course of investigation. With an overwhelming sense of pride and genuine obligation, I take this opportunity and proud privilege to extend my heartfelt and deep sense of gratitude towards my esteemed teacher and chairman of my advisory committee, ***Dr.D.Nuthan****, Associate Director of Research (HQ), for his thought provoking, inspiring, valuable and expert guidance, timely suggestion, close counsel, critical evaluation, everlasting patience and constant encouragement at every step of the present investigation and in finalization of this manuscript. It was indeed a great opportunity of mine to work under his guidance.*

I avail this opportunity to express my deep sense of gratitude and grateful regards to ***Dr.K.P.Chinnaswamy****, Professor & Head of Department, Dept. of Sericulture,* ***Dr.D.Dayal Doss****, Professor, Dept. of Biotechnology,* ***Dr.Fathima Sadutalla****, Associate Professor, Dept. of Sericulture and* ***Dr.Vasundhara****, Professor, Dept. of Horticulture, University of Agricultural Sciences, Bangalore the members of my advisory committee for their edifying counsels and statutory advices during the course of investigation. It is my proud privilege to express my gratitude and sincere thanks for their encouragement, benevolent guidance and kind cooperation extended to me during this study.*

With utmost reverence, I wish to place on record my respectful regards and gratitude to ***Dr.K.P.Chinnaswamy****, Professor and Head of Dept. Sericulture, UAS, Bangalore for his whole hearted encouragement and steadfast inspiration in my successful accomplishment of the study. I extend my sincere thanks to other staff members of the Dept. of Sericulture for their help rendered in many ways.*

Inspite of all these, I lack in words to express my deep sense of gratitude to my beloved parents ***Sri.Venkateshaiah*** *and* ***Smt.Lakshmidevamma****, brothers* ***Jayaprakash Narayan, Harish Kumar****, sisters* ***Umaganesh, Chaitra*** *and* ***Ammu****. I avail this opportunity to express my deep sense of reverence and gratitude to my uncles* ***Gangadhar, Krishnappa, Soma*** *aunty* ***Lakshmi*** *and* ***Gangadevi*** *and family members, who encouraged me to pursue higher studies, their selfless persuasion and sacrifices, heartfelt blessings, love, care and constant inspiration have made this manuscript a little remuneration to translate their dreams in to reality.*

I empathetically feel infact fortunate to extend my immense amount of gratitude to ***Dr.D. Dayal Doss****, Professor, Dept. Of Biotechnology, for provided the laboratory facilities throughout my research work, for finishing my master's programme successfully with out any interruptions and for his valuable advise and constant encouragement and at this outset I would also thank* ***Raju*** *for his assistance.*

My heart is joyous to express feelings with thanks to my roommates ***Divya*** *and* ***Indira*** *batch-mates* ***Shy, Mammu, Manju, Mohan, Murthy, Somu****, senior friends* ***Hadimani, Amarnath, Rashmi, Saritha, Murari, Soori*** *and* ***Sudhakar*** *for their help during my research work.*

I would like to remember my close friends ***Amul Reddy, Kavitha, Nirmala, Nimmi, Sajju, Sandeep, Swapna, Reddy*** *who were mainly responsible for the successful completion of my experience and ocean of friends for being source of my strength and corners of spirit. Any omission in this brief acknowledgement does't mean lack of gratitude.*

Ending is inevitable for any kind of work. Though acknowledging is an endless task, I end by saying endless thanks to all those whom I am able to recall here and also those whom I might have left unknowingly.

Place: Bangalore.
Date: August, 2007

Reddy Laksh

(Reddy Lakshmi V.)

Standardization of hardening protocol of tissue cultured mulberry plants (*Morus* spp.)

REDDY LAKSHMI, V.

Abstract

Present investigation on "standardization of hardening protocol of tissue cultured mulberry plants (*Morus* spp.)" was carried out to standardize the hardening protocol for mulberry genotypes through nodal explants using five genotypes *viz.*, V_1, S_{36}, (Irrigated); S_{13}, RFS_{135} (Rainfed) and Mysore local (Control).

Nodal explants of all the genotypes exhibited survival of 79.36 per cent when treated with 0.1 per cent $HgCl_2$ for about 8 minutes. S_{36} recorded maximum callus initiation (60.76%) compared to the other genotypes, when treated with 2.0mg/L 2, 4-D along with 0.75 or 1.00mg/L BAP, 2, 4-D therefore it was identified as the best auxin for callus induction. Callus differentiation into shoots was also maximum in S_{36} variety, when MS medium used was fortified with combination of BAP (2mg/L) and NAA (0.1mg/L). Significantly higher rooting percentage (48.07%, 40.82%) was recorded when shoots were transferred to MS media having 1.0 mg/L and 1.5 mg/L NAA respectively as compared to control (22.50 %). Maximum number of leaves were recorded when 50 per cent and 75 per cent glycerol were applied as antitransparents (4.40, 4.13), followed by low melting paraffin wax (4.00) and 50 per cent grease petroleum (3.93) compared to control (2.93). Shoot length recorded maximum when grown on potting mixture (Peat: perlite: vermiculite (1:1:1)+ MS+1 ppm of NAA+1ppm IBA +1ppm IAA+(5.46) while sand: soil: perlite +1/2 MS+1ppm IAA+1ppm IBA+1ppmIAA stood next (5.40). Maximum plant height was recorded significantly with the 50 per cent shading (3.69%) and 75 per cent shading (3.42%). Survival rate was recorded significantly higher in mulberry with 45 days (81.94%) and 30 days after planting (80.89%). Considering callus initiation, shoot, internodal length, root formation, plant height, number of leaves and survival per cent, genotype S_{36} performed best out of all other varieties tried.

Date: 10-09-2007
Place: Bangalore

Dr. D. Nuthan
Major Advisor

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

INTRODUCTION

I. INTRODUCTION

India is the second largest producer of raw silk (11,487 M.T) with an area 3,13,109 hectares under mulberry cultivation. Karnataka is the pioneering state contributing about 54% of the Indian silk production with a land area of 1, 66,000 hectares under mulberry cultivation. (Annon, 1992).

Mulberry has its economic importance in the sericulture industry because its foliage is used as food for silkworms. Besides being an important host plant, mulberry is also one of the important multipurpose trees and referred to as *"Kalpavaruksha"* (Dandin and Ramesh, 1987). Incidentally the production cost of mulberry leaves covers more than 60% of the cost of cocoon production in sericulture. (Das and Krishna Swami, 1965).

Mulberry is represented by more than a hundred wild and evolved tropical genotypes characterized by high leaf yield, tolerant to biotic and abiotic stresses. Much work has been done on large- scale production of plants involving techniques such as automation of existing producers, (Levin, 1988) and the production of somatic embryos (Arima *et al.*, 1989; Stuart *et al.*, 1990; Zimmerman, 1993; Dave and Batra, 1995; Tomar *et al.*, 1999).

Mulberry is conventionally propagated through cuttings. Goel *et al.*, (1998) studied variability in rooting parameters in 150 Indian and exotic mulberry germplasm accessions at sapling stage and categorized them as poor, medium and good survivors. Many desired cultivars do not root easily or have low rooting ability. Such difficult varieties could be multiplied by using tissue culture techniques (Bonga, 1977, 1981; Vasil and Vasil, 1980; Pal, 1983 and Uematsu *et al.*, 1987). Propagation *via* cuttings is restricted to only certain months of the years, and the saplings obtained by cuttings when

compared with micro propagated plants also show inferior morphogenesis vigor (Zaman *et al.,* 1997).

Mulberry propagation through seeds is undesirable owing to cross-pollination and heterozygosis (Das, 1983), polyploidy in the plants and the dioecious nature of the genus is a serious barrier to genetic improvement by conventional hybridization technique. Asexual multiplication is preferred over sexual means as the genetic characters of the parent are maintained and population variation is minimized. Clonal propagation of mulberry using tissue culture techniques has many useful applications in silk industry (Ravindaran *et al.,* 1988).

Micro propagation technique is effectively utilized for clonal propagation of elite genotypes of a number of economic tree species *viz,* Teak (Gupta *et al.,* 1980), *Eucalyptus* (Gupta *et al.,* 1981) and *Sandalwood* (Rao and Bapat, 1978).

In France, Nitsch succeeded in the culture of excised ovaries and tissues of fruit trees. This was the 1st attempt of the culture of fruits *in vitro.* Ohyama (1970) was the first to report tissue culture in Mulberry plants. *Morus alba, M .bombycis* and *M. indica* have since been cultured (Ohyama, 1970); Ghugale *et al.,* 1971; Oka and Ohyama, 1981; Vijayan *et al.,* 2000; Machii,1990; Saito and Katagiri, 1989); Hossain *et al.,* 1992; lvanica, 1987; Jain and Datta, 1992), cotyledon (Wang *et al.,* 1996) and anther explants were carried out and indirect regeneration (Shoukang *et al.,* 1987; Srinivasa *et al.,* 2001; Narayan *et al.,* 1989) was also reported.

Recently, tissue culture technology has been applied to mulberry and fruitful results have been obtained. There are reports that regarding the development of a highly efficient protocol for direct regeneration from auxiliary buds of some elite Indian mulberry

cultivars, namely RFS_{175}, S_1, K_2, DD and a Japanese cultivar *Morus multicaulis*. cv. Goshoerami in the media supplemented with these thidiazuron and activated charcoal (Tewary *et al.*, 1999; Bhatnagar *et al.*, 2001).

In recent years tissue culture has been extensively tried in economically important tree species (Sinha and Mallik, 1987). Recently, Mhatre *et al.*, 1985 has done considerable work on direct regeneration of plants from the culture of leaf and axillary buds in *Morus indica* L. Joarder *et al.* (1988) have also observed rapid multiplication of mulberry through culture of shoot tips and lateral buds. The use of *in vitro* techniques for mass clonal propagation is the most advanced application of plant tissue culture.

Tissue culture technology is used as a valuable tool for large-scale *invitro* multiplication of commercial crops (George, 1996). In this direction, varieties of plants were tried based on existing production parameters in commercial laboratories. It is necessary to know the production cost per plant and various strategies to lower the cost of production per plant basis (Prakash and Pierik, 1993). Further, the input per liter of the medium generally reflects on the cost of the plant produced such as the price of sucrose and agar.

Asexual multiplication is preferred over sexual means as the genetic characters of the parent are maintained and population variation is minimized (Ravindaran *et al.*, 1988). By using micro-propagation techniques, approximately 10^8 plants can be produced from a single nodal explant with in a year. Such high rate of plant multiplication could not be achieved by any other traditional method of vegetative propagation (Sharma and Thorpe; 1990).

Tremendous progress has been made in mulberry tissue culture which is mainly confined to micropropagation through direct and

indirect regeneration using different explants. *In vitro* rooting techniques was practically an additional step to obtain complete plants under culture conditions (Ohyama, 1970; Oka and Ohyama, 1974; Patel *et al.*, 1983; Chung, 1985; Ivanicka, 198; Oshigane, 1989; Narayan *et al.*, 1989; Tewary and Subba Rao, 1990; Rao and Raghunath, 1993 and Srinivasa, *et al.*, 2001).

Several attempts have been made to utilize tissue culture techniques for micro propagation and regeneration using non-embryogenic vegetative propagules of different varieties of mulberry to overcome constraints (Ohyama, 1970; Ghugale *et al.*, 1971; Oka and Ohyama, 1981; Kim *et al.*, 1985; Jain *et al.*, 1990; Hossain *et al.*, 1992; Pattnaik and Chand, 1997; Tewary and Oka, 1999 and Srinivasa, *et al.*, 2001).

Though Mulberry responds well to tissue culture techniques, little or no information exists on use of nodal explants and hardening process of tissue cultured mulberry plants. Therefore, the present investigation, "Standardization of hardening protocol of tissue cultured mulberry plants (*Morus* spp)." was carried out with the following objectives:

1. Micropropagation and standardization of hardening protocol for tissue cultured plants.
2. To maximize survival rate in tissue cultured plants.
3. To enhance the plant growth and establishment of tissue cultured plants.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Direct and indirect regeneration of mulberry using shoot tips, axillary buds, internodes, leaf petiole, hypocotyls, cotyledons, and leaf as explants material are reviewed in this chapter.

2.1 Sterilization of Explants

To initiate aseptic culture, the nodal segments (0.5-1.0cm) from six month old aforesaid cultivars were rinsed in 5% Teepol for 3min., disinfected in a 0.1% (w/v) aqueous $HgCl_2$ solution for 5min., thoroughly in sterile distilled water and cultured in MS medium (Tewary *et al.*, 1989; Kathiravan *et al.*, 1995; Pavan *et al.*, 1999 and Bhatnagar *et al.*, 2002).

Seeds of mulberry (*Morus alba*), were washed thoroughly in running tap water, surface sterilized with 0.1% mercuric chloride for 5 min, and washed several times with sterile distilled water (Kim *et al.*, 1985; Kapur *et al.*, 2001; Das *et al.*, 2003 and Vijayan *et al.*, 2000).

The shoot apex explants were sterilized in chlorine for 5-8min and washed several times in sterile water (Tewary and Subba Rao, 1990).

Seeds of Mulberry (*Morus alba*), were surface sterilized with 0.2% mercuric chloride for 8 min, and rinsed several times with sterile distilled water (Ohyama, 1970; Sharma and Thorpe, 1990; Shajahan *et al.*, 1995).

The auxiliary buds were washed first with the detergent "*Teepol*" (5ml/100ml distilled water) for 8-10 min and removed by washing the buds 5-6 times with distilled water. Later, buds were surface sterilized with HgCl2 (0.15%) for 8 min and washed 7-8 times with autoclaved double distilled water (Jain *et al.*, 1990).

The explants were washed with running tap water for 20-30 min and then treated with 1 % v/v Cetavlon (20%w/v cetrimide, a detergent and antiseptic) for 5 min. After washing with water several times, explants were passed through an ultraviolet sterile laminar flow for sterilization. Explants were treated by successive immersion in 70% ethanol for 30 sec. and 0.05% $HgCl_2$ for 2 min, followed by rinsing 5-6 times in sterile water (Yadav *et al.*, 1990).

Shoot node explants were washed first with Labolene (liquid detergent) for 15 min followed by through washing in running tap water. The cleaned material was then treated with 0.1% Bavistin for 20 minutes followed by 0.1% streptomycin for 10 minutes and then given several washes in distilled water. The cleaned material was surface sterilized with 5% sodium hypochlorite solution for 5 min followed by thorough rinsing with autoclaved distilled water (Tewary *et al.*, 1996).

Pavan *et al.*, 1999 reported that nodal explants of K_2 mulberry variety were thoroughly washed in running tap water, and then with 5 per cent Teepol and sterile distilled water. Explant was surface sterilized with aqueous solution of $HgCl_2$ (0.1%) for 5 min followed by rinsing four to five times in sterile distilled water.

Explants were surface sterilized by immersing and washing in solution of Labolene (1:20) for 10 min, rinsed in sterile water and transferred to 70% alcohol for 20-30 seconds followed by the treatment of 12% Mercuric chloride for 10 min, then washed for 4-5 times with sterile water (Revanasiddaiah *et al.*, 1999).

Viable seeds of variety S-799 were treated initially with 70% ethanol for 1min and washed thoroughly with sterile distilled water and then surface sterilized with mercuric chloride (0.1% w/v) 4-5 minutes and then washed several times with sterile distilled water under aseptic conditions (Vijayan *et al.*, 1998: Wang *et al.*, 1996).

2.2 Regeneration Response of Different Mulberry Genotypes

Kathiravan *et al.*, 1995 reported that regeneration in tissue culture is a genetically controlled trait (Bhojwani *et al.*, 1984: Tempeton-Somers and Collins, 1986) in mulberry, frequency of shoot regeneration and the number of shoots per explant varied among the genotypes. The maximum frequency of regeneration was observed in S_{34} followed by MR_2, M_5 and Ichinose (92%, 86%, 82% and 74% respectively).

The optimum BAP concentration for shoot bud differentiation and subsequent shoot growth was found to be 1 mg/1 for Sujanpur-5 and 2 mg/1 for S-799. Whereas, the increased concentration of BAP i.e., 3 mg/1 was found giving highest sprouting percentage and optimum growth in K_2 and LF_2 varieties. Raising the BAP concentration further up to 5 mg/1 failed to produce any response in the tested genotypes. Of various concentrations tried, IBA combined with 1 mg/1 of BAP induced maximum sprouting (70-90%) with optimum shoot elongation (3-4cm) in Tr_{10}, MS_3, RFS_{135}, and MR_2 (varieties). Addition of GA_3 at 0.5 mg/1 in the above combination of IBA + BAP was found essential to give optimum results in S_{-41} and C_{763} (Tewary *et al.*, 1996).

In *Morus indica* BAP (0.25-0.5mg/1), in *M. alba* and *M. rotondifolia* GA_3 (0.5-1.0mg/1) were found to induce sprouting. Two genotypes of *M. bombycis*, namely Schmanochi and Mizusawa, developed healthy shoots on the incorporation of 2,4-D (0.5-1.0 mg/1) and BAP (0.5-2.0 mg/1), respectively (Jain *et al.*, 1990).

2.3 Effect of Source of Explants on Regeneration Response

Kim *et al.*, 1985 reported that cotyledons excised from embryos cultured on medium without cytokines did not produce buds under any of the culture conditions. The first formed leaves were excellent

explants for adventitious bud formation with younger (10-15 days old) leaves showing higher frequencies of bud formation than older leaves. Hypocotyl segments showed very poor response to the culture treatments. Shoot tips bearing a few leaf primordial, however, were very good explants. The Murashige and Skoog's medium (1962) was more effective in bud induction and development than Von Arnold and Eriksson (1981) medium although 2 mg/1 BAP alone was sufficient to induce buds in most explants. Addition of low levels of NAA concentration there was a reduction in frequency of bud formation in all explants. Addition of NAA at 0.1 mg/1 caused callus formation and completely inhibited bud formation. Increasing the levels of BAP in the medium above 2 mg/1 did not improve bud formation, instead, had adverse effects on elongation of the buds.

The position of the nodal segments on the parent shoot exerted a notable effect on the rate of shoot multiplication. More mature segments formed more vigorous shoots, characterized by greater shoot length and number of nodes per shoot. Consequently, the nodes from the basal portion of the donor shoot were distinctly superior to all other explants for shoot multiplication. The amount of callus formed at the cut end of the shoot also increased with the maturity of the nodal segment (Sharma and Thorpe, 1990).

2.4 Callus Induction

Shoot apex explants when placed on MS medium supplemented with 2mg/l BAP after about a month of culture, produced friable callus (Tewary *et al.*, 1990).

Christena *et al.* (1992) reported that somatic embryogenesis, was induced in hypocotyl of explants of Geranium (Pelargonium x Hortorum) cultured on media supplemented with various concentrations of N-phenyl-N'-1,2,3-thaidiazol-5-yl urea(thidiazuron) ranging from 0.2 to 1.0μM . The use of N^6 –benzyl amino -purine in

combination with Indole –3-acetic acid also evoked embryogenesis, but the efficiency of somatic embryo production was significantly lower than that obtained with TDZ.

Machii, 1992 reported that callus induction under light conditions, almost the same number of calli was formed on the MS and B5 media. On the contrary, under dark conditions, a larger number of calli was formed on the B5 medium than MS medium.

Calli was induced by 100% addition of 5mg /l Coumarine or 0.1 to 0.25mg/l 2, 4-D to the medium. The application of 10mg/l Coumarine to the medium containing 2, 4-D showed extensive promotive effects on the proliferation of calli tissues .On the other hand, application of 1.0 mg/l brassinolide showed a similar effect as cytokine (Kuno and Ji, 1996).

Combinations of IAA and TDZ , NAA and TDZ ,2,4-D and TDZ, the efficiency of callus formation was ++ except for + min high levels (10µM)of Naphtalene Acetic Acid (NAA). Furthermore, in case of Indole Acetic Acid (IAA) and BAP, NAA and BAP, efficiency was +, but the combination of 2,4-D and BAP showed the callus formation of +++ (high levels of 2,4-D),and ++(low levels of 2,4-D) (Tohjima *et al.*,1996).

Vijayan *et al.*, 1998 reported that hypocotyl segments callused within 14-16 days of inoculation on MS medium containing 2mg/l 2, 4-D and 0.5 mg/l BAP. Callus was very friable on medium containing auxin alone and whereas compact callus obtained on media containing higher concentrations of 2, 4-D and lower concentration of BAP.

Srinivasa *et al.*, 2001, reported that callus was induced form the aseptic segments on Murashige and Skoog's Basal Media (MSBM) supplemented with 2,4-D (2mg/l), CM 10% and CH (1mg/l). After

three weeks of culture cut end of the explants proliferated into profuse compact callus with initiation of roots.

Kavyashree *et al.* (2005a) reported that high frequency (83%) of callus was induced from the cut ends of *in vitro* petiole explant derived through direct –organogenesis of mulberry variety-S-54,when cultured on Linsmaier and Skoog's. Basal Medium (1965) fortified with 2,4-D (2.5mg/l) when compared to low frequency (15%) of callus induction on LSBM fortified with NAA(1.0mg/l). After 21 days of culture, profuse light yellowish compact and moderate creamish white friable callus was observed on respective media.

2.5 Shoot Formation (Differentiation)

Chitra and Padmaja (1999) reported that 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 (0.3mg/l). *In-vitro* proliferated shoots were multiplied rapidly by culture of shoot tips on MS medium with BAP (0.5 and 1.0mg/l), which produced the greatest multiple shoot formation. Multiplication was also achieved by culture of shoot tips on MS medium with BAP (4.0mg/l) and GA_3.

Ohnishi *et al.* (1986), reported that addition of both abscissic acid (ABA) and p-amino benzoate (PABA), led to the suppression of the callus proliferation and the callus could be preserved for 20 weeks without loosing the freshness in the dark at 28°C. At a low temperature and over a long period of time, the callus was found to be alive but growth was realized when the callus was exposed to ambient conditions.

The explant cultured on semisolid medium with different concentrations of growth hormones, callusing was observed after 4-5 weeks on MS supplemented with BAP (2mg/l) and 2, 4-D (0.5mg/l).

Browning of callus was observed due to phenolic accumulation after certain age. BAP + 2, 4-D were found suitable for long term callus cultures and the growth rate revealed increasing growth indices with aging of callus and growth was maximum in about 4 months duration (Tewary *et al.*, 1989).

Chattopadhyay *et al.* 1990 reported that most distinguishable multiple shoots were noted on MS medium containing IAA+BAP (1+2mg/l). Initiation of 40 per cent rooted shoots from auxillary bud explant from a mature plant was also noted after 15 days of culture period on Murashige and Skoog's medium (1962) having NAA+Kn (1+0.5mg/l). Various concentrations of IAA and BAP in different combinations were also considered to promote the frequency of multiple shoot formation from a single auxillary bud. Maximum number of shoots were obtained after 30 days of culture.

Addition of BA to MS with 3 per cent sucrose favoured shoot growth. Among the various BA concentrations tested, 2.5µM BA was significantly superior, both in terms of shoot length and the number of nodes formed per shoot .While the presence of 0.5 and 1.0 µM BA, in both the above parameters of shoot multiplication remained lower however, levels greater than 2.5 µM either inhibited both shoot length and number of nodes or only shoot length at 7.5µM BA. Sucrose at 3% was optimum for shoot length. Decreasing the sucrose concentration to 1 to 2 per cent or increasing it to 5 per cent inhibited shoot growth, although the rate of shoot multiplication was not affected at 5 per cent sucrose. This was mainly due to the formation of multiple auxillary shoots (Sharma and Thorpe, 1990).

Shoot apex explants cultured on MS medium fortified with different concentrations of BAP, regenerated multiple number of shoot along with a proliferation of callus (Tewary *et al.*, 1996).

Yadav *et al.* (1990) reported that shoot proliferation (80%) and growth increased when a cytokinin (BAP or Kn) was present in the medium, depending on the concentration. Maximum number of shoots was observed at 1.0mg/l of BAP, where as maximum shoot length was observed at 0.5mg/l BAP. BAP was better than Kn for shoot multiplication and growth.

Saito, 1992 reported that the largest number of shoots was obtained from the adventitious buds, when 1mg/l of BA was added to the MS medium. "Hayatesakari" developed the largest number of shoots, followed by "Minamiskari" and "Kenmochi" by sub culturing the adventitious bud clusters derived from immature leaves on MS medium.

Optimum proliferation of shoots was obtained on MS medium supplemented with 1.0mg/l benzyl- aminopurine (BAP) and 2.0 percent fructose under a light intensity of 3000-6000lux at 25°c. Proliferation was maximum after 30 days of culture (Katase, 1993a).

Multiple bud bodies (MBB) were induced after 1-2 months in the presence of 2mg/l 4PU, a cytokinin-like urea derivative. BA and Zeatin promoted the development of few leaves and the formation of elongated shoots, but did not induce MBB. The MBBs from the mulberry produced normal shoots when cultured on a solid medium containing 1mg/l BA. Fructose stimulated the shoot formation from MBB more effectively than sucrose (Hayashi and Oka, 1995).

Kathiravan *et al.* (1995) reported that MS (Murashige and Skoog) medium containing benzyl-aminopurine (BAP) or Kinetin (K) induced multiple shoots in nodal explants, but the maximum number of regenerants per explant was induced by the addition of BAP. The efficiency of BAP for shoot multiplication was not improved when it was not supplemented with Naphthalene Acetic Acid (NAA) or Indole Butyric Acid (IBA).

Shajahan *et al.* (1995) reported that Murashige and Skoog's (MS) salts containing 1.0mg/l Benzyl Amino Purine is a suitable medium for *in vitro* shoot multiplication. Mature nodal segments (third or forth node from shoot tip) formed more vigorous shoot than the shoot tip and youngest nodal segments.

Tewary *et al.* (1996) reported that the maximum morphogenic response was elicited by BAP (2mg/l) alone for S_{34} where green shoot buds were inducted within three weeks of culture. Four to six shoot number however increased during each passage. This increase is due to the optimal hormonal concentrations used in subsequent passages, which promote the growth of meristematic tissue in regenerating cultures. The rate of multiplication plotted at 20 days interval indicated a continuous increase up to 100 days and thereafter, it remained almost static during subsequent sub-cultures.

Wang *et al.* (1996) reported that a medium containing MS mineral salts, vitamins, 3.0mg/l of BAP, 0.3mg/l IAA, 30g/l of sucrose or glucose, 500mg/l lactalbumine hydrolysate, solidified with 6g/l of agar powder was most favourable for inducing adventitious buds on mulberry cotyledons, with this medium, the induction rate reached over 80%.

Kamili *et al.* (1998) reported that addition of growth adjuvants like Yeast extract(YE),Coconut milk(CM), Malt extract(ME), peptone and casamino acids with 2,4-D (1mg/l) promoted hundred per cent sprouting of auxillary buds in both *Morus alba* and *Morus latifolia.*

High frequency bud break and multiple shoots were induced in apical shoot buds and nodal explants of *Morus cathayan*, *M.ithou* and *M. serrata* on MS medium containing 0.5-1.0mg/l BAP. Addition of gibberellic acid (0.4mg/l) along with BAP induced faster bud break both in apical shoot buds and nodal explants and also enhanced the

frequency of bud break in all three species (Pattnaik and Chand, 1997).

Sugimura *et al.* (1998) reported that high frequency of bud formation was achieved by using the medium supplemented with cytokinin (6-benzyl amino purine or thidiazuron) and 2, 3, 5-triiodobenzoic acid (TIBA) throughout the culture.

Combination of BAP (2mg/l) and NAA (0.1mg/l) was found most effective in inducing higher percentage (64.00+_7.00) of multiple shoot buds (38.66+-7.50) from callus. The presence of BAP in the germinating medium could have reduced the endogenous auxin content in the hypocotyl explant while the higher amount of endogenous auxin present in the explant was found inducing rhizogenesis in Mulberry callus (Vijayan *et al.*, 1998).

Multiple shoots were regenerated from the basal leaf in the presence of the high concentrations of thidiazuron (TDZ) and low concentrations of 2, 3, 5-triiodobenzoic acid (TIBA) (Adachi *et al.*, 1999).

Adventitious bud formation and plant regeneration from immature leaf cultures varied considerably with genotype .Several genotypes having high capacity for adventitious bud formation, like Kanadasansou-A showed the highest percentage(72%) of adventitious bud formation, while Oyutaka formed the highest number (6.3) of adventitious buds per explant and showed high percentage of shoot formation(Machii,1999).

Revanasiddaiah *et al.* (1999) reported that MSBM was found to be best when compared to LSBM for bud multiplication. After the

results showed that *in vitro* formed shoots produced 80-85% rooted shoots when the MS was supplemented with 1 and 2mg/l NAA for T_1 and T_2 explants respectively (Chattopadhyay *et al.*,1990).

The shoots were rooted on half strength MS medium containing the combination of IAA, IBA and Indole –3-propionic acid each at 1.0mg/l. The plantlets were successfully acclimatized and eventually established in soil (Pattnaik and Chand, 1997).

The shoots maintained in the proliferation medium (MS+2.5mg/l BA + 3% sucrose) for over 4 weeks developed adventitious roots at the callused basal end. Addition of 0.05 per cent to 0.1 per cent activated charcoal to the shoot proliferation medium did not affect the rate of shoot multiplication or the formation of roots. However, the presence of activated charcoal initiated roots were within 2 weeks of culture as compared with 4-5 weeks in controls (Sharma and Thorpe, 1990).

Two to three roots were produced by each shoot on MS medium containing an auxin (IBA, IAA, and NAA) after 3 weeks of culture. Among the three auxins tested, IBA was found to be the best for root induction, followed by IAA and NAA. Shoots rooted best in 0.25-1.0mg/l of IBA. The maximum number of roots per shoots was recorded at 0.25mg/l of IBA (Yadav *et al.*, 1990).

Regenerated shoots were rooted on MS basal medium with lower levels of IBA but an increase in concentration of IBA inhibited root development (Kathiravan *et al.*, 1995).

Rooting was better in 0.5mg/l NAA, where 89 per cent of the explants initiated vigorous roots. Root system developed from NAA was larger than those from IBA. Higher concentrations of NAA were inhibitory to root induction where as higher concentration of IBA

weeks the auxillary buds of aseptic plant gave more multiple shoots on MS medium supplemented with 2.5mg/l BAPcompared to the explant of field grown plants.

Regeneration frequency varied from 1.67+- 0.51 to 15.00+-2.88% with 1-3 shoots per explant in sucrose .In fructose, regeneration frequency varied from 6.67+-1.66 to23.33+-4.11% with 1-6 buds per explant. In glucose, the frequency of regeneration was very high, showing a range of 5.00+-2.88 to 80.00+-5.77 with 1-12 buds per explant indicating the superiority of glucose over the other two carbohydrate sources. (Vijayan *et al.*, 2000).

Chakravarthy *et al.* (2001) reported that thidiazuron (TDZ), a substituted phenyl urea with cytokine activity was significantly more effective in inducing nodule like structures from leaf cultures. The optimal TDZ and IAA concentrations for organogenesis from leaf cultures were 4.54M and 1.71M, respectively.

Kapur *et al.*, 2001 reported that regeneration percentage was maximum (up to 60%) from leaf explants of field grown plants followed by the leaf explants taken from *in vitro* cultures (up to 40%).The highest percentage of regeneration(61.4%) was observed on MS+IAA (2mg/l) + TDZ (1.1mg/l) + $AgNO_3$ (2mg/l) in both K-2 and DD cultivars. Addition of BAP to TDZ, reduced the response, but presence of IAA and $AgNO_3$ enhanced the regeneration potential of TDZ from both the *in vitro* raised and field grown explants, in the cultivars K-2 and DD. The results indicated that TDZ is better than BAP in inducing multiple shoots. The presence of $AgNO_3$ is not only beneficial but also improves the quality of regenerants.

2.6 ROOTING

After 4 weeks of culture periods, *in vitro* raised multiple shoots were excised and transferred to MS liquid medium individually. The

induced more roots but the growth was very slow (Vijayan *et al.*, 1998).

Adventitious roots were induced from the basal part of shoots using a media with 0.1µM 2, 4-D and hormone free medium. Increasing the concentration of 2, 4-D did not promote rooting response. Approximately 60% of inoculated shoots regenerated into complete plantlets with profuse roots when 0.1µM 2, 4-D was added to the medium (Adachi *et al.*, 1999).

Chakravarthy *et al.* (2001) reported that rooted shoots were obtained on MS basal medium and elongated shoots were rooted on MS medium with 4.9µM indole butyric acid (IBA).

With the addition of activated charcoal to the NAA supplemented medium, the rooting frequency increased appreciably. The rooting percentage was 72 for C_{176} and 66 for C_{776} (Bhatnagar *et al.*, 2002).

Kavyashree *et al.* (2005a) reported that well developed elongated, healthy, multiple shoots derived from light yellowish compact callus were subjected to ex-vitro rooting technique to obtain a complete plantlet.

2.7 Hardening

2.7.1 Effect of Antitransparents in Hardening Process of Tissue Cultured Mulberry Plants

Spraying of plantlets with impermeable materials (50% aqueous glycerol, low melting point paraffin or petroleum grease, dissolved in diethyl ether, growth retardants and anti-transparent (abscisic acid) is a method of reducing water loss from newly planted material. The plantlets so treated could be transferred directly into pots and no

establishment phase was necessary (Smith *et al.*, 1990; Smith *et al.*, 1992; Ghaxhigae *et al.*, 1992 and Pospisilova, 1996).

2.7.2 Performance of Potting Mixture and Shade Levels on Survival Rate of Tissue cultured Mulberry Plants

In vitro shoots of *Morus indica* variety S_{54} were transferred to bottles containing potting mix, peat: perlite: vermiculate (1:1:1 v/v) with different concentrations per *ex-vitro* rooting then transferred to tissue cultured grade soilrite mix, peat: perlite (3:1 v/v) for profuse rooting and acclimatization and then transferred to the field, where survival was 98 per cent (Kavyashree, *et al.*, 2005a).

Multiple shoots proliferated from axillary buds of a mature tree of mulberry (S_{54}) cultured on LSBM supplemented with BAP. Shoots were transferred to rooting medium containing TIBA. Then plantlets were transferred to soilrite mix, peat: perlite: vermiculate (1:1:1 v/v) with half strength of MS with NAA. Plantlets were successfully established in soil (Kavyashree *et al.*, 2005b).

The nodal explants of *Morus alba* L produced multiple shoots. This when transferred to rooting media, best rooting was observed in presence of activated charcoal. The rooted shoots could be transplanted to sand-vermiculate (1:1) mixture in the green house with 100 per cent transplantation success (Sharma *et al.*, 1990).

Proliferated shoot cutting inserted directly into plastic trays containing vermiculate moistened with 0.5ml/L. Hyponex were placed in the green house and exposed to mist for 15 minutes after. Every 30 minutes after 0.25 days of mist treatment rooted plantlets were grown under natural day light in the same green house (Katase, 1994).

Abnormal changes (protuberant, opened stomata), caused by the high humid atmosphere within the culture vessel can be

minimized by transferring the plantlets into the culture vessel with ventilation and acclimatized *in vitro* for 15 days and transferred to pots and maintained in outdoor conditions for better survival rate (Adachi *et al.*, 1999).

Plantlets with well developed roots were transferred to earthen pots containing soilrite: autoclaved soil (1:1) and moistened with 0.5 per cent MS salts initially on alternate days and thereafter twice a week for a month and then transferred to field. After hardening, the survival rate was 80 to 85 per cent (Kapur *et al.*, 2001).

Among all the combinations of potting mixtures tried it was found that potting mixture peat: perlite: sand in ratio of 50: 25: 25 (v/v) was found to be the best combination for hardening of mulberry. The survival percentage of *in vitro* raised plants was found to be 90 to 95 per cent in the above potting mixture (Srinivasa *et al.*, 2001).

Multiple shoots proliferated from shoot tip and nodal explants of a mature type of mulberry, cultured on Murashige and Skoog's medium supplemented with BAP. Shoots were transferred to rooting medium containing IAA. Plantlets rooted within 4 weeks. The plantlets are successfully transfered to soilrite mix for acclimatization (Usha Yadav *et al.*, 1990).

MATERIAL AND METHODS

III. MATERIAL AND METHODS

The present investigation on 'Standardization of Hardening Protocol of Tissue Cultured Mulberry Plants' was carried out in the plant protection laboratory of the Department of Biotechnology, GKVK, UAS, Bangalore-560065 during the year 2006-07. For this study five genotypes namely V_1, S_{36}, S_{13}, RFS_{135} and Mysore local (control) were chosen. These mulberry genotypes were collected from the germplasm maintained in the Department of Sericulture, GKVK, UAS, Bangalore-560065.

3.1 Laboratory Requirements

3.1.1 Glasswares

Various types of glassware like conical flasks, tissue cultured bottles, petriplates, test tubes, funnels, beakers, measuring cylinders and pipettes were used Borosil India Ltd., Mumbai.

Glassware rinsed with water were soaked in 0.15 per cent chromic acid over night followed by rinsing with tap water and cleaning with detergent solution. Before use, the glassware were thoroughly washed with tap water and rinsed with distilled water. All the material was oven dried and stored in a dust proof area for further use.

3.1.2 Chemicals

All the salts, vitamins used in the preparation of tissue culture media were of analytical reagent grade from M/s.Hi Media Co., Mumbai. Growth hormones used were of tissue culture grade and standards obtained from M/s.Sigma Chemicals Co. USA. The gelling agent used for the media was certified agar obtained from M/s.Titan Biotech Ltd., Bhiwandi. Sterilizing agents used for sterilizing the plant material were obtained from M/s.NICE, Cochin.

3.1.3 Sterilization of Vitamins and Hormones

The heat-liable substances like; vitamins, hormones etc., can not be autoclaved. So these substances were sterilized by membrane filter of pore size 0.22µ. The filter holders were autoclaved before use. The substance to be sterilized was inserted into the filter using a disposable syringe. The filtered substance was collected in a sterile vial and kept in the refrigerator.

3.2 Culture Medium

3.2.1 Murashige and Skoog's (MS) Medium Composition

The composition of MS Media used for the present experiment (Murashige and Skoog, 1962) is given in the Table.1

The MS basal medium was freshly prepared whenever required by drawing appropriate amount of nutrient solutions from stock solution prepared and stored. The stock solutions were stored in refrigerator maintained at 4-5°C.

Sucrose	:	20 to 30g/L
Agar	:	8 to 10g/L
pH	:	5.8

3.3 Research Material for the Study

Five mulberry genotypes namely S_{36}, V_1, S_{13}, RFS_{135} and Mysore local were collected from the germplasm maintained in the Department Sericulture, UAS, GKVK, Bangalore.

Antitransparents: Glycerol, paraffin wax, grease petroleum.

Potting Mixtures: Pots, soil, peat, perlite, vermiculate, FYM, compost.

3.3.1 Preparation of Stock Solution

(a) 2, 4-D Stock Solution (1mg/ml)

100mg of 2, 4-D was weighed and dissolved in 100µl of 0.2N NaOH. Then the volume made up to 100ml with sterile double distilled water, filtered and stored at 4°C.

Table-1: Composition of Murashige and Skoog's basal medium (1962) stock solutions

Constituents	Mg/Lit	Stock solution (mg/10lit)	Group	Volume made	Ml/lit medium
Macronutrients					
NH_4NO_3	1650	16500	I	250ml	25ml
KNO_3	1900	19000			
$MgSO_4$	370	3700			
KH_2PO_4	170	1700			
$CaCl_2\ 2H_2O$	440	4400	II	100ml	10ml
Micronutrients					
$FeSO_4.\ 7H_2O$	27.85	278.50	III	50ml	5ml
Na_2EDTA	37.25	372.50			
H_3BO_3	6.20	62.00	IV	100ml	10ml
$MnSO_4.\ 4H_2O$	22.30	223.0			
$ZnSO_4.\ 7H_2O$	8.60	86.00			
KI	0.83	8.30			
$Na_2MoO_4.2H_2O$	0.25	2.50			
$CuSO_4.5H_2O$	0.025	0.25			
$CoCl_2.\ 6H_2O$	0.025	0.25			
Vitamins					
Myoinositol	100	1000	V	100ml	10ml
Nicotinic acid	0.50	5.00			
Pyridoxine HCl	0.50	5.00			
Thiamine HCl	0.10	1.00			
Glycine	2.00	20.00			

(b) Benzyl Adenine Purine (BAP) Stock Solution (2mg/ml)

200mg of BAP was weighed and dissolved in 1ml of 0.2N HCl and the volume was made up to 100ml with hot sterile double distilled water, filtered and stored at 4°C.

(c) Per cent $HgCl_2$ Stock Solution

Take 2.0ml of 5.0 per cent $HgCl_2$ and dilute to 100ml with double distilled water.

(d) Naphthalene Acetic Acid (NAA) Stock Solution (1mg/ml)

100mg of NAA weighed and dissolved in 100µl of 0.2N NaOH. Then the volume was made upto 100ml with sterile double distilled water, filtered and stored at 4°C.

(e) Indole Butric Acid (IBA) Stock Solution (1mg/ml)

100mgof IBA was weighed and dissolved in 100µl of 0.5N HCl. Then the volume made upto 100ml with sterile double distilled water, filtered and stored at 4°C.

(f) Indole Acetic Acid (IAA) Stock Solution (1mg/ml)

100mg of IAA was weighed and dissolved in 100µl of 0.2N NaOH. Then the volume made upto 100ml with sterile double distilled water, filtered and stored at 4°C.

(g) 1.0 Per cent Bavistin Stock Solution

1gm of wettable powder of Bavistin was weighed and dissolved in 100ml of double distilled water.

(h) 2.0 Per cent Sodium Hypochlorite (NaClO)

50ml of 4 per cent concentrated NaClO was diluted to 100ml with double distilled water.

(i) 1.0N HCl Stock Solution

8.33ml of concentrated HCl diluted to 100ml by using double distilled water.

(j) 10N NaOH Stock Solution

4.0 grams of NaOH was weighed and dissolve in double distilled water and make up the volume to 10ml.

3.3.2 MS Media Preparation

While preparing culture media, the macro nutrients, micronutrients, vitamins and sucrose were dissolved accordingly and make-up the volume to one liter by adding double distilled water. The pH of the media was adjusted to 5.8 by adding of 1.0N HCl or 1.0N NaOH. Agar was added at the rate of 8 to 10g/L and melted by heating, then dispensed the media of about 30ml to the tissue cultured bottles and autoclaved at 121°C at a pressure of 15l/PSI for 15 minutes.

3.4 Standardization of Sterilization Protocol for Explants of Mulberry

To know the optimum concentration of $HgCl_2$ and time of treatment for 100 per cent survivability of explants. Different concentrations of $HgCl_2$ were tried for different durations. The treated explants were placed on basal MS medium and observed for contamination.

3.4.1 Preparation of Explant

- Mulberry cutting 15 to 20cm in length were collected during cool hours.
- Nodal buds were extracted from the healthy cuttings and washed thoroughly under running water for 4 to 5 times.
- The explant material was treated with 1 to 2 drops of Tween-20 and washed thoroughly with tap water for 3 to 4 times.

- The material was treated with 1 per cent Bavistin for 20 minutes, washed with sterile water till all the residues are washed out and
- The explant material was treated with 0.1 per cent $HgCl_2$.
- Rinsed with sterile water 4 to 5 times.
- Explants were treated with 2.5 per cent sodium hypochlorite (NaClO) for 3 minutes.
- Finally the plant material was thoroughly washed with sterile water for 4 to 5 times. All these operations were done under laminar airflow cabinet.
- Observations for per cent survivability were recorded.

The concentration at which the per cent survivability was 100 was standardized for sterilization of nodal explants was found to be at 0.1 per cent $HgCl_2$ for 8 minutes (Table 2).

3.4.2 Selection of material for Explant preparation (Plate 1)

Nodal Bud Explants

Nodal buds were collected from the healthy plants, 15 to 20cm long and pencil thick cuttings from the middle portion of the mature plant are procured during cool hours (Plate 2).

3.4.3 Preparation of Explants

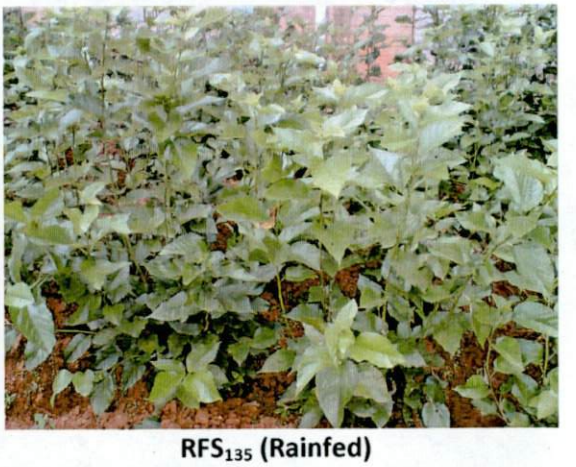
Nodal Bud Explants

Surface sterilized nodal buds were taken and the edges cut to eliminate the immature plant part as well as the $HgCl_2$ affected tissues at the base. Then the scales around the bud were removed and cut to make two portions namely, upper and lower portions and placed in culture media with cut ends facing the media.

S_{36} (Irrigated)

V_1 (Irrigated)

S_{13} (Rainfed)



RFS_{135} (Rainfed)

Mysore Local (Control)

Plate 1: Mulberry Genotypes

3.5 Preparation of Transfer Area for Aseptic Manipulation

All the aseptic manipulations like, surface sterilization, preparation and inoculation of explants and their sub-culturing were carried out in a laminar air flow cabinet. Before using the laminar air flow bench, the working surface was sterilized with 70 per cent ethyl alcohol. Later, the UV light was switched on for 20 minutes. Then the airflow was switched on and left for at least 15 minutes before use. During the course of transfer, between each transfer the surgical instruments used were dipped in 70 per cent, ethyl alcohol followed by dipping in glass bead sterilizer for 15 to 20 seconds and cooled before use. After the completion of sterile transfer operation the laminar hood was cleaned and sprayed with 70 per cent ethyl alcohol and kept closed.

3.6 Incubation Room

The culture bottles were closed with plastic and sealed with parafilm wrap and were maintained in the culture room at a temperature of 25±2°C and relative humidity at 55 to 60 per cent under built in white fluorescent light at a photon flux density of 30 to 50$\mu m^{-2}s^{-1}$/3000 LUX under a photoperiod of 16 hours of light and 8 hours of darkness.

3.7 Standardization of Callus Induction from Nodal Buds

This experiment was conducted to find out the optimum concentration of growth regulators for callus induction. Ten treatment were formulated using MS Medium as basal media with different concentrations of 2, 4-D and BAP. The treatments are as follows: -

T_1 : MS + 2.0mg/L of 2, 4-D + 0.5mg/L BAP

T_2 : MS + 2.0mg/L of 2, 4-D + 0.75mg/L BAP

T_3 : MS + 2.0mg/L of 2, 4-D + 1.00mg/L BAP

T_4 : MS + 2.5mg/L of 2, 4-D + 0.5mg/L BAP

T_5 : MS + 2.5mg/L of 2, 4-D + 0.75mg/L BAP

T_6 : MS + 2.5mg/L of 2, 4-D + 1.00mg/L BAP

T_7 : MS + 3.0mg/L of 2, 4-D + 0.5mg/L BAP

T_8 : MS + 3.0mg/L of 2, 4-D + 0.75mg/L BAP

T_9 : MS + 3.0mg/L of 2, 4-D + 1.00mg/L BAP

T_{10} : Plain MS (Control)

Observations Recorded

1. Number of explants responding
2. Per cent response
3. Days taken for swelling
4. Days taken for callus induction in numbers
5. Callus colour – white, creamy, yellowish, brown, greenish etc.
6. Callus type – Friable, compact or modular
7. Proliferation of (callus intensity) represented by amount of callus was recorded by scoring as follows.

   \- = No. callus

   \+ = Low

   ++ = Good

   +++ = Profuse Callusing

8. Callus differentiation (Per cent)

   Each treatment had 3 replications with five genotypes and observations were recorded at 4 days intervals.

3.9 Standardization of Regeneration from Nodal Buds

This experiment was conducted to find out the optimum concentration of growth regulators for producing maximum multiple shoots. Friable callus obtained from nodal buds explants of five mulberry genotypes were transferred to the MS media containing different concentrations of BAP and IAA for morphogenesis. Ten treatments followed are as follows.

T_1 : MS + 2.0mg/L BAP + 0.10mg/L IAA

T_2 : MS + 2.0mg/L BAP + 0.15mg/L IAA

T_3 : MS + 2.0mg/L BAP + 0.20mg/L IAA

T_4 : MS + 2.5mg/L BAP + 0.10mg/L IAA

T_5 : MS + 2.5mg/L BAP + 0.15mg/L IAA

T_6 : MS + 2.5mg/L BAP + 0.20mg/L IAA

T_7 : MS + 3.0mg/L BAP + 0.10mg/L IAA

T_8 : MS + 3.0mg/L BAP + 0.15mg/L IAA

T_9 : MS + 3.0mg/L BAP + 0.20mg/L IAA

T_{10} : MS (Control)

Each treatment had three replications with five genotypes and observations were recorded at 5 days intervals.

Observations Recorded

1. Mean number of shoots / explants
2. Shoot length (cm)

3.10 Standardization of Root Induction for the Explants of Mulberry

For faster and better induction of roots NDA, IBA and IAA were used. The well grown, elongated shoots were transferred to the rooting media containing different concentrations of NAA, IBA and IAA and response was observed. Treatments are as follows: -

T_1 : MS + 0.5mg/L NAA

T_2 : MS + 1.0mg/L NAA

T_3 : MS + 1.5mg/L NAA

T_4 : MS + 0.5mg/L IBA

T_5 : MS + 1.0mg/L IBA

T_6 : MS + 1.5mg/L IBA

T_7 : MS + 0.5mg/L IAA

T_8 : MS + 1.0mg/L IAA

T_9 : MS + 1.5mg/L IAA

T_{10} : MS (Control)

Each treatment had three replications with five genotypes and observations were recorded at 5 days intervals.

Observations Recorded

1. Rooting per cent
2. Mean number of roots / explants

3.11 Effect of Antitransparents in hardening process tissue cultured mulberry plants

This experiment was conducted to find out the optimum concentrations of antitransparents to avoid the water loss (Transpiration rate) of the mulberry plant. Ten treatments were formulated with different concentrations. Treatments are as follows: -

T_1 : Spray of Glycerol 25%

T_2 : Spray of Glycerol 50%

T_3 : Spray of Glycerol 75%

T_4 : Spray of low melting paraffin wax

T_5 : Spray of medium melting paraffin wax

T_6 : Spray of high melting paraffin wax

T_7 : Application of 25% grease petroleum

T_8 : Application of 50% grease petroleum

T_9 : Application of 75% grease petroleum

T_{10} : Control without antitransparent

Each treatment had three replications with five genotypes and observations were recorded at 5 days intervals.

Observation Recorded

Number of leaves / plant.

3.12 The Performance of potting mixture in hardening techniques of mulberry

This experiment is done to find out the best potting mixture for the tissue cultured mulberry plantlets in presence of different concentrations of growth hormones and regulators. Treatments are as follows:

T_1 : Peat: Perlite: Vermiculate (1:1:1) + 1ml MS + 0.5ppm of NAA + 0.5ppm of IBA + 0.5ppm IAA

T_2 : Peat: Perlite: Vermiculate (1:1:1) + 1ml MS + 1ppm of NAA + 1ppm of IBA + 1ppm IAA

T_3 : Peat: Perlite: Vermiculate (1:1:1) + 1ml MS + 2ppm of NAA + 2ppm of IBA + 2ppm IAA

T_4 : Sand: Soil: Perlite + 1ml MS + 0.5ppm of NAA + 0.5ppm of IBA + 0.5ppm IAA

T_5 : Sand: Soil: Perlite + 1ml MS + 1ppm of NAA + 1ppm of IBA + 1ppm IAA

T_6 : Sand: Soil: Perlite + 1ml MS + 2ppm of NAA + 2ppm of IBA+ 2ppm IAA

T_7 : Sand: Soil: FYM / Compost

T_8 : Sand: Soil (Control)

Each treatment had 3 replications with five genotypes and observations were recorded at 5 days intervals.

Observations Recorded

1. Inter nodal length (cm)
2. Shoot length (cm)
3. Root length (cm)

3.13 Effect of shading percentage in hardening process of mulberry plants

This experiment was done to find out the optimum percentage of shading levels necessary for survival of the tissue cultured mulberry plants. Treatments are as follows:

T_1 : 100% shading

T_2 : 75% shading

T_3 : 50% shading

T_4 : 25% shading

T_5 : Control (Direct sunlight)

Each treatment had 3 replications with five genotypes and observations recorded at 5 days intervals.

Observation Recorded

Plant height (cm)

3.14 Field Treatment for *in vitro* mulberry Plants

This experiment was done to find out the optimum days required for the well development and good survival per cent for the tissue cultured plant under field conditions.

Survival percentage for 20 days

Survival percentage at 30 days

Survival percentage at 45 days

Observations Recorded

1. Survival Percentage
2. General Appearance of Plant

3.15 Standardization of hardening protocol of *in vitro* grown plants

To establish *in vitro* plants *in vivo*, plants with well developed shoots and roots were taken out from the bottles. Then thoroughly washed with sterile water to remove traces of agar, and then dipped in an antifungal solution like Dithane M-45 (0.25 per cent). Then plantlets were treated with antitransparents like glycerol to avoid transpiration rate. The plantlets were then transferred to clean plastic cups containing pasteurized garden soil (Peat: Perlite : Vermiculate) 1:1:1 1ml strength of MS liquid media was added in each cup. The

cups were then covered with thin, transparent polythene covers and kept in the culture room for a week. The temperature and humidity of the culture room were maintained at 25±2°C and 80 per cent RH.

After a week the plants were transferred to field. Shade was provided initially for 2 weeks using coconut leaves. Watering was done every day initially for 1 week and then the plants were watered once in two days. Survival percentage was recorded for all five genotypes.

3.16 Statistical Analysis

The data collected in this study were statistically analysed by subjecting to ANOVA as described by Sunder Raj *et al.* (1972) with factorial Completed Randomized Design (CRD).

EXPERIMENTAL RESULTS

IV. EXPERIMENTAL RESULTS

Experiment was conducted to "**Standardize the Hardening Protocol of Tissue Cultured Mulberry Plants (*Morus* spp.)**". The results of this experiment are presented under the following headings.

4.1 Standardization of Sterilization Protocol for Explants of Mulberry

Survival per cent of explants were found to be significantly different with the duration of treatments. The survival rate of explants ranged between 22.01 to 79.36 per cent with different durations and at different concentrations. Among the different durations, the survival per cent of explants was significantly more at 8 minutes (79.36%) followed by 6 minutes (51.38%) at a concentration of 0.1 per cent of $HgCl_2$. The treatments, duration and interactions were all significant (Table 2 and Fig. 1).

4.2 Effect of Growth Hormones on Callus Initiation

The per cent response of callus initiation in the nodal explants of mulberry was found to be significantly different among the treatments and genotypes. Among the genotypes the per cent response varied form 27.04 (Mysore local) to 60.76 S_{36} per cent. Among the irrigated mulberry genotypes S_{36} showed significantly highest response for callus initiation (60.76%) than V_1 (60.06%). Among rainfed mulberry genotypes, maximum response was recorded in S_{13} (45.86%) than RFS_{135} (31.12%) compared to the check Mysore local (27.04%) (Table 3 and Fig. 2).

Significantly maximum response of callus initiation was observed in the nodal explants of mulberry when cultured on MS-medium supplemented with 2.0mg/L 2, 4-D along with 1.00, 0.75 and 0.50mg/L BAP (51.06, 50.65 and 45.73% respectively compared to control (46.10%).

Table 2: Effect of $HgCl_2$ concentration and duration of treatment on the survival of explants

$HgCl_2$ Concentration %	Survival (%) of Explants				
	4 Min.	6 Min.	8 Min.	10 Min.	Mean
0.05	54.05 (47.32)	41.50 (40.10)	34.35 (35.88)	21.36 (27.52)	37.81 (37.70)
0.1	53.42 (46.96)	61.05 (51.38)	96.56 (79.36*)	46.00 (42.70)	64.26 (55.10)
0.3	45.77 (42.57)	34.25 (35.82)	21.41 (27.56)	33.46 (35.34)	33.72 (35.32)
0.5	32.46 (34.75)	23.13 (28.74)	14.05 (22.01)	14.21 (22.14)	20.96 (26.91)
Mean	46.42 (42.89)	39.92 (39.01)	41.60 (41.20)	31.43 (31.93)	39.19 (38.76)

Test of Significant	F-test	SEm±	CD at 5%
Treatment Duration (A)	*	0.19 (0.13)	0.55 (0.38)
$HgCl_2$ Concentration (B)	*	0.19 (0.13)	0.55 (0.38)
A x B	*	0.38 (0.26)	1.10 (0.76)

*Significant at 5%

Note: Figures in the parenthesis indicate Arc sign transformation

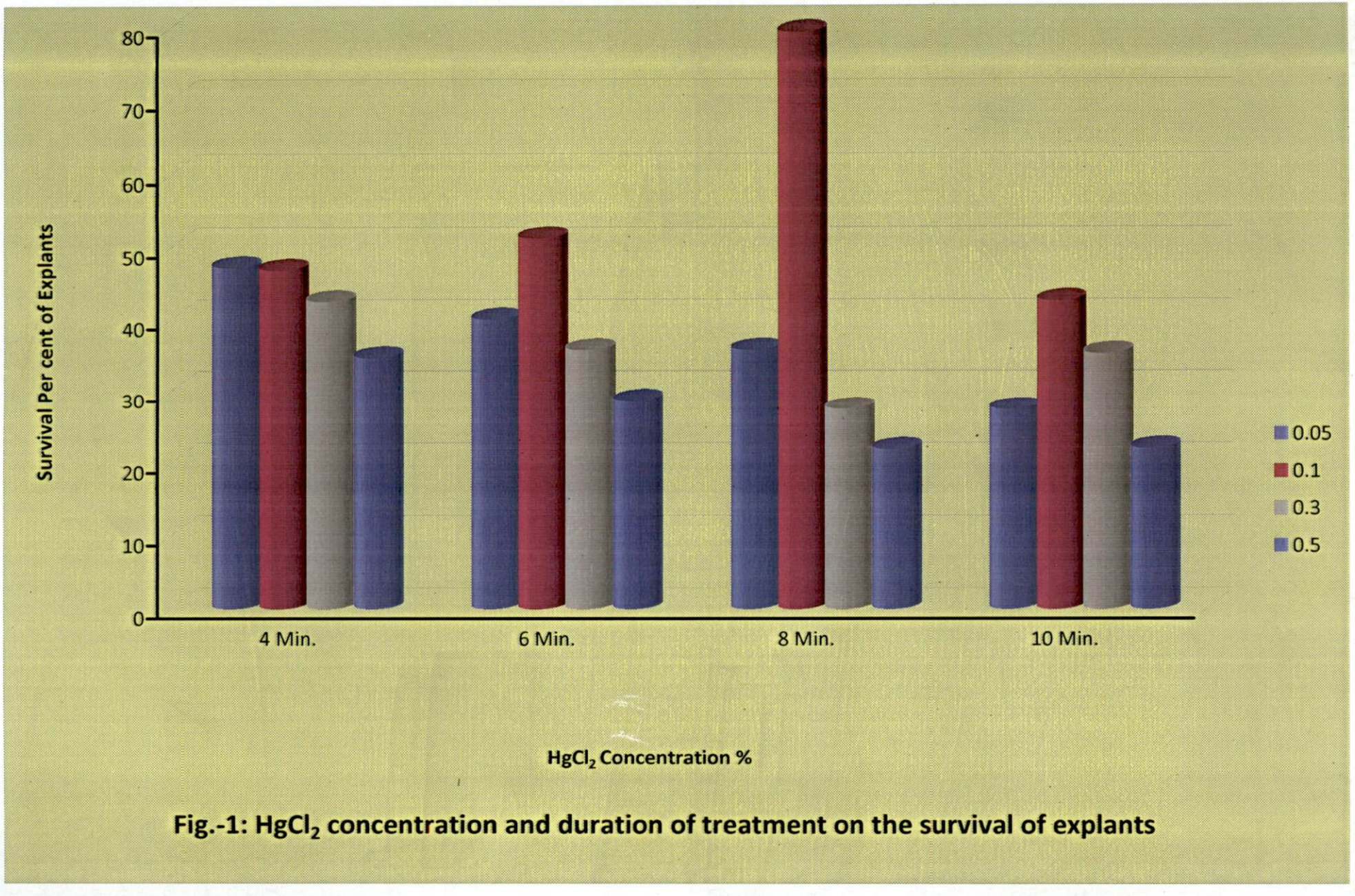


Fig.-1: $HgCl_2$ concentration and duration of treatment on the survival of explants

Table 3: Effect of Growth Harmones on Per cent Callus Initiation Response of Mulberry Genotype

Treatments MS + 2, 4-D + BAP (mg/L)	S-36	V-1	S-13	RFS-135	Mysore Local	Mean
T_1 : MS+2.0+0.50	71.06 (57.46)	63.41 (52.79)	51.89 (46.08)	52.13 (46.22)	19.40 (26.13)	51.58 (45.73)
T_2 : MS+2.0+0.15	84.30 (66.67)	88.70 (71.16)	(44.30) 48.80	(40.84) 42.78	(30.28) 25.47	(50.65) 58.01
T_3 : MS+2.0+1.00	(74.63) 92.90	(58.46) 72.63	(58.30) 72.15	(34.79) 32.57	(29.15) 23.78	(51.06) 58.81
T_4 : MS+2.5+0.50	(67.61) 85.31	(59.44) 74.11	(51.42) 61.10	(37.05) 36.32	(32.36) 28.66	(49.57) 57.10
T_5 : MS+2.5+0.75	(65.18) 82.35	(56.51) 69.53	(46.93) 53.36	(38.49) 38.75	(28.12) 22.23	(47.05) 53.24
T_6 : MS+2.5+1.00	(67.94) 85.85	(66.86) 84.49	(46.32) 52.30	(39.06) 39.72	(24.34) 17.05	(48.90) 55.89
T_7 : MS+3.0+0.50	(57.66) 71.38	(63.66) 80.21	(46.99) 53.46	(28.60) 22.95	(21.48) 13.53	(43.68) 48.31
T_8 : MS+3.0+0.75	(35.35) 33.50	(55.05) 67.17	(41.12) 43.26	(30.51) 25.83	(24.06) 16.73	(37.22) 37.30
T_9 : MS+3.0+1.00	(60.76) 76.13	(53.52) 64.63	(35.78) 34.22	(35.33) 33.46	(23.21) 15.63	(41.72) 44.81
T_{10} : Control (Plain MS)	(54.33) 65.92	(63.19) 79.17	(41.39) 43.73	(40.32) 41.89	(31.29) 27.02	(46.10) 51.54
Mean	(60.76) 74.87	(60.06) 74.40	(45.86) 51.43	(31.12) 36.64	(27.04) 20.95	(46.17) 51.66

Test of Significant	F-Test	SEm±	CD at 5%
Mulberry Genotypes (A)	*	(0.46) 0.67	(1.29) 1.89
Treatments (B)	*	(0.65) 0.95	(1.83) 2.68
A x B	*	(1.47) 2.14	(4.10) 6.00

Significant at 5%

Note: Figures in the parenthesis indicate Arc sign transformation.

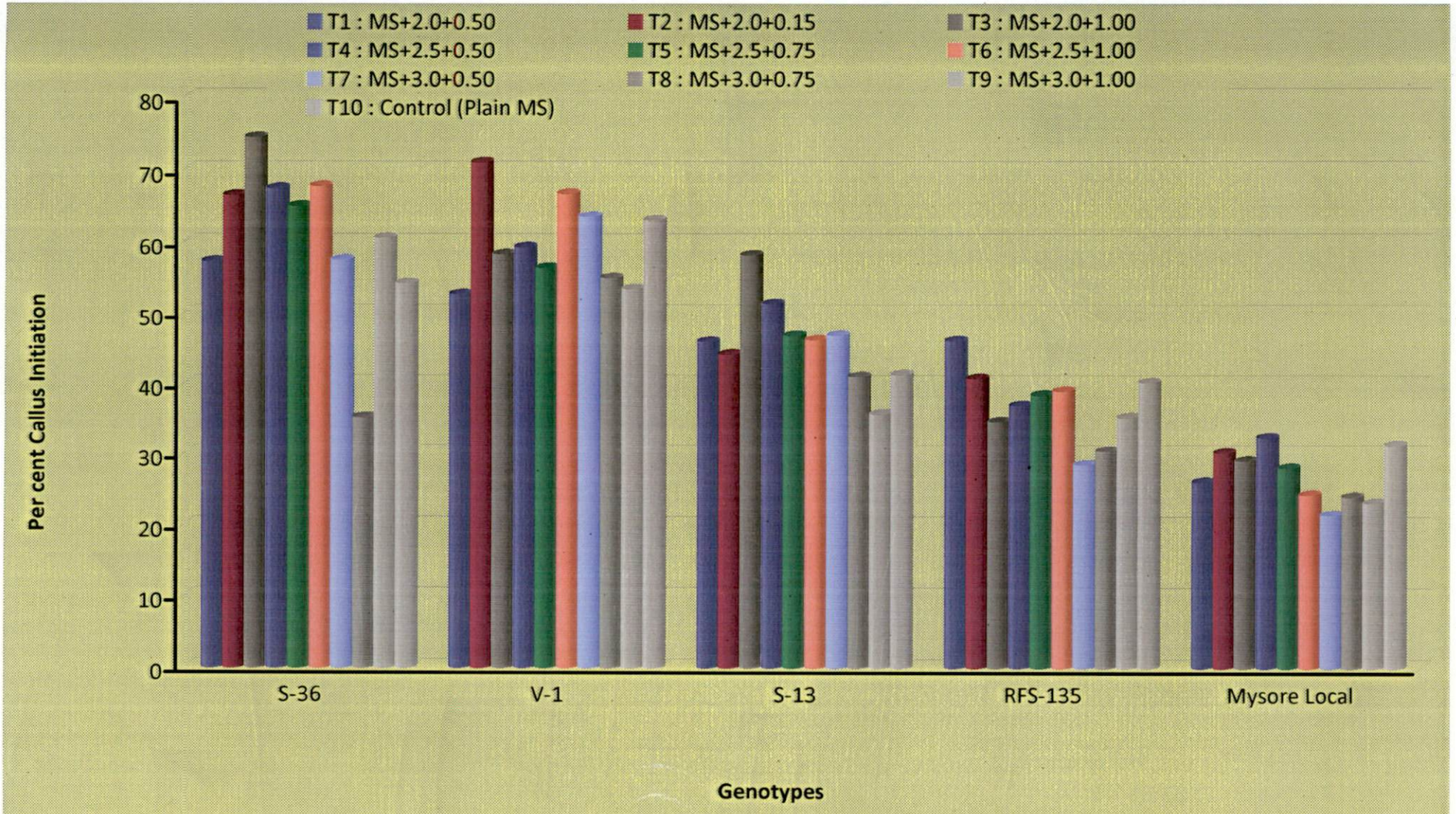


Fig.-2: Growth Harmones on Per cent Callus Initiation Response of Mulberry Genotype

Among the interactions, nodal explants obtained from the irrigated mulberry genotypes S_{36} recorded significantly higher response (60.76%) compared to V_1 (60.06%) when cultured on MS-Medium supplemented with 2.0mg/L 2, 4-D + 1.00mg/L BAP and 2.0mg/L 2, 4-D + 0.75mg/L BAP respectively. Whereas in case of rainfed genotypes S_{13} showed significantly higher response (45.86%) compared to RFS_{135} (31.12). When MS was fortified with 2.0mg/L 2, 4-D + 1.00mg/L BAP and 2.5mg/L 2, 4-D + 0.50mg/L BAP respectively compared to Mysore local (27.04%) on plain MS with and growth regulators.

Explants obtained from the nodal buds of mulberry genotypes have taken 4.00 to 7.00 days (Table 4) for swelling among different varieties and for different treatments. Among the varieties, V_1 has taken less number of days (4.93 days) for swelling of the explants compared to RFS_{135} (5.93 days), whereas in case of irrigated variety, S_{36} responded significantly faster (5.10 days than the rainfed variety S_{13} (5.83 days) compared to Mysore local (5.86 days) (Fig. 3).

Faster response was recorded in the genotypes (4.86 days) when cultured on MS medium supplemented with 2.0mg/L 2, 4-D and 0.75mg/L BAP compared to the control (6.40 days) (Plate 3).

The number of days taken for callus initiation in the nodal explants of mulberry was found to significantly varying from 10.33 to 14.33 days (Table 5). Among the varieties, callus was initiated significantly faster in V_1 (10.92 days) than RFS_{135} (13.26 days), while irrigated genotypes V_1 responded faster (10.92 days) than S_{36} (10.93 days) compared to Mysore local (13.46 days) (Fig. 4 and Plate 4).

Among the different treatments, MS supplemented with 2.5mg/L 2, 4-D and 0.50mg/L BAP showed non significant faster

Table 4: Effect of Growth Hormones on Number of Days taken for Swelling in Mulberry Genotypes

Treatments MS + 2, 4-D + BAP (mg/ml)	S-36	V-1	S-13	RFS-135	Mysore Local	Mean
T_1 : MS+2.0+0.50	5.33 (2.41)	4.66 (2.26)	4.66 (2.26)	5.00 (2.33)	5.66 (2.48)	5.06 (2.35)
T_2 : MS+2.0+0.75	5.00 (2.33)	4.66 (2.66)	5.33 (2.41)	5.00 (2.33)	4.33 (2.19)	4.86 (2.31)
T_3 : MS+2.0+1.00	4.66 (2.26)	4.33 (2.19)	5.33 (2.41)	6.00 (2.54)	5.66 (2.46)	5.20 (2.37)
T_4 : MS+2.5+0.50	5.66 (2.46)	4.33 (2.19)	6.66 (2.67)	5.33 (2.41)	7.00 (2.73)	5.80 (2.49)
T_5 : MS+2.5+0.75	4.33 (2.19)	4.66 (2.27)	5.00 (2.33)	6.66 (2.67)	6.33 (2.61)	5.40 (2.41)
T_6 : MS+2.5+1.00	4.00 (2.12)	4.33 (2.19)	6.33 (2.61)	6.66 (2.67)	5.00 (2.33)	5.26 (2.38)
T_7 : MS+3.0+0.50	4.66 (2.27)	4.66 (2.27)	6.66 (2.67)	6.33 (2.60)	6.00 (2.53)	5.66 (2.47)
T_8 : MS+3.0+0.75	5.33 (2.40)	5.33 (2.40)	5.6 (2.47)	6.66 (2.67)	6.33 (2.60)	5.86 (2.51)
T_9 : MS+3.0+1.00	5.33 (2.40)	6.66 (2.67)	6.00 (2.54)	5.66 (2.48)	5.33 (2.40)	5.80 (2.50)
T_{10} : Control	6.66 (2.67)	5.66 (2.48)	6.66 (2.67)	6.00 (2.54)	7.00 (2.73)	6.40 (2.62)
Mean	5.10 (2.35)	4.93 (2.32)	5.83 (2.50)	5.93 (2.50)	5.86 (2.51)	5.53 (2.44)

Test of Significant	F-Test	SEm±	CD at 5%
Mulberry Genotypes (A)	*	0.16 (0.03)	0.47 (0.09)
Treatments (B)	*	0.23 (0.04)	0.66 (0.06)
A x B	NS	0.53 (0.10)	1.49 (0.30)

* - Significant at 5%

NS – Nonsignificant

Note: Figures in the parenthesis indicate square root transformation.

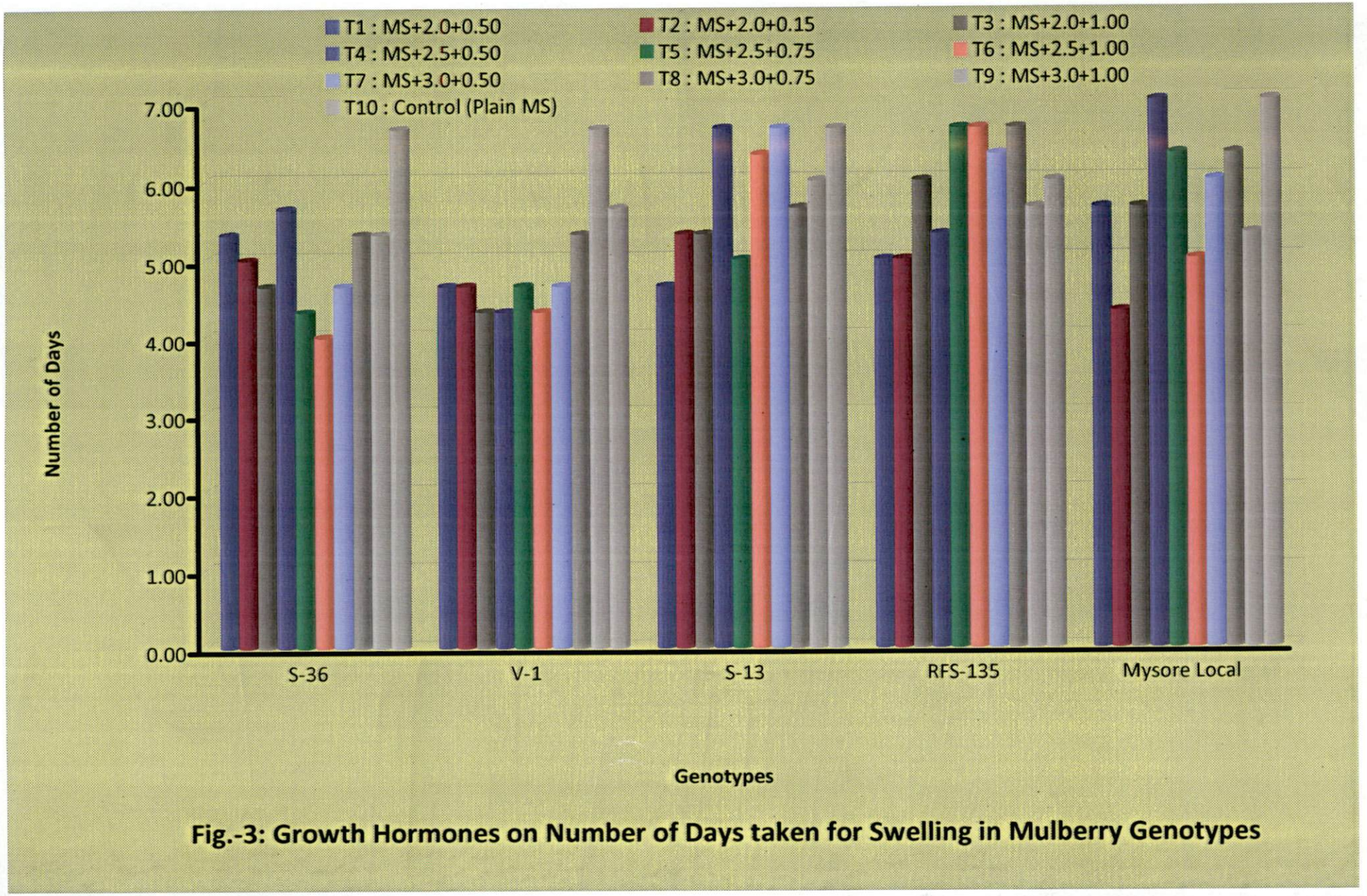


Fig.-3: Growth Hormones on Number of Days taken for Swelling in Mulberry Genotypes

Table 5: Effect of Growth Hormones on Number of Days taken for Callus Initiation in Mulberry Genotypes

Treatments MS + 2, 4-D + BAP (mg/L)	S-36	V-1	S-13	RFS-135	Mysore Local	Mean
T_1 : MS+2.0+0.50	12.33	11.00	12.33	13.33	13.66	12.53
T_2 : MS+2.0+0.75	11.00	11.00	12.66	13.66	14.33	12.53
T_3 : MS+2.0+1.00	10.33	10.66	12.00	13.00	14.33	12.06
T_4 : MS+2.5+0.50	16.00	10.66	12.66	12.00	12.66	11.80
T_5 : MS+2.5+0.75	10.33	10.66	12.66	14.00	14.00	12.33
T_6 : MS+2.5+1.00	11.00	11.00	12.33	13.00	12.66	12.00
T_7 : MS+3.0+0.50	11.00	11.33	12.00	12.66	13.00	12.00
T_8 : MS+3.0+0.75	10.66	10.66	11.33	14.00	14.00	12.13
T_9 : MS+3.0+1.00	10.66	10.66	12.33	14.00	13.00	12.13
T_{10} : Control	11.00	11.66	12.00	13.00	13.00	12.13
Mean	10.93	10.92	12.23	13.26	13.46	12.16

Test of Significant	F-Test	SEm±	CD at 5%
Mulberry Genotypes (A)	*	0.178	0.50
Treatments (B)	NS	0.25	0.70
A x B	NS	0.56	1.58

* - Significant at 5%

NS – Nonsignificant

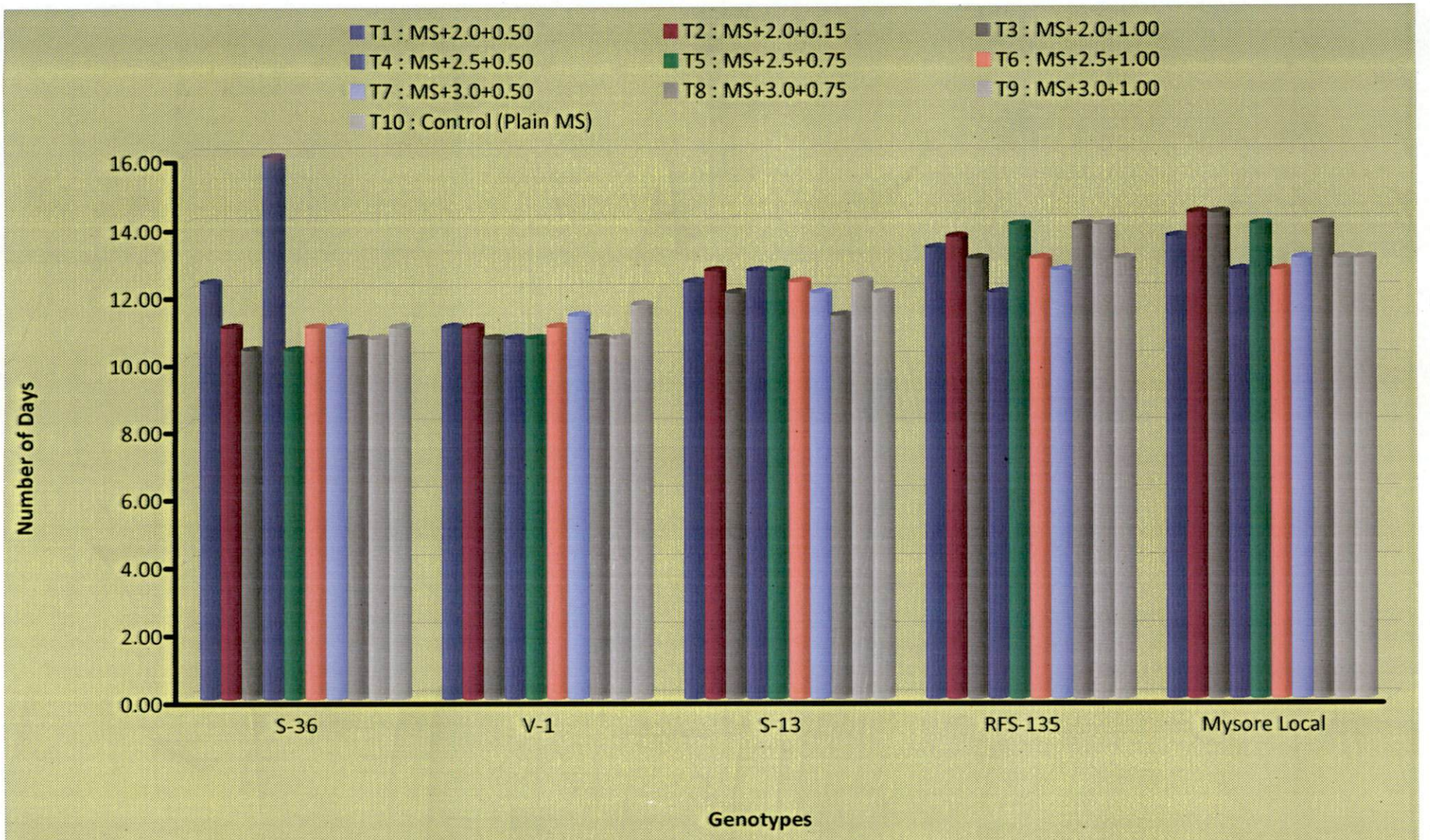


Fig.-4: Growth Hormones on Number of Days taken for Callus Initiation in Mulberry Genotypes

response (11.80 days) compared to control (12.13 days) without growth regulators.

Among the interactions, nonsignificant faster response (11.08 days) was recorded in V_1 when MS was supplemented with 2.5mg/L 2, 4-D and 1.00mg/L BAP compared RFS_{135} (12.53 days) on plain MS without growth regulators (Table 5).

Yellow friable callus was obtained on media containing higher concentration of 2, 4-D and lower concentration of BAP and highest per cent (85%) (Table 6) of callus formation was found on medium containing 2, 4-D (2mg/L) alone.

4.3 Effect of Growth Hormones on Morphogenesis (Differentiation, Shoot, Shoot Length)

Per cent callus differentiation significantly varied from 18.95 to 75.12 per cent (Table 7). Among the irrigated genotypes S_{36} recorded significantly maximum callus differentiation (60.91) compared to V_1 (51.50%) (Fig. 6 and Plate 5).

Among the rainfed genotype, RFS_{135} recorded higher callus differentiation (33.72%) compared to S_{13} (31.12%). The maximum differentiation was recorded when callus was cultured on MS medium supplemented with 2.5mg/L 2, 4-D + 1.00mg/L BAP (53.26%) compared to the control (26.63%) i.e., with out regulator.

Among the interactions, significantly higher callus differentiation was recorded in S_{36} when fortified with 2.5mg/L 2, 4-D + 1.00mg/L of BAP (75.12%) followed by V_1 on MS with 2.5mg/L 2, 4-D + 0.75mg/L BAP (75.11%) compared to Mysore local on plain MS without growth regular 18.95 per cent.

Table 6: Effect of Growth Regulators on Colour, Texture and Intensity of Callus

Treatments MS + 2, 4-D + BAP (mg/L)	S-36			V-1			S-13			RFS-135			Mysore Local		
	Clr	Tex	Int	Clr	Tex	Int	Clr	Tex	Int	Clr	Tex	Int	Clr	Tex	Int
T_1 : MS+2.0+0.50	Br	F	++	Br	F	++	Br	F	++	Br	F	++	Br	F	+
T_2 : MS+2.0+0.75	Br	F	++	Br	F	+++	Br	F	++	Br	F	++	Br	F	+
T_3 : MS+2.0+1.00	Br	F	+++	Br	F	+++	Br	F	++	Br	F	++	Br	F	+
T_4 : MS+2.5+0.50	Br	F	+++	Br	F	+++	Br	F	++	Br	F	++	Br	F	++
T_5 : MS+2.5+0.75	Br	F	++	Br	F	++	Br	F	++	Br	F	++	Br	F	++
T_6 : MS+2.5+1.00	Br	F	+++	Br	F	++	Br	F	++	Br	F	++	Br	F	+
T_7 : MS+3.0+0.50	Br	F	++	Br	F	+	Br	F	++	Br	F	+	Br	F	+
T_8 : MS+3.0+0.75	Br	F	++	Br	F	+++	Br	F	++	Br	F	+	Br	F	+
T_9 : MS+3.0+1.00	Br	F	+++	Br	F	+	Br	F	+++	Br	F	+	Br	F	+
T_{10} : Control	Br	F	+	Br	F	+	Br	F	+	Br	F	+	Br	F	+

Clr : Callus Colour
Tex : Callus Texture
Int : Callus Intensity
Br : Brown
F : Fraible

\+ : Low → No Callus
++ : Good
+++ : Profuse callusing

Table 7: Effect of Growth Hormones on Callus Differentiation (%) in Mulberry Genotypes

Treatments MS + 2, 4-D + BAP (mg/L)	S-36	V-1	S-13	RFS-135	Mysore Local	Mean
T_1 : MS+2.0+0.50	51.51 (67.63)	75.53 (60.47)	12.54 (20.70)	42.44 (40.65)	22.66 (28.41)	47.73 (43.57)
T_2 : MS+2.0+0.75	85.68 (67.77)	76.20 (60.80)	23.29 (28.84)	37.31 (37.63)	29.31 (32.78)	50.36 (45.57)
T_3 : MS+2.0+1.00	73.79 (59.21)	72.68 (58.51)	17.48 (24.69)	33.41 (35.30)	24.81 (29.86)	44.43 (41.51)
T_4 : MS+2.5+0.50	76.80 (61.23)	86.98 (68.88)	21.90 (27.88)	33.94 (35.63)	23.22 (28.77)	48.57 (44.48)
T_5 : MS+2.5+0.75	74.53 (59.72)	93.35 (75.11)	24.70 (29.78)	33.45 (35.33)	19.06 (25.88)	49.02 (45.16)
T_6 : MS+2.5+1.00	93.36 (75.12)	73.45 (59.01)	75.98 (60.65)	46.55 (43.02)	22.77 (28.40)	62.42 (53.26)
T_7 : MS+3.0+0.50	83.52 (66.06)	70.70 (57.23)	36.67 (37.25)	36.13 (36.94)	26.32 (30.86)	50.67 (45.67)
T_8 : MS+3.0+0.75	86.22 (68.27)	64.50 (53.43)	35.42 (36.63)	28.78 (32.44)	24.32 (29.54)	47.85 (44.04)
T_9 : MS+3.0+1.00	55.43 (48.11)	52.18 (46.24)	22.27 (28.15)	16.80 (24.16)	23.41 (28.93)	34.02 (35.12)
T_{10} : Control	34.52 (35.98)	33.43 (35.32)	20.30 (26.77)	7.71 (16.11)	11.00 (18.95)	21.39 (26.63)
Mean	74.93 (60.91)	69.90 (51.50)	29.05 (32.12)	31.65 (33.72)	22.69 (28.25)	45.64 (42.50)

Test of Significant	F-Test	SEm±	CD at 5%
Mulberry Genotypes (A)	*	0.39 (0.28)	1.11 (0.80)
Treatments (B)	*	0.56 (0.40)	1.57 (1.13)
A x B	*	1.25 (0.90)	3.51 (2.53)

* - Significant at 5%

Note: Figures in the parenthesis indicate Arc sign transformation.

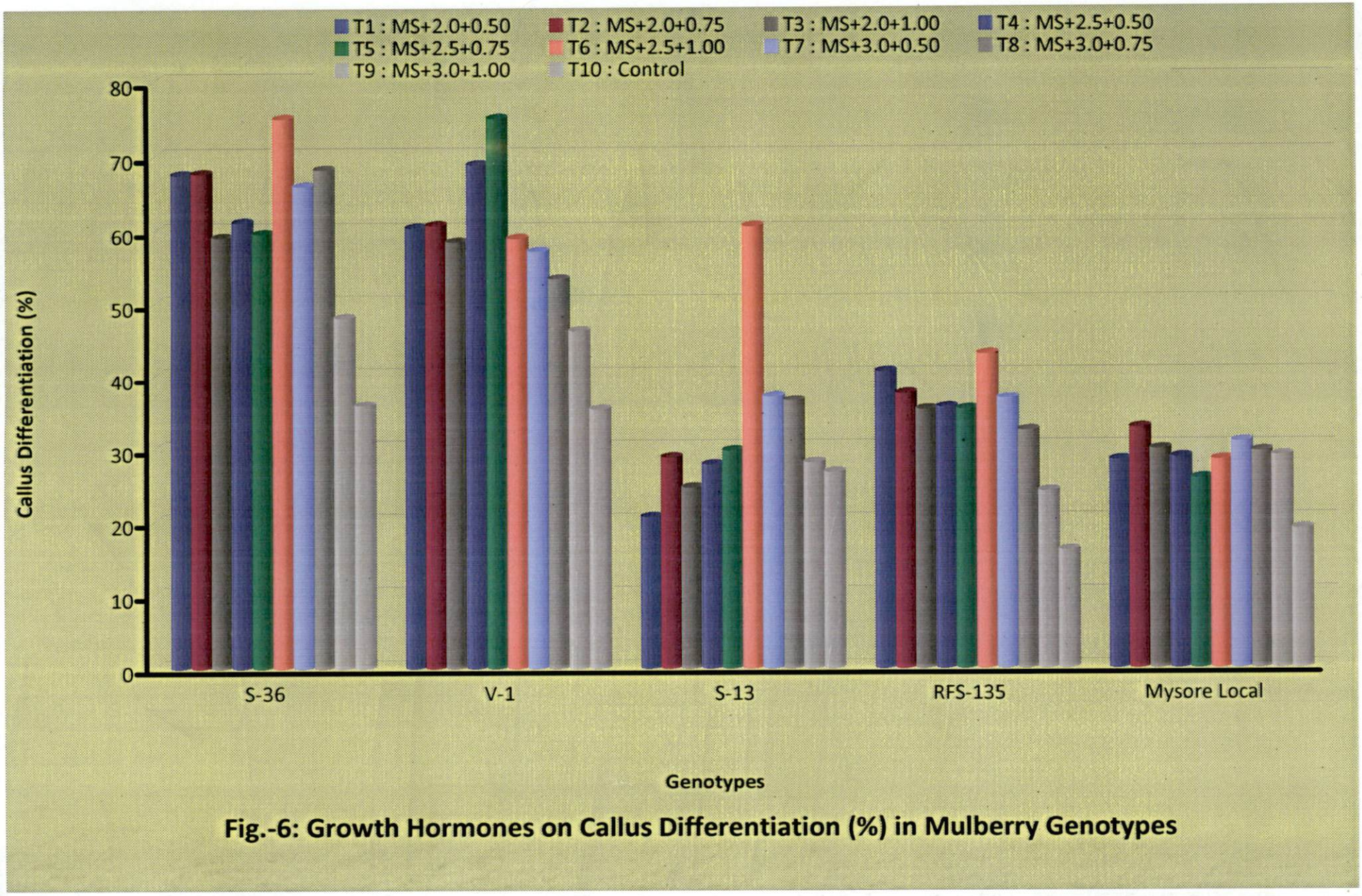


Fig.-6: Growth Hormones on Callus Differentiation (%) in Mulberry Genotypes

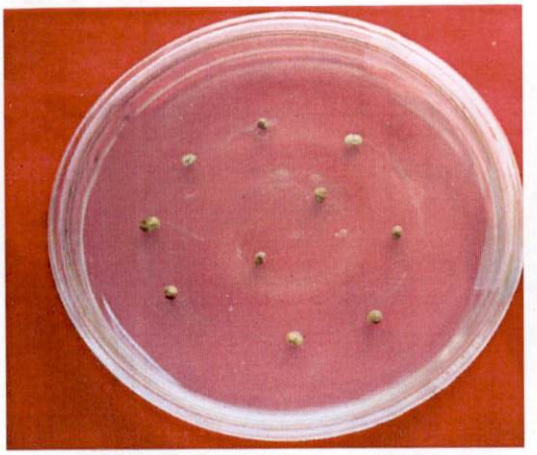


Plate 2: Nodal bud explants

Plate 3: Swelling of nodal buds

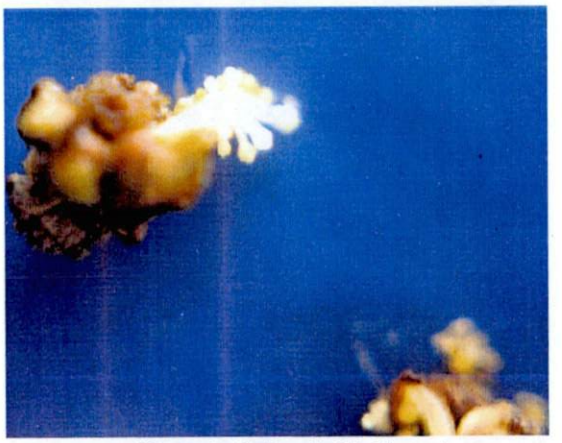


Plate 4: Callus initiation

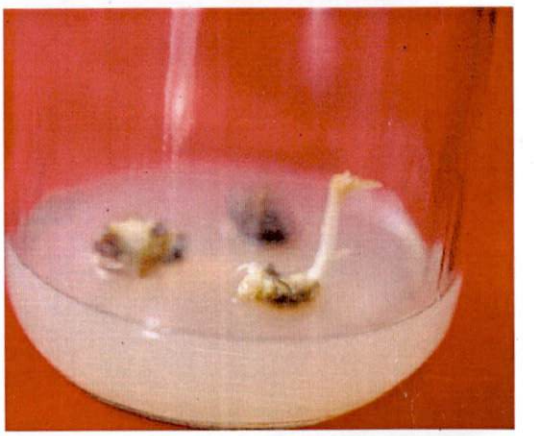


Plate 5: Shoot initiation

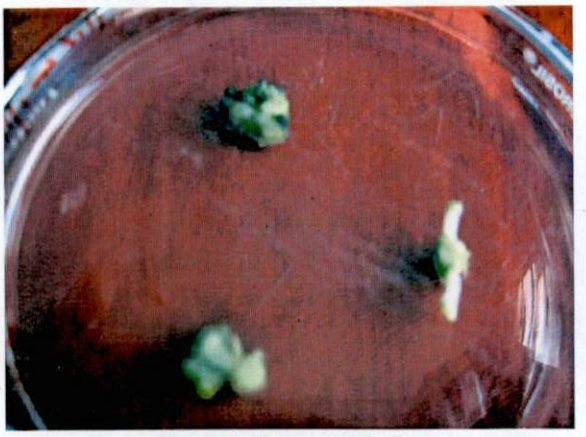


Plate 6: Shoot Initiation

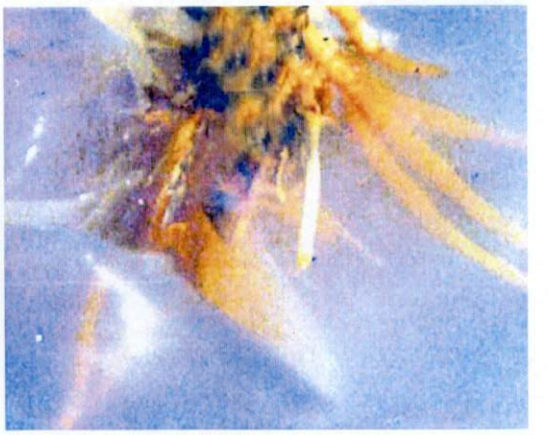


Plate 7: Root Induction

Mean number of shoots per explant recorded significant difference ranging from 3.58 to 36.60 (Table 8). Among the irrigated varieties V_1 recorded more number of shoots (26.72) than S_{36}, whereas in the rainfed genotypes, S_{13} recorded significantly more (14.40) number of shoots compared to the RFS_{135} (13.00) as compared to Mysore local (6.29) (Plate 8).

Among the different treatments, more number of shoots regenerated from callus cultured on MS having 2.5mg/L BAP and 0.20mg/L IAA (23.40%) followed by 2.5mg/L BAP and 0.10mg/L IAA (20.50%) compared to the control (4.28%) without growth regulator.

Among the interactions, more number of shoots regenerated in V_1 (26.72%) when the callus was transferred to MS medium having 2.5mg/L BAP and 0.20mg/L of IAA followed S_{36} (22.97%) where callus was transferred to MS with 2.5mg/L BAP and 0.15mg/L of IAA compared to the Mysore local (6.29%) in control (Fig. 7).

Length of shoots obtained from the callus cultures significantly varied among the treatments and genotypes ranging from 1.20 to 3.80cm (Table 9).

Among the irrigated varieties, V_1 recorded more length of 2.45cm than S_{36} (2.37cm) whereas among the rainfed genotypes, S_{13} showed maximum length (2.41cm) than RFS_{135} (2.26cm) when compared to Mysore local (1.93cm).

The maximum shoot length of 2.97cm was recorded with MS media when fortified with 2.5mg/L BAP and 0.20mg/L IAA followed by MS when fortified with 2.5mg/L BAP and 0.10mg/L IAA, and followed by MS when supplemented by MS when fortified with 2.5mg/L BAP and 0.15mg/L IAA (2.89cm) compared to control (1.27cm).

S_{36}

V_1

S_{13}

RFS_{135}

Mysore Local (Control)

Plate 8: Genotype differentiation

Table 8: Effect of Growth Hormones on Mean Number of Shoots Per Explant in Mulberry Genotypes

Treatments MS + 2, 4-D + BAP (mg/L)	S-36	V-1	S-13	RFS-135	Mysore Local	Mean
T_1 : MS+2.0+0.10	10.51	15.84	6.88	11.33	4.93	9.90
T_2 : MS+2.0+0.15	20.94	39.40	7.77	13.03	7.56	17.74
T_3 : MS+2.0+0.20	28.20	33.63	16.05	16.66	5.40	19.99
T_4 : MS+2.5+0.10	36.51	33.50	15.17	15.33	8.33	21.77
T_5 : MS+2.5+0.15	36.60	33.90	15.99	9.00	5.88	20.27
T_6 : MS+2.5+0.20	35.11	34.22	25.26	13.55	8.89	23.40
T_7 : MS+3.0+0.10	24.28	32.22	24.80	14.77	6.44	20.50
T_8 : MS+3.0+0.15	18.04	21.82	13.08	16.92	6.27	15.23
T_9 : MS+3.0+0.20	14.36	18.22	14.71	15.88	5.34	13.70
T_{10} : MS (Control)	5.12	4.47	4.34	3.58	3.89	4.28
Mean	22.97	26.72	14.40	13.00	6.26	16.68

Test of Significant	F-Test	SEm±	CD at 5%
Mulberry Genotypes (A)	*	0.32	0.92
Treatments (B)	*	0.46	1.30
A x B	*	1.03	2.91

* - Significant at 5%

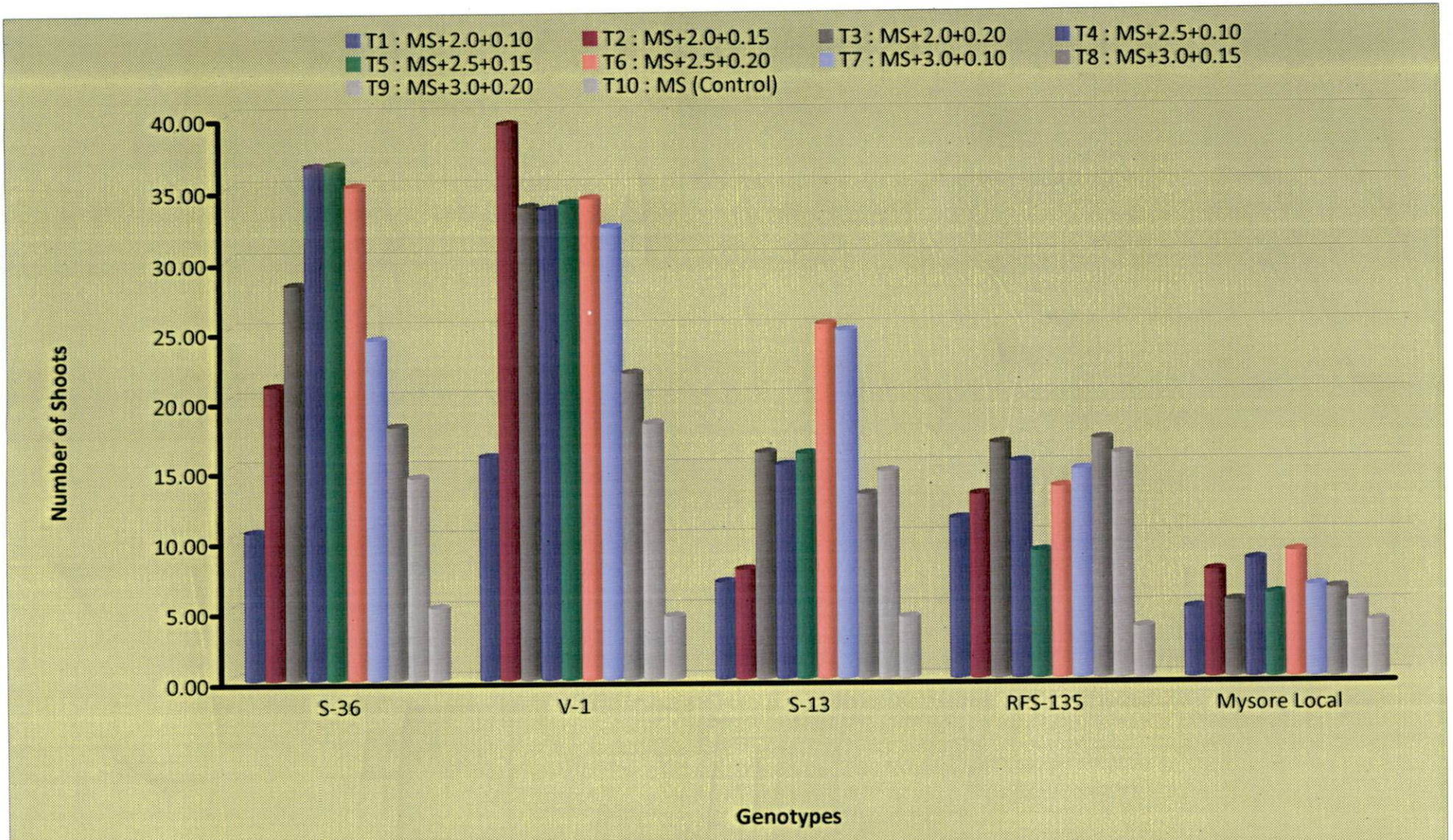


Fig.-7: Growth Hormones on Mean Number of Shoots Per Explant in Mulberry Genotypes

Table 9: Effect of Growth Hormones on Shoot Length (cm) Induced from the Callus of Mulberry Genotypes

Treatments MS + 2, 4-D + BAP (mg/L)	S-36	V-1	S-13	RFS-135	Mysore Local	Mean
T_1 : MS+2.0+0.10	2.09	1.62	1.29	1.65	1.46	1.62
T_2 : MS+2.0+0.15	1.86	1.88	2.15	2.35	2.13	2.08
T_3 : MS+2.0+0.20	2.29	2.16	2.26	2.38	2.38	2.29
T_4 : MS+2.5+0.10	3.43	3.14	2.80	3.37	2.14	2.97
T_5 : MS+2.5+0.15	3.80	2.57	3.21	2.56	2.36	2.29
T_6 : MS+2.5+0.20	3.31	3.19	3.06	3.24	2.10	2.98
T_7 : MS+3.0+0.10	1.70	3.13	3.06	1.52	2.24	2.33
T_8 : MS+3.0+0.15	2.22	2.85	2.60	2.63	1.65	2.39
T_9 : MS+3.0+0.20	1.87	2.66	2.50	1.62	1.60	2.05
T_{10} : MS (Control)	1.23	1.30	1.20	1.30	1.33	1.27
Mean	2.38	2.45	2.41	2.26	1.94	2.28

Test of Significant	F-Test	SEm±	CD at 5%
Mulberry Genotypes (A)	*	0.0209	0.0586
Treatments (B)	*	0.0295	0.0828
A x B	*	0.0660	0.1852

* - Significant at 5%

Among the interactions, maximum shoot length 3.80cm was recorded in S_{36} with the treatment of 2.5mg/L BAP and 0.15mg/L IAA followed by V_1 (3.18) with the treatment of 2.5mg/L BAP and 0.20mg/L IAA (Fig. 8).

4.4 Effect of Growth Hormones on Root Formation

Shoots obtained from the callus rooted when cultured with rooting hormones and the per cent response varied significantly higher among the genotypes and treatments (Table 10 and Plate 7). The response ranged from 17.57 to 55.40 per cent. Among the irrigated genotypes, S_{36} recorded high rooting per cent (37.29%) than V_1 (37.19%), whereas among the rainfed genotypes, S_{13} recorded higher rooting per cent (39.26%) than the RFS_{135} (32.61) compared to Mysore local (33.16%) (Fig. 9).

Shoots rooted well in treatments, when MS supplemented with 1.0mg/L NAA, 1.5mg/L NAA, 1.5mg/L IBA and 1.0mg/L IAA i.e., NAA followed by IBA and IAA. Among the different concentrations, significantly higher rooting percentage (48.07, 40.82%) were recorded when shoots were transferred to MS having 1.0mg/L and 1.5mg/L NAA, respectively (Plate 6).

The interaction effects were found significant. Number of roots induced from the shoots of different mulberry genotypes with different treatments significantly varied form 1.64 to 8.03 (Table 11 and Fig. 10).

Among the rainfed genotypes, more number of roots were induced in RFS_{135} (4.57 than S_{13}) (4.55), where as among the irrigated genotypes V_1 (5.22) recorded more number of roots than S_{36} (5.05) as compared to control Mysore local (4.28).

More number of roots were obtained when shoots were transferred to MS having NAA followed by IBA and IAA. Maximum

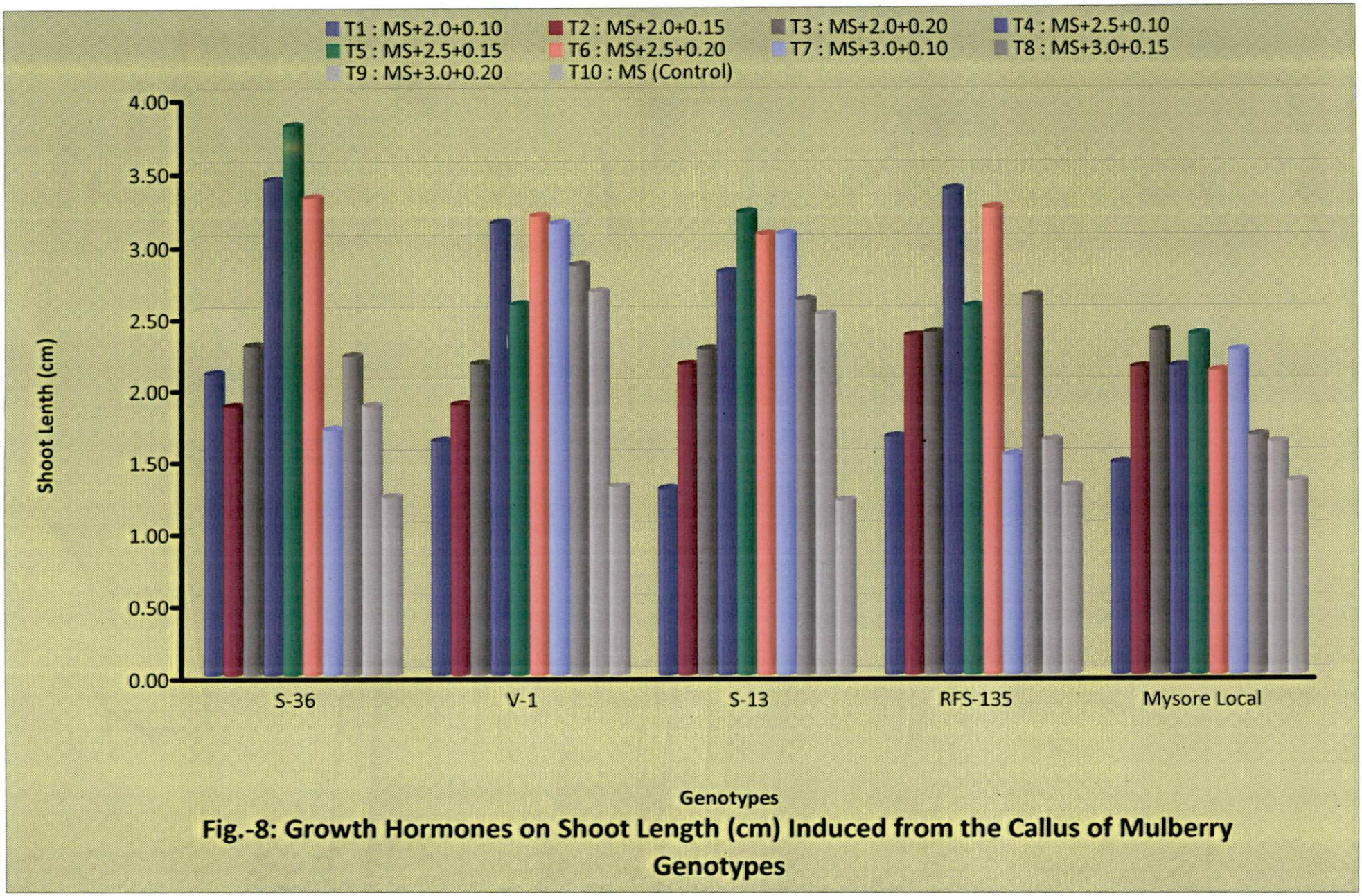


Fig.-8: Growth Hormones on Shoot Length (cm) Induced from the Callus of Mulberry Genotypes

Table 10: Effect of Growth Hormones on Per cent Rooting Induced from the Callus of Mulberry Genotypes

Treatments MS + 2, 4-D + BAP (mg/L)	S-36	V-1	S-13	RFS-135	Mysore Local	Mean
T_1 : MS+0.5mg/L NAA	43.23 (41.10)	47.17 (43.37)	51.75 (46.00)	33.81 (35.55)	25.67 (30.44)	40.32 (39.29)
T_2 : MS+1.0mg/L NAA	67.75 (55.40)	62.90 (52.47)	64.66 (53.52)	43.78 (41.42)	37.11 (37.53)	55.24 (48.07)
T_3 : MS+1.5mg/L NAA	50.94 (45.54)	40.18 (39.33)	49.47 (44.69)	35.71 (36.60)	37.62 (37.83)	42.78 (40.82)
T_4 : MS+0.5mg/L IBA	24.74 (29.82)	26.15 (30.75)	32.55 (34.78)	24.74 (29.83)	33.62 (35.43)	28.36 (32.12)
T_5 : MS+1.0mg/L IBA	37.37 (37.68)	40.54 (39.54)	37.42 (37.71)	31.26 (33.98)	37.18 (37.57)	36.75 (37.30)
T_6 : MS+1.5mg/L IBA	36.17 (36.96)	40.73 (39.65)	43.60 (41.32)	33.83 (35.56)	33.69 (35.47)	37.60 (37.79)
T_7 : MS+0.5mg/L IAA	22.27 (28.16)	21.83 (31.19)	33.81 (35.55)	22.64 (28.40)	27.50 (31.62)	26.61 (30.98)
T_8 : MS+1.0mg/L IAA	42.49 (40.67)	40.09 (39.28)	37.55 (37.79)	26.43 (30.93)	35.00 (36.27)	36.31 (36.99)
T_9 : MS+1.5mg/L IAA	37.07 (37.51)	25.52 (30.34)	33.74 (35.51)	25.84 (30.55)	27.84 (31.84)	36.31 (33.15)
T_{10} : Control	11.74 (20.03)	19.13 (25.94)	18.86 (25.73)	15.53 (23.21)	9.11 (17.57)	14.87 (22.50)
Mean	37.38 (37.29)	36.92 (37.19)	40.34 (39.26)	29.36 (32.61)	30.43 (33.16)	34.89 (35.90)

Test of Significant	F-Test	SEm±	CD at 5%
Mulberry Genotypes (A)	*	0.18 (0.11)	0.51 (0.31)
Treatments (B)	*	0.25 (0.15)	0.72 (0.44)
A x B	*	0.57 (0.35)	1.62 (0.98)

* - Significant at 5%

Note: Figures in the parenthesis indicate Arc sign transformation

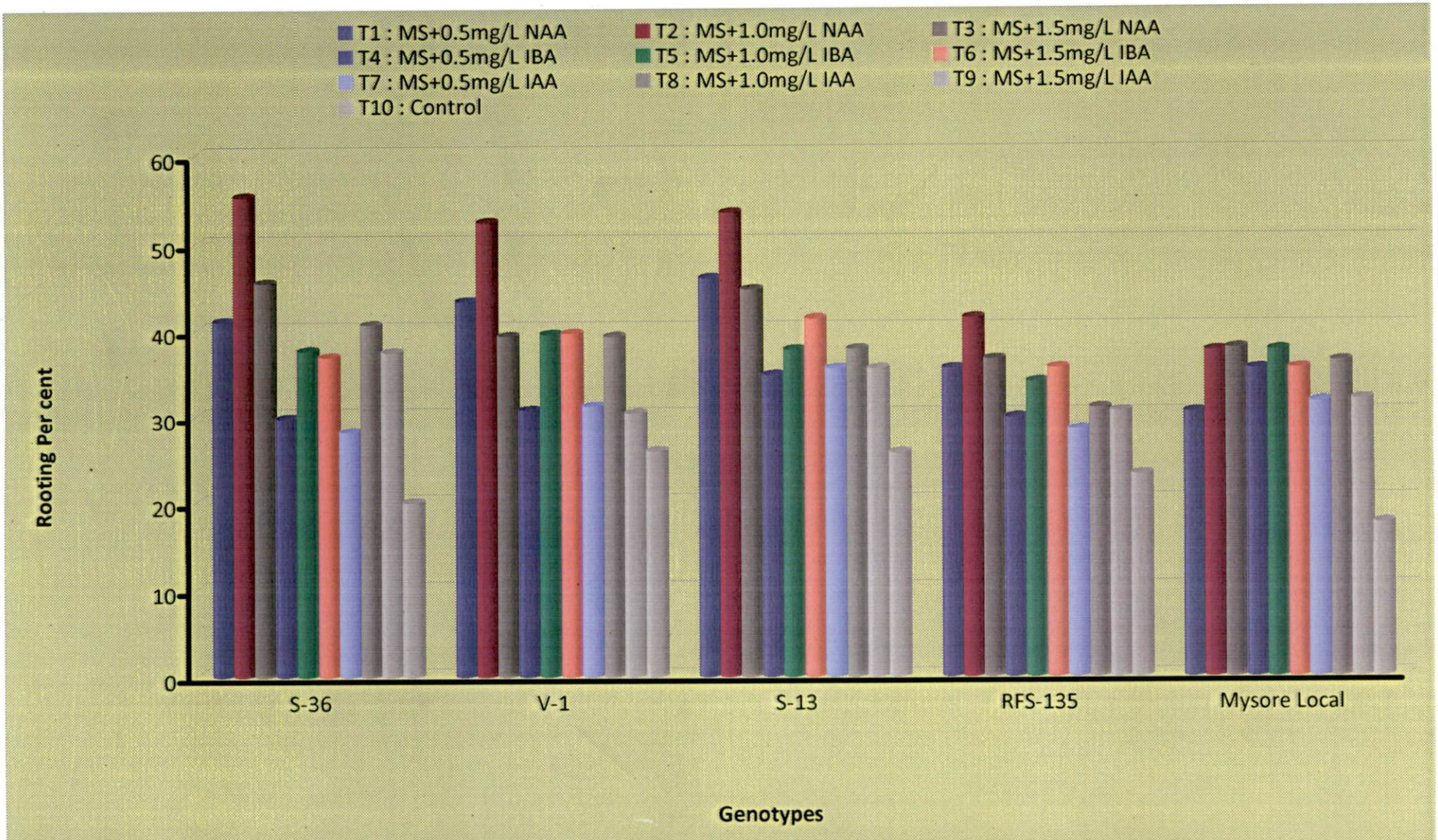


Fig.-9: Growth Hormones on Per cent Rooting Induced from the Callus of Mulberry Genotypes

Table 11: Effect of Growth Hormones on Mean Number of Roots Explants in Mulberry Genotypes

Treatments MS + 2, 4-D + BAP (mg/L)	S-36	V-1	S-13	RFS-135	Mysore Local	Mean
T_1 : MS+0.5mg/L NAA	7.03	7.25	7.05	4.26	3.05	5.73
T_2 : MS+1.0mg/L NAA	6.05	5.03	5.06	5.99	3.56	5.14
T_3 : MS+1.5mg/L NAA	7.98	6.09	4.26	5.18	8.03	6.31
T_4 : MS+0.5mg/L IBA	6.05	5.06	4.50	4.23	4.03	4.77
T_5 : MS+1.0mg/L IBA	6.07	6.55	4.33	4.30	4.06	5.06
T_6 : MS+1.5mg/L IBA	4.93	5.06	5.03	5.04	4.20	4.85
T_7 : MS+0.5mg/L IAA	4.06	4.63	3.21	5.10	5.03	4.40
T_8 : MS+1.0mg/L IAA	3.03	5.03	4.33	4.36	5.03	4.35
T_9 : MS+1.5mg/L IAA	3.21	4.96	5.43	4.92	4.20	4.54
T_{10} : Control	2.13	2.53	2.31	2.31	1.64	2.19
Mean	5.05	5.22	4.55	4.57	4.28	4.73

Test of Significant	F-Test	SEm±	CD at 5%
Mulberry Genotypes (A)	*	0.025	0.07
Treatments (B)	*	0.03	0.09
A x B	*	0.07	0.22

* - Significant at 5%

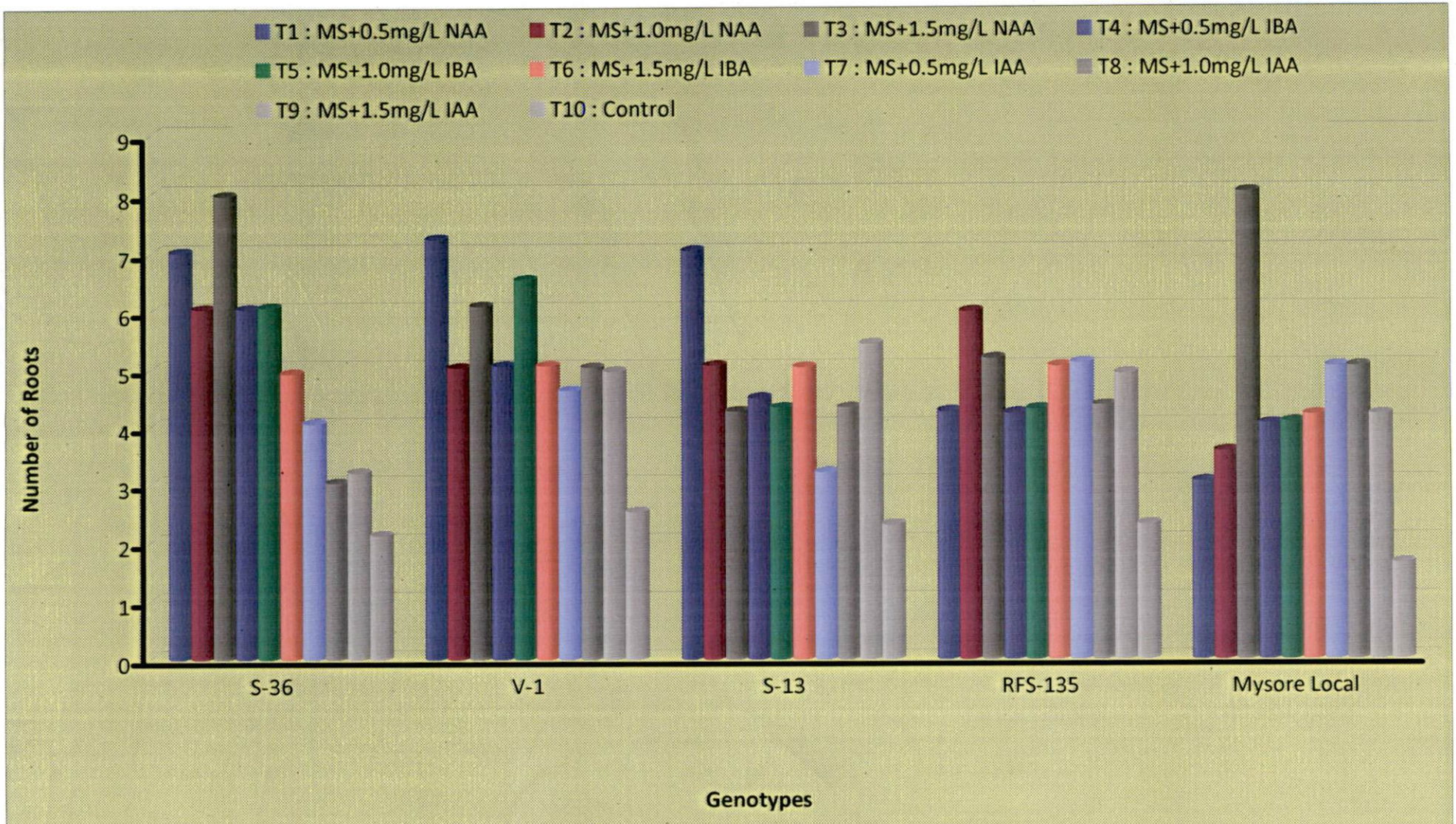


Fig.-10: Growth Hormones on Mean Number of Roots Explants in Mulberry Genotypes

number of roots were recorded of MS having 1.5mg/L NAA, 0.5, 1.0mg/L NAA (6.31, 5.73, 5.14) followed by MS with 1.5mg/L IBA, 0.5mg/L IBA, 1.5mg/L IAA (4.85, 4.77, 4.54) as compared to control (2.19).

Among interactions, maximum number of roots (7.98) were recorded on S_{36} kept in MS having 1.5mg/L NAA followed by S_{13} (7.05) also under the same treatment.

4.5 Effect of Anti transparent on Number of Leaves

Number of leaves recorded from the shoots of different mulberry genotypes with different treatments significantly varied from 1.66 to 6.33 (Table 12 and Fig. 11).

Among the rainfed genotypes more number of leaves recorded in S_{13} (3.66) than RFS_{135} (2.86), whereas among the irrigated genotypes more number of leaves recorded in S_{36} (4.83) than (4.60) as compared to control Mysore local (2.13).

More number of roots were obtained when shoots were treated with 50 per cent of spray of glycerol (4.40) followed by 75 per cent of spray of glycerol (4.13), followed by spray of low melting paraffin wax (4.00), followed by application of 50 per cent grease petroleum (3.93) as compared to control (2.93) without anti transparents.

4.5 Effect of Potting Mixture on Internodal Length (cm)

Intermodal length was recorded from the explants were significantly varied among the treatments and genotypes which ranged form 1.30 to 3.73cm (Table 13 Fig. 12).

Among the irrigated varieties, S_{36} recorded the length of 2.75cm than V_1 (2.55cm), whereas among the rainfed varieties S_{13} showed maximum length (2.39) than RFS_{135} (2.31cm) as compared to Mysore local (1.99cm).

Table 12: Effect of Antitransparent on Number of Leaves in Hardening Process of Tissue Cultured Mulberry Plants

Treatment	S-36	V-1	S-13	RFS-135	Mysore Local	Mean
T_1 : Spray of Glycerol 25%	3.66 (2.03)	3.33 (1.95)	3.00 (1.85)	3.00 (1.85)	1.66 (1.46)	2.93 (1.83)
T_2 : Spray of Glycerol 50%	6.33 (2.61)	5.33 (2.41)	4.33 (2.19)	3.66 (2.03)	2.33 (1.67)	4.40 (2.18)
T_3 : Spray of Glycerol 75%	5.33 (2.41)	5.00 (2.33)	4.33 (2.19)	3.66 (2.03)	2.33 (1.67)	4.13 (2.13)
T_4 : Spray of Low Melting Paraffin Wax	5.66 (2.48)	5.33 (2.40)	4.00 (2.11)	2.66 (1.76)	2.33 (1.67)	4.00 (2.08)
T_5 : Medium Melting Paraffin Wax	5.00 (2.34)	5.33 (2.41)	4.33 (2.19)	2.66 (1.76)	1.66 (1.46)	3.80 (2.03)
T_6 : High Melting Paraffin Wax	4.33 (2.19)	3.66 (2.01)	3.66 (2.03)	2.66 (1.76)	2.33 (1.67)	3.33 (1.93)
T_7 : Application of 25% Grease Petroleum	4.33 (2.19)	4.66 (2.01)	3.00 (2.03)	2.66 (1.76)	2.33 (1.67)	3.40 (1.95)
T_8 : 50% Grease Petroleum	6.00 (2.54)	5.00 (2.34)	3.66 (2.03)	2.33 (1.67)	2.66 (1.77)	3.93 (2.07)
T_9 : 75% Grease Petroleum	4.66 (2.27)	4.33 (2.19)	3.00 (1.85)	2.66 (1.77)	2.00 (1.58)	3.33 (1.93)
T_{10} : Control Without Anti-transparents	3.00 (1.85)	4.00 (2.11)	3.33 (1.94)	2.66 (1.77)	1.66 (1.46)	2.93 (1.82)
Mean	4.83 (2.29)	4.60 (2.24)	3.66 (2.02)	2.86 (1.82)	2.13 (1.61)	3.62 (2.00)

Test of Significant	F-Test	8cm 1	CD
Mulberry Genotypes (A)	*	(0.13) 0.03	(0.38) 0.10
Treatments (B)	*	(0.19) 0.05	(0.54) 0.14
A x B	NS	(0.43) 0.11	(1.22) 0.31

* - Significant at 5%

NS – Nonsignificant

Note: Figures in the parenthesis indicate square root transformation.

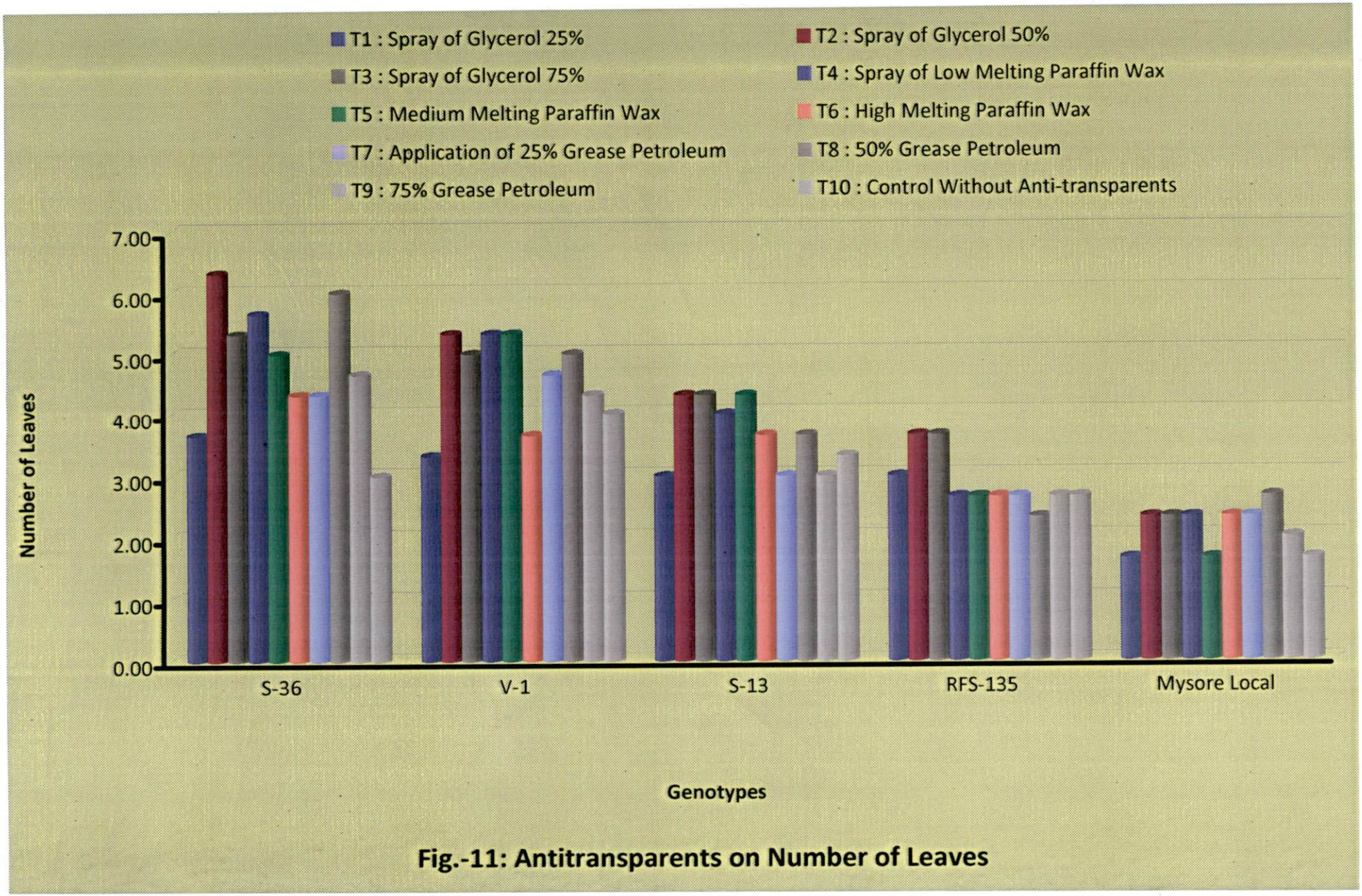


Fig.-11: Antitransparents on Number of Leaves

Table 13: Effect of Potting Mixture on Internodal Length (cm) of Mulberry Plant

Treatment	S-36	V-1	S-13	RFS-135	Mysore Local	Mean
T_1: Peat: Perlite: Vermiculate (1:1:1) + 1ml MS + 0.5ppm of NAA + 0.5ppm of IBA + 0.5ppm IAA	3.31	3.05	2.92	2.59	2.10	2.79
T_2: Peat: Perlite: Vermiculate (1:1:1) + 1ml MS + 1ppm of NAA + 1ppm of IBA + 1ppm IAA	3.73	3.54	2.51	2.63	2.33	2.95
T_3: Peat: Perlite: Vermiculate (1:1:1) + 1ml MS + 2ppm of NAA + 2ppm of IBA + 2ppm IAA	2.30	2.25	2.16	2.10	1.81	2.12
T_4: Sand: Soil: Perlite + 1ml MS + 0.5ppm of NAA + 0.5ppm of IBA + 0.5ppm IAA	3.31	2.31	2.12	2.16	1.99	2.38
T_5: Sand: Soil: Perlite + 1ml MS + 1ppm of NAA + 1ppm of IBA + 1ppm IAA	3.32	3.16	3.00	2.93	2.21	2.92
T_6: Sand: Soil: Perlite + 1ml MS + 2ppm of NAA + 2ppm of IBA+ 2ppm IAA	2.16	2.25	2.83	2.64	2.33	2.44
T_7: Sand: Soil: FYM / Compost	2.02	1.99	1.99	1.93	1.85	1.95
T_8: Sand: Soil (Control)	1.88	1.85	1.61	1.53	1.30	1.63
Mean	2.75	2.55	2.39	2.31	1.99	2.40

Test of Significant	F-Test	SEm±	CD at 5%
Mulberry Genotypes (A)	*	0.02	0.05
Treatments (B)	*	0.02	0.07
A x B	*	0.05	0.16

* - Significant at 5%

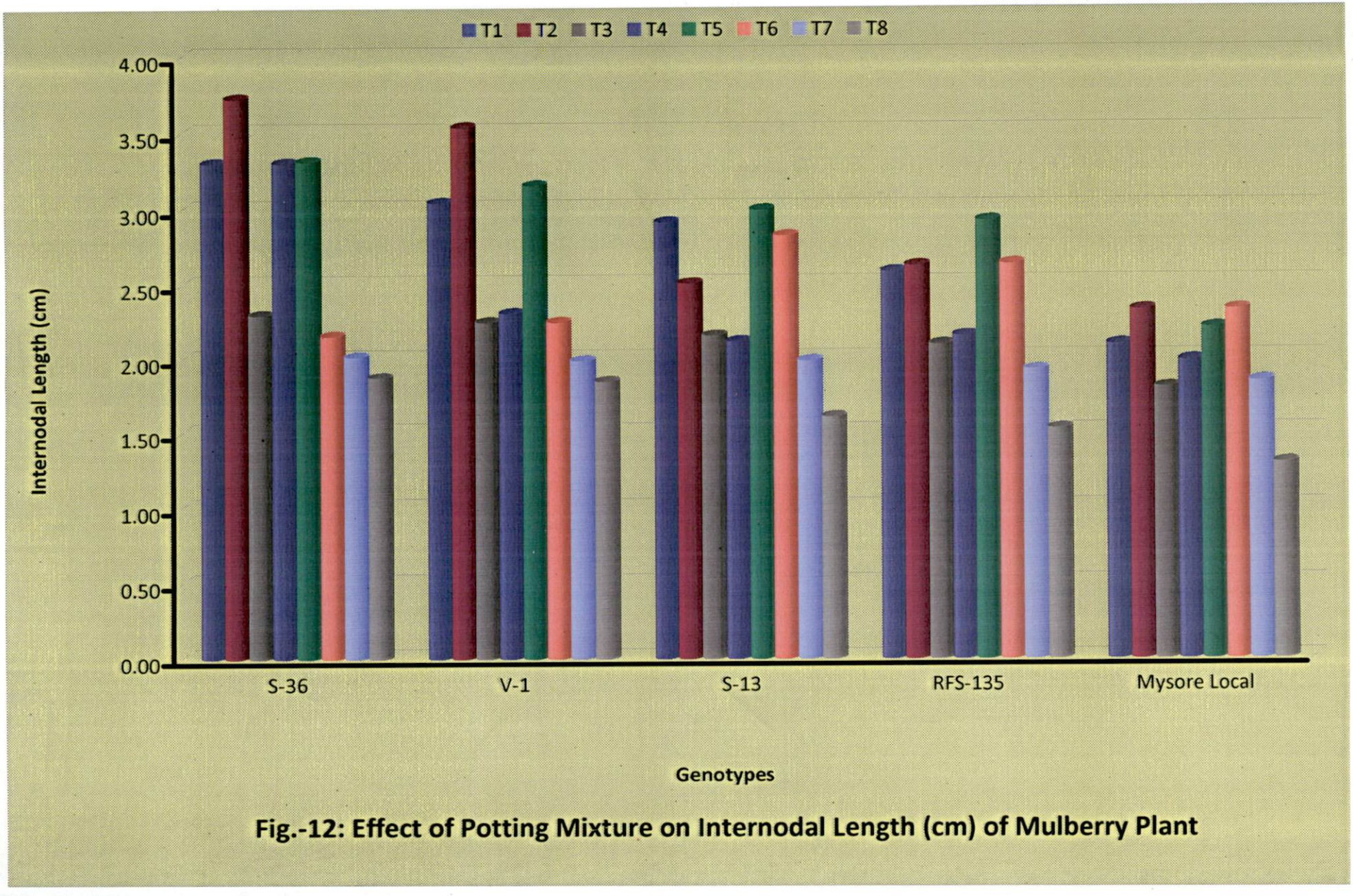


Fig.-12: Effect of Potting Mixture on Internodal Length (cm) of Mulberry Plant

The maximum internodal length of 2.95cm was recorded with potting mixture peat : perlite : vermiculate (1:1:1) + ½ MS + 1ppm of NAA + 1ppm IBA + 1ppm IAA, followed by 2.92cm with sand : soil : perlite + ½ MS + 1 ppm of NAA + 1ppm IBA + 1ppm of IAA as compared to control (1.63cm) i.e., Sand and Soil.

Among the interactions maximum internodal length was recorded in 12 treatments (3.73cm) of S_{36} followed by V_1 (3.54cm) also under the same treatment.

4.5.2 Effect of Potting Mixture on Shoot Length (cm)

Shoot length obtained from the explants were significantly varied among the treatments and genotypes which ranged from 4.06 to 6.00cm (Table 14 Fig. 13).

Among the irrigated varieties S_{36} recorded the shoot length of 5.27cm than V_1 (5.25cm), whereas among the rainfed varieties S_{13} recorded maximum length (5.15cm) than RFS_{135} (5.08cm) as compared to Mysore local (4.97cm).

The maximum shoot length of 5.46cm recorded with potting mixture peat : perlite : vermiculate (1:1:1) + ½ MS + 1 ppm of NAA + 1ppm IBA + 1 ppm IAA followed by sand : soil : perlite + ½ MS + 1ppm of NAA + 1ppm IBA + 1ppm of IAA (5.40cm) as compared to control (4.36cm).

Among the interaction maximum shoot length was recorded in potting mixture peat : perlite : vermiculate (1:1:1) + ½ MS + 1ppm of NAA + 1ppm IBA + 1ppm IAA (6.00cm) in S_{36} followed by V_1 (5.73cm) also under the same treatment.

4.5.3 Effect of Potting Mixture on Root Length (cm)

Root lengths obtained from the explants were significant among the treatments, genotypes and with interactions of treatments and

Table 14: Effect of Potting Mixture on Shoot Length (cm) of Mulberry Plant

Treatment	S-36	V-1	S-13	RFS-135	Mysore Local	Mean
T_1: Peat: Perlite: Vermiculate (1:1:1) + 1ml MS + 0.5ppm of NAA + 0.5ppm of IBA + 0.5ppm IAA	5.06	5.70	5.50	5.30	5.20	5.35
T_2: Peat: Perlite: Vermiculate (1:1:1) + 1ml MS + 1ppm of NAA + 1ppm of IBA + 1ppm IAA	6.00	5.73	5.33	5.13	5.10	5.460
T_3: Peat: Perlite: Vermiculate (1:1:1) + 1ml MS + 2ppm of NAA + 2ppm of IBA + 2ppm IAA	5.16	5.20	5.36	5.43	5.50	5.33
T_4: Sand: Soil: Perlite + 1ml MS + 0.5ppm of NAA + 0.5ppm of IBA + 0.5ppm IAA	5.40	5.16	5.40	5.36	5.16	5.30
T_5: Sand: Soil: Perlite + 1ml MS + 1ppm of NAA + 1ppm of IBA + 1ppm IAA	5.80	5.63	5.36	5.20	5.00	5.40
T_6: Sand: Soil: Perlite + 1ml MS + 2ppm of NAA + 2ppm of IBA+ 2ppm IAA	5.10	5.20	5.06	5.10	4.93	5.08
T_7: Sand: Soil: FYM / Compost	5.03	5.00	4.83	4.90	4.80	4.91
T_8: Sand: Soil (Control)	4.66	4.43	4.36	4.26	4.06	4.36
Mean	5.27	5.25	5.15	5.08	4.97	5.15

Test of Significant	F-Test	SEm±	CD at 5%
Mulberry Genotypes (A)	*	0.02	0.05
Treatments (B)	*	0.02	0.07
A x B	*	0.05	0.16

* - Significant at 5%

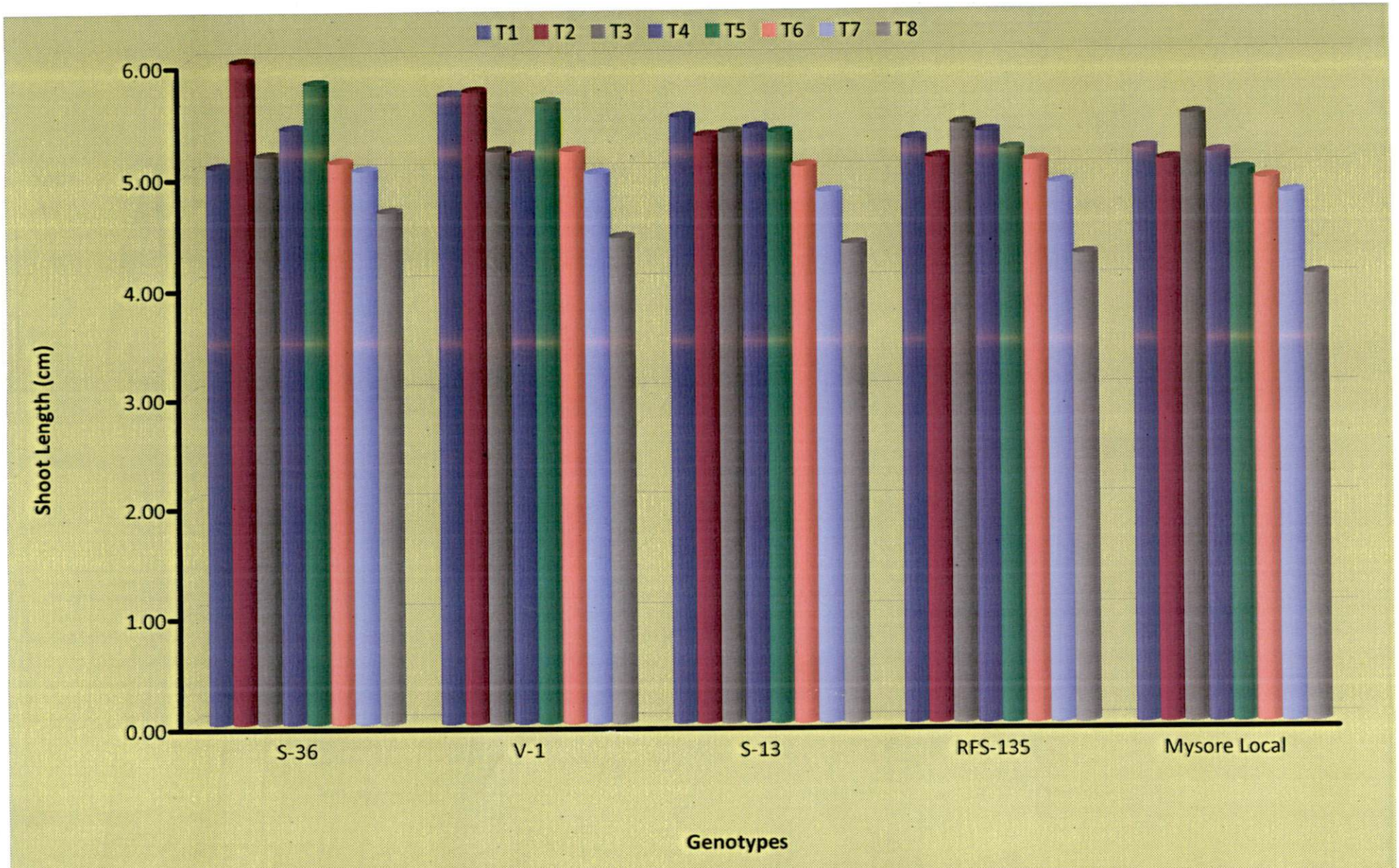


Fig.-13: Potting Mixture on Shoot Length (cm) of Mulberry Plant

genotypes which ranged from 5.16 to 8.20cm (Table 15, Fig. 14 and Plate 9).

Among the irrigated varieties S_{36} recorded the root length of 7.02cm than V_1 (6.96cm), whereas among the rainfed varieties S_{13} recorded maximum length (6.75cm) than RFS_{135} (6.69cm) as compared to control Mysore local (6.53cm).

The maximum root length was recorded with potting mixture peat : perlite : vermiculate (1:1:1) + ½ MS + 1ppm of NAA + 1ppm IBA + 1ppm IAA (7.32cm) followed by peat : perlite : vermiculate (1:1:1) + ½ MS + 2ppm of NAA + 2ppm IBA + 2ppm IAA (7.30cm) as compared to control (5.58cm).

Among the interaction maximum root length was recorded in potting mixture peat : perlite : vermiculate (1:1:1) + ½ MS + 1ppm of NAA + 1ppm IBA + 1ppm IAR (8.20cm) in S_{36} followed by V_1 (7.70cm) under the treatment of peat : perlite : vermiculate (1:1:1) + ½ MS + 2ppm of NAA + 2ppm of IBA + 2ppm IAA.

4.6 Effect of Shading Percentage on Plant Height (cm)

Plant height recorded were significantly varied among the treatments and genotypes but non significant with interactions of treatments and genotypes which ranged from 2.41 to 3.85cm (Table 16 and Fig. 15).

Among the irrigated varieties S_{36} recorded the plant height (3.18cm) than V_1 (3.10cm), where as among the rainfed varieties S_{13} recorded maximum length (3.07cm) than RFS_{135} (2.96cm) as compared to control (2.90cm).

The maximum plant height was recorded with 50 per cent shading percentage (3.69cm) followed by 75 per cent shading

Table 15: Effect of Potting Mixture on Root Length (cm) of Mulberry Plant

Treatment	S-36	V-1	S-13	RFS-135	Mysore Local	Mean
T_1: Peat: Perlite: Vermiculate (1:1:1) + 1ml MS + 0.5ppm of NAA + 0.5ppm of IBA + 0.5ppm IAA	7.30	7.06	7.03	7.06	7.00	7.09
T_2: Peat: Perlite: Vermiculate (1:1:1) + 1ml MS + 1ppm of NAA + 1ppm of IBA + 1ppm IAA	8.20	7.26	7.10	7.10	6.96	7.32
T_3: Peat: Perlite: Vermiculate (1:1:1) + 1ml MS + 2ppm of NAA + 2ppm of IBA + 2ppm IAA	7.00	7.70	7.60	7.30	6.93	7.30
T_4: Sand: Soil: Perlite + 1ml MS + 0.5ppm of NAA + 0.5ppm of IBA + 0.5ppm IAA	7.20	7.40	7.10	6.90	6.80	7.08
T_5: Sand: Soil: Perlite + 1ml MS + 1ppm of NAA + 1ppm of IBA + 1ppm IAA	7.60	7.50	7.23	7.16	7.03	7.30
T_6: Sand: Soil: Perlite + 1ml MS + 2ppm of NAA + 2ppm of IBA+ 2ppm IAA	6.50	6.40	6.30	6.43	6.30	6.38
T_7: Sand: Soil: FYM / Compost	6.40	6.50	6.20	6.20	6.10	6.28
T_8: Sand: Soil (Control)	6.00	5.86	5.50	5.40	5.16	5.58
Mean	7.02	6.96	6.75	6.69	6.53	6.79

Test of Significant	F-Test	SEm±	CD at 5%
Mulberry Genotypes (A)	*	0.02	0.05
Treatments (B)	*	0.02	0.07
A x B	*	0.05	0.16

* - Significant at 5%

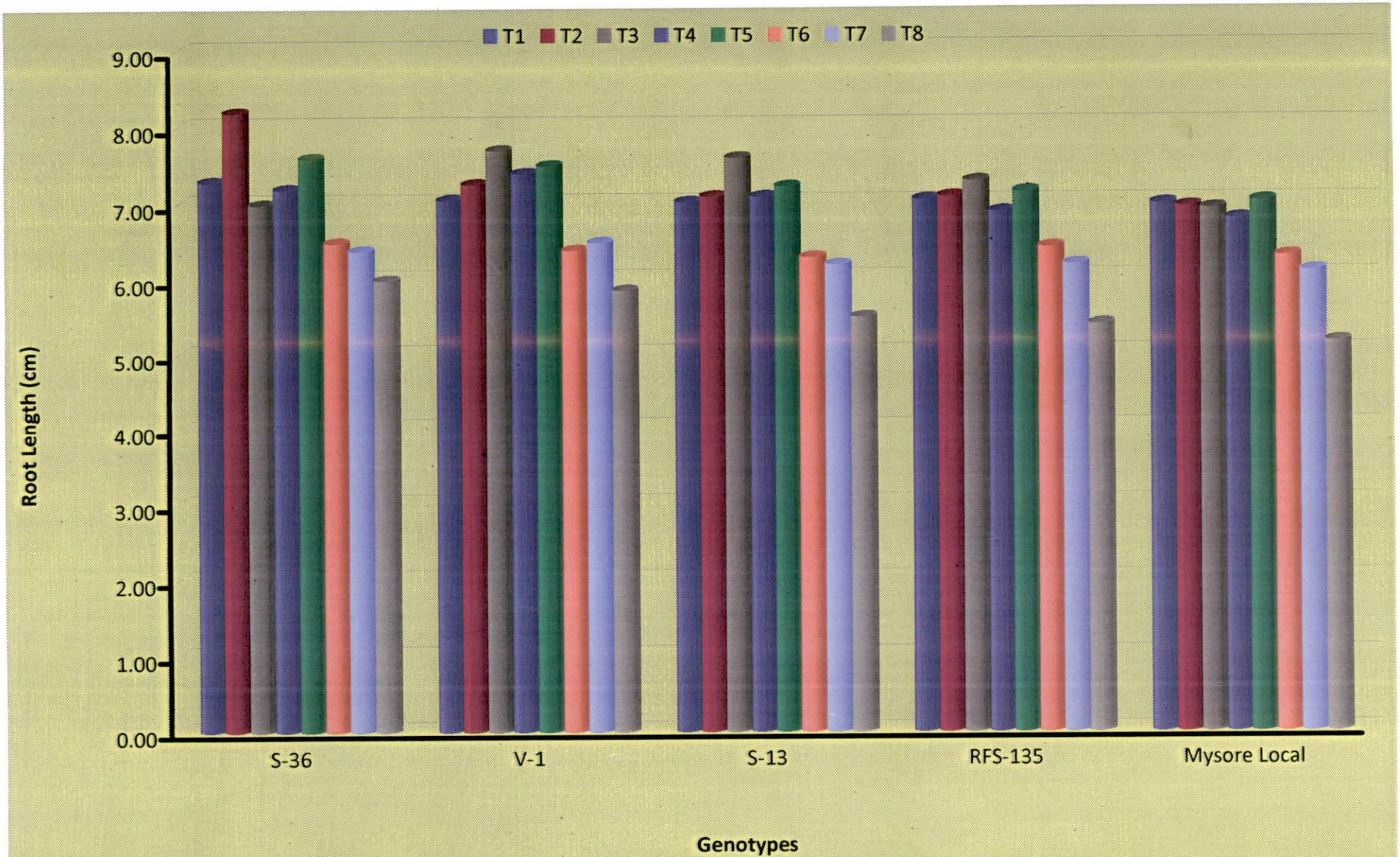


Fig.-14: Potting Mixture on Root Length (cm) of Mulberry Plant

Table 16: Effect of Percentage Shade on Plant Height (cm)

Treatment	S-36	V-1	S-13	RFS-135	Mysore Local	Mean
T_1 : 100%	2.73	2.61	2.54	2.41	2.34	2.53
T_2 : 75%	3.53	3.53	3.43	3.34	3.29	3.42
T_3 : 50%	3.85	3.76	3.67	3.62	3.58	3.69
T_4 : 25%	3.13	3.07	3.08	2.97	2.91	3.03
Control	2.67	2.54	2.61	2.48	2.41	2.54
Mean	3.18	3.10	3.07	2.96	2.90	3.04

Test of Significant	F-Test	SEm±	CD at 5%
Mulberry Genotypes (A)	*	0.02	0.05
Treatments (B)	*	0.02	0.07
A x B	NS	0.05	0.15

* - Significant at 5%

* - NS-Nonsignificant

Note: Figures in the parenthesis indicate square root transformation

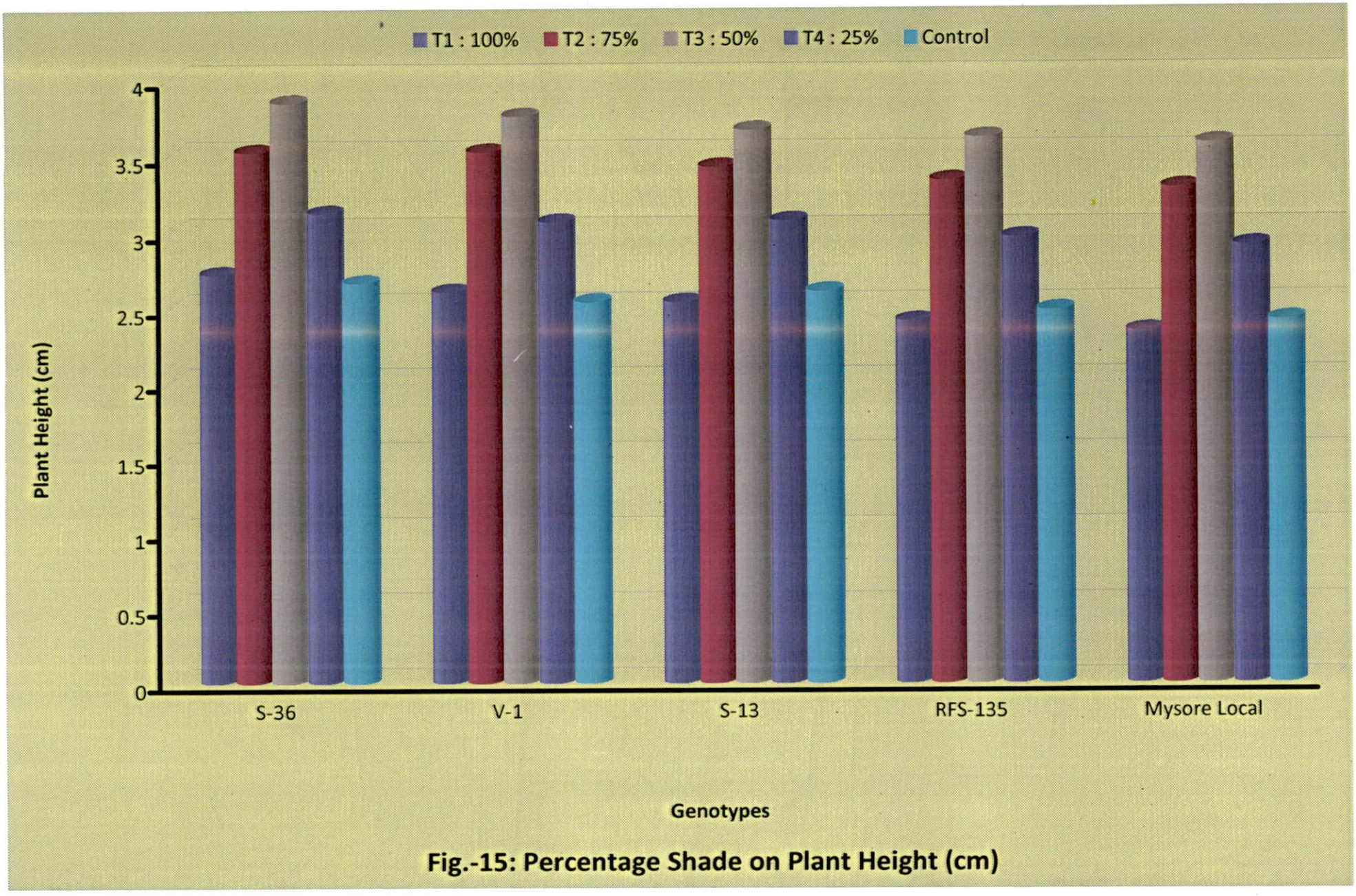


Fig.-15: Percentage Shade on Plant Height (cm)

percentage (3.42cm) as compared to control (2.54cm). The interactions were however non significant.

4.7 Survival Rates (%)

Survival rates recorded varied significantly among the treatments and genotypes but non-significant among interactions of treatments and varieties which ranged from (79.50 to 84.26%) (Table 17, Fig. 16, Plate 10, 11 and 12).

Among the irrigated varieties S_{36} recorded (83.46%) survival followed by V_1 (81.78%), whereas among the rainfed varieties S_{13} recorded maximum percentage (81.05%) followed by RFS_{135} (80.08%) as compared to control (78.67%). The maximum survival rate was recorded with 45 days (81.94%) followed by 30 days (80.89%) as compared to 25 days (80.19%). The interaction were however non-significant (Table 18).

Table 17: Effect of Percentage Shade on Survival Rate (%)

Treatment	S-36	V-1	S-13	RFS-135	Mysore Local	Mean
25 Days	98.33 (82.66)	97.33 (80.64)	97.00 (80.02)	97.00 (80.11)	95.33 (77.54)	96.99 (80.19)
30 Days	98.66 (83.46)	97.66 (81.25)	97.66 (81.25)	96.66 (79.50)	96.33 (78.98)	97.39 (80.89)
45 Days	99.00 (84.26)	98.66 (83.46)	98.00 (81.86)	97.33 (80.64)	97.66 (79.50)	97.93 (81.94)
Mean	98.66 (83.46)	97.83 (81.78)	97.55 (81.05)	96.99 (80.08)	96.21 (78.67)	97.43 (81.01)

Test of Significant	F-Test	SEm±	CD at 5%
Mulberry Genotypes (A)	*	0.36 (0.34)	1.38 (1.00)
Treatments (B)	*	0.27 (0.26)	0.96 (0.77)
A x B	NS	0.61 (0.60)	1.99 (1.73)

* - Significant at 5%
NS-Nonsignificant

Note: Figures in the parenthesis indicate Arc sign transformation.

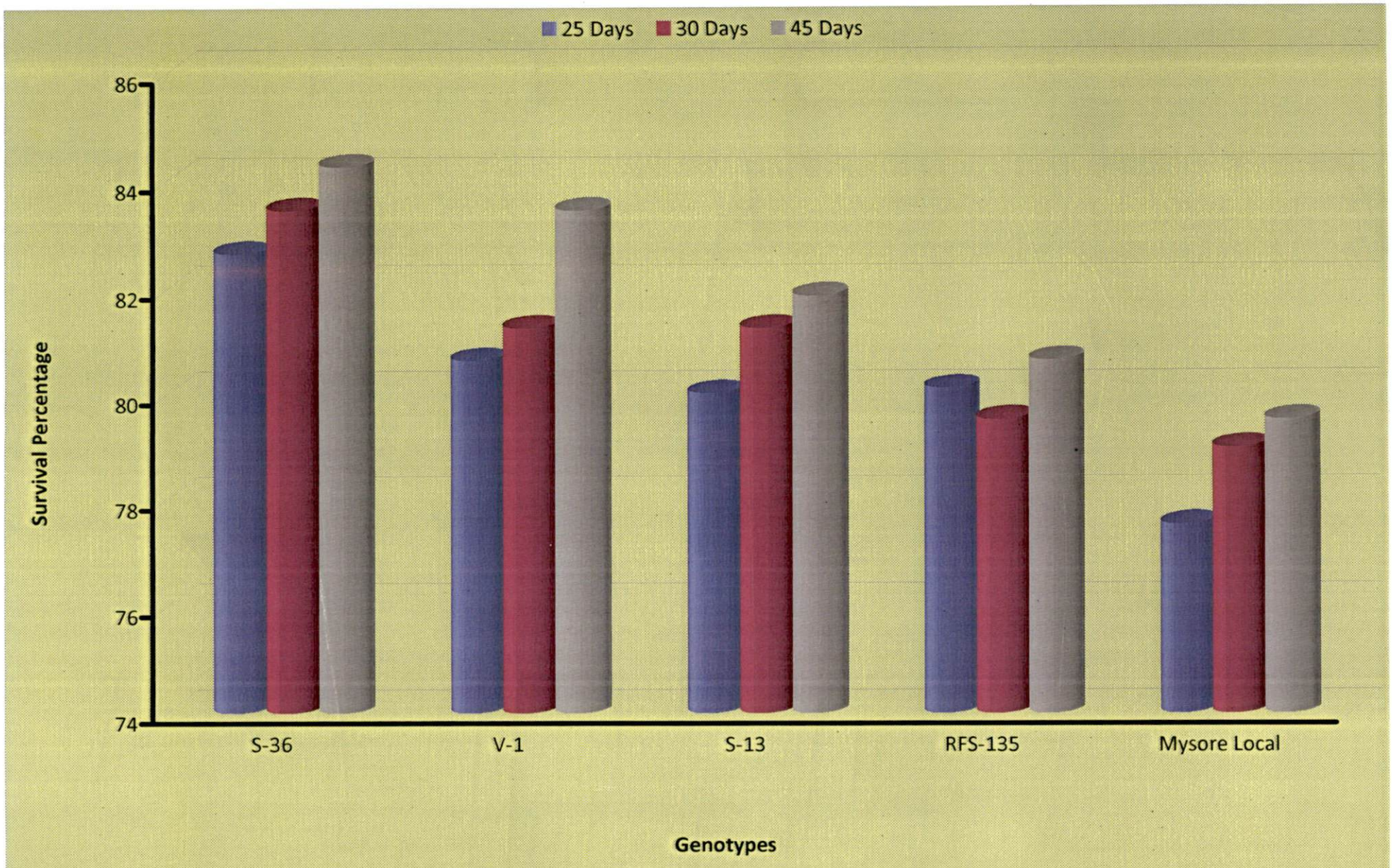


Fig.-16: Percentage Shade on Survival Rate (%)

Table 18: General appearance of tissue cultured mulberry plants

Genotypes	Parameters						
	Leaf Nature	**Leaf Colour**	**Shoot Length**	**Root Length**	**Inter Nodal**	**Plant Height**	**General Appearance**
S-36	Unlobed	Pale Green	Medium	Longer	Medium	Shorter	Healthy
V-1	Unlobed	Pale Green	Medium	Medium	Medium	Longer	Healthy
S-13	Unlobed	Pale Green	Shorter	Medium	Shorter	Medium	Healthy
RFS-135	Unlobed	Green	Medium	Shorter	Shorter	Longer	Healthy
Mysore Local	Lobed & Unlobed	Dark Green	Shorter	Shorter	Shorter	Medium	Healthy

Plate 9: Potted *in vitro* raised plants in different combinations of soil rite mix

Plate 10: Hardened S_{36} variety ready for transfer to field

Plate 11: Hardened V_1 variety ready for transfer to field

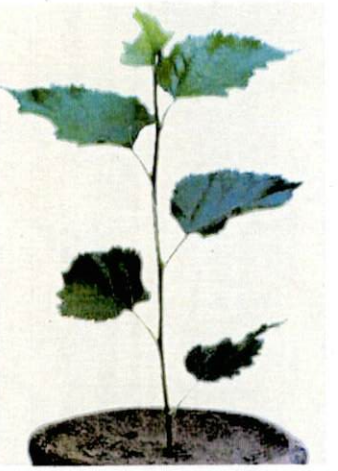


Plate 12: Hardened Mysore local (Control) variety ready for transfer to field

DISCUSSION

V. DISCUSSION

The results of the present investigation have revealed interesting facts regarding **"Standardization of Hardening Protocol of Tissue Cultured Mulberry Plants (*Morus* spp.)"** for tissue cultured plantlets are discussed in this chapter.

5.1 Sterilization Protocol for Explants of Mulberry

The present study revealed that the survival per cent of the explants was significantly maximum (79.36%) at 8 minutes ($HgCl_2$) at the concentration of 0.1 per cent and it was significantly minimum at the duration of 10 minutes (22.14) at the concentration of 0.5 per cent of $HgCl_2$. These results are in agreement with the results of Kim *et al.* (1985), Tewary *et al.* (1989), Kathiravan *et al.* (1995), Wang *et al.* (1996), Vijayan *et al.* (1998 and 2000), Wang *et al.* (1999), Kapur *et al.* (2001), Bhatnagar *et al.* (2002) where the maximum survival per cent was recorded when explants were treated with 0.1 per cent (w/v) aqueous $HgCl_2$ solution for 5 minutes.

5.2 Effect of Growth Regulators on Callus Initiation

Significantly maximum response of callus initiation was recorded in S_{36} (60.76%) followed by V_1 (60.06%), S_{13} (45.86%), RFS_{135} (31.12%) and Mysore local (27.04%). Regeneration in tissue culture is a genetically controlled trait (Bhojwani *et al.*, 1984, Templeton-Somers and Collins, 1986).

Maximum response of callus iniation was observed with 2.0mg/L 2, 4-D along with 0.75 and 1.00mg/L BAP (60.76% and 60.06%), respectively. The results are in confirmation with the findings of earlier reports (Ohyama, 1970; Tewary *et al.*, 1996, Jain *et al.*, 1990, Srinivasa *et al.*, 2001). Yellow friable callus was obtained on media containing higher concentration of 2, 4-D and lower concentration of BAP and highest per cent (85%) (Table 6) of callus

formation was found on medium containing 2, 4-D (2mg/L) alone (Kavyashree *et al.*, 2005a) or in conjugation with 0.5mg/L BAP (Vijayan *et al.*, 1998). From the wide range of auxins and cytokinins used for induction of callus, 2, 4-D was identified as the best auxin for callus induction (Komo *et al.*, 1994) and cytokinin supplementation along with auxins resulted in better growth of callus (Ketchum *et al.*, 1995).

Induced callus was maintained on MSBM supplemented with 2, 4-D (2mg/L and CM 10 per cent for a period of six months (Srinivasa *et al.*, 2001).

MS supplemented with 2.5mg/L 2, 4-D and 0.50mg/L BAP showed faster response (11.80 days) compared to control (12.13 days) with the growth regulators. Hypocotyl segments cultured on MS-medium formed callus within 12 days (Kavyashree *et al.*, 2005), 14-16 days (Vijayan *et al.*, 1998), 3-4 weeks (Tewary *et al.*, 1989) a month (Tewary *et al.*, 1990) of inoculation.

5.3 Effect of Growth Hormones on Shooting

Significantly maximum callus differentiation was recorded in S_{36} (60.91%) followed by V_1 (5.50%), RFS_{135} (33.72%), S_{13} (32.12) and Mysore local (28.25%). Regeneration in tissue culture is a genetically controlled trait (Bhojwani *et al.*, 1984, Templeton-Somers and Collins, 1986). Frequency of shoot regeneration per explant varied among the genotypes (Kathiravan *et al.*, 1995).

Significantly higher differentiation was recorded in S_{36} when MS fortified with 2.5mg/L 2, 4-D and 1.00mg/L BAP 75.12%) followed by V_1 on MS and 2.5mg/L 2, 4-D and 0.75mg/L BAP (75.11%) compared to Mysore local (25.88%). Genotypic difference in their response to auxin and cytokinin concentrations and combinations as revealed by

the variation in callus differentiation and shoot formation (Tewary *et al.*, 1996). Combination of BAP (2mg/L) and NAA (0.1mg/L) were found most effective in inducing higher per cent of multiple shoots (Kim *et al.*, 1985); Vijayan *et al.*, 1998). The presence of BAP in MSBM with auxin promotes the rate of shoot multiplication in mulberry which is also in support of Oka and Ohyama (1974), Mhatre, (1985), Kim *et al.*, (1985) and Rao and Raghunath (1993) in different varieties of mulberry.

Shoot length was significantly more in mulberry with 1.0mg/L BAP and 0.5mg/L IBA (Tewary *et al.*, 1996). Present results coincide with the above and also support the results of Vijayan *et al.* (1998).

5.4 Effect of Growth Hormones on Rooting

Shoots rooted well in MS medium having NAA followed by IBA and IAA. Significantly higher rooting percentage (48.07%, 40.82%) was recorded with 1.0mg/L and 1.5mg/L NAA respectively followed by 1.0mg/L IBA (37.30%) compared to control. The results are in agreement with the results of Chattopadhyay *et al.* (1990), Vijayan *et al.* (1998), Chakravarthy *et al.* (2001), Bhatnagar *et al.* (2002), and Kavyashree *et al.* (2005b). It is observed that in mulberry NAA is better than IBA for root induction and growth, though IBA, IAA, 2, 4-D and NAA are commonly used for root induction in plantlets (Narayan *et al.*, 1989, Yadav *et al.*, 1990, Jain *et al.*, 1992, Rao and Vidyanath, 1997).

More number of roots arose on MS having NAA followed by IBA and IAA. Maximum number of roots were recorded with 1.5, 0.5mg/L NAA (6.31, 5.73) followed by 1.0mg/L IBA (5.06) compared to the control (2.27). These findings are supported by reports of Yadav *et al.* (1990), Vijayan *et al.* (1998), and Kavyashree *et al.* (2005b).

5.5 Effect of Anti transparents on Number of Leaves

More number of leaves were found with spray of glycerol followed by spray of low melting paraffin wax and grease petroleum. Maximum number of leaves were recorded with 50 per cent, 75 per cent glycerol (4.40, 4.13) followed by low melting paraffin wax (4.00) and 50 per cent grease petroleum (3.93) compared to the control (2.93). These findings are supported by the reports of Smith *et al.*, 1990, Smith *et al.*, 1992, Ghaxhigae *et al.*, 1992 and Pospisilova, 1996).

5.6 Effect of Potting Mixture on Inter nodal Length, Shoot Length and Root Length (cm)

Internodal length was significantly more in mulberry with peat: perlite: vermiculate (1:1:1) + ½ MS + 1ppm of NAA + 1ppm IBA + 1ppm IAA (2.95) and Sand: Soil: Perlite + ½ MS + 1ppm of NAA + 1ppm IBA + 1ppm IAA (2.92cm). Shoot length was significantly maximum in mulberry with peat: perlite: vermiculate (1:1:1) + ½ MS + 1ppm of NAA + 1ppm IBA + 1ppm IAA (5.46) and sand: soil: perlite + ½ MS + 1ppm of NAA + 1ppm IBA + 1ppm IAA (5.40).

Root length recorded was significantly more in mulberry with peat: perlite: vermiculate (1:1:1) + ½ MS + 1ppm of NAA + 1ppm of IBA + 1ppm IAA (7.32) and sand: soil: perlite + ½ MS + 4.00ppm of NAA + 1.00ppm IBA + 1.00ppm IAA (7.30). The present results are supported by the results of Adachi *et al.* (1990), Katase, 1994, Kapur *et al.*, 2001, Kavyashree *et al.*, 2005a, Kavyashree *et al.*, 2005b, Srinivasa *et al.*, 2001).

5.7 Effect of Shade on Plant height (cm)

Plant height recorded was significantly more in mulberry with 50 per cent shading (3.69%) and 75 per cent shading (3.42%). The present results are supported by the results of Desjardins *et al.* (1988).

5.8 Survival Rate of tissue cultured mulberry plant

Survival rate recorded was significantly more in mulberry with 45 days (81.94%) and 30 days (80.89%). Present results were supported by the Adachi *et al.* 1990, Katase 1994, Kavyashree *et al.* 2000a, Kavyashree *et al.* 2005b, Kapur *et al.* 2001, Srinivasa *et al.* 2001, and Usha Yadav *et al.* 1990.

SUMMARY

VI. SUMMARY

The results of the present investigation have revealed interesting facts regarding **"Standardization of Hardening Protocol of Tissue Cultured Mulberry Plants (*Morus* spp.)"** was carried out to standardize the protocol for mulberry genotypes through nodal explants using five mulberry genotypes *viz.*, V_1, S_{36} (Irrigated); S13, RFS_{135} (Rainfed) and Mysore local (Control).

Salient features of the investigation are as follows.,

Survival per cent of the explants were significantly higher in (79.36%) at 8 minutes ($HgCl_2$) at the concentration of 0.1 per cent compared to (14.05%) at 8 minutes ($HgCl_2$) at the concentration of 0.5 per cent.

The highest response of callus initiation was recorded in S_{36} (60.76%) followed by V_1 (60.06%), S_{13} (45.86%), RFS_{135} (31.12%) and Mysore local (27.04%).

Among media maximum response of callus iniation was observed with 2.0mg/L 2, 4-D along with 0.75 and 1.00mg/L BAP (60.76% and 60.06%), respectively. 2, 4-D was identified as the best auxin for callus induction.

Higher values for callus differentiation was recorded in S_{36} (60.91%) followed by V_1 (51.50%), RFS_{135} (33.72%), S_{13} (32.12) and Mysore local (28.25%). Combination of BAP (2mg/L) and NAA (0.1mg/L) were found most effective in inducing higher per cent of multiple shoots.

Higher rooting percentage (48.07%, 40.82%) was recorded with 1.0mg/L and 1.5mg/L NAA respectively followed by 1.0mg/L IBA (37.30%) compared to control.

Maximum number of leaves were recorded with 50 per cent, 75 per cent glycerol (4.40, 4.13) followed by low melting paraffin wax (4.00) and 50 per cent grease petroleum (3.93) compared to the control (2.93).

Shoot length was recorded significantly maximum in mulberry with the peat: perlite: vermiculate (1:1:1) + ½ MS + 1ppm of NAA + 1ppm IBA + 1ppm IAA (5.46) and sand: soil: perlite + ½ MS + 1ppm of NAA + 1ppm IBA + 1ppm IAA (5.40).

Plant height was recorded highest in mulberry with the 50 per cent shading (3.69%) and 75 per cent shading (3.42%). Survival rate was recorded significantly maximum in mulberry with 45 days (81.94%) and 30 days (80.89%).

Future Line of Work: -

Micropropogation techniques can be effectively utilized for clonal propogation of popular mulberry varieties, this filling the demand and supply gap for e.g., V_1 variety. Tissue culture technology can be commercialized for large scale *in vitro* multiplication of popular mulberry genotypes, especially the poor rooting varieties can be propogated by tissue culture technique. *In vitro* rooting techniques should be standardized.

Production cost per plant should be worked out, and possibilities of reducing cost of production of mulberry plants from nodal explants should be worked out.

REFERENCES

VII. REFERENCES

ADACHI, T., SUGIMURA, Y., KOTANI, E. AND FURUSAWA, T., 1999, Adaptability of mulberry plantlets regenerated *in vitro* to outdoor conditions. *J. Seric. Sci. Jpn.*, **68** (2): 161-163.

ANNONYMOUS, 1992, *Silk in India; Statistical Biennial*, CSB, Bangalore, p. 69.

*ARIMA, Y., OKII, M. AND KAI, M., 1989, Propagation of somatic plant embryos- with production of embryogenic tissue in presence of ancymidol. European Patent Applic. No. 328424.

BHATNAGAR, S., DAS, M. AND KHURANA, P., 2002, Rapid, *in vitro* TDZ-mediated micropropagation of *Morus indica* C_{176} and C_{776} through axillary buds. *Indian J. Seric.*, **41**: 80-83.

BHATNAGAR, S., KAPUR, A. AND KHURANA, P., 2001, TDZ mediated differentiation in commercially valuable Indian mulberry cultivars K2 and DD. *Plant Biotechnol.*, **18**(1): 61-64.

BHOJWANI, S. S., MULLINS, K. AND COHEN, D., 1984, Intervarietal variation for *in vitro* plant regeneration in the genus *Trifolium*. *Euphytica*, **33**: 915-921.

BONGA, J. M., 1977, In: *Applied and Fundamental Aspects of Plant Cell, Tissue and Organ culture*. J. Reinert and Y. P. S. Bajaj (eds). Springer Verlag, New York, 93-108.

*BONGA, J. M., 1981, In: Tissue Culture of Economically Important Plants, A. N. Rao (ed). Proc. of the COSTED Symp. Singapore, 191-196.

CHAKRAVARTHY, R., PAVAN, U., RAMESH, M., SRINIVASA REDDY, K. AND SADANANDAM, A., 2001, Thidiazuron-induced organogenesis from mature leaf cultures in mulberry (*Morus indica* L.) Cv S_{54}. *Sericologia*, **41** (2): 269-273.

CHATTOPADHYAY, S., CHATTOPADHYAY, S. AND DATTA, S.K., 1990, Quick *in vitro* production of mulberry (*Morus alba* L.) plantlets for commercial purpose. *Indian J. Exp. Bio.*, **28**: 522-525.

CHITRA, D. S. V. AND PADMAJA, G., 1999, Clonal propagation of mulberry (*Morus indica* L. cultivar M-5) through *in vitro* culture of nodal explants. *Sci. Hort.*, **80** (3): 289-298.

CHRISTENA, V., JAVED, A. Q., RAVIDER, G. AND PRAVEEN, K. S., 1992, Morphoregulatory role of thidiazuron. *Pl. Physiol.*, **99**: 1704-1707.

DANDIN, S.B. AND RAMESH, S.R., 1987, A *Kalpavaruksha* called mulberry. *Indian silk*, **87**: 49-53.

DAS, B. C., 1983, Mulberry taxonomy, Cytogenetics and breeding, in: *National Seminar on silk research and development.* March 10-13, Bangalore, India.

DAS, B.C. AND KRISHNASWAMI, S., 1965, Some observations on interspecific hybridization in mulberry. *Indian J. Seric.*, **4**: 1-8.

DAS, M., KUMRI, S. AND KHURANA, P., 2003, Utility of mature tissue explants for regeneration and transformation studies in Indian mulberry, *Adv. in tropic. Seri.*,

DAVE, A. AND BATRA, A., 1995, Somatic tissues lead in to embryogenesis in cumin. *Curr. Sci.*, **68**(7): 754-755.

*DESJARDINS, Y.A., LAFORAGE, F., LESSIER, C. AND GOSSELIN, A. 1988, Acclimatization of micropropagated horticultural plants. *Acta. Hort.* **230**: 47-57.

*GEORGE, E. F., 1996, In: George, E. F. (Ed), Plant propagation by tissue culture. Part 2: In Practices Exegetics Ltd., Edington, England.

*GHAXHIGAE, J., BRENCKMANN, F. AND SAUGIER, B. 1992. *Plant Cell, Tissue and Organ Culture*, **30**: 51-57.

GHUGALE, D. D., KULKARNI, D. AND NARSIMHAN, R., 1971, Clonal propagation of *Morus alba*, *Indian J. Exp. Biol.*, **9**: 381-384.

GOEL, A. K., RAVINDRAN, S., ANANDARAO, A., NAIK, V. G., TIKADER, A., MUKERJEE, P. AND SEKAR., 1998, Variability in rooting parameters at juvenile stage in mulberry (*Morus* spp.) germplasm. *Indian J. Seric.*, **37**: 109-112.

*GUPTA, P. K., MASCARENHAS, A. F. AND JAGANNATHAN, V., 1981, Tissue culture of forest trees: Clonal propagation of mature tree of eucalyptus. *Plant Sci. Lett.*, **20**: 195-201.

*GUPTA, P. K., NADGIR, A. L., MASCARENHAS, A. F. AND JAGANNATHAN, V., 1980, Tissue culture of forest trees: Clonal multiplication of *Tectona grandis* (Teak) by tissue culture. *Plant Sci. Lett.*, **17**: 259-268.

HAYASHI, K. AND OKA, S., 1995, Formation of multiple bud bodies and plant regeneration in the mulberry, *Morus alba*. *J. Seric. Sci. Jpn.*, **64** (2): 117-123.

HOSSAIN, M., RAHMAN, S. M., ZAMAN, A., JORDER, N. AND ISLAM, R., 1992, Micropropagation of *Morus laevigata* Wall. From mature trees. *Plant Cell Rep.*, **11**: 522-524.

ISLAM, R., ZAMAN, A., HOSSAIN, M., JORDER, N., BARI, M.A. AND BARMAN, A. C., 1993, *In vitro* propagation of mulberry (*Morus alba* L.): Effect of cytokinin types and tyrosine. *Bull. Seric. Res.*, **4**: 6-9.

IVANICKA, J., 1987, *In vitro* micropropagation of mulberry, *Morus nigra* L. *Sci. Hort.*, **32**:33-39.

JAIN, A. K. AND DATTA, R. K., 1992, Shoot organogenesis and plant regeneration in mulberry (*Morus bombycis* Koidz), *Plant Cell Tiss. Org. Cult.*, **29**: 43.

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** (12): 737-740.

*JOARDER, O. I., HOSSAIN, N. AND JOARDER, N., 1988, Rapid multiplication of *Morus alba* L. through tissue culture. *Abstract proceedings of International Conference of Plant Research and it's relevance in future*, 154.

KAMILI, A. N. AND SHAH, A. M., 1998, Regeneration of plants from axillary buds in mulberry species. *J. Seric.*, **6**: 29-33.

KAPUR, A., BHATNAGAR, S. AND KHURANA, P., 2001, Efficient regeneration from mature leaf explants of Indian mulberry *via* organogenesis. *Sericologia*, **41**: 207-214.

KATASE, M., 1993a, Factors affecting proliferation of shoot in mulberry axillary bud culture. *J. Seric. Sci. Jpn.*, **62**: 152-161.

KATASE, M., 1993b, *Ex vitro* rooting and acclimatization of mulberry shoots. *J. Seric. Sci. Jpn.*, **62** (5): 420-427.

KATASE, M., 1994, Root initiation and acclimatization of mulberry shoots by the *ex vitro* rooting method with mist treatment. *J. Seric. Sci. Jpn.*, **63** (4): 293-298.

KATHIRAVAN, K., GANAPATHI, A. AND SHAJAHAN, A., 1995, Evaluation of *in vitro* response of mulberry genotypes. *Sericologia*, **35**: 305-317.

KAVYASHREE, R., GAYATRI, M. C. AND REVANASIDDAIAH, H.M., 2005a, A protocol for *in vitro* regeneration of mulberry variety-S_{54} through callus phase. *Sericologia*, **45** (1): 103-109.

KAVYASHREE, R., GAYATRI, M. C. AND REVANASIDDAIAH, H.M., 2005b, *Ex vitro* rooting and acclimatization of *in vitro* shoots of mulberry variety S_{54}. *Sericologia*, **45**: 81-85.

KETCHUM, R. E. B., GIBSON, D. M. AND GALLO, L. G., 1995, Media optimization for maximum biomass production in cell culture of Pacific Yew. *Plant Cell Tissue and Organ Cult.*, **42**: 185-193.

KIM, H. R., PATEL, K. R. AND THORPE, T. A., 1985, Regeneration of mulberry plantlets through tissue culture. *Bot. Gaz.*, **146**: 335-340.

KIRAN, K., SHARMA AND TREVORA. THORPE., 1990, *In vitro* propagation of mulberry through nodal segments *Scientia Hortic.* **42**: 307-320.

*KOMO, K., 1990, Effect of phytohormones from callus of gadiolus cultivar Jenny lee. *In vitro Cell Dev. Biol.*, **30**: 265-331.

KUNO, K. AND JI, D., 1996, Characteristics of mulberry callus proliferated by the application of brassinolide and coumarin. *J. Seric. Sci. Jpn.*, **65** (5): 347-351.

LEVIN, R., 1988, Plant tissue cell cultivation containing meristematic areas aseptically transported to sieve, then propagules transferred to culture vessel. European Patent No. 132414.

MACHII, H., 1990, Leaf disc transformation of mulberry plant (*Morus 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.

MACHII, H., 1999, Screening mulberry (*Morus spp.*) genotypes for adventitious bud formation and plant regeneration from immature leaf culture. *J. Seric. Sci. Jpn.*, **68** (6): 479-489.

MHATRE, M., BAPAT, V. A. AND RAO, P. S., 1985, Regeneration of plants from the callus of the leaves and axillary buds in mulberry (*Morus indica* L.). *Plant Cell Rep.*, **4**: 78-80.

MURASHIGE, T. AND SKOOG, F., 1962, A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol. Plant.*, **15**: 473-497.

NARAYAN, P., CHAKRABORTHI, S. AND SUBBA RAO, G., 1989, Regeneration of plantlets from callus of stem segments of mature plant of *Morus alba* L.. *Proc. Indian Natl. Sci. Acad.*, B_5: 469-472.

*OHNISHI, T., YASUKURA, F. AND TAN, J., 1986, Preservation of mulberry callus by the addition of both Abscissic acid (ABA) and p-Aminobenzoate (PABA) in culture medium. *J. Seric. Sci. Jpn.*, **55** (3): 252-255.

*OHYAMA, K. AND OKA, S., 1981, Effect of N- (2-chloro-4-pyridyl)-N-phenyl urea on bud formation in hypocotyl segments of mulberry trees. *Proceedings of the International Workshop on in vitro Cultivation of Forest Tree Species. AFOCEL*, 277-280.

OHYAMA, K., 1970, Tissue culture in mulberry tree, *JARQ.*, **5**: 30-34.

OKA, S. AND OHYAMA, K., 1974, Studies on *in vitro* culture of excised buds in mulberry tree. I. Effect of growth substances on the development of shoots and organ formation from winter buds. *J. Seric. Sci. Jpn.*, **43**: 230-235.

OKA, S. AND OHYAMA, K., 1975, Studies on *in vitro* culture of excised buds in mulberry tree. II. Effect of growth substances on the development of shoots from axillary bud. *J. Seric. Sci. Jpn.* **44**: 444-450.

OKA, S. AND OHYAMA, K., 1981, *In vitro* initiation of adventitious buds and its modification by high concentration of benzyladenine in leaf tissue of mulberry (*Morus alba* L). *Can. J. Bot.*, **59**: 68-74.

*OKA, S., 1985, *In vitro* culture of isolated buds and organ formation in mulberry. *Bull. Seric. Exp. Stn.*, **29** (6): 747- 752.

OSHIGANE, K., 1989, Studies on formation of adventitious bud and growth of plantlet in the hypocotyl callus of mulberry. *J. Fac. Text.Sci. Tech. Sinshu Univ. E Agric. Seric.* ***107***:53-54.

*PAL, A., 1983, *In vitro* callus development and shoot bud multiplication from rhizomes of *Costus specious* (Koen.), *Sm. Cell Chr. Res.*, **6** (3): 68-72.

*PATEL, G. K., BAPAT, V. A. AND RAO, P. S., 1983, *In vitro* culture of organ explants of *Morus indica*: 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 cathyana* Hemsi., *M. thoukoiz* 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., 1996, Micropropagation of a fruit tree, *Morus australis* Poir, Syn. *M. Acidosa* Griff. *Plant Cell Rep.*, **15**: 841-845.

PAVAN, U., RABBANI, M., YASHODHARA, V., RAMASWAMY, N. AND SADANANDAM, A., 1999, Effect of antibiotics on the *in vitro* growth of mulberry, *M. indica* L. *Indian J. Seric.*, **38** (2): 168-170.

POSPISILOVA, J. 1996. *Biol. Plant.* **38**: 605-609

*PRAKASH, J. AND PIERIK, R. L. M., 1993, In: Prakash, J. and Pierik, R. L. M. (Eds.), *Plant Biotechnology – Commercial prospects and problems*, Oxford and IBH Publishing Co., Pvt., Ltd., New Delhi : 1.

RAO, P. S. AND BAPAT, V. A., 1978, Vegetative propagation of sandalwood plants through tissue culture. *Can. J. Bot.*, **56** (9): 1153-1156.

*RAO, R. K. AND VIDYANATH, K., 1997, Induction of multiple shoots from seedling shoot tips of different varieties of sesamum. *Indian J. Pl. Physiol.*, **2**: 257-261.

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

RAVINDARAN, S., DANDIN, S.B. AND JAIN, A.K., 1988, Application of plant cell tissue and organ culture in mulberry improvement programmes. *Indian silk.*, (Dec. Ed.), 37-39.

REVANASIDDAIAH, H.M., SRINIVASA, M.M. AND GAYATRI, M.C., 1999, Micropropagation of mulberry *Morus albaL.* (Berhampore local) through axillary buds of aseptic and field grown plant. *Proc. Natl. Sem. Tropic. Seric.*, pp.1-3.

SAITO, H AND KATAGIRI, K., 1989, Adventitious bud induction in leaves isolated from winter buds of mulberry. *J. Seric. Sci. Jpn.*, **58**: 197.

SAITO, H., 1992, *In vitro* propagation of mulberry through adventitious bud induction system. *J. Seric. Sci. Jpn.*, **61** (1): 46-51.

SHAJAHAN, A., KATHIRAVAN, K. AND GANAPATHI, A., 1995, Economic micro propagation for mulberry. *Indian silk*, **8**:

SHARMA, K. AND THORPE, T.A., 1990, *In vitro* propagation of mulberry (*Morus alba* L.) through nodal segments. *Scientia Horticulturae*, **42**: 307-320.

*SHOUKANG, L., DONFENG, J. AND JUN, Q., 1987, *In vitro* production of haploid plants from mulberry (*Morus*) anther culture. *Sciencia Sinica*, **30**: 853.

SINHA, R. K. AND MALLIK, R., 1987, *In vitro* initiation, maintenance and growth rate study of cotyledonary derived callus culture of *Acacia catechu* Wild. *Cell Chr. Res.*, 10 (1): 8-12.

*SMITH, E.F., GRIBANDO, I., ROBERTS, A.V. AND MOTTLY, J. 1992, Comprehensive micropropagation of horticultural crops. *Hort. Sci.*, **27**: 111-113.

*SMITH, E.F., ROBERTS, A.V. AND MOTTLY, J. 1990. *Plant Cell. Tissue and Organ Culture*, **21**: 141-145.

SRINIVASA, M. N., REVANASIDDAIAH, H.M., GAYATRI, M. C. AND KAVYASHREE, R., 2001, Indirect organogenesis through stem callus in mulberry variety Berhampore Local. *Sericologia,* **41**: 101-104.

*STUART, D. A., MCCALL, C. M. AND SLADE, D., 1990, Improved somatic embryogenesis using induction medium containing synthetic auxin analogues. World Patent No. 9001058.

SUGIMURA, Y., ADACHI, T., KOTANI, E. AND FURUSAWA, T., 1998, Shoot bud formation and plantlet regeneration from the basal tissues of mulberry leaves. *J. Seric. Sci. Jpn.*, **67** (5): 421-424.

*TEMPLETON-SOMERS, K. M. AND COLLINS, W. W., 1986, Heritability of regeneration in tissue of sweet potato (*Ipomia batatus*). *Theor. Appl. Genet.*, **71**: 835-841.

TEWARY, A., BHATNAGAR, S. AND KHURANA, P., 1999, *In vitro* response of commercially valuable varieties of *Morus* sp. to thidiazuron and activated charcoal. *Plant Biotechnol.*, **16**: 413-417.

TEWARY, P. K., AND SUBBA RAO, G., 1990, Multiple shoot formation through shoot apex culture of mulberry. *Indian J. Forestry*, **13** (2): 109-111.

TEWARY, P. K., GUPTA, B. K. AND SUBBA RAO, G., 1989, *In vitro* studies on growth rate of callus of mulberry (*Morus alba*). *Indian J. Fore*, **12**: 34-35.

TEWARY, P.K., RAGHUNATH, M.K. AND SARKAR, A., 2001, Effect of some cheap gelling agents on Micropropagation of mulberry. *Indian J. Seric.*, **40**: 188-191.

TEWARY, P.K., RAGHUNATH, M.K., VENKATESWARLU, M. AND SARKAR, A., 1996, Genotypic differences in response to *in vitro* shoot development of mulberry (*Morus* spp.). *Indian J. Seric.*, **35**: 104-106.

TEWARY, P.K., SARKAR, A., KUMAR, V. AND CHAKRABORTI, S., 1995, Rapid *in vitro* multiplication of high yielding mulberry (*Morus* sp.) genotypes V_1 and S_{34}. *Indian J. Seric.*, **34**: 133-136.

THORPE, T. A. AND PATEL, K. R., 1984, Clonal propagation: adventitious buds. *Cell culture and Somatic Cell Genetics of Plant Cells*, **1**: 49-60.

TOHJIMA, F., YAMANOUCHI, H., KOYAMA, A. AND MACHI, H., 1996, Effects of plant hormones on the callus induction from mulberry cotyledons. *J. Seric. Sci. Jpn.*, **65** (6): 510-513.

*TOMAR, A., RATHODE, R. S., CHAND, L. AND GARG, G. K., 1999, Organogenesis and somatic embryogenesis from callus cultures of chickpea (*Cicer arietinum*), In: Abst. Vol. Of Natl. Symp. On role of plant tissue culture in biodiversity, conservation and economic development, G. B. Pant Institute of Himalayan Environment and Development, Almora, pp: 16.

*UEMATSU, C., AKIHAMA, T. AND INOKUMA, C., 1987, Propagation of almond by shoot tip culture. *Jpn.,, J. Breed.*, **37**: 464-468.

USHA YADAV, MADAN LAL AND VIJAI S. JAISWAL., 1990, Micropropagation of *Morus nigra* L. From shoot tip and nodal explants of mature trees. *Scientia Hortic.*, **44**: 61-67.

*VASIL, I. K. AND VASIL, V., 1980, Perspectives in Plant Cell ans Tissue Culture. *Int. Rev. Suppl.* **11 A**: 145-173.

VIJAYAN, K., CHAKRABORTHI, S. P. AND ROY, B. N., 1998, Regeneration of plantlets through callus culture in mulberry. *Indian J. Plant Physiol.*, **3**: 310-313.

VIJAYAN, K., CHAKRABORTI, S.P. AND ROY, B.N., 2000, Plant regeneration from leaf explants of mulberry: Influence of sugar, genotype and 6-benzyladenine. *Indian J. Exp. Bio.*, **38**: 504-508.

*VON ARNOLD, S., AND ERIKSSON, T., 1981, In vitro studies of adventitious shoot formation in *Pinus contorta*. *J. Bot.*, **59**:870-874.

WANG, Y., CHEN, A. Y. AND NI, G. E., 1996, Factors influencing the formation of adventitious buds on mulberry cotyledons. *Sericologia*, **36**: 321-331.

YADAV, U., LAL, M. AND JAISWAL, V. S., 1990, Micropropagation of *M. nigra* L. from shoot tip and nodal explants of mature trees. *Scientia Horticulturae*, **44**: 61-67.

ZAMAN, A., ISLAM, R. AND JOARDER, O. I., 1997, Field performance and bio chemical evaluation of micropropagated mulberry plants. *Plant Cell Tissue Org. Cult.*, **51**: 61-64.

*ZIMMERMAN, J. L., 1993, Somatic embryogenesis: a model for early development in higher plant. *The plant Cell*, **5**:1411-1423.

---

*Original not seen.