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# Interaction Between time of Nodal Explant Collection and Growth Regulators Determines the Efficiency of *Morus alba* Micropropagation

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## Abstract

**The hormonal requirement suiting micropropagation of *Morus alba* during any season throughout the year was studied. Sprouting frequency from axillary buds of *M. alba* was greatly influenced by the time of explant collection, the highest value was achieved when nodal explants were collected at the end of bud dormancy period (late in March) and cultured on Murashige and Skoog (MS) medium supplemented with low concentration (0.5 mg/L) of BAP, kinetin or IBA (85-68%). In addition, they showed higher axillary bud sprouting on growth-regulators-free medium (49%) than others collected in autumn or winter and cultured on medium supplemented with various growth regulators (47-48%). Regardless of that period, young explants with greenish buds collected in summer exhibiting high sprouting frequency (66%) on MS medium supplemented with 0.5 mg/L kinetin and 0.5 mg/L GA3. Shoot multiplication via adventitious bud formation was achieved when the nodal explants were cultured on MS medium supplemented with 2 mg/L BAP and 0.2 mg/L IBA. Further multiplication via nodal explants of *in vitro* grown shoots was obtained on MS medium supplemented with 0.5 mg/L BAP and 0.5 mg/L GA3. While half strength MS medium supplemented with low concentration (0.5 mg/L) of IBA, IAA or 2,4-D stimulated adventitious root formation, IBA was the best. After transfer the plantlets to the soil, acclimatization for three weeks was essential prerequisite for survival in high frequency (92%). Peroxidase activity is related to break of bud dormancy where maximum enzyme activity was detected when the lateral buds were induced to commence growth under field condition (early in spring) or *in vitro*.**

**Key words:** Bud dormancy, growth regulators, mulberry, tissue culture

## Introduction

Mulberry is a fast growing tree and the sole feed of mulberry silkworm (*Bombyx mori*). Many elite varieties have poor rooting ability through stem cuttings and it is restricted to few months throughout the year (Narayan et al. 1989). In addition, seed propagation of mulberry is not recommended due to the heterogeneous nature of the seedlings owing to its cross pollination (Das 1983; Hossain et al. 1992). Tissue culture techniques provide a fast and dependable method for mass production of uniform plantlets in short time throughout the year. Propagation of different *Morus* species from axillary buds has been reported by many workers (Jain et al. 1990; Ponchia and Cardiman 1992; Rao and Bapat 1993; Kathiravan et al. 1997). It was induced on medium supplemented with BAP, kinetin, 2,4-D, IBA and GA3 (Jain et al. 1990; Anuradha and Pullaiah 1992; Vijaya Chitra and Padmaja 1999; Vijaya Chitra and Padmaja 2002).

The success of internodal segment culture is affected by season during which the explants are collected (Hu and Wang 1983; Endress 1994; Vijaya Chitra and Padmaja 2002). Altman and Goren (1974) found that both bud dormancy and *in vitro* bud sprouting were influenced by field conditions of the donor plant material such as temperature, light intensity and photoperoid. Also, the sprouting from axillary buds of *Mours* ssp. is greatly dependent on the genotype and growth regulators

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(Ohyama and Durjan 1987; Sharma and Thorpe 1990; Pattnaik and Chand 1997; Ohyama and Chand 2000; Bhau and Wakhlu 2001; Vijaya Chitra and Padmaja 2002). In the previous reports, the growth regulators have been investigated separately without examining extensively the interaction between them during different season on *in vitro* propagation of *M. alba* or growth of *in vitro* grown shoots. Therefore, in this work, the hormonal requirement suiting micropropagation during any season throughout the year was investigated to determine the most suitable period for *M. alba* micropropagation.

In many plant species, the initiation of buds is always proceeded by an increase in peroxidase activity (Thorpe et al. 1978; Kevers et al. 1981; Booij et al. 1993), it may be due to the involvement of peroxidases in auxin metabolism and morphogenesis processes (Hassanein et al. $1999_{8a,b}$). Consequently, the relative peroxidase activity during the break of bud dormancy of *M. alba* under field (at the end of bud dormancy period) or tissue culture conditions in comparison to conditions retard growth of lateral buds during other seasons was investigated.

In addition, multiplication of *in vitro* grown shoots via adventitious bud formation and nodal explants of *in vitro* grown shoots for mass propagation was also investigated. Rooting of *Morus* ssp. was induced by various types of auxins, but a high frequency of rooting with development of healthy roots was observed on medium supplemented with 1.0 mg/L 2,4-D (Vijaya Chitra and Padmaja 1999, 2002).

The present studies differ from previous studies as the high frequency of shoot differentiation from nodal explants depended strongly on the time of nodal explant collection, the best time was at the end of bud dormancy period at the end of Match, and the root induction was depended on auxin types, 0.5 mg/L IBA in half strength MS medium was the best. Also, this work study the effect of acclimatization period on the efficiency of plantlets transfer to the soil, three weeks was an essential prerequisite to establish high survival frequency.

## Materials and Methods

The natural sprouting of axillary buds of *Morus. alba* tree begins early in April. Therefore, the nodal explants with axillary buds were collected from lateral branches of a 20 years old tree (it grows in South Valley University Garden in Sohag) in late March (21-31), June, September and November. The explants (1.5-2.5 cm long) were washed thoroughly under running tap water for 20 min and surface sterilized in 75% alcohol for 2 min followed by 0.1% mercuric chloride for 10 min. Then, they were washed 6 times in sterile distilled water, 5 min each. The nodal explants were cultured on MS medium (Murashige and Skoog 1962) supplemented with different types of growth regulators to examine percentage of axillary bud sprouting (see: Table 1). In each experiment, 45 explants were cultured on each medium. The experiment was repeated thrice, in successive three years (1999-2002). After a month culture, the appearance of shoot meristems from axillary buds was taken into consideration for calculating the sprouting frequency (%).

To identify the most suitable culture condition for growth of regenerated shoots, three replicates (each with 10 shoots) of healthy grown shoots (45 days age) were cut from the initial explants (collected at different seasons) and subcultured for further 45 days on the same medium. The length of shoots and number of internodes were recorded.

To stimulate shoot proliferation from the base of initiated shoots on the original explants, the explants showing growth of adventitious shoots and inflorescence were subcultured on MS medium supplemented with 2 mg/L kinetin or BAP in combination with 0.2 mg/L IBA. The number of shoots/ explant was counted after four weeks culture.

MS medium supplemented with low concentration of growth regulators (0.5-1.0 mg/L BAP, 0.5 mg/L BAP and 0.5 mg/L GA3,

**Table 1.** The effect of different growth regulators on sprouting frequency from axillary buds of *Morus alba* after 30 days culture. Means ± standard deviation of three independent experiments. 45 explants were used for each experiment. * Significant differences at $p>0.05$ between the best hormonal requirements in each period in relation to corresponding control (a: The hormonal requirement induced maximum values of explants collected at the end of bud dormancy period).

| Treat. (mg/L) | Sprouting frequency (%) | | | |
|---|---|---|---|---|
| | Mar. | Jun. | Sept. | Dec. |
| Free | 49 | 11 | 9 | 12 |
| 0.1 2,4-D | 49 | 39 | 31 | 25 |
| 0.3 2,4-D | 59 | 38 | 26 | 28 |
| 0.6 2,4-D | 38 | 20 | 21 | 15 |
| 1.0 2,4-D | 15 | 13 | 21 | 11 |
| 0.5 BAP | 85 a | 50 | 34 | 30 |
| 1.0 BAP | 76 | 48 | 39 | 32 |
| 2.0 BAP | 65 | 41 | 27 | 28 |
| 4.0 BAP | 29 | 24 | 23 | 24 |
| 0.5 kinetin | 73 | 48 | 39 | 35 |
| 1.0 kinetin | 70 | 60 | 39 | 37 |
| 2.0 kinetin | 49 | 62 | 38 | 36 |
| 0.5 BAP + 0.5 GA3 | 80 | 53 | 35 | 32 |
| 0.5 kinetin + 0.5 GA3 | 74 | 66* | 42 | 38 |
| 0.5 BAP + 0.3 2,4-D | 41 | 16 | 20 | 19 |
| 0.5 kinetin + 0.3 2,4-D | 29 | 12 | 19 | 19 |
| 0.5 IBA | 68 | 45 | 24 | 26 |
| 0.5 IBA + 0.5 BAP | 78 | 51 | 33 | 33 |
| 0.5 IBA + 0.5 GA3 | 65 | 49 | 28 | 28 |
| 0.5 IBA + 0.5 BAP + 0.5 GA3 | 79 | 51 | 48* | 47* |

and 0.5 mg/L IBA and 0.3 mg/L 2,4-D) was used to detect the most suitable growth regulator for multiplication via nodal explant of *in vitro* grown shoots. Ninety nodes (30 nodes/ year) were subcultured for 6 weeks. Then the percentage of nodes showing shoot formation was determined.

Adventitious root formation of healthy shoot cuttings was induced on half strength MS medium supplemented with 1. 5% sucrose and two concentrations (0.5 or 1.0 mg/L) of each IBA, IAA or 2,4-D. The frequency of rooting (%) and the number as well as the length (cm) of root system/ shoot cuttings were determined after 5 weeks.

Since preliminary experiment indicated that number of roots and root length were not limiting factors for plantlets transfer to the soil, rooted shoots irrespective the number or length of root system were transferred to soil and subjected to different periods of acclimatization (1, 2, 3 or 4 weeks). It was accomplished by covering the pots with plastic bags. At the last week, two pores were made and their size was increased daily for gradual decrease of humidity around the plantlets. After one month, the number of plantlets showed further growth was counted to study the effect acclimatization period on plantlets survival.

To determine the relative peroxidase activity, half gm of buds and surrounded bark zone were homogenized in 0.5 mL buffer contained 40 mM potassium phosphate pH 7.2, 0.1 mM EDTA, 5 mM guaiacol, 0.3 mM hydrogen peroxide (Wakamatsu and Takahama 1993). The extracted samples were assayed spectrophotometrically at 470 nm. The peroxidase activity of buds collected early in March was considered to be a control (100%).

Unless otherwise stated, the following conditions were applied: Cultures were maintained under white florescent tubes, providing an irradiance of 65 $\mu$mol $m^{-2}s^{-1}$, in a tissue culture room at 25°C ± 2 with a 16 h daily photoperiod without humidity control. MS medium supplemented with 30 g/L sucrose and 0.8% (w/v) agar, pH 5.8 (prior to autoclaving for 20 min at 121°C ) was used in all experiments. The vitamin part was (expressed as mg/L): Myoinositol (100), Vitamin B1 hydrochloride (4), nicotinic acid (4), pyridoxal hydrochloride (0.7), biotin (0.04) and folic acid (0.5). Where used the growth regulators were added prior to adjusting pH and autoclaving, but GA3 was filter sterilized and added into medium after autoclaving. Media were dispensed into 90 mm diameter Petri dishes or 250 mL jars.

All data were subjected to analysis of variance using statistical package. The significant difference of the average was tested using Least Significance Differences (L. S. D) at significant levels 5%.

## Results

A progressive increase in contamination of nodal explants with the age of the plant material was recorded. Contamination of explants obtained from newly formed branches harvested in June was lower (10%) than those of explants harvested in other periods (March 23%, September 14%, December 18%).

The axillary buds sprouted into shoots in 7 days when the nodal explants were collected in late March and cultured on MS medium with or without growth regulators. On the other side, sprouting delayed when explants were collected during the other seasons and it depended on the type of inducer used. BAP at low concentration (0.5- 1 mg/L) induced faster sprouting (12 days) than kinetin, IBA and BAP in high concentration (16 days) as well as 2,4-D (20 days).

The sprouting frequency from axillary buds varied strongly depending on the period of explant collection and hormonal component of the media (Table 1). In general, high sprouting frequency was observed in early spring (85%), when the nodal explants were collected at the end of dormancy period and cultured on MS medium supplemented with 0.5 mg/L BAP. This period is at the end of March (21-31). During that period, explants cultured on growth-regulator free MS medium showed *in vitro* sprouting (49%) more than those collected in autumn or winter and cultured on MS medium supplemented with various growth regulators, where combination between 0.5 mg/L BAP, 0.5 mg/L IBA and 0.5 mg/L was necessary to induce relatively high sprouting frequency (47%-48%). In summer, where explants with green buds were used, 0.5 mg/L kinetin and 0.5 mg/L GA3 gave the best results (66%). It's worthy to mention that the data obtained from explants collected before and after the end of March was similar to those collected in winter and summer, respectively.

In general, kinetin was better than BAP in inducing axillary bud sprouting, vise versa was detected in early spring. Addition of 0.5 mg/L GA3 did not improve sprouting frequency of that obtained by 0.5 mg/L BAP, but it improved sprouting frequency when it used in combination with 0.5 mg/L kinetin or IBA.

Shoots of *M. alba* grew with an average of 3.9-10.5 cm/ shoot in three months (Table 2) with an average of 2.8-8.8 nodes per shoot (Table 3). The length of shoots (7.6-9.5 cm/shoot) as well as the number of nodes (6.1-7.8 nodes/ shoot) was high when BAP at low concentrations (0.5-1.0 mg/L) was used alone or in combination with other growth regulators to induce bud sprouting. The number of internodes was proportional to the shoot length. Combination of BAP, GA3 and IBA in low concentration (0.5 mg/L) suited the shoot growth, resulting high number of internodes, at any period throughout the year.

Shoots and inflorescence were detected on *M. alba* explant

irrespective of time of explant collection and growth regulator used. Upon the removal of inflorescence and brown tissue from the original explants and transferring them to medium supplemented with 2 mg/L BAP or kinetin in combination with 0.2 mg/L IBA, adventitious shoots was formed in two weeks. Combination between BAP and IBA was better than that of kinetin and IBA in inducing shoot multiplication and it was dependent on the time of explant collection (Table 4). Explants collected at the end of bud dormancy period was better (10.3 shoot/ explant) than fresh-green explants collected in June (7.3 shoot/ explant) as well as those collected in other periods. Growth of those shoots was diminished due to the formation of fast growing callus from the original explant. For continuous growth, the resulted shoots were transferred after removal of callus to medium supplemented with 0.5 mg/L BAP alone or in combination with 0.5 mg/L of GA3 and / or IBA. Further multiplication through nodal explants of *in vitro* initiated shoots was accomplished in high frequency (85%) on MS medium supplemented with 0.5 mg/L BAP and 0.5 mg/L GA3 (Table 5).

The effect of several auxins (IBA, IAA and 2,4-D) in two concentrations (0.5 and 1 mg/L) in half strength MS medium on root induction of *M. alba* was studied (Table 6). High rooting frequency was obtained when IBA at concentration of 0.5 mg/L was used. The length of root system was strongly influenced by the type of auxin used. 2,4-D induced higher number of

**Table 2.** The effect of different growth regulators on growth of shoots obtained from *M. alba* explants after 90 days culture.
Means±standard deviation of three independent experiments. 45 explants were used for each experiment. * Significant differences at p> 0.05 between the best hormonal requirements in each period in relation to corresponding control (a: the hormonal requirement induced maximum values of explants collected at the end of bud dormancy period).

| Treat. (mg/L) | Sprouting frequency (%) | | | |
|---|---|---|---|---|
| | Mar. | Jun. | Sept. | Dec. |
| Free | 7.5±0.3 | 6.2±0.2 | 6.2±0.2 | 5.1±0.1 |
| 0.1 2,4-D | 8.9±0.2 | 6.9±0.4 | 6.2±0.2 | 6.5±0.3 |
| 0.3 2,4-D | 9.2±0.1 | 7.0±0.5 | 8.2±0.2 | 7.1±0.5 |
| 0.6 2,4-D | 5.2±0.3 | 4.1±0.1 | 4.5±0.1 | 4.0±0.5 |
| 1.0 2,4-D | 5.2±0.3 | 4.0±0.2 | 4.1±0.3 | 4.2±0.9 |
| 0.5 BAP | 10.2±0.3 | 8.6±0.1 | 9.4*±0.3 | 7.6±0.4 |
| 1.0 BAP | 10.5 a±0.3 | 8.5±0.2 | 9.1±0.1 | 8.4±0.2 |
| 2.0 BAP | 5.7±0.5 | 4.7±0.1 | 4.3±0.1 | 4.5±0.2 |
| 4.0 BAP | 5.2±0.3 | 4.0±0.3 | 4.5±0.3 | 4.5±0.3 |
| 0.5 kinetin | 6.4±0.3 | 4.5±0.5 | 4.5±0.2 | 4.4±0.3 |
| 1.0 kinetin | 6.7±0.3 | 6.2±0.3 | 6.4±0.1 | 5.3±0.2 |
| 2.0 kinetin | 5.7±0.2 | 6.0±0.3 | 6.7±0.2 | 4.5±0.5 |
| 0.5 BAP + 0.5 GA3 | 10.5±0.3 | 8.4±0.3 | 9.0±0.3 | 8.9±0.1 |
| 0.5 kinetin + 0.5 GA3 | 8.1±0.9 | 6.9±0.3 | 8.6±0.2 | 9.2*±0.3 |
| 0.5 BAP + 0.3 2,4-D | 10.1±0.2 | 8.6±0.1 | 8.1±0.1 | 8.0±0.2 |
| 0.5 kinetin + 0.3 2,4-D | 5.7±0.2 | 3.9±0.5 | 4.9±0.2 | 4.1±0.2 |
| 0.5 IBA | 9.1±0.2 | 7.5±0.3 | 7.9±0.3 | 7.3±0.3 |
| 0.5 IBA + 0.5 BAP | 10.2±0.1 | 8.9±0.3 | 9.2±0.1 | 8.4±0.3 |
| 0.5 IBA + 0.5 GA3 | 9.6±0.1 | 9.1±0.3 | 9.1±0.1 | 8.7±0.3 |
| 0.5 IBA + 0.5 BAP +0.5GA3 | 10.2±0.3 | 9.2*±0.1 | 9.2±0.2 | 9.0±0.2 |

**Table 3.** The effect of different growth regulators on number of buds initiated on explants of *Morus alba* after 90 days culture.
Means±standard deviation of three independent experiments. 45 explants were used for each experiment. * significant differences at p> 0.05 between the best hormonal requirements in each period in relation to corresponding control (a: the hormonal requirement induced maximum values of explants collected at the end of bud dormancy period).

| Treat. (mg/L) | Number of buds/ shoot | | | |
|---|---|---|---|---|
| | Mar. | Jun. | Sept. | Dec. |
| Free | 6.3±0.2 | 4.4±0.3 | 5.3±0.1 | 5.0±0.3 |
| 0.1 2,4-D | 7.2±o.2 | 5.2±0.6 | 6.3±0.1 | 6.0±0.1 |
| 0.3 2,4-D | 7.3±0.3 | 5.2±0.2 | 4.3±0.2 | 5.4±0.3 |
| 0.6 2,4-D | 5.3±0.4 | 4.3±0.2 | 4.0±0.3 | 4.1±0.5 |
| 1.0 2,4-D | 3.8±0.6 | 2.9±0.3 | 3.7±0.5 | 4.1±0.2 |
| 0.5 BAP | 8.8a±0.3 | 7.0±0.1 | 7.5*±0.3 | 6.1±0.4 |
| 1.0 BAP | 8.6±0.4 | 6.2±0.3 | 7.5*±0.2 | 6.0±0.4 |
| 2.0 BAP | 5.5±0.3 | 4.5±0.3 | 4.5±0.2 | 4.4±0.1 |
| 4.0 BAP | 4.9±0.4 | 3.7±0.3 | 4.5±0.3 | 4.4±0.2 |
| 0.5 kinetin | 6.0±0.4 | 3.6±0.3 | 4.3±0.2 | 4.4±0.3 |
| 1.0 kinetin | 6.4±0.3 | 6.2±0.3 | 6.3±0.3 | 5.2±0.4 |
| 2.0 kinetin | 7.2±0.2 | 6.2±0.2 | 6.5±0.2 | 4.6±0.2 |
| 0.5 BAP + 0.5 GA3 | 7.2±0.2 | 6.2±0.2 | 6.3±0.3 | 6.4±0.2 |
| 0.5 kinetin + 0.5 GA3 | 7.1±0.2 | 6.1±0.2 | 5.9±0.4 | 6.3±0.3 |
| 0.5 BAP + 0.3 2,4-D | 7.5±0.1 | 6.5±0.1 | 6.1±0.2 | 6.2±0.3 |
| 0.5 kinetin + 0.3 2,4-D | 4.7±0.1 | 3.7±0.1 | 4.1±0.2 | 3.9±0.2 |
| 0.5 IBA | 7.5±0.5 | 6.5±0.5 | 6.3±0.2 | 6.3±0.4 |
| 0.5 IBA + 0.5 BAP | 7.9±0.2 | 6.9±0.2 | 7.2±0.5 | 6.8±0.3 |
| 0.5 IBA + 0.5 GA3 | 8.2±0.2 | 7.2±0.2 | 7.1±0.2 | 6.8±0.3 |
| 0.5 IBA + 0.5 BAP +0.5 GA3 | 8.5±0.4 | 7.5*±0.4 | 7.4±0.2 | 7.5*±0.4 |

**Table 4.** The effect of MS medium containing 2 mg/L BAP and 0.2 mg/L IBA on adventitious shoot formation on nodal explants collected at different periods.
Each value represents the mean±SD of three experiments. * Means significantly different in relation to control.

| Month | Number of adventitious shoot/ explant |
|---|---|
| March (Control) | 10.33±0.6 |
| June | 7.33*±1.5 |
| September | 4.00*±1.0 |
| October | 4.50*±1.7 |

**Table 5.** Percentage of shoot formation from axillary buds of *in vitro* grown shoots (obtained from explants collected in March) on MS medium supplemented with different growth regulators.
Each value represents the mean±SD of three experiments. * Means significantly different in relation to control.

| Growth regulators conc. | Frequency (%)of growth of axillary buds of segments obtained from *in vitro* grown shoots |
|---|---|
| 0.5 mg/L BAP (Control) | 69 |
| 1.0 mg/L BAP | 59* |
| 0.5 mg/L BAP + 0.5 mg/L GA3 | 85* |
| 0.5 mg/L IBA | 51* |
| 0.3 mg/L 2,4-D | 13* |

**Table 6.** Effect of different auxins on adventitious root formation of mulberry shoot cuttings (obtained from explants collected in March) cultured on half strength MS medium supplemented with 1.5% sucrose and IBA, IAA or 2,4-D.
Each value represents the mean±SD of three experiments. * Means significantly different in relation to control.

| Auxin conc. | Root frequency (%) | Number of roots/ shoot cutting | Length of roots/ root system |
|---|---|---|---|
| 0.5 mg/L IBA (Control) | 81 | 3.3±0.6 | 6.2±1.2 |
| 1.0 mg/L IBA | 68* | 3.7±0.6 | 5.9±0.4 |
| 0.5 mg/L IAA | 63* | 2.3±0.6 | 11.3*±0.9 |
| 1.0 mg/L IAA | 46* | 2.3±0.6 | 11.8*±0.5 |
| 0.5 mg/L 2,4-D | 45* | 5.3*±1.5 | 9.4*±0.7 |
| 1.0 mg/L 2,4-D | 47* | 6.0*±1.0 | 10.3*±0.2 |

roots and they were thicker and stronger than those formed under the influence of other auxins. Furthermore, 2,4-D induced the formation of adventitious roots in short time (10 days) in comparison to those induced under the influence of both IBA and IAA (2 weeks).

It's worthy to mention that survival frequency was not proportional to the number of roots or root length but it was proportional to the length of acclimatization period. Increasing the acclimatization period increased the survival frequency up to three weeks (Table 7), further increase of acclimatization period was not necessary.

Peroxidase activity of buds and their surrounded areas (0.5-0.75 cm radius of bark) were determined at the end of bud dormancy period as well as during other periods throughout the year (Table 8). The data indicated that maximum activity was registered in buds and their surrounded zone at the time of proceeded the initiation of bud growth if the growth was induced under field condition or *in vitro*.

**Table 7.** Affect of acclimatization period under plastic bags on the survival frequency of mulberry plantlets in soil.
Each value represents the mean±SD of three experiments. * Means significantly different in relation to control.

| Acclimatization period (weeks) | Survival frequency (%) |
|---|---|
| 1 (Control) | 9 |
| 2 | 39* |
| 3 | 92* |
| 4 | 92* |

**Table 8.** Relative peroxidase activity of axillary buds and surrounded bark during different periods throughout the year as well as that of *in vitro* cultured explants.
Each value represents the mean±SD of three experiments. * Means significantly different in relation to control.

| Time | Relative peroxidase activity |
|---|---|
| March early (Control) | 100 |
| March late | 175* |
| April early | 117* |
| Summer | 120* |
| Autumn | 98 |
| Winter | 92 |
| *In vitro* (March early) | 163* |

## Discussion

Mulberry is a highly heterozygous plant and it's *in vitro* propagation through nodal cuttings would ensure genetic uniformity among the regenerants (Vijaya Chitra and Padmaja 2002). Data in this work indicate that *in vitro* propagation of *M. alba* via nodal explant is strongly affected by the period during which the explants is collected. Distinct period was better than others, it was at the end of the bud dormancy period, it was in late March. During that time, the endogenous growth inhibitor level may be low in parallel to break the bud dormancy (Divlin and Witham 1983). Furthermore, the endogenous growth substances at the end of dormancy period become in state stimulating valuable sprouting frequency even on growth-regulator free medium. Also, it explains way sprouting of axillary buds at the end of dormancy period appeared in short time irrespective the growth regulators were used or not.

The data of early spring showed that BAP was more effective than kinetin in inducing axillary bud sprouting and shoot differentiation in mulberry. On the other side, results in summer, autumn and winter showed that kinetin induced higher sprouting frequency than BAP. Therefore, the results in this work indicate that the hormonal requirements inducing valuable micropropagation of *M. alba* dependent on the period dur-

ing which the explants were collected.

If we excluded the period proceeds the natural sprouting, at the end of bud dormancy period, summer was better than any other periods throughout the year, where the explants have green buds and they were recommended by many authors (Oka and Ohyama 1975; Anuradha and Pullaiah 1992; Zaman et al. 1997; Vijaya Chitra and Padmaja 2002). These explants showed the highest frequency of aseptic cultures (10%) and valuable sprouting frequency (66%) on MS medium supplemented with 0.5 mg/L kinetinand 0.5 mg/L GA3. Vijaya Chitra and Padmaja (2002) reported that sprouting of axillary buds and the frequency of aseptic cultures obtained in summer was the highest.

During dormancy periods, in September and December, combination between IBA, BAP and GA3 in low concentration (0.5 mg/L of each) was necessary to induce sprouting from axillary buds. While, GA3 did not improve the sprouting frequency of explants collected at the end of dormancy period, it impro-ved the sprouting frequency of explants collected during dormancy periods and cultured on MS medium supplemented with various growth regulators. It indicates that, the concentration of endogenous gibberllins remains low during the dormant period, but rises thirty fold after sprouting begins (Divlin and Witham 1983). Under tissue culture condition, the differential response of nodal explants collected during different seasons may be due to the differences in plant physiology (Quraishi et al. 1996). Also, it could be linked to the dynamics of endogenous substances in the donor plant material (Mhatre et al. 1985; Civinova and Sladsky 1990).

BAP at low concentration (0.5 mg/L) was found to stimulate growth of regenerated shoots as well as the number of nodes per shoot, where it used alone or in combination with 0.5 mg/L GA3 and/or 0.5 mg/L IBA. *In vitro* grown shoots could be used as a material for mass production via nodal explant. Multiplication via adventitious shoot formation was induced on MS medium with 0.5 mg/L BAP and 0.5 mg/L GA3 and it influenced by the time of explant collection, the period at the end of dormancy period was the best. Furthermore, nodal explants with green buds collection in June was better than those collected during the dormant periods in September and December.

IBA was the best auxin for *M. alba* rooting due to its ability to induce high rooting frequency in comparison to 2,4-D and IAA. In other reports, IBA was reported to be the most favorable auxin for root formation in *M. alba* (Kathiravan et al. 1997; Bhau and Wakhl 2001). On the other side, many authors reported that 2,4-D and NAA were suitable auxin for adventitious root formation in mulberry (Jain et al. 1990; Yadav et al. 1990; Hossain et al. 1992; Vijaya Chitra and Padmaja 2002).

This study indicated that the efficiency of plantlets transfer to the soil was not influenced by the number or length of root system but it was affected by the acclimatization period, three weeks was an essential prerequisite to establish high survival frequency.

The internodal segments of *M. alba* on MS medium supplemented with various growth regulators showed the formation of shoot and floral premordia. It was accompanied with increase of peroxidase activity (Table). These results are in accordance with studies of other investigators (Nakanishi and Fajii 1992; Booij et al. 1993; Arezki et al. 2001). Also, As in case of many plant species, the formation of buds under field condition is always proceeded by an increase of peroxidase activity (Thorpe et al. 1978; Kevers et al. 1981; Booij et al. 1993), it may be due to the involvement of peroxidases in the auxin metabolism and other morphological processes. Booij et al. (1993) reported that increase the peroxidase activity resulted in lowering the endogenous auxin level and consequently an auxin-cytokinin ratio which favorable bud formation or bud growth.

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