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Full Length Research Paper

Factors controlling micropropagation of *Myrica esculenta* buch. – Ham. ex D. Don: a high value wild edible of Kumaun Himalaya

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Various factors such as browning, season, media type and plant growth regulators influence the micropropagation of female trees of *Myrica esculenta*. These factors have successfully been addressed after addition of some media additives, collection of the explant at right season, standardizing the media type and use of plant growth regulators at varying concentrations. Polyvinylpyrollidone (PVP - 0.5%) was found effective for successful partial removal of phenolic compounds and obtaining maximum percent survival of explants. The explant collection season played an important role in reducing phenol induced browning and winter season was found best for explant establishment. The maximum number of shoots (4-5/explant) was obtained in Woody Plant Medium (WPM) supplemented with 10µM kinetin and 0.1µM naphthalene acetic acid (NAA). Of all the media types tried, Woody Plant Medium was found to be the best. Kinetin was found superior to benzyl amino purine and N^6(γ,γ-dimethylallyamino)purine (2iP) for explant establishment and multiplication. NAA induced rooting to 45.8% of explants in 1/2 strength Woody Plant Medium. However, *ex vitro* survival percentage was low.

Key Words: Browning, *in vitro*, plant regeneration, tissue culture, woody plant medium.

INTRODUCTION

Myrica esculenta Buch.-Ham. ex D. Don (Myricaceae) is a wild tree with highly valued edible fruit distributed between 900-2100 m asl in the Indian Himalaya from Ravi eastwards to Assam, Khasi, Jaintia, Naga and Lushi Hills and extending to Malaya, Singapore, China and Japan (Osmaston, 1927). In the Western Himalaya, the species occurs mainly in *Pinus roxburghii*, *Quercus leucotrichophora* and mixed oak forests and is widely accepted among local people for its delicious fruits and processed products (Dhyani and Dhar, 1994; Bhatt et al., 2000a). The popularity of the species can be judged from the fact that local people of the region can earn over Rs. 14 .00 lakh /season from selling the fruits of the species (Bhatt et al., 2000a). Besides, the species is considered a good fuel source and used as fodder and a source of medicine and oil (Singh et al., 1986; Rastogi and Mehrotra, 1991).

Remarkable variability in morphological characters (fruit yield, fruit and seed size) among different populations and circumference at breast height (CBH) classes was observed. Kalika population was found to be elite. This was further strengthened by biochemical analysis (seed protein, carbohydrate, moisture percent,

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Abbreviations: BA (6-(benzyl amino) purine); B_5 – (Gamborg`s medium, Gamborg et al., 1968); 2iP ($N^6$(γ,γ-dimethylallyamino) purine); IBA (indole-3-butyric acid); MS (Murashige and Skoog, 1962 medium); NAA (ά-(naphthalene acetic acid); PVP (polyvinylpyrollidone); WPM (Lloyd and McCown`s (1980) Woody Plant Medium).

SDS-PAGE and isozyme) of the fruit and seeds across the populations (Bhatt, 2000). This variability can be attributed to self incompatibility of the species, thus making it obligatory outcrossing and preventing true to type propagation.

The species is generally propagated via seeds but physical dormancy caused by impermeable seed coat results in unreliable germination pattern (Bhatt et al., 2000b). Also, the seed germination method yields a progeny of highly heterozygous plants and may produce male or female trees. The species under investigation is a dioecious fruit tree and female trees are economically preferred much more than the male ones. The male trees are not recognizable males until they reach sexual maturity, therefore, preferred trees can be propagated by cloning of sexual mature female specimen. Vegetative propagation with cuttings carried out in different seasons with various auxin concentrations did not induce rooting (Bhatt, 2000). Therefore, micropropagation seems to be the only way for cloning of selected trees. Although clonal propagation using tissue culture has been carried out in *M. esculenta* (Nandwani 1994), the report did not describe the sex of the cloned material. The present study was carried out to investigate the influence of medium additives on explant establishment, season of explant collection, effect of auxin-cytokinin addition on shoot proliferation and effect of auxin on rooting of shoots of mature female trees of *M. esculenta*.

MATERIALS AND METHODS

Plant material and preparation of explant

Shoot segments were collected from female elite individuals of *M. esculenta* from Kalika population (altitude 1775 m asl; latitude 29°38' N and longitude 79°50' E). After trimming the leaves, shoots were cut into small pieces (2.0-2.5 cm long) each with a single node. These nodal segments were used as explant. Explants were washed with teepol (2% w/v) for 5 min followed by 15 min savlon (an antiseptic containing 3% antimicrobial agents centrimide + detergent: Johnson and Johnson, India). They were further rinsed with distilled water, put in Bavestin (BASF India) (0.25% w/v) solution and placed in a rotary shaker for 1 h for vigorous shaking. Thereafter, the explants were thoroughly rinsed with double distilled water and treated with different concentrations of $HgCl_2$ (0.05 or 0.1%) for various durations (6, 8, 10 or 12 min) under aseptic conditions in Laminar Flow System (Klenzaids, Bangalore India) for obtaining aseptic explants. These explants were then thoroughly rinsed with double distilled water (4-5 times). Both ends of the explants exposed to sterilants were trimmed and explants were placed aseptically in 25x150 mm test tubes (Borosil, India Ltd.) containing 15 ml WPM (Lloyd and McCown, 1980) supplemented with 3% sucrose, kinetin (10 µM) and NAA (0.1 µM). The medium was solidified with 0.8% agar (Bacteriological grade, Qualigens India Ltd.) and pH adjusted at 5.8 prior to autoclaving at 121°C and 108 kPa for 20 min. All chemicals used were of analytical grade (Sigma Chemical Co. USA, Merck, Hi-Media and Qualigens India Ltd.). The cultures were maintained at 25 ± 2°C and 60 ± 5% relative humidity in a culture room under a 16 h photoperiod of 40 µmol m^2 s^{-1} light intensity provided by cool white fluorescent tubes.

Effect of pretreatments and media additives on explant browning

Experiments were designed on the basis of response of explants inoculated on the WPM. In these trials, explant browning and media staining was noticed due to severe leaching of phenol exudates in the medium. Since explant establishment was difficult, pretreatment became necessary. Therefore, various experiments were conducted to circumvent this problem.

To study the effect of various pretreatments and media additives on explant browning and survival percentage, different sets of sterilized explants were dipped in antioxidant solution [ascorbic acid (50 mg l^{-1}) and citric acid (75 mg l^{-1})] filter sterilized using 0.22 µM pore size filter, polyvinylpyrollidone (PVP 0.5%), sucrose (2%), sucrose (2%) + PVP (0.5%) and distilled water for 2-3 h, separately. One set of explants were serially transferred (ST) to the freshly prepared WPM medium after every 24 h interval. Another set of the treatment solution was also incorporated into the medium separately. Pretreated explants were cultured in 25 x 150 mm test tubes containing 15 ml WPM supplemented with 10 µM kinetin and 0.1 µM NAA. Sterilized explants without pretreatment were directly cultured into the medium containing media additives. One set of explants were kept for chilling treatment at 4^0C for 24 or 48 h. Observations were recorded after 21 days in growth chamber.

Effect of collection time on explant browning

To study the seasonal response of explant establishment, explants were collected in the first week of every month from female tree. Sterilized explants were then inoculated on WPM containing 10 µM kinetin combined with 0.1µM NAA. The medium was supplemented with 0.5% PVP to reduce explant browning. Records on percent survival of explants, shoot number and shoot length were made after two weeks of incubation.

Effect of cytokinin concentration on explant establishment

To determine the most suitable hormonal combination for explant establishment and multiple shoot production, sterilized explants were inoculated into test tube each containing 15 ml WPM supplemented with various concentration of kinetin (1.0-10 µM) and NAA (0.01-0.1 µM). The medium was supplemented with 0.5% PVP to reduce explant browning. One set of explants cultured on WPM without growth regulators served as control. After four weeks, newly developed shoots were transferred into 100 ml conical flask (Borosil India Ltd.) each containing 35 ml WPM. Records on shoot number and shoot length were made after two weeks of incubation.

Effect of media type on explant establishment

To standardize most suitable media for explant establishment and multiple shoot induction, sterilized explants were inoculated into different media type: WPM, MS (Murashige and Skoog 1962), B_5 (Gamborg et al., 1968) and 1/2 MS, containing kinetin (10 µM) and NAA (0.1µM). All the media were supplemented with 0.5% PVP to reduce explant browning. The sucrose concentration was 3% in all the media tested. After two weeks of incubation, percent survival, shoot number and shoot length per explant were recorded.

Effect of cytokinin type on explant establishment

To identify the most suitable cytokinin type for explant establishment and multiple shoot induction, different sets of

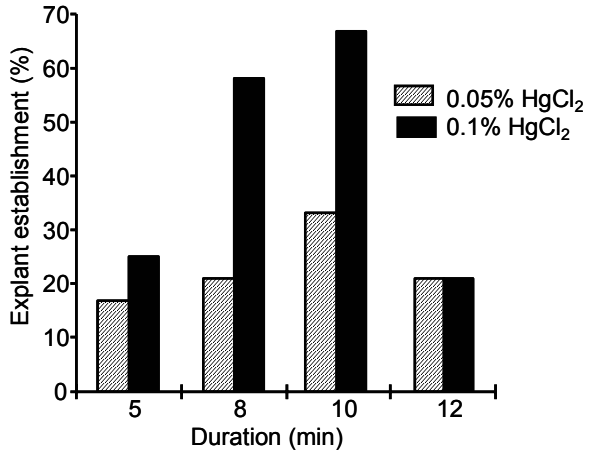

Figure 1. Effect of $HgCl_2$ concentration and treatment duration on explant establishment of *Myrica esculenta.*

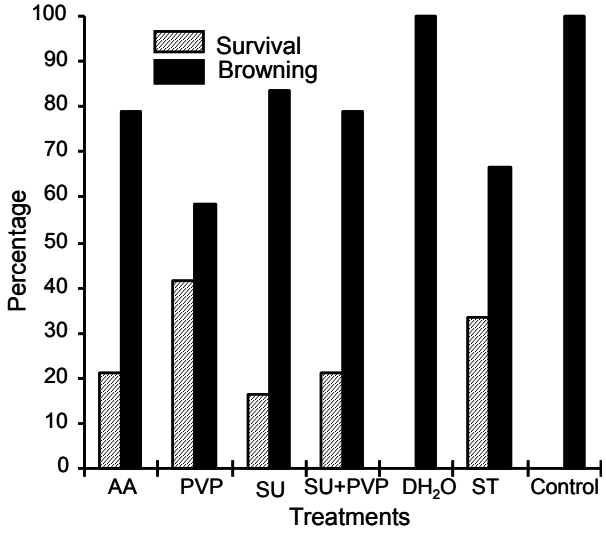

Figure 2. Effect of various pretreatments on explant establishment and percentage browning of explants (AA-Ascorbic acid 50 mg/l + Citric acid 75 mg/l, PVP – Polyvinylpyrollidone (0.5%), SU – Sucrose (2%), SU+PVP, DH_2O – Distilled Water, ST- Serial transfer, Control)

sterilized explant were inoculated on WPM supplemented with kinetin, BA or 2iP (10 µM), each combined with NAA (0.1µM). Each medium was supplemented with 0.5% PVP. After two weeks of incubation, percent survival, shoot number and shoot length per explant were recorded.

Effect of auxin type and concentration on in vitro rooting

After four weeks of incubation in multiplication medium, shoots >1.5 cm long were harvested for rooting experiment. A two step procedure was adopted for standardizing most suitable auxin type and concentration for root induction. In the first step, shoots >1.5 cm were excised from explants of multiplication medium and inoculated on half strength WPM containing various concentrations of IBA or NAA (10-40 µM). After one week of incubation, treated shoots were then transferred into 1/2 strength WPM without growth regulators. Shoots showing >5 mm long roots were considered as rooted. Data on percent rooting, root number and root length were recorded after four weeks of transfer in rooting medium without growth regulators.

Well-rooted shoots were removed from culture tubes after four weeks in rooting medium. Rooted shoots were washed thoroughly with tap water to remove the adhering medium and transferred to thermacaul pots (5 cm diameter) containing 120 g (w/v) soilrite + sand + soil (1:1:1). Transparent polyethylene bag or 250 ml glass beaker was inverted over each plantlet to maintain high humidity and watered with 1/4 strength WPM salt solution on alternate days. Plantlets were monitored under the same environmental conditions as in vitro cultures for two weeks and thereafter transferred to polyhouse for further growth. The transparent polyethylene bag or beaker was removed permanently upon new leaf appearance.

Experimental design and statistical analysis

Three replicates (8 explants/replicate) were inoculated in each treatment of an experiment. After four weeks of culture period, records were made on percentage of explant initiating shoots, shoot number and shoot length per explant. For rooting experiment percent rooting, root number and root length were measured.

Experiments were carried out in a completely randomized block design (CRBD). Data scored on percentage were subjected to arcine transformation before analysis and then converted back to percentage for presentation in table (Snedecor and Cochran, 1968). Significant differences among mean values were separated using Fisher's least significant differences (F-LSD $P<0.05$) test with SYSTAT (Wilkinson, 1986).

RESULTS AND DISCUSSION

Of the various pretreatments tried, 0.1% $HgCl_2$ for 10 min gave the maximum aseptic cultures (Figure 1). Duration of treatment exceeding 10 min was found deleterious. Lower concentration of $HgCl_2$ (0.05%) was found less effective. Explant browning and media staining were major impediments in the establishment phase of culture of *M. esculenta* in WPM. The medium turned brown within 24 h due to release of brown exudates. To avoid the injurious effect of exudates on explant establishment various possible protectants were tried, among which 0.5% PVP for 2-3 h prior to inoculation was found effective and significantly better compared to control, but still explant browning percentage was quite high (Figure 2). The other widely used pretreatments such as antioxidants, sucrose, sucrose + PVP, DH_2O and ST were less effective. Dipping the explants in sterilized distilled water failed to reduce phenol leaching thereby preventing explant establishment. Pretreatment with PVP was also found effective in controlling phenol exudation from mature explants of guava (Amin and Jaiswal, 1988). Although ST and antioxidant solution reduced explant

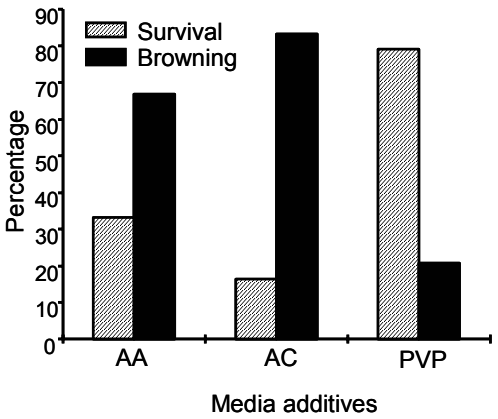

Figure 3. Effect of various media additives on explant establishment and percentage browning of explants (AA- Ascorbic acid 50 mg/l + Citric acid 75 mg/l, AC – Activated Charcoal, PVP – Polyvinylpyrollidone).

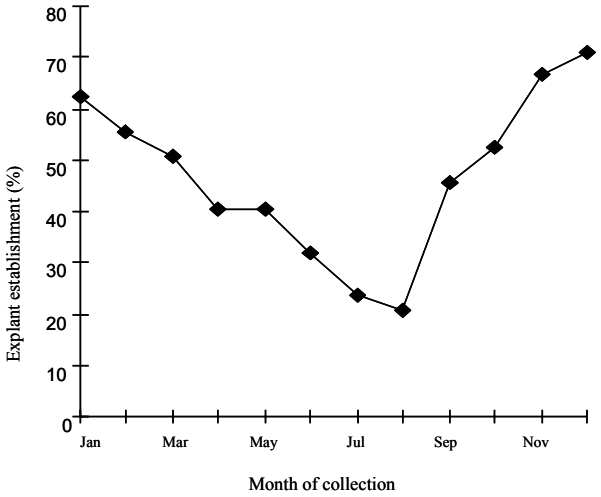

Figure 4. Effect of collection season on explant establishment (Jan –January, Mar–March, Jul–July, Sep–September, Nov–November; WPM + 10μM kinetin + 0.1 μM NAA + 0.5% PVP).

browning significantly as compared to control, the results were not encouraging when compared with PVP solution. The effectiveness of PVP is also reported in *Tectona grandis* (Gupta et al., 1980). Sucrose alone or in combination with PVP did not yield encouraging results in the present study. Dipping the explants in distilled water failed to reduce explant browning. The purpose of this treatment was to dissolve the water soluble phenol and reduce browning in explants. PVP (0.5%) when added into medium was found most effective resulting in 79.16% explant establishment. Addition of antioxidants or activated charcoal could not significantly reduce percent explant browning (Figure 3). Control set also failed to reduce percent browning with none of the explants established. PVP added in the medium was also found effective in explant browning prevention from earlier reports on guava (Amin and Jaiswal, 1988), *Tectona grandis* (Gupta et al., 1980) and *Cleistanthus collinus* (Quraishi and Mishra, 1998).

Shoot collection season proved to play important role in reducing percent browning and inducing bud break. Explants collected during winter (November - December) gave the maximum response as measured by percentage of explants with axillary bud break *in vitro* (Figure 4). Seasonal changes greatly influence explant establishment (Siril and Dhar, 1997). Maximum bud break was observed in winter season (November to January). Cultures could not establish in rainy season due to heavy fungal and bacterial contamination. The actively growing season was known to be more responsive for bud break than others (Dhar and Upreti, 1999), which is contrary to the present report where maximum bud break was achieved in winter season. In the spring season maximum explants died due to phenolics. This is perhaps due to higher phenolic content in growing shoots.

Sterilized explants were inoculated in various concentrations of kinetin (1-10 μM) with or without NAA (0.05 - 0.2 μM). The incubation of 10 μM kinetin and 0.1 μM NAA was found most effective. Maximum explants (70.8%) established after four weeks of culture in WPM containing 10 μM kinetin with 0.1 μM NAA (Table 1). Newly developed shoots transferred in the same medium produced 5 shoots/explant. ANOVA revealed significant interaction of auxin and cytokinin in shoot number. Presence of high cytokinin (>10 μM) considerably reduced percent establishment. Lower kinetin concentration (5 μM) increased shoot length but significantly lowered shoot number. Explants failed to survive in control set. Addition of 10 μM kinetin with 0.1 μM NAA was found most suitable for explant establishment. The effectiveness of cytokinin in promoting axillary bud development *in vitro* in forest trees is well documented (Zaerr and Mapes, 1982; McCown and Sellmer, 1987). Addition of low level of auxin along with cytokinin has been known to increase percent establishment as well as shoot number in forest trees (Rathore et al., 1991). In several tree species the combination of auxin and cytokinin seems to be positive for number and length of shoots whereas in others it was found inhibiting shoot growth and stimulating only shoot multiplication. Torres and Carlisi (1989) found that the number of *in vitro* derived shoots of *Camellia sasanqua* was influenced only by the action of cytokinins whereas length increased in the presence of both auxin and cytokinin. However, in micropropagation of *Pittosporum napaulensis,* concentration of cytokinin and auxin had a positive effect on the shoot number and elongation in other tree species as well (Dhar et al., 2000). Nandwani (1994) reported that 2.5 -5.0 mg l^{-1} kinetin with 0.05 mg l^{-1} NAA is essential for micropropagation of *M. esculenta.* This is supported by the present study, where kinetin and NAA were found most effective in explant establishment and axillary shoot formation.

Table 1. Effect of plant growth regulators on explant establishment and multiple shoot induction in *Myrica esculenta*; WPM, 3% sucrose and 0.5%PVP.

Plant growth regulator (µM) Kinetin	NAA	Percent establishment	Shoot number	Shoot length (cm)
1.0	0.00	16.6	2.0	1.2
1.0	0.10	20.8	2.3	1.4
1.0	0.50	26.6	1.3	0.8
2.5	0.00	25.0	2.3	1.4
2.5	0.10	41.6	2.6	1.6
2.5	0.50	25.0	2.6	1.7
5.0	0.00	33.3	4.0	1.4
5.0	0.10	66.6	4.3	2.2
5.0	0.50	54.1	3.0	1.9
10.0	0.00	41.6	4.6	1.7
10.0	0.10	70.8	5.0	1.9
10.0	0.50	58.3	2.6	1.6
15.0	0.00	33.3	3.6	0.8
15.0	0.10	41.6	4.3	1.0
15.0	0.50	25.0	2.6	0.9
00.0	0.00	0.00	0.0	0.0
LSD P< 0.05		13.7	1.87	0.60
F		28.69**	5.41**	6.46**

Level of significance: ** p<0.01

Table 2. Effect of media type on explant establishment and multiple shoot induction in *Myrica esculenta*; *All media contain kinetin 10µM + NAA 0.1 µM + PVP 0.5%.

Media type	Percent establishment	Shoot number/explant	Shoot length (cm)
WPM	66.6	5.0	2.26
MS	33.3	2.6	1.90
B5	20.8	1.6	1.26
1/2 MS	41.6	2.0	0.96
LSD P<0.05	12.12	1.03	0.70
F	5.93*	14.29**	0.31^{ns}

Level of significance: * p<0.05, ** p<0.01, ns - not significant

Nodal segments showed highest response in WPM as compared to MS and B_5. Cultures grown in WPM gave 5 shoots/explant, which was significantly higher than MS and B_5 (Table 2). Reducing the salt concentration in MS resulted in poor performance with regard to percent establishment, shoot number and shoot length as compared to WPM. Some species give similar response in all media while others show preference for a specific medium for explant establishment and growth (McCown and Sellmer, 1987). In the present study, WPM gave better results than B_5, MS and 1/2 MS. This is contrary to the earlier report (Nandwani, 1994) who found best results in MS medium. Reports suggest that in B_5, explants do not respond well due to the presence of high ammonium and nitrate content (Constabel, 1984), which inhibit percent establishment and shoot multiplication. In the present study, the best results in WPM, were probably due to lower concentration of salts and sucrose compared to B_5 and MS. Low concentration of sucrose is reported to reduce browning in *Pseudotsuga menziesii* cultures (Evers, 1984) and weaker salt formulations promote axillary bud development in forest trees (McCown and Sellmer, 1987).

To compare the response of various cytokinin types, 10 µM kinetin responded better as compared to other cytokinins, BA and 2iP (Table 3). ANOVA revealed a significant effect of treatment with respect to percent establishment and shoot number. Shoot number in case of kinetin was significantly (P<0.05) higher than other cytokinins tried. However, BA was found second best

Table 3. Effect of cytokinin type on explant establishment and multiple shoot induction in *Myrica esculenta*; WPM + NAA 0.1 µM + PVP 0.5%.

Cytokinin type (10 µM)	Percent establishment	Shoot number/explant	Shoot length (cm)
BA	45.6	3.2	1.73
Kinetin	60.9	5.0	2.06
2iP	32.4	2.3	1.26
LSD P< 0.05	13.6	2.08	0.79
F	10.33**	9.48**	0.49^{ns}

Level of significance: ** p<0.01, ns - not significant.

Table 4. Effect of auxin type and concentration on root induction of *Myrica esculenta* on 1/2 WPM.

Auxin type	Concentration (µM)	Percent rooting	Root number	Root length (cm)
NAA	5.0	0.0	0.0	0.0
	10.0	16.6	2.0	3.3
	20.0	45.8	2.6	3.5
	40.0	33.3	1.7	2.2
IBA	5.0	0.0	0.0	0.0
	10.0	20.8	2.3	2.8
	20.0	29.1	1.4	2.5
	40.0	16.6	1.3	2.0
Control	0.0	0.0	0.0	0.0
LSD P<0.05		13.94	1.22	1.31

cytokinin for shoot induction. Low rate of explant establishment as well as shoot number was found in 2iP supplemented medium. This cytokinin also inhibited shoot elongation. Superiority of kinetin over other cytokinins has been demonstrated in woody perennials (Dhar and Upreti, 1999).

Shoots >1.5 cm were tried for rooting experiments. Shoots were harvested from multiplication medium and placed in 1/2 WPM supplemented with different concentration of NAA and IBA (5-40 µM). The shoots failed to root in auxin supplemented medium even after three weeks of culture. A two step procedure was adopted for root induction as suggested by Gasper and Coumans (1987). In the first step, microcuttings were inoculated in 1/2 WPM supplemented with various concentrations of NAA or IBA (10-40 µM) for two weeks. In the second step, microcuttings were removed and planted in growth regulator free 1/2 strength WPM. Shoots started to root after 20 days of culture in the above medium. Two weeks treatment in 20 µM NAA resulted in 45.8% rooting whereas 20 µM IBA gave only 29.1% rooting (Table 4). About 2-3 roots formed per microcutting grew 3-3.5 cm long within six weeks at 10-20 µM NAA. Lower concentrations of NAA, IBA or the control set failed to induce roots. Higher auxin concentration reduced rooting percentage as well as root number. Half - strength WPM mineral salt formulation was found most suitable for rooting in *M. esculenta*. This is contrary to the findings of Nandwani (1994) who reported 3/4 MS suitable for root induction. WPM (½ strength) has also been found suitable for the rooting in other tree species such as *Cercis canadensis* (Mackay et al., 1995).

Well rooted shoots were transferred in thermacaul pots containing soil+soilrite +sand (1:1:1), which was found to be the most suitable mixture for *ex vitro* survival and growth. Immediate transfer of plants from low light intensity and high humidity to controlled temperature and normal atmospheric humidity conditions caused death of regenerants. A gradual transfer procedure was found imperative. Pots were covered with polyethylene bags or glass beaker to maintain high humidity and were kept in growth chamber 25 ± 2°C for four weeks. Covers were removed upon growth of new leaves appearance. Only 20% of plantlets survived in a mixture of soilrite + sand + soil. In order to ensure higher *ex vitro* survival rate, experiments are being carried out to standardize acclimatization and hardening system.

Micropropagation protocol, detailed above, is highly effective to produce large number of plants. It is an ideal technique for cloning dioecious woody plants such as *M. esculenta,* where female trees are preferred due to higher economical value.

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