African Journal of Agricultural Research Vol. 8(2), pp. 216-223, 17 January, 2013
Available online at http://www.academicjournals.org/AJAR
DOI: 10.5897/AJAR10.1159
ISSN 1991-637X ©2013 Academic Journals

*Full Length Research Paper*

# Direct induction of somatic embryogenesis and plant regeneration from cotyledon explants of *Myrica rubra* Sieb. & Zucc.

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Accepted 24 August, 2011

**Somatic embryogenesis was induced on woody plant medium (WB) supplemented with Thidiazuran (TDZ) alone or in combination with 2,4-D from mature cotyledon explants of *Myrica rubra*. All concentrations of TDZ except 1.0 mgL$^{-1}$ induced somatic embryos and adventitious shoots simultaneously within two months of culture. Addition of 2,4-D in the medium significantly improved induction of somatic embryos. Frequency of embryogenesis was only 3.34% with 7.00 embryos per explants when TDZ was fortified as a single growth regulator which was improved to 22.00% with the addition of 0.1 mgL$^{-1}$ 2,4-D in the media. Repetitive embryogenesis was induced on optimized concentrations (0.5 mgL$^{-1}$ BA and 0.05 mgL$^{-1}$ TDZ) of two cytokinins in combinations with various concentrations of 2, 4-D. Continuous culture of the explants with cluster of embryos on the induction media did not induce repetitive embryogenesis. On repetitive embryogenesis induction media, most of the embryos induced were smaller in size than those of the primary embryos during their induction stage. TDZ in combination with IBA induced adventitious shoots on the surface of somatic embryos explants. TDZ (0.2 mgL$^{-1}$) plus IBA (1.0 mgL$^{-1}$) was the most effective combination with maximum number (8.5) of shoots per explant. Shoot elongation was achieved on the media supplemented with 0.5 mgL$^{-1}$ BA concentration plus 0.1 mgL$^{-1}$ NAA. Root induction on micro-shoots was directly related to the media strength. Microshoots rooted on BW medium fortified with 0.5 mgL$^{-1}$ IBA with highest rooting efficiency. Rooted plants were successfully hardened and grown in the greenhouse. The protocol reported here for *M. rubra* is efficient, reproducible and could be used for genetic transformation experiments.**

**Key words:** *Myrica rubra*, Red bayberry, direct somatic embryogenesis, cotyledon explants, Thidiazuran (TDZ).

## INTRODUCTION

Somatic embryogenesis is the development of embryos from somatic cells. This is accomplished *via* a series of developmental stages, most of which are similar to zygotic embryogenesis (Hana et. al., 1999). Since the first report in 1968, somatic embryogenesis has been reported in more than 200 plant species (Attila et al., 2003). It provides an alternative approach for improving plant traits (Haoro et al., 2000). Somatic embryos can be germinated to form plants and can multiply to produce many more somatic embryos through a process referred to as secondary or repetitive embryogenesis. Repetitive somatic embryos have a unicellular origin in the epidermis (Polito et al., 1989), a most important property of somatic embryos that has been exploited for avoiding chimeric transformation of many woody plants (Dandekar et al., 1992). The plants through somatic embryogenesis

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are non-chimeric and show greater genetic uniformity and clonal fidelity (Martin, 2003).

Somatic embryogenesis is one of pathway of plant propagation that results in the production of non chimeric and true to type plants that comprise clonal populations, and have been used to develop efficient methods for rapid clonal propagation of a wide variety of plant species (Martin 2003; McGranahan et al., 1990). Secondary somatic embryogenesis has been reported in many tree species (Daigny et al., 1996; Benelli et al., 2001).

The mass multiplication of embryogenic propagules is the most attractive application of *in vitro* somatic embryos, which can be utilized directly in various studies such as genetic transformation, somatic hybridization and soma-clonal variation. Somatic embryos can be used for biotechnological applications including genetic modification of trees to select desired traits through gene transfer technology (Ananthakrishnan et al., 1999). Thidiazuron (TDZ), a substituted phenylurea (*N*-phenyl-1, 2,3 thidiazol-5-ylurea) is a potent bioregulant of *in vitro* morphogenesis (Dolendro et al., 2003). TDZ induced somatic embryogenesis in geranium, peanut and neem (Murthy et al., 1998). Using TDZ both shoot organogenesis and somatic embryogenesis has been induced simultaneously in white ash (Bate et al., 1992) and chickpea (Murthy et al., 1996; George and Eapen, 1994; Sreenivasu et al., 1998).*Myrica rubra* is a dioecious species and its progeny is highly heterozygous. Conventional vegetative propagation methods such as air layering and grafting are not rapid to meet the need of elite varieties. Somatic embryogenesis provides great promise of mass propagation and could be used as genetic engineering vehicle to develop non-chimeric transgenic plants. This present study accomplishes plant regeneration through direct somatic embryogenesis from cotyledon explants of *Myrica rubra*. This is the first report on plant regeneration through direct somatic embryogenesis from cotyledon explants of this species. Being a suitable material for *agrobacterium* mediated genetic transformation, cotyledon explants could be successfully used for introducing new traits in *Myrica* species through gene transformation technology.

## MATERIALS AND METHODS

### Plant material and preparation of explants

Mature seeds of *Myrica rubra* cultivar “Biji” were obtained from Cixi county, Zhejiang province during the month of June, 2004 and stored less than 5°C for four months. Seeds were treated with concentrated sulfuric acid ($H_2SO_4$) for 4 min and thoroughly washed with tap water for 2 h to wash out the residual effects of the acid. Seeds were soaked in distilled water for 48 h and cracked with pincers to remove the endocarp. In the laminar hood chamber, seeds along with testa were disinfected with 60% alcohol for 30 s per minute and rinsed 3 to 5 times with double distilled water and sterilized with 0.05% $HgCl_2$ for 6 to 7 min followed by 5 to 7 rinses. Seed testa was removed and explants were prepared by giving a single cut to each seed, vertically using a sharp knife.

### *In vitro* culture conditions and induction of somatic embryogenesis

Explants were incubated on basic BW medium (Sugawara et al., 1994) under 50 $\mu E.m^{-2}.s^{-1}$ of illumination provided by cool white fluorescent tubes, 16/8 h light/dark and 26 ± 2°C. The basal medium was supplemented with various concentrations of TDZ (0.2, 0.4, 0.6, 0.8 and 1.0 $mgL^{-1}$) alone or in combination with 2, 4-D (0.05, 0.1, 0.15, 0.2 and 0.25 $mgL^{-1}$) for induction of somatic embryogenesis. All the plant growth regulators were obtained from Sigma Chemical, USA. Growth regulators were filter sterilized and added to the media in the aseptic conditions. There were ten treatments in this experiment, each treatment with 50 explants replicated 5 times. Experiment was laid out in a randomized design in the vessels of jam size bottle, each bottle with 30 ml of media, each bottle with 10 explants. This experiment was repeated at least two times.

### Repetitive embryogenesis

To induce repetitive or secondary embryogenesis, explants with clusters of somatic embryos were transferred to the optimal concentrations of TDZ (0.05 mg/L) and BA (0.5 $mgL^{-1}$ BA) in combination with various concentration of 2,4-D (0.025, 0.5 and 0.75 $mgL^{-1}$). There were nine treatments, each with 36 explants replicated 4 times. The experiment was repeated at least two times.

### Embryo maturation and multiple shoot regeneration on embryos

For maturation and germination, the well-developed somatic embryos were transferred to Agar medium, Basic BW, ½ and ¼ strength BW, basic BW with 0.5 $mgL^{-1}$ ABA, and basic BW with 1.0 $mgL^{-1}$ indole-3-butyric acid (IBA) media without growth regulators or BW full strength salts with 1.0 $mgL^{-1}$ IBA. Culture conditions were the same as mentioned above and observations were made after 5 weeks of culture.

### Multiple shoot regeneration on somatic embryos and plantlet formation

Effects of various concentrations of IBA (0.5 to 2.0 $mgL^{-1}$) in combination with 0.2 $mgL^{-1}$ TDZ were evaluated for adventitious shoot induction on well developed mature embryos to be referred hereafter as “somatic embryos explants”. Explants with adventitious multiple shoots were transferred to 0.5 $mgL^{-1}$ BA with 0.1 $mgL^{-1}$ NAA for elongation. Shoots were transferred to the rooting media containing basic BW supplemented with 0.5 $mgL^{-1}$ IBA. After 4 weeks of culture, plantlets recovered were washed with running tap water and plantlets were planted in the trays containing 1:2 of perlite and sand. Plants were maintained under tissue culture conditions for 3 weeks covered with thin plastic sheet and then transferred to the green house. After 15 days, slits were made in the plastic to lower the humidity and sheets were completely removed on shifting them to the green house. Plants were maintained in the greenhouse for 6 months.

### Statistical analysis

Statistical analysis in one-way ANOVA using General Linear Model procedure in SAS statistical package (SAS Inst., Cary, N.C.) was carried out and standard deviation for each mean was worked out. Means were compared using Least Significant Difference test at $p \leq 0.05$.

**Table 1.** Induction of embryogenesis from cotyledon explants of *Myrica rubra* cultured on BW medium with various concentration of TDZ and 2, 4-D. (Observations were made after 7 weeks in culture) .

| TDZ | 2,4-D | Explant | Frequency | Number of embryos per explant |
|---|---|---|---|---|
| 0.2 | | 50 | 2.00±2.64$^{f}$ | 2.67±3.05$^{efg}$ |
| 0.4 | | 50 | 2.67±2.88$^{f}$ | 6.00±2.64$^{def}$ |
| 0.6 | | 50 | 3.34±1.52$^{ef}$ | 7.00±2.64$^{cde}$ |
| 0.8 | | 50 | 0.33± 0.58$^{f}$ | 1.00±1.73$^{gf}$ |
| 1.0 | | 50 | 0.00$^{f}$ | 0.00$^{g}$ |
| 0.6 | 0.05 | 50 | 14.33± 3.51$^{bc}$ | 9.34±2.51$^{cd}$ |
| 0.6 | 0.1 | 50 | 22.00± 5.00$^{a}$ | 23.33± 5.13$^{b}$ |
| 0.6 | 0.5 | 50 | 18.34 ±4.04$^{ab}$ | 33.00± 4.58$^{a}$ |
| 0.6 | 1.0 | 50 | 12.67 ±4.04$^{cd}$ | 11.40±2.51$^{c}$ |
| 0.6 | 1.5 | 50 | 8.33 ±4.16$^{de}$ | 4.33± 2.51d$^{efg}$ |

Means in the same column followed by the same letter are not significantly different by LSD at p≤0.05.

## RESULTS

### Induction of somatic embryogenesis

Most of the explants turned green within two weeks of culture on somatic embryos induction media. Somatic embryogenesis and adventitious shoots were simultaneously induced directly from the surface of cotyledon explants. Adventitious shoots appeared earlier than somatic embryos. Frequency of embryogenesis was higher in explants which remain dormant in the first two weeks. White globular embryos appeared from the inner layers of cotyledons on each side of the explants. Somatic embryos appeared directly in either cluster or single form with different sizes and shapes but most of them were in globular form. All concentrations of TDZ except 1.0 $mgL^{-1}$ induced somatic embryos within 7 wk of culture (Table 1 and Figures 1A and B). TDZ concentration above 0 .8 $mgL^{-1}$ severely suppressed the growth of adventitious shoots and induction of somatic embryogenesis.

Addition of 2, 4-D in the medium significantly improved induction of somatic embryos. Frequency of embryogenesis was only 3.34% with 7.00 embryos per explants when TDZ was fortified as a single growth regulator. Embryogenesis was improved to 22.00 % with the addition of 0.1 $mgL^{-1}$ 2,4-D in combination of 0.6 $mgL^{-1}$ TDZ. Similarly, number of somatic embryos per explant was only 7, which were improved to 33.00 embryos per explant when media was fortified with 0.5 $mgL^{-1}$ 2, 4-D in combination with 0.6 $mgL^{-1}$ TDZ. Higher concentrations of 2, 4-D had an adverse effect on frequency and number of somatic embryos.

### Repetitive embryogenesis

For induction of repetitive or continuous embryogenesis, optimized concentrations (0.5 and 0.05 $mgL^{-1}$) of TDZ and BA were supplemented in combinations with various concentrations of 2, 4-D (Table 2). Continuous culture of the explants with cluster of embryos on the induction media ceased to proliferate. On repetitive embryogenesis media most of the embryos induced were smaller in size than those of the primary embryos during their induction stage. All the primary embryos culture on repetitive embryo induction media was physiologically matured. Both TDZ and BA were able to induce the repetitive embryogenesis without 2, 4-D, however their combined effect was very positive on the induction of repetitive embryogenesis. BA in combination with 2, 4-D gave better results in inducing repetitive somatic embryogenesis than TDZ (Figure 1C). In repetitive embryogenesis, BA induced somatic embryos at a high frequency of 75.00% than 51.75% by TDZ. Similarly, maximum number of embryos per explant (19.08) was induced by BA than 12.83 embryos by TDZ. Highest frequency (83.00%) and number of embryos (28.00) were achieved with 0.5 $mgL^{-1}$ 2, 4-D concentrations in combination with the optimum 0.5 $mgL^{-1}$ BA. With the best combination of 2, 4-D (0.75 $mgL^{-1}$) plus 0.05 mg/L TDZ, highest frequency and number of embryos (57.00% and 17.00) were recorded.

### Maturation and multiple shoot regeneration on embryos

For maturation and germination, single or cluster of embryos were transferred to basic, ½, ¼ strength BW media or basic media containing 0.5 $mgL^{-1}$ ABA and 1.0 $mgL^{-1}$ IBA and their response was noted after 5 weeks in culture (Table 3). In all the treatments, embryos turned green (Figure 1 E) except the media containing ABA. In the media containing 0.5 $mgL^{-1}$ ABA, only rhizogenesis was obtained and with 0.1 $mgL^{-1}$ IBA, embryos turned green with rhizogenesis (Figures 1D and F). On the agar medium, the embryos turned green in the first two weeks

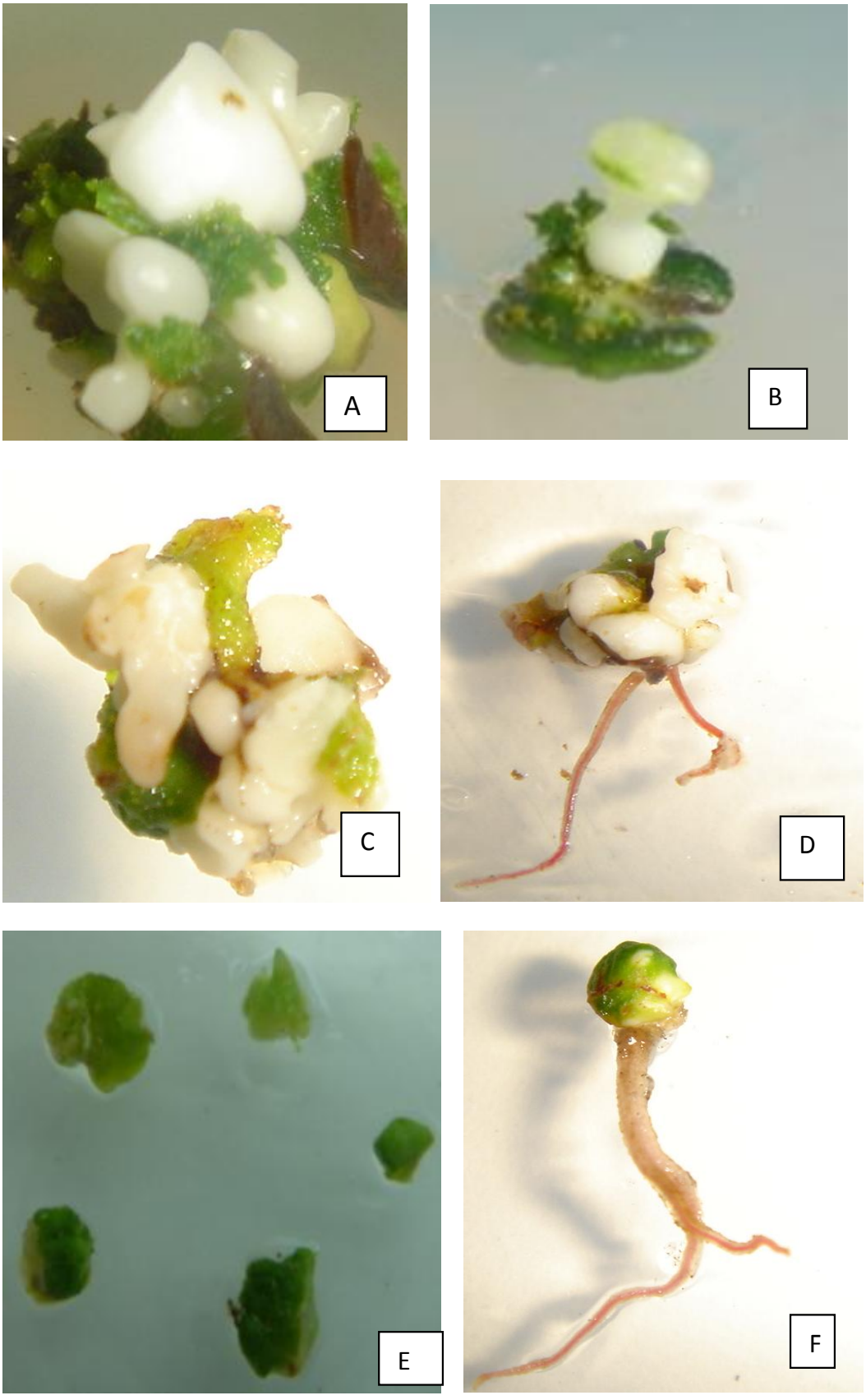
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**Figure 1.** Different stages of direct somatic embyrogenesis from cotyledon explants of *M. rubra.* A. Cluster of globular somatic embryos and regeneration of adventitious shoots on BW medium containing 0.6 mg $L^{-1}$ TDZ in combination with 0.1 mg $L^{-1}$ 2, 4-D after 7 weeks in culture. B. A single torpedo stage somatic embryo. C. Repetitive or continuous embryogenesis on the media containing 0.5 mg $L^{-1}$ BA in combination with 0.5 mg $L^{-1}$ 2,4-D after 7 weeks in culture. D. Rhizogenesis on the somatic embryos on the medium containing 0.5 mg $L^{-1}$ ABA. E. Single mature somatic embryos. F. Rhizogenesis fron the single embryo. G. Single somatic embryos swallowed on the media containing 0.2 mg $L^{-1}$ TDZ and 0.1 mg $L^{-1}$ IBA after the 3$^{rd}$ week of the culture. H. Regeneration of shoots on somatic embryos. I. Micro-shoot regeneration from somatic embryos after 35 days of culture. J. Rooted plants were transferred on the media containing 1:2 of perlite and sand. K. Acclimatized plants.

and later on most of the embryos turned brown and died.

**Multiple shoot regeneration on somatic embryos and plantlet formation**

Already green and well mature somatic embryos explants were selected and cultured on the media containing 0.2mg$L^{-1}$ TDZ in combination with various levels of IBA (Table 4). Explants became swollen and flattened (Figure 1G) on the media during the third week of culture. After 5 weeks of culture, adventitious shoots emerged on the explants (Figure 1H). Maximum number of shoots (8.50) were recorded when somatic embryos explants were cultured on 0.2 mg/L TDZ in combination with 1.0 mg/L IBA. Increasing IBA concentration (>1.0 mg$L^{-1}$) not only decreased number of shoots per explant but also induced callus on the lower surface adjacent to the media. Based on our previous experiment, explants with adventitious multiple shoots (Figure 1I) were transferred to optimal BA

**Table 2.** Induction of repetitive embryogenesis from the primary single or cluster of embryos of *Myrica rubra* cultured on BW medium with various concentration of TDZ, BA and 2, 4-D (Observations were made after 5 weeks in culture).

| TDZ | 2,4-D | Explant | Frequency (%) | Number of embryos |
|---|---|---|---|---|
| 0.6 | 0.15 | 45 | 6.67±2.51$^{c}$ | 3.00±1.00$^{d}$ |
| 0.05 | 0.00 | 45 | 50.33±5.03$^{ab}$ | 9.33±2.51$^{c}$ |
| 0.05 | 0.025 | 45 | 46.33±5.68$^{b}$ | 10.00±4.16$^{bc}$ |
| 0.05 | 0.5 | 45 | 53.34±2.08$^{Aab}$ | 15.00±2.64$^{ab}$ |
| 0.05 | 0.75 | 45 | 57.00±6.24$^{a}$ | 17.34±3.05$^{a}$ |
| | | **Mean** | 51.75 | 12.83 |
| **BA** | | | | |
| 0.5 | 0.00 | 45 | 67.33± 4.72$^{b}$ | 6.34 ± 2.51$^{c}$ |
| 0.5 | 0.025 | 45 | 78.67± 3.21$^{A}$ | 21.67± 3.78$^{b}$ |
| 0.5 | 0.5 | 45 | 83.00± 1.00$^{A}$ | 28.00± 1.00$^{a}$ |
| 0.5 | 0.75 | 45 | 71.00 ± 3.60$^{B}$ | 20.33± 4.16$^{b}$ |
| | | **Mean** | 75.00 | 19.08 |

Means in the same column followed by the same letter are not significantly different by LSD at p≤0.05.

**Table 3.** Effect of various treatments on maturation and germination of somatic embryos from cotyledon explants of *M. rubra*.

| Treatment | Response |
|---|---|
| Agar | First two weeks greening and then turned brown |
| Basic BW media without growth regulators | Greening |
| ½ BW without growth regulators | Greening |
| ¼ BW medium without growth regulatoris | Greening |
| Basic BW media with 0.5 mg $L^{-1}$ ABA | Rhizogenesis |
| Basic BW media with 1.0 mg $L^{-1}$ IBA | Greening and rhizogenesis |

**Table 4.** Effect of TDZ in combination with 2, 4-D on adventitious shoot on mature somatic embryos explants of *Myrica rubra* after 8 weeks in culture.

| TDZ | IBA | Number of explants | Number of shoots per explants |
|---|---|---|---|
| 0.2 | 0.5 | 65 | 6.00 ± 1.58$^{b}$ |
| 0.2 | 1.0 | 65 | 8.50 ± 1.51$^{a}$ |
| 0.2 | 1.5 | 65 | 5.60 ± 1.81$^{b}$ |
| 0.2 | 2 | 65 | 4.60 ± 1.67$^{b}$ |

Means in the same column followed by the same letter are not significantly different by LSD at p≤0.05.

concentration (0.5 $mgL^{-1}$) in combination with NAA (0.1 $mgL^{-1}$) for shoot elongation and cultured for 5 weeks. Elongated shoots (1.5 to 2 cm) were transferred to the rooting media consisting basic BW supplemented with 0.5 $mgL^{-1}$ IBA. Root induction started within 3 to 4 weeks in rooting medium with a high efficiency of 90%. Rooted plants were removed from the jars; residues of the media were washed out (Figure 1J) and planted in trays containing 1:2 of perlite and sand. Plants were maintained under tissue culture conditions for 3 weeks covered with thin plastic sheet and then transferred to the green house. Within 3 to 4 weeks, plastic sheets were completely removed, plants were watered twice a day in a high humidity (60 to 70%) conditions. Plants were maintained in the greenhouse for 6 months and showed normal growth and development. (Figure 1K).

## DISCUSSION

Results of this study indicated that somatic embryogenesis and adventitious shoots were induced simultaneously on BW medium supplemented with TDZ alone or in combination with 2, 4-D from mature cotyledon explants of *M. rubra*. TDZ has always been classified as a synthetic cytokinin, however, it brings about some

physiological changes associated with auxins. Cotyledon explants treated with TDZ undergo a different morphological route of development than that induced by purine cytokinins (Victor et al., 1999). Saxena et al. (1992) suggested that TDZ is a successful substitute for auxins, normally required to induce somatic embryogenesis in peanut and *Pelargonium*. This was proved by the findings of Murthy et al. (1995), where the level of cytosolic indole acetic acid increased in cotyledons and hypocotyls of TDZ-treated peanut seedlings. TDZ exhibits cytokinin-like activity in physiological responses usually mediated by adenine-based cytokinins (Mok et al., 1982; Thomas and Katterman, 1986). Induction of direct somatic from cotyledon explants of *Myrica rubra* on TDZ containing media suggest that TDZ probably helps in establishing an optimum endogenous hormones ratio, which is a prerequisite for the induction of somatic embryogenesis. Gill et al. (1993) reported that in geranium direct embryogenesis from cotyledon explants only by TDZ explains the phenomenon that it establishes a balance of endogenous hormone level required for the induction of somatic embryogenesis. TDZ dual role in the embryogenesis and morphogenesis of the tissues in the *in vitro* conditions speculate a potential role in the induction of somatic embryogenesis: a cytokinin-like activity that promotes cell division and differentiation and a minor auxin-like activity that seems to be crucial for induction of embryogenic competence (Victor et al., 1999).

In this study, repetitive embryogenesis was achieved on the media supplemented with a certain level of BA and TDZ alone or in combination with 2, 4-D. Prolonged culture of somatic embryos to induce repetitive embryogenesis on induction media was not encouraging. Effect of BA in combination 2, 4-D was better on the induction of repetitive embryogenesis than TDZ plus 2,4-D. Scott et al. (1998) also reported that TDZ successfully induced somatic embryogenesis from sweetgum seed explants; however, repetitive embryogenesis was not achieved on the induction medium with the same TDZ concentrations. They reported that BA and 2, 4-D was the best combination for repetitive embryogenesis in sweet gum. Similar findings in *Vigna radiata*, where somatic embryos were induced on media containing 2, 4-D in combination with BA resulted in the highest frequency of repetitive embryogenesis (Prathibha et al., 2004).

In this study, maturation, greening and rhizogenesis of embryos was achieved on the media containing IBA. Embryos were matured only on the media without growth regulators or with ABA. Similar results were reported by Daren et al., (2000), in *Narcissus pseudonarcissus*, where IBA significantly improved germination of somatic embryos. It has been reported that abscisic acid (ABA) promote maturation of somatic embryo in *in vitro* conditions and is an effective supplement in the medium supporting growth of globular and early heart-shaped somatic embryos (Capuana and Debergh, 1997). ABA also synchronises the cultures and inhibits aberrant development during transition from globular to later stage (Ammirato, 1983), however in our study, ABA did not improve germination of somatic embryos of *M. rubra*. In this study, mature and well developed embryos when cultured on the medium containing TDZ and IBA induced multiple shoots. There is no report that describes multiple shoot induction from the surface of somatic embryos.

This protocol of plant regeneration through direct somatic embryogenesis would facilitate the mass propagation of *M. rubra* and would pave new ways in improving this fruit tree at the single cell level. Repetitive somatic embryogenesis could be a useful tool to insert new traits through *Agrobacterium*-mediated transformation in *Mryica* species. Frequency of somatic embryogenesis from cotyledon explants in *M. rubra* and their subsequent germination needs to be refined through further intensive research work.

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