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RESEARCH ARTICLE

In vitro mass propagation and greenhouse establishment of *Munronia pinnata* (Wall) Theob. *(Binkohomba)*

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Abstract: *Munronia pinnata* (Wall). Theob is a valuable medicinal plant which is widely used in Ayurvedic medicine in Sri Lanka. Due to over exploitation and low percentage of seed germination of *M. pinnata* it has become necessary to adopt *ex situ* conservation methods *via in vitro* propagation techniques. The objectives of this study was mass propagation, greenhouse establishment and comparison of *in vitro* propagated plants with seed raised plants. Callus cultures were initiated from different explant types such as apical buds, nodal segments, petioles, petals and leaf discs in Murashige and Skoog (MS) medium supplemented with 1.1 mg L^{-1} 2,4- dichlorophenoxy acetic acid (2,4-D) and 0.5 mg L^{-1} benzyl amino purine (BAP) at 25 ± 1^{0}C in complete darkness. Leaf discs (6.0 mm) showed significantly higher calli production (mean fresh weight 0.167± 0.04 g) than other tested explants. The best growth regulator combination for callus initiation was 1.1 mg L^{-1} 2,4-D and 0.3 mg L^{-1} BAP which produced a mean of 0.30 ± 0.06 g callus while other treatments produced lesser amount of callus. Leaf discs taken from first fully opened leafs, produced the highest amount of callus (0.06 ± 0.01 g) and a decreasing trend was observed with increased maturity of the leaf. Tissues along the midrib area have a higher potential to give high yield of callus. Shoot proliferation was higher (32.9 ± 2.2) in MS medium containing 3.0 mg L^{-1} naphthalene acetic acid (NAA) and 3.0 mg L^{-1} BAP. Light intensity at 3000 lx showed significantly higher number of shoots (33.4 ± 2.84) than other tested light intensities. Half MS minerals supplemented with 0.2 mg L^{-1} indole acetic acid (IAA) was the most effective treatment for root induction (75%) with the highest mean root length (15.05 ± 0.5 mm) and 3.15 ± 0.03 of mean number of roots. A suitable potting mixture for acclimatization was determined using different ratios of compost and sand. Higher percentage of survival (60%) was achieved with compost: sand 1:3. When morphological features of seed raised plants were compared with tissue cultured plants, mean height increment was higher in seed raised plants while mean number of leaves was higher in tissue cultured plants and branching habit was observed only in tissue cultured plants. A comparison of physiological parameters between the two types of plants showed that the rate of photosynthesis was higher in tissue cultured plants whereas stomatal resistance was higher in seed raised plants.

Keywords: Cold treatment, commodity treatments, de-topping, hormonal regulation, shoot morphology.

INTRODUCTION

Munronia pinnata (Wall). Theob. (Sin. *Binkohomba,* Family *Meliaceae*) is a valuable medicinal plant which is widely used in Ayurvedic medicine in Sri Lanka. The specific threats such as over exploitation and low percentage of seed germination, necessitates the adoption of *ex situ* conservation methods *via* micropropagation techniques. Although there are few records on *in vitro* propagation of *M. pinnata*[1-4], the number of plants produced was low and also establishment of *in vitro* propagated plants on mass scale has not been tested. The objectives of this study were mass propagation, greenhouse establishment and comparison of micropropagated plants with seed raised plants grown in the greenhouse. Comparisons are important to observe morphological and physiological pattern of *in vitro* raised plant growth that is affected by the culture conditions such as growth hormones, light, sugar, high relative humidity etc.

METHODS AND MATERIALS

Mature plants of *M. pinnata* were maintained in the net house under 70% shade. They were treated with N:P:K (9:8:6) (Baur 14™) fertilizer mixture fortnightly. A solution

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of Captan™ (1.0 g L^{-1}) was sprayed to mother plants every 10 days to reduce fungal attacks. Plants were watered daily. All quantitative experiments were carried out using 7-leaflet stage plants.

Explants were collected from the mother stock maintained in the greenhouse and washed with Dettol™ soap water for 5 min. The leaves were kept under running tap water for 1 h. They were washed in 10% Clorox for 5 min and then in 70% (v/v) ethanol for 2 min followed by two successive washings in sterile distilled water. For all experiments, *in vitro* cultures were incubated in a growth room at 25 ± 1ºC. Light was provided with white cool fluorescent tubes, which provided (wavelength range between 400 – 700 nm) Photosynthetically Active Radiation (PAR). For light incubation 16 h/d was used and for callus induction cultures were incubated in complete darkness. Glass jam jars (200 mL) were used as culture vessels.

Completely Randomized Design was used in all experiments. Twenty replicates were used in each treatment unless otherwise stated in the text. Culture vessels were randomized every seven days. Analysis of Variance was used to identify whether there is any significant difference between different treatments and the Least Significant Difference (LSD) was used as the mean separation technique.

Selection of explants: Physiological state and year-round availability are some of the important factors to be considered in selection of explants[5,6]. Therefore, in order to select the best explant source for callus initiation, apical buds, nodal segments, petioles, floral parts (petals) and leaf discs (6.0 mm in diameter) were cultured on Murashige and Medium Skoog (MS) supplemented with 1.1 mg L^{-1} 2,4- dichlorophenoxy acetic acid (2.4-D) and 0.5 mg L^{-1} benzyl amino purino (BAP). Number of days taken for callus initiation and the percentage of callus production were measured.

Selection of the best growth regulator combination for callus initiation: Results of the previous experiment showed that leaf discs are the best explant source for callus induction. Thus leaf discs (6.0 mm) were cultured in MS medium supplemented with a range of 2.4-D (0.9 – 1.3 mg L^{-1}) and BAP (0.3 – 0.7 mg L^{-1}). Callus fresh weight was measured after 2, 4, 6, 8 and 10 wks of incubation.

Determination of the effect of maturity stage of leaves on callus formation: Leaf disc explants from first, second, third, fourth and fifth fully opened leaves from the apical bud were cultured separately on media containing 1.1 mg L^{-1} 2, 4-D and 0.3 mg L^{-1} BAP, which was the best growth regulator combination for callus induction. Callus fresh weight was measured after 6 wks of incubation.

Determination of effect of the location of the leaf disc on callus production: Since explants from the first fully opened leaf produces more callus they were selected for this experiment. Leaf disc explants were cut along the midrib of the leaflet and the lateral parts of the leaflet. They were cultured separately on MS medium supplemented with 1.1 mg L^{-1} 2, 4-D and 0.3 mg L^{-1} BAP. Callus fresh weight was measured after 6 wks of inoculation.

Determination of best growth regulator combination for shoot initiation and multiplication: Approximately 1.0 cm^2 pieces of calli were transferred into MS medium supplemented with a range of naphthalene acetic acid (NAA) and BAP. Mean number of shoots, mean shoot height (mm) and mean number of leaves per shoot were measured after 8 wks of inoculation.

Effect of light intensity on shoot induction from callus: MS medium supplimeted with 3.0 mg L^{-1} NAA and 3.0 mg L^{-1} BAP was selected for the experiment. *In vitro* produced calli were transferred into the above medium and maintained under four different light intensities (1000, 2000, 3000, 4000 lx) in order to determine the effect of light on the production of green callus which further developed into shoots. Mean number of shoots, mean shoot height and mean number of leaves per shoot were measured after eight wks of inoculation.

Determination of the best growth regulator combination for rooting of shoots: Elongated shoots were transferred into a full or half strength MS medium containing different concentrations of indole butyric acid (IBA) (0.1 – 0.3 mg L^{-1}) or indole acetic acid (IAA) (0.1 – 0.5 mg L^{-1}). Cultures were incubated at light intensity of 3000 lx. Root type (single or many roots per shoot), percentage of root production and mean root length (mm) were measured after 10 wks of incubation. Some of the elongated shoots (> 5 cm long) were transferred into a potting mixture of sand : compost (2 : 1) for *ex vitro* rooting with or without treating with commercial rooting powder. Secto™, Rooton™, Clolnex™ were used as commercial rooting powders.

Determination of a suitable potting mixture for acclimatization and selection of suitable growth conditions for growing in vitro produced plants: Rooted plants were kept in the same medium for further development of roots. They were carefully removed from the culture medium without breaking roots and washed carefully with sterile water twice to remove all traces of

agar. They were then dipped in a fungicide solution of 1.0 gL^{-1} Captan™ for 5 min. and transferred to different potting mixtures (compost: river-sand). Potted plants were covered with sterile polypropylene bags (propagator units). They were either directly transferred to the greenhouse or kept in the incubation room for further two wks or four wks before transferring to the greenhouse. Survival percentage was measured at six wks of keeping plants in the greenhouse.

Comparison of tissue cultured plants with plants raised from seeds: Seedlings from seeds collected from mother plants were used for this experiment. Both seed raised and tissue cultured plants of same height (6.0 cm) were selected for the experiment. All plants were potted using the same potting mixture and maintained under similar environmental conditions. Selected morphological (height of the plant, number of leaves per plant and number of branches) and physiological (photosynthetic rate and stomatal resistant) parameters were measured over a period of nine months.

Measurements were taken after six and nine months. No measurements were taken after three months due to small size of the leaves which could not be placed in the apparatus chamber. Photosynthetic rate was measured twice a day while stomatal resistance was measured three times a day. Photosynthetic rate was measured using portable photosynthesis system LI-6400 (LI-COR Inc., USA) and the stomatal resistance was measured using AP_4 porometer (Delta-T Devices Ltd., U.K.).

RESULTS AND DISCUSSION

Selection of explant

Callus provides an important culture system that can be maintained more or less indefinitely. These cultures are potentially useful as a method for commercial propagation due to high rates of multiplication[7]. All tested explant types responded positively in MS medium with hormone combination of 1.1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP at 25 ± 1ºC in complete darkness. Calli were creamy white and fragile in texture. Callus weight of the leaf discs is the highest (0.18 ± 0.04 g) and it is significantly higher than that of all the other explants except petals. Nodal segments produced the lowest callus fresh weight with a mean value of 0.04 ± 0.04 g (Table 1).

Although many researchers have used MS as the basal medium, growth regulators and the concentrations used were not comparable for callus induction from leaf discs. Combination of BAP (1.25 mg L^{-1}) and NAA (4.5 mg L^{-1})[1] and BAP and IAA (2.0 mg L^{-1} and 0.2 mg L^{-1} respectively)[3] were used in order to obtain callus from leaf discs. In some cases 2,4- D (1.1 mg L^{-1}) and BAP (0.2 mg L^{-1}) were also used for callus induction from leaf discs[4].

Selection of the best growth regulator combination for callus initiation

Callus initiation was first observed after six days of incubation. Although callus initiation was observed in all growth regulator combinations tested, Analysis of Variance showed that there was a significant difference between the mean callus fresh weights in different growth regulator combinations. There was a significantly high amount of callus production in 1.1 mg L^{-1} 2,4-D, and 0.3 mg L^{-1} BAP out of the tested combinations, with the mean of 0.30 ± 0.06 g of fresh callus (Table 2).

Calli in the medium turned from creamy white to brown with time and they became hard in texture showing a decrease of active cells in the cell mass after six weeks (Figure 1a). Many authors cited that the regenerative capacity of cells and callus tissues may reduce or completely lost if growth is continued for too long[6,8]

Determination of the effect of maturity stage of leaves on callus formation

There was a significant difference in callus formation among the explants from leaves at different stages of maturity. The first fully opened leaf gave the highest mean callus weight (0.06 ± 0.01 g). Mean callus weight decreased with the increase in maturity of leaves. In general, more juvenile material is preferred for *in vitro* propagation[9,10] (Table 3). Although the first fully opened leaf produced higher amount of calli, it was non - significant with the

Table 1: Mean calli production from different explant sources in 1.1 : 0.5 mg L^{-1} 2,4-D: BAP medium after 5 weeks of incubation at 25 ± 1ºC in complete dark.

Explant type	Mean callus fresh weight (g)	% callus induction
Nodal segment	0.05 ± 0.01[b]	80.0
Petals	0.13 ± 0.02[a]	75.0
Leaf disc	0.18 ± 0.04[a]	100.0
Petiole	0.09 ± 0.04[b]	80.0
Apical bud	0.06 ± 0.01[c]	80.0
LSD	0.07	

* Measurements are the means of twenty replicates ± SE.

* Means within columns with the same letter are not significantly different.

Table 2: Mean callus fresh weights as a response to time and different growth regulator combinations of 2,4-D and BAP on *M. pinnata* leaf disc explants.

Growth regulators	Mean callus frsh weight (g)				
2,4-D : BAP (mg L^{-1})	Second week	Fourth week	Sixth week	Eighth week	Tenth week
0.9:0.3	0.03 ± 0.00	0.08 ± 0.01	0.21 ± 0.03 [b]	0.19 ± 0.02	0.22 ± 0.23
1.1:0.3	0.06 ± 0.00	0.13 ± 0.00	0.30 ± 0.06 [a]	0.21 ± 0.03	0.24 ± 0.38
1.3:0.3	0.06 ± 0.02	0.06 ± 0.01	0.15 ± 0.02	0.19 ± 0.02	0.24 ± 0.02
0.9:0.4	0.04 ± 0.00	0.05 ± 0.01	0.08 ± 0.01	0.16 ± 0.03	0.21 ± 0.03
1.1:0.4	0.03 ± 0.00	0.08 ± 0.01	0.13 ± 0.01	0.21 ± 0.03	0.20 ± 0.02
1.3:0.4	0.02 ± 0.00	0.09 ± 0.01	0.13 ± 0.01	0.19 ± 0.02	0.19 ± 0.02
0.9:0.5	0.06 ± 0.02	0.08 ± 0.01	0.17 ± 0.02 [b]	0.21 ± 0.03	0.25 ± 0.04
1.1:0.5	0.04 ± 0.00	0.06 ± 0.00	0.17 ± 0.04 [b]	0.18 ± 0.1	0.25 ± 0.05
1.3:0.5	0.05 ± 0.00	0.09 ± 0.02	0.13 ± 0.03	0.15 ± 0.02	0.18 ± 0.01
0.9:0.6	0.04 ± 0.00	0.07 ± 0.02	0.13 ± 0.03	0.26 ± 0.01	0.20 ± 0.05
1.1:0.6	0.03 ± 0.00	0.07 ± 0.01	0.12 ± 0.03	0.14 ± 0.04	0.28 ± 0.04
1.3:0.6	0.03 ± 0.00	0.07 ± 0.01	0.14 ± 0.04	0.18 ± 0.03	0.18 ± 0.03
0.9:0.7	0.03 ± 0.00	0.12 ± 0.03	0.11 ± 0.03	0.19 ± 0.04	0.22 ± 0.01
1.1:0.7	0.02 ± 0.01	0.09 ± 0.01	0.19 ± 0.04 [b]	0.16 ± 0.03	0.21 ± 0.04
1.3:0.7	0.03 ± 0.00	0.11 ± 0.02	0.05 ± 0.01	0.10 ± 0.03	0.20 ± 0.04
LSD	0.06	0.01	0.09	0.02	0.03

* Values are the means of twenty replicates

* Means within columns with the same letter are not significantly different.

Table 3: Variation of callus production with the maturity stage of the leaf.

Maturity stage of the leaf	Mean callus fresh weight (g)
First leaf	0.06 ± 0.01 [a]
Second leaf	0.06 ± 0.01 [a]
Third leaf	0.05 ± 0.00 [b]
Fourth leaf	0.05 ± 0.01 [b]
Fifth leaf	0.04 ± 0.01 [c]
LSD	0.01

* Measurements are the means of thirty replicates ± SE.

* Means within columns with the same letter are not significantly different.

Table 4: The effect of the location of the leaf disc on callus production.

Leaf area	Mean callus fresh weight (g)
Along the midrib	0.11 ± 0.02[a]
Lateral parts	0.02 ± 0.03[b]
LSD	0.11

* Measurements are the means of twenty replicates ± SE.

* Means within columns with different letters are significantly different.

values obtained from second leaf. Since the area of the first leaf was not adequate for obtaining sufficient amounts of leaf discs for further experiments, discs from second fully opened leaves were used for other experiments.

Determination of effect of the location of the leaf disc on callus production

Fresh weight of callus produced by leaf discs taken along the midrib of the leaflet was significantly higher (0.10 ± 0.02 g) than the explants taken from the lateral parts (0.02 ± 0.03 g) (Table 4). This revealed that tissues along the midrib area also have potential to give high yield of callus.

Determination of best growth regulator combination for shoot initiation and multiplication

Mean number of shoots, mean shoot length, and mean number of leaves per shoot were significantly different among the tested growth regulator combinations ($p<0.05$)

Greenish colour and green bud like structures appeared after fourteen days of transferring the cultures into shoot initiation media (Figure 1b). Almost all the treatments tested induced shoots from calli. Requirement for exogenous auxin and cytokinin in the process of bud differentiation varies with the tissue system and apparently depends on the endogenous levels of the two hormones in the tissue[6]. Medium containing 3.0 mg L^{-1} NAA and 3.0 mg L^{-1} BAP showed the highest shoot formation ability with the mean of 32.9 ± 2.2 shoots. The highest mean shoot length of 31.63 ± 1.86 mm and the highest number of leaves per shoot were 6.12 ± 0.34 also observed in this medium. Medium containing 2.0 mg L^{-1} NAA and 4.0 mg L^{-1} BAP was the second best growth regulator combination for shoot initiation, which gave 24.31 ± 1.78 mean number of shoots, 28.55 ± 1.22 mm shoot length and 4.87 ± 0.33 numbers of leaves per shoot. The lowest shoot formation ability was observed in the treatment with 1.0 mg L^{-1} NAA and 1.0 mg L^{-1} BAP which gave 0.95 ± 0.33 of mean number shoots, 1.2 ± 0.38 mm mean shoot length and 0.9 ± 0.30 mean number of leaves (Table 5).

Figure 1: a) Greenish bud initials on callus, b) Multiple shoots from callus, c) Rooting in different media, d) Acclimatized plants

Effect of light intensity on shoot induction from callus

Light intensity at 3000 lx showed significantly higher production of shoots with the mean of 33.40 ± 2.84, mean shoot length 30.52 ± 2.67 mm and mean number of leaves 6.32 ± 0.41 when compared with other tested light intensities. Although not tested for a range, 2500-3000 lx light intensity was applied by other researchers[3] in their effort in micropropagation of *M. pinnata*. The result of this study are similar to those in reported information. Light intensity at 4000 lx was the second best treatment with 23.60±2.33 of mean number of shoots, 28.82±3.48 mm of mean shoot length, and 4.37±0.36 of mean number of leaves. The light intensity of 1000 lx gave the lowest values for measured parameters (Table 6).

Determination of the best growth regulator combination for rooting of shoots

Shoots developed during the multiplication stage did not show spontaneous rooting. There was a significant difference between treatments in the mean root length and number of roots per shoot after 8 weeks of incubation. Apart from growth regulator free MS medium and MS medium supplemented with 0.3 mg L^{-1} IAA, all other tested treatments produced roots (Figure 1c). Half strength MS supplemented with 0.2 mg L^{-1} IAA was the most effective treatment in stimulating roots (70-75%) (Table 7).

Half strength basal medium performed better than full strength medium specially in the presence of IBA. Many researchers suggested that lowering the concentration of mineral salts in rooting media helps in adapting the *in vitro* propagated plants to photoautotrophic condition in the greenhouse[6-8]. Although no contaminations were observed in the shoots when transplanted in a potting mixture after treatment with commercial rooting powders, they dried off after 3 - 4 days.

Table 5: The effect of BAP and NAA on shoot induction from callus of *M. pinnata* leaf explants after eight weeks of incubation at 25±1 °C.

NAA: BAP (mg L^{-1})	No. ofshoots	ShootLength (mm)	No ofleaves
1:1	0.95 ± 0.33	1.20 ± 0.38	0.90 ± 0.30
2:1	1.15 ± 0.32	2.02 ± 0.49	1.53 ± 0.37
3:1	2.45 ± 0.53	3.02 ± 0.59	3.00 ± 0.59
4:1	3.90 ± 0.67	4.40 ± 0.69	4.22 ± 0.66 [f]
5:1	8.40 ± 0.91	9.39 ± 0.70	4.34 ± 0.23 [f]
1:2	6.60 ± 1.07	5.85 ± 0.85	3.78 ± 0.57 [f]
2:2	8.25 ± 1.26	6.83 ± 1.06	4.00 ± 0.58 [f]
3:2	12.50 ± 0.96	10.42 ± 0.93	4.91 ± 0.36 [f]
4:2	23.85 ± 1.82 [b]	28.46 ± 1.92 [d]	4.74 ± 0.26 [f]
5:2	12.45 ± 0.92	10.35 ± 0.90	5.03 ± 0.36 [f]
1:3	3.35 ± 0.90	2.76 ± 0.65	2.49 ± 0.58
2:3	18.65 ± 2.45	9.92 ± 1.30	4.76 ± 0.59 [f]
3:3	32.90 ± 2.20 [a]	31.63 ± 1.86 [c]	6.12 ± 0.34 [e]
4:3	18.91 ± 2.50	9.86 ± 1.30	4.80 ± 0.60 [f]
5:3	1.05 ± 0.34	0.30 ± 0.48	1.02 ± 0.32
1:4	7.00 ± 1.13	7.45 ± 1.20	3.81 ± 0.61
2:4	24.30 ± 1.78 [b]	28.55 ± 2.02 [d]	4.87 ± 0.33 [f]
3:4	6.90 ± 1.12	7.49 ± 1.22	3.85 ± 0.61
4:4	2.70 ± 0.45	3.82 ± 0.47	2.88 ± 0.42
5:4	3.50 ± 0.54	4.35 ± 0.54	3.93 ± 0.50 [f]
1:5	8.35 ± 0.93	8.82 ± 0.98	4.13 ± 0.46 [f]
2:5	4.35 ± 0.61	4.95 ± 0.62	4.71 ± 0.58 [f]
3:5	8.05 ± 1.01	6.73 ± 0.90	4.49 ± 0.50 [f]
4:5	6.30 ± 0.91	6.52 ± 0.85	3.91 ± 0.50
5:5	4.45 ± 0.84	4.36 ± 0.69	3.82 ± 0.60
LSD	4.62	4.60	0.70

* Measurements are the means of thirty replicates ± SE.

* Means within columns with the same letter are not significantly different.

Table 6: Variation of shoot initiation under four different light intensities at 16 hour photoperiod.

Light intensity (lx)	Mean number of shoot	Mean shoot length (cm)	Mean number of leaves
1000	10.90 ± 0.74	8.77 ± 0.84 [e]	4.29 ± 0.28 [g]
2000	19.70 ± 1.73 [b]	11.19 ± 0.89 [e]	4.71 ± 0.19 [g]
3000	33.40 ± 2.84 [a]	30.52 ± 2.67 [c]	6.32 ± 0.41 [f]
4000	23.60 ± 2.33 [b]	28.82 ± 3.48 [d]	4.37 ± 0.36 [g]
LSD	16.33	16.33	1.36

* Measurements are the means of twenty replicates ± SE.

* Means within columns with the same letter are not significantly different.

Determination of a suitable potting mixture for acclimatization and suitable growth condition for growing in vitro produced plants:

Maintaining plantlets in the culture room for four weeks before being transferred to outdoor conditions increased the survival rate significantly when compared with those kept in the culture room for 2 weeks. Highest percentage of survival (60%) was achieved with the potting mixture containing compost: sand (1:3) Plants showed 50% survival in complete sand and 20% in the mixture of compost : sand mixture of 1:2 (Table 8). A lower survival rate was observed with the increase of the amount of compost in the potting mixture. This may be due to retention of extra amount of water in compost, which provides favorable conditions for microbial growth (Figure 1d). Keeping plants in a confined atmosphere with high relative humidity for few days for the formation of new roots allowing plants to feed itself normally under natural conditions is recommended[7,8].

Comparison of tissue cultured plants with plants raised from seeds

Morphology

Plant height increment is defined as the difference between the measurements of initial measurement and final measurement (i.e. after 9 months). Analysis of variance showed a significant difference in mean height increment, mean leaf number increment, and mean number of branches between the two plant types. As shown in Table 9, tissue cultured plants had a significantly higher number of leaves (12.20 ± 1.20). This phenomenon is referred to as 'bushy habit' in tissue cultured plants[12]. Branching is a very rare character in field grown *M.*

Table 7: Initiation of roots with different growth regulator concentrations of IBA and IAA after eight weeks of inoculation.

Basal medium + the type of growth regulator	Growth regulator (mg L^{-1})	Mean root length (mm)	Mean no. of roots/shoot	% root initiation
MS	0.0	0.00 ± 0.00	0.00 ± 0.00	0.0
MS+IBA	0.1	0.75 ± 0.20	0.50 ± 0.11	5.0
MS+IBA	0.2	0.85 ± 0.20	0.65 ± 0.13	5.0
MS+IBA	0.3	3.51 ± 1.15	0.85 ± 0.17	25.0
MS+IBA	0.4	3.50 ± 0.90	1.50 ± 0.30	30.0
MS+IBA	0.5	2.75 ± 0.93	1.00 ± 0.16	10.0
MS+IAA	0.1	2.65 ± 1.02	1.30 ± 0.43	10.0
MS+IAA	0.2	5.66 ± 1.18	3.30 ± 0.78	40.0
MS+IAA	0.3	0.00 ± 0.00	0.00 ± 0.00	0.0
MS+IAA	0.4	4.69 ± 1.53	1.05 ± 0.26	20.0
MS+IAA	0.5	3.51 ± 1.38	0.75 ± 0.12	15.0
1/2MS	0.0	3.49 ± 0.86	1.70 ± 0.48	20.0
1/2MS+IBA	0.1	6.46 ± 1.67	2.45 ± 0.74	40.0
1/2MS+IBA	0.2	9.68 ± 1.11	3.25 ± 0.35	75.0
1/2MS+IBA	0.3	8.57 ± 1.73	2.05 ± 0.25	55.0
1/2MS+IBA	0.4	8.29 ± 2.06	1.65 ± 0.29	40.0
1/2MS+IBA	0.5	4.53 ± 1.21	1.45 ± 0.49	40.0
1/2MS+IAA	0.1	2.43 ± 0.90	0.85 ± 0.18	15.0
1/2MS+IAA	0.2	15.05 ± 0.50	3.15 ± 0.30	70.0
1/2MS+IAA	0.3	3.40 ± 0.95	1.35 ± 0.27	25.0
1/2MS+IAA	0.4	3.33 ± 1.29	0.85 ± 0.20	15.0
1/2MS+IAA	0.5	5.40 ± 1.53	1.35 ± 0.29	30.0
LSD		2.08	0.56	

* Measurements are the means of twenty replicates ± SE.

pinnata. Branching is a type of transient phenotypic variation which is a common characteristic of many herbaceous ornamentals propagated by tissue culture when compared with the conventionally propagated plants. This character is considered as highly beneficial factor for some species[7,8,12]. Flowering behavior of tissue cultured plants was similar to those of natural plants when both types of plants were grown under same conditions.

Table 8: Percentage survival of *in vitro* produced plants in different types of potting mixtures, when they were maintained in an incubator for two weeks or four weeks before being transferred to the green house.

Soil mixture (Compost:Sand)	% survival	
	2 weeks	4 weeks
1 : 0	0	0
0 : 1	30	50
1 : 1	10	10
1 : 2	20	30
1 : 3	40	60
2 : 1	0	0
2 : 3	10	20
3 : 2	0	0
3 : 1	0	0

Physiology

Rate of photosynthesis was higher in tissue-cultured plants than in seed raised plants at six and nine months of growth (Table 10). Although the rate of photosynthesis was always higher in tissue cultured plants than others a marked difference cannot be claimed between them. Photosynthetic rate increased with time in both plant types showing further establishment under the greenhouse environment.Once transplanted in soil, *in vitro* plants have to change their mode of nutrition from heterotrophic to photoautotrophic. This needs a well functional root system for anchoring to the substrate, absorbing water and nutrients from the soil and a developed photosynthetic apparatus. Poor development of such an apparatus is a major constraint for better acclimatization[8,13].

Stomatal resistance of seed raised plants was higher than that of tissue cultured plants. One reason for tissue cultured plants showing lower stomatal resistance to the water loss may be due to less developed stomata and cuticle of the leaves. After nine months both plant types showed a slight increase in stomatal resistance over the value at six months, which can be considered as a positive sign of establishment of the (Table 11) plant. Introduction of micropropagated plants to natural conditions is a critical step because of the malfunctioning of stomata[14, 15].

Table 9: Increments of mean height, leaf number and number of branches in tissue cultured plants and seed- raised plants of *M.pinnata* for nine months.

Plant type	Mean height increment (cm)	Mean leaf number increment	Mean number of branches
Tissue cultured plants	3.34 ± 0.40 [a]	12.20 ± 1.20	1.30 ± 0.37
Seed raised plants	5.31 ± 0.26 [a]	9.50 ± 0.81	0.00 ± 0.00
LSD	2.02	1.52	1.30

* Measurements are the means of ten replicates ± SE.

* Means within columns with the same letter are not significantly different.

Table 10: Rate of photosynthesis at six and nine months of growth.

Rate of photosynthesis ($\mu molCO_2 m^{-2} s^{-1}$)	At six months		At nine months	
	Morning	Noon	Morning	Noon
Tissue cultured plants	6.66 ± 0.39 [a]	7.27 ± 0.32 [b]	7.01 ± 0.46	8.27 ± 0.28
Seed raised plants	5.13 ± 0.164 [a]	6.68 ± 0.27 [b]	6.15 ± 0.16	6.97 ± 0.29 [f]
LSD	2.18	0.83	1.23	1.85

* Measurements are the means of ten replicates ± SE.

* Means within columns with the same letter are not significantly different.

Table11: Mean stomatal resistance (scm^{-1}) at six and nine months

Plant type	Stomatal resistance, scm^{-1}					
	At six month			At nine month		
	Morning	Noon	Evening	Morning	Noon	Evening
Tissue cultured	4.79 ± 0.40	4.79 ± 0.40 [a]	4.30 ± 0.37	6.24 ± 0.64	4.09 ± 0.44	4.49 ± 0.47
Seed raised	8.05 ± 0.66	5.72 ± 0.39 [a]	7.84 ± 0.62	8.85 ± 0.51	7.33 ± 0.59	8.02 ± 0.76
LSD	4.6	3.54	5.0	3.69	4.58	4.98

* Measurements are the means of ten replicates ± SE.

* Means within columns with the same letter are not significantly different.

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