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# Micropropagation of *Myrica rubra* Sieb. and Zucc. using shoot tips and nodal explant

**Ssyed Asghari[1,2]*, Sayed Jaffar Abbas, Ligeng Chen[1], Xinhua He[1] and Yonghua Qin[1]**

[1]Key Laboratory of Horticultural Plant Growth, Development and Biotechnology, Ministry of Agriculture, Zhejiang University, Hangzhou, 310029, P. R. China.
[2]Agricultural Research Institute, Tarnab, Peshawar, Pakistan.

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***Myrica rubra* is an important horticultural, forestry and ornamental tree. Its micropropagation was achieved by using shoot tip and nodal explants from two commercial cultivars "Biji and Dongkui" on BW medium supplemented with Thidiazuron (TDZ) and/or 2,4-Dichlorophenoxyacetic acid (2,4-D). TDZ induced multiple shoots but stunted growth. Maximum number of shoots (5.75) was recorded for both cultivars with 0.6 mg $L^{-1}$ TDZ concentration. Shoot length was significantly improved with optimum concentration of TDZ (0.6 mg $L^{-1}$) and with different concentrations of 2,4-D. BW medium was found as suitable medium for micropropagation of both the cultivars of *M. rubra* with maximum number and longer shoots. WPM and MS medium ranked second in multiple shoot regeneration of both cultivars. Single microshoot cultured on plant growth regulator free medium for 3 weeks initiated roots on the medium containing Indole butyric acid (IBA) only with reduced (¼ and ½) medium strength. Roots were induced with a high frequency of 95% in Biji and 87% in Dongkui with 0.3 mg $L^{-1}$ IBA, respectively. No callus was observed on shoots in rooting media. All the concentrations of indole-3-acetic acid (IAA) either on reduced medium or full strength did not initiate any roots. Rooted plants were successfully acclimatized.**

**Key words:** *Myrica rubra*, Chinese bay berry, micropropagation, plant growth regulators.

## INTRODUCTION

*Myrica rubra* with a common name of red bayberry is an important member of *Myricaceae* family. It is one of the endemic plants species of far East Asia grown under warm and humid climatic conditions. *Myricaceae* is also considered as first *actinorhizal* family emerged during the late cretaceous period (Maggia and Bousquet, 1994). This plant can form a symbiosis with the actinomycetes *Frankia* and produce nitrogen fixing root nodules (Schweitzer and Lancelle, 1983). It is a stone fruit with a berry like shape and edible portion developed from the exocarp. It also consists of capsule that looks like cellules termed flesh segments (He et al., 2004). This species is highly valued in Japan and China for its medicinal properties (Jing et al., 2002). Fruit, seed, bark, leaf, and root of red bayberry are uses as medicines. Fruits and leafs are beneficial for treating congestion, cough, digestive problems, and diarrhea. The bark is used for the treatment of arsenic poisoning, skin diseases, wounds, and ulcers (Chen et al., 2004; He et al., 2002; Li, 2002). In recent years, a number of flavonoids and polyphenols have been extracted from its different parts (Yang et al., 2003; Chi et al., 2002; Gao et al., 2001).

It is domesticated in China, Japan, Thailand, South Korea and Philippines as fruit tree while in America and Europe as an ornamental tree (Chen et al., 2004). Due to

*Corresponding author. E-mail: sayedjaffarabbas@hotmail.com.

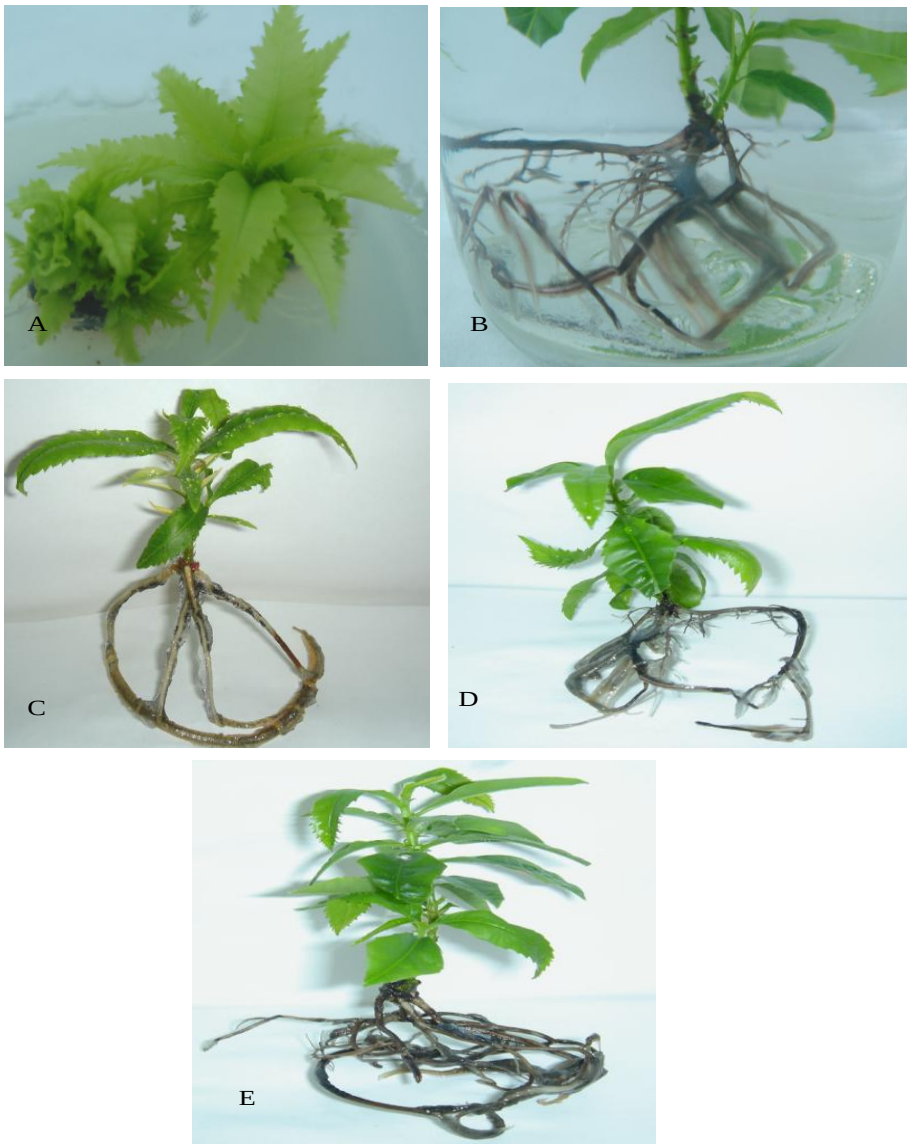


**Figure 1.** Different stages of micropropagation from shoot tips and nodal explants of *M. rubra*, A, Multiple shoots growing in BW medium containing 0.6 mg $L^{-1}$ TDZ after 6 weeks in culture; B, well established root system on shoots from cultivar Biji in the media containing 0.3 mg $L^{-1}$; C, roots developed on shoot after 4 weeks in culture from cultivar Dongkui, D. roots developed after 8 weeks in culture cv. Biji; E, roots developed after 8 weeks in culture on rooting medium on shoots from cv. Dongkui.

long its lifespan, great economic value, low production costs and nitrogen-fixing activity, red bayberry is considered as “green factory” while due to its high economic output it is called “money-making tree” (He et al., 2002; Li, 2002).

Micropropagation is used for faster multiplication of true-to-type genotypes in the minimum possible time (Vengadesan et al., 2002). Plants through micropropagation show greater genetic uniformity and clonal fidelity (Martin, 2003). In China, Biji and Dongkui are the most promising cultivars of *M. rubra* with promising characteristics and are commercially planted throughout the country (Zhuang and Pan, 2001). It is highly cross-pollinated and progeny is heterozygous (Horikawa, 1972) and also its vegetative propagation techniques such as air layering and grafting are not rapid to meet the need of elite varieties demand in time (He et al., 2002). There is only one report that describes its micropropagation procedure from the seedling tissues of *Myrica gale* (Fernando et al., 1998). To our knowledge, protocol for it *in vitro* propagation of *M. rubra* has not yet developed (Figure 1).

**Table 1.** Effect of TDZ on multiple shoot regeneration from *M. rubra* shoot tips and nodal explants after 9 weeks in culture.

| TDZ | Explants | Survival (%) | | Shoots per explant | | Shoot length (cm) | |
|---|---|---|---|---|---|---|---|
| | | Biji | Dongkui | Biji | Dongkui | Biji | Dongkui |
| 0.2 | 48 | 95 | 87 | 2.75 ± 0.96$^{c}$ | 2.75 ± 1.71$^{b}$ | 1.15 ± 0.17$^{a}$ | 2.18 ± 0.29$^{a}$ |
| 0.4 | 48 | 98 | 89 | 3.50 ± 1.29$^{bc}$ | 3.0 ± 0.82$^{b}$ | 0.80 ± 0.32$^{b}$ | 1.97 ± 0.27$^{a}$ |
| 0.6 | 48 | 100 | 90 | 5.75 ± 0.95$^{a}$ | 5.75 ± 1.70$^{a}$ | 0.63 ± 0.13$^{bc}$ | 1.38 ± 0.29$^{b}$ |
| 0.8 | 48 | 94 | 88 | 4.25 ± 0.71$^{abc}$ | 4.50 ± 1.29$^{ab}$ | 0.55 ± 0.12$^{bc}$ | 1.15 ± 0.20$^{b}$ |
| 1 | 48 | 90 | 88 | 5.00 ± 1.82$^{ab}$ | 4.50 ± 1.29$^{ab}$ | 0.45 ± 0.13$^{c}$ | 1.00 ± 0.18$^{b}$ |

Means within each row followed by the same letter are not significantly different by LSD at p ≤ 0.05.

**Table 2.** Effect of TDZ and 2,4-Dichlorophenoxyacetic acid (2,4-D) on shoot regeneration and elongation from *M. rubra* shoot tips and nodal explants after 9 weeks in culture.

| TDZ | 2,4-D | Survival (%) | | | Number of shoots per explant | | Shoot length (cm) | |
|---|---|---|---|---|---|---|---|---|
| | | Explant | Biji | Dongkui | Biji | Dongkui | Biji | Dongkui |
| 0.6 | 0.025 | 48 | 98 | 87 | 5.50 ± 1.29$^{abc}$ | 5.25 ± 1.26$^{a}$ | 3.62 ± 0.28$^{ab}$ | 4.29 ± 0.42$^{a}$ |
| 0.6 | 0.05 | 48 | 97 | 91 | 6.25 ± 1.50$^{ab}$ | 4.75 ± 1.50$^{a}$ | 4.02 ± 0.17$^{a}$ | 4.92 ± 0.41$^{a}$ |
| 0.6 | 0.075 | 48 | 100 | 86 | 7.00 ± 1.83$^{a}$ | 5.00 ± 1.41$^{a}$ | 3.80 ± 0.32$^{ab}$ | 4.67 ± 0.33$^{ab}$ |
| 0.6 | 0.1 | 48 | 97 | 89 | 4.00 ± 1.82$^{bc}$ | 4.25 ± 1.70$^{a}$ | 3.45 ± 0.34$^{bc}$ | 4.25 ± 0.38$^{bc}$ |
| 0.6 | 0.2 | 48 | 94 | 90 | 3.50 ± 1.29$^{c}$ | 4.00 ± 1.42$^{a}$ | 3.10 ± 0.40$^{c}$ | 3.87 ± 0.53$^{c}$ |

Means within each row followed by the same letter are not significantly different by LSD at p ≤ 0.05.

The aim of this study was to develop a micropropagation protocol for “Biji and Dongkui” cultivars of *M. rubra*. This technique would facilitate large scale clonal production of these two commercially important cultivars.

## MATERIALS AND METHODS

### Plant materials

Shoot tips and nodal explants were taken from 5 years old *M. rubra* plants of Biji and Dongkui cultivars grown in the green house of Horticulture Department, Zhejiang University. The excised explants were soaked in Tween-20 (1-2 drops/100 ml $H_2O$) for 15 min and was washed thoroughly with tap water for 30 min. Surface disinfection of shoot tips was done with 60% (v/v) ethanol for 1-½ min and sterilized with 0.05% (w/v) $HgCl_2$ for 7 min While the nodal explants was washed with 75% (v/v) ethanol for 1 to 2 min and sterilized with 0.1% (w/v) $HgCl_2$ for 8 min under laminar hood conditions. All the explants were rinsed for 3 to 5 times with autoclaved double distilled water by agitator. Every explant size was about 0.4 to 0.6 cm also having one or two buds.

### Media and culture conditions

The basal medium of BW (Sugawara et al., 1994) with the addition of mineral salts, vitamins and 30 g sucrose was used. Medium pH was adjusted to 5.8 prior the addition of agar (8 g) and autoclaved at 121°C for 20 min. Shoot tips and nodal explants were placed separately in different jars containing 30 ml of medium and after 4 weeks, all the cultures were transferred to fresh medium of the same composition. All cultures were maintained at 26 ± 1°C under fluorescent day light tubes with a photon flux density of 50 µmol·m$^{-2}$·s$^{-1}$ of 16 h photo period.

### Induction and elongation of multiple shoots

For induction of multiple shoots various concentrations (0.2, 0.4, 0.6, 0.8, and 1 mg L$^{-1}$) of TDZ were used. Once the optimum level of TZD (Table 1) was known then all the shoot tips and nodal explants were cultured on the medium having a constant level of TDZ with different concentrations (0.025, 0.050, 0.075, 0.1, and 0.2 mg L$^{-1}$) of 2,4-D (Table 2). The total period of culture was 9 weeks with an interval of 4 weeks subculture.

### Effect of media on induction of multiple shoots

The effects of five different media for example; BW medium (Sugawara et al., 1994), WPM (Lloyd and McCown, 1980), MS medium (Murashige and Skoog, 1962), NN medium (Nitsch and Nitsch, 1969) and DKW medium (McGranahan et al., 1987) with the constant combination of 0.6 mg L$^{-1}$ TDZ and 0.075 mg L$^{-1}$ 2,4-D were compared on induction of multiple shoot. Cultures were evaluated for callus formation and shoot induction after 9 weeks of culture.

### Rooting

Before transferring microshoots for root induction, single shoot was transplanted into growth regulators free medium and cultured for 3 weeks with the aim to eliminate the effect of growth regulators. Healthy shoots (3 to 4 cm) were transferred to ¼, ½ and full strength BW medium containing various concentrations of IBA (0.05, 0.1, 0.3, 0.6 mg L$^{-1}$) and indole-3-acetic acid (IAA) (0.3, 0.6 and 0.9 mg L$^{-1}$). Effects of these auxins on were examined after in

**Table 3.** Effect of media on number of shoots and shoot length after 9 weeks in culture.

| Medium | Explant | Varieties | | Number of shoots per explant | | Shoot length (cm) | |
|---|---|---|---|---|---|---|---|
| | | Biji | Dongkui | Biji | Dongkui | Biji | Dongkui |
| BW | 48 | 100 | 91 | 6.80 ± 1.48[a] | 5.60 ± 1.14[a] | 5.00 ± 0.50[a] | 5.02 ± 0.46[a] |
| NN | 48 | 56 | 64 | 3.40 ± 1.14[b] | 2.60 ± 1.15[b] | 2.64 ± 0.55[c] | 2.58 ± 0.58[b] |
| WPM | 48 | 87 | 90 | 5.00 ± 1.58[ab] | 5.80 ± 0.83[a] | 3.98 ± 0.53[b] | 4.56 ± 0.67[a] |
| MS | 48 | 94 | 89 | 5.00 ± 1.58[ab] | 5.20 ± 1.48[a] | 4.18 ± 0.59[b] | 4.32 ± 0.63[a] |
| DKW | 48 | 69 | 87 | 3.80 ± 1.48[b] | 2.80 ± 1.49[b] | 2.04 ± 0.63[c] | 2.20 ± 0.58[b] |

Means within each row followed by the same letter are not significantly different by LSD at $p \leq 0.05$.

8 weeks culture. Once roots were initiated plantlets were transferred to full strength BW medium without the addition of growth regulators.

#### Plant acclimatization

Rooted plants were removed from the jars and the residues of the media were washed out. All the plants were individually planted in pots containing 1:2 of perlite and sand medium and covered with thin plastic sheet. After 7 days, slits were made in the plastic to lower the humidity level and the sheets were completely removed on shifting them to the green house. Plants were maintained under this controlled condition for 2 weeks and then transferred to the green house. Plants were maintained in the greenhouse for 2 months and all of them showed normal growth and development without any morphological abnormalities.

#### Statistical procedures and analysis

Per treatment was replicated 12 times in a completely randomized design. Each replica consisted of four explants and all the experiments were repeated 3 times. Data were analyzed using SAS statistical package (SAS Inst., Cary, N.C.) and treatments means were compared by using LSD at $p \leq 0.05$.

## RESULTS

### Effect of TDZ and/or 2,4-D on shoot regeneration and elongation

Data was recorded on the basis of viability of the explants. Nodal explants were medium tender having greenish axillary buds responded efficiently for bud sprouting and multiple shoot regeneration, while shoot tip explants which were much tender turned brown after normal treatment of sterilization and disinfection. The survival percentage of explants and their subsequent development into shoots varied from 90 to 100% for variety Biji and 87 to 90% for Dongkui on BW medium supplemented with various levels of TDZ (Table 1). Numbers of shoots were significantly affected by different concentrations of TDZ. Both the cultivars responded almost similar in producing maximum number of shoots (5.75) as compared to the Minimum number of shoots (2.75) by 0.6 mg $L^{-1}$ TDZ and 0.2 mg $L^{-1}$ TDZ, respectively. A significant difference in shoot length was observed between two cultivars. Dongkui gave longer but fewer shoots than Biji. Maximum and minimum shoot length in Biji was 1.15 and 0.45 cm as compared to 2.18 and 1.00 cm in Dongkui, respectively. Longer shoots were obtained with the lower concentrations of TDZ that subsequently decreased with the higher TDZ concentrations. With the addition of 0.075 and 0.05 mg $L^{-1}$ 2,4-D, maximum number (7.00) and longer shoots (4.92) were obtained in cultivars Biji and Dongkui, respectively. Shoot length and number of shoots in both the cultivars were significantly improved with the addition of 2,4-D.

### Effect of media on multiple shoot organogenesis

BW medium gave good results for shoot organogenesis with highest shoot survival percentage, number of shoots per explants, and shoot length. The effects of WPM and MS media on micropropagation of *M. rubra* were observed almost at the same time, while NN and DKW gave poor results. In this study, significant differences among the media were observed for shoots per explant and shoot length. Shoot regeneration responses of both the cultivars to all media were observed similar. The highest survival percentage of explants was 100 and 91% for variety Biji and Dongkui, respectively on BW medium supplemented with 0.6 mg $L^{-1}$ TDZ and 0.075 mg $L^{-1}$ 2,4-D (Table 3). Media significantly affected the number of shoots formation and shoot length elongation. Maximum number of shoots 6.80 and 5.60 was obtained for Biji and Dongkui, respectively by BW medium as compared to the lowest 3.40 and 2.60 by NN media. Longer shoots were obtained in BW medium for both the cultivars as compared to the shortest in NN and DKW media.

### Root induction

Root induction started within 3 to 5 weeks in the rooting medium. Rooting proved to be mainly dependent on medium strength, since roots were observed on maximum shoots cultured on reduced strength medium

**Table 4.** Effect of IBA and IAA on microshoots rooting from *M. rubra* after 8 weeks, cultured in ¼ strength of BW medium.

| Rooting (%) | | | Number of roots per shoot | | Root length (cm) | |
|---|---|---|---|---|---|---|
| **IAA** | **Biji** | **Dongkui** | **Biji** | **Dongkui** | **Biji** | **Dongkui** |
| 0.3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.6 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.9 | 0 | 0 | 0 | 0 | 0 | 0 |
| IBA | | | | | | |
| 0.05 | 34 | 24 | $1.97 \pm 0.49^{b}$ | $1.27 \pm 0.27^{b}$ | $5.12 \pm 0.56^{b}$ | $5.40 \pm 0.45^{c}$ |
| 0.1 | 75 | 66 | $2.75 \pm 0.26^{b}$ | $1.82 \pm 0.48^{b}$ | $5.22 \pm 0.61^{b}$ | $6.40 \pm 0.51^{b}$ |
| 0.3 | 95 | 87 | $4.02 \pm 0.75^{a}$ | 2.65 ± 0.38a | $5.80 \pm 0.28^{ab}$ | $7.25 \pm 0.42^{a}$ |
| 0.6 | 88 | 84 | $4.02 \pm 0.40^{a}$ | $2.95 \pm 0.42^{a}$ | $6.02 \pm 0.26^{a}$ | $7.10 \pm 0.53^{ab}$ |

Means within each row followed by the same letter are not significantly different by LSD at $p \leq 0.05$.

**Table 5.** Effect of iba and iaa on rooting from *M. rubra* microshoots after 8 weeks cultured in ½ strength BW medium.

| Rooting (%) | | | Number of roots per shoot | | Root length (cm) | |
|---|---|---|---|---|---|---|
| **IAA** | **Biji** | **DongKui** | **Biji** | **Dongkui** | **Biji** | **Dongkui** |
| 0.3 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.6 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.9 | 0 | 0 | 0 | 0 | 0 | 0 |
| **IBA** | | | | | | |
| 0.05 | 21 | 26 | $2.00 \pm 0.66^{b}$ | $1.20 \pm 0.761^{b}$ | $3.50 \pm 0.25^{a}$ | $3.92 \pm 0.37^{a}$ |
| 0.1 | 65 | 35 | $2.70 \pm 0.29^{b}$ | $1.70 \pm 0.45^{b}$ | $3.52 \pm 0.30^{a}$ | $4.30 \pm 0.74^{a}$ |
| 0.3 | 74 | 45 | $3.60 \pm 0.80^{a}$ | $2.62 \pm 0.43^{a}$ | $3.82 \pm 0.33^{a}$ | $4.60 \pm 0.50^{a}$ |
| 0.6 | 75 | 54 | $3.57 \pm 0.33^{a}$ | $2.68 \pm 0.60^{a}$ | $3.65 \pm 0.42^{a}$ | $4.37 \pm 0.82^{a}$ |

Means within each row followed by the same letter are not significantly different by LSD at $p \leq 0.05$.

(¼ and ½ BW) (Tables 4 and 5). Medium strength greatly affected rooting efficiency, number of roots and root length. Roots did not occur with the addition of IAA in the ¼, ½ and full strength medium in both the cultivars. On ¼ strength medium, rooting efficiency and number of roots were observed higher in Biji than Dongkui, while longer roots were obtained in Dongkui by IBA concentrations. Roots were induced with a high frequency of 95 and 87% in the medium supplemented with 0.3 mg $L^{-1}$ IBA in Biji and Dongkui, respectively as compared to the lowest 34 and 24% with 0.05. Maximum roots of 4.02 were obtained by 0.3 and 0.6 mg $L^{-1}$ IBA in cultivar Biji as compared to the minimum 1.97 with 0.05 mg $L^{-1}$ IBA in the same cultivar. The longest root which is 7.25 cm Dongkui ranked first as compared to 6.02 cm in Biji. After induction of roots under various concentrations of IBA in both ¼ and ½ strength media, subsequent root development and differentiation in adventitious roots was very slow.

However, following transfer to full strength medium without growth regulators further elongation and differentiation into adventitious roots was easily achieved within 3 weeks culture. No callus was observed during shoot rooting. On ½ strength medium, rooting efficiency, number of roots, and root length were greatly reduced in both cultivars as compared to ¼ strength medium. On this medium also, micro shoots did not induce roots with all tested concentrations of IAA. Rooting efficiency was increased with an increase in IBA concentration and reached at peak (75 and 54%) with the application of 0.6 mg $L^{-1}$ IBA. Maximum roots of 3.60 were obtained by 0.3 in cultivar Biji as compared to the minimum of 2.00 with 0.05 mg $L^{-1}$ IBA in the same cultivar. On ½ strength medium, Dongkui ranked first with longest roots of 4.60 cm as compared to 3.82 cm obtained with the same concentration in cultivar Biji.

## DISCUSSION

Results of this study indicated that multiple shoots were regenerated from shoot tips and nodal explants from 5 years *M. rubra* plants with scion cultivars of Biji and Dongkui. TDZ induced multiple shoots formation but with stunted growth. Its lower concentrations produced fewer shoots while higher concentrations arrested shoot growth. TDZ in combination with 2,4D induced better shoot regeneration with maximum number and longer

shoots. Nemeth (1986) reported that multiple shoots regeneration depends on many endogenous and exogenous factors such as genetic background, physiological influences, age, and the ontogenetic phase of the mother plant, environment, and composition of the nutrient medium. Organogenesis is commonly induced by manipulation of exogenous phytohormone levels and occurs either directly from explant tissue or through callus (Vengadesan et al., 2002). Our results are in accordance with previous studies, suggesting that TDZ is among the most active cytokinin-like growth substances for tissue culture of woody plant species and its low concentrations induces axillary shoot proliferation but may inhibit shoot elongation and high concentrations promotes formation of adventitious shoots (Heutteman and Preece, 1993). Woody plants have responded to lower concentrations of TDZ in a similar manner to higher concentrations of other cytokinins (Donna and Preece, 2004). In the current study, all concentrations of TDZ stimulated callus formation on the lower ends of the explants and similar results were reported by Heutteman and Preece (1993) in *Gymnema sylvestr*.

In this study, BW medium was found as a suitable medium for the micropropagation of *M. rubra* followed by WPM and MS media. Several other studies suggest that type and composition of the nutrient medium play a basic role in *in vitro* propagation system of plants (Arafeh, 1999; Shimomura and Kitazawa, 1991). Diverse media types evoked regeneration response in numerous plant species. In *A. koa*, MS medium produced maximum number of shoots (Skolmen and Mapes, 1976; Xie and Hong, 2001). In *Acacia catechu*, WPM and half strength WPM were successfully used in inducing shoot regeneration (Das et al., 1996). Adventitious shoot regeneration in *Acacia mangium* was obtained in *Juglans* on DKW medium by Douglas and McNamara (2000). In *A Acacia koa*, successful shoot regeneration system was obtained on Schenk and Hildebrandt medium (Skolmen and Mapes, 1976).

Root induction on microshoots proved to be mainly dependent on medium strength and type of auxin used. Roots were only initiated in the medium containing IBA with reduced medium strength (¼ and ½ BW). Concentrations of IBA with full strength of BW medium did not induce roots in *M. rubra* in the stipulated period of 8 weeks. All concentrations of IAA either on reduced medium or full strength did not initiate roots. These results are in accordance with previous studies suggesting that low salt concentrations promote rooting of woody plants (Nemeth, 1986). According to Liu et al. (2000) IBA was shown to promote more and stronger root formation in *Salvia sclarea* than IAA. Sahoo et al. (1997) also reported that, of the three auxins tested (IBA, IAA, and NAA) with *Ocimum basilicum*, IBA was most effective in inducing rooting and obtaining the highest number and length of roots. For root initiation, IAA was reported as the least successful auxin in *Salvia valentine* (Cuenca and Amo-Marco, 2000). Helior et al. (1997) have reported that IBA serves as a suitable auxin for *in vitro* rooting of *Vitis vinifera* cv. Pinot noir. Contrary to our results, microshoots of *Salvia blancoana*, *S. valentina* (Cuenca and Amo-Marco, 2000) and *Salvia miltiorrhiza* (Morimoto et al., 1994) were able to form roots in the absence of auxin in the medium. Hosoki and Tahara (1993) also concluded that IBA was not necessary for rooting of *Salvia miltiorrhiza* since percent rooting and root number were high irrespective of IBA concentrations.

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