MICROPROPAGATION OF *MICROCOS PANICULATA L*.USING SHOOT TIP EXPLANTS

Abstract

To develop protocols for micropropagation of *Microcos paniculata L* several experiments were carried out by using shoot tip explants from seedling. Murashige and Skoog (MS) medium supplemented with various concentrations of Benzyl amino purine (BA), NAA and Kn singly or in combination was used to determine the rate of shoot proliferation. Among the tested cytokynins, Kn proved to be most effective. For rooting, NAA, IBA and IAA were used as plant growth regulators in MS media. The response of NAA was superior to IAA and IBA for root induction. MS media is supplemented with BAP, 24D & TDZ was used for trial of Callus Induction. 24D in combination with BAP and TDZ alone were found to be best for Callus Induction. The present study confirms that micropropagation of *M. paniculata* L can be efficiently preformed by using cytokinins of low concentrations.

Keywords: Micropropagation, Multiple shoot induction, Root Induction, Callus, *Microcos paniculata* L

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I. INTRODUCTION

Microcos paniculata L of the family Tiliaceae is an indigenous wild fruit which is used for commercial purpose during season in Manipur, India. Although this fruit is not found utilized very commonly like apple, banana etc. it is found to make pickles and dry fruits in some Asian countries and having high antioxidant properties according to the findings. (Haripyaree *et al*, 2010). Moreover seeds of this species lose viability within a short time. The most preferred tissues are seeds and bud wood (Novaro, 1981). However, various countries have limitations on the introduction of bud wood because of the threats of familiarizing new and virulent diseases and it is controlled only through seeds. Rapid cloning of the leading genotypes through *in vitro* adventitious shoot proliferation is widely used for many fruit species (Zimmerman, 1986). Though when the seed is used as the genetic material for germplasm exchange, shriveling and viability loss during packing, storage and transfer are the main difficulties. The problems associated with the germplasm exchange and conservation of germplasm of this indigenous fruit plant having high antioxidant property, we proposed a protocol for regeneration of *Microcos paniculata L*. from shoot tip explants and may be effectively used for germplasm exchange.

1. **Traditional Uses:** The leaves, fruits, barks and roots of *Microcos paniculata* extracts were Traditionally used by various tribal groups in many countries for medicinal purpose (Editorial Com., 1994; He, 1999)Not only in India the traditional application of *M.paniculata* L leaves, fruits, barks and roots was also reported in other countries like Bangladesh, Sri Lanka, India, China, Indonesia, Myanmar, Malaysia, Andaman Islands, Vietnam, Cambodia and Thailand (Krtikar & Basu, 1987; Ghani 1998;Pavel & Hossain; 2012) . In these countries, the plant was originally used for eczema, stomachache, injuries, itch, typhoid fever, hepatitis, small-pox, heat blow, dysentery and syphilitic ulceration of the mouth. Stewed *M. paniculata* L leaves along with shell of snail and turmeric powder were also used for treatment of jaundice (Sinha 1996).

II. MATERIAL & METHOD

1. **Preparation of Plant Material:** For shoot tip induction, nodal shoot tip explants of mature field grown plants and *in vitro* generated and germinated seedlings were taken. For callusing, cotyledon, shoots and roots from in vitro generated seedlings were used.

2. **In Vitro Multiplication:** Murashige and Skoog's (1962) medium with 3% sucrose, 0.8% agar, enriched by the supplementation of auxin and cytokinin was used. 6-benzylaminopurine (BAP; 0.25mg/l – 2mg/l) singly of in combination with naphthalene acetic acid (NAA 0.25mg/l) and Kinetin (Kn 0.5mg/l) were tested. About 30 ml of medium was dispensed into separate tubes (32 x 200mm), which were enclosed with double layered aluminum foil and autoclaved at 15 lb for 20 min. After cooling to room temperature, the explants were inoculated into the media and incubated at 25±2ºC under light intensity of 3000 lux (fluorescent tubes) for 7 hr and 0 hr photoperiod in a 24 hr cycle.

   The pH of all the media (at each treatment) was adjusted to 5.8 with 1N NaOH prior 1N HCL prior to autoclaving for 20 min at 121ºC. Fifteen replicated were at each treatment.

3. **Sterilization of Explants:** Shoot tips and axillary shoots from the field grown plants as well as from *in vitro* generated plants were taken for the experiment. Seed, shoot tips and axillary buds were first washed with labolene then treated with 0.1% Dhanustin-50 (fungicide) for 10-15 min. This was followed by surface sterilization with 70% ethanol for 4 min. then 0.02% $HgCl_2$ solution for 7 min. and rinsed 5 times with sterile distilled water.

4. **Culture Media and Condition:** The explants were cultured in MS medium containing 3% (w/v) sucrose supplemented with growth regulators. The media were adjusted to pH 5.8 by using 1N NaOH and 1N HCl before adding 0.8 % (w/v) agar. About 20 ml of the media were then disposed in culture tubes and closed with aluminium foil before autoclaving at 121ºC for 20 min. The cultures were then inoculated with single explants and then incubated in a growth chamber at a temperature of 25±2ºC and 16 hr photoperiod provided by white fluorescent tubes (30µ mol $m^{-2}s^{-1}$).

5. **Shoot Bud Induction:** The shoot tip explants were inoculated in bud induction medium consisting of MS medium containing 3% (w/v) sucrose, 0.8% (w/v) agar and supplemented with BAP (6 benzyl amino purine) alone or in combination with kinetin (kn) and NAA (α-naphthalene acetic acid) for shoot induction. The number of shoot buds developing on the shoot tip explants was recorded after four weeks of culture and the effect of different shoot induction media was evaluated.

6. **Rooting and Elongation of Shoot Buds:** Buds induced were then placed on medium containing MS medium supplemented with different concentrations of 1AA (Indole acetic acid),1BA (Indole 3 butyric acid) or α NAA (Naphthalene acetic acid) for rooting and elongation. The cultures were incubated in a growth chamber at 25±2ºC and 16 hr photoperiod. Date on percentage of rooting and length of regenerated shoots were recorded after six weeks.

7. **Germination and Callus Induction:** The fruits were harvested in February. Seeds were extracted and sterilized as mentioned above. Both seed coat and cover were removed aseptically. The seeds were kept on MS medium and MS medium supplemented with growth regulators. Germinating seedling were aseptically cut into pieces, different explants viz shoot, root and cotyledon were kept on MS medium containing 2,4D (2,4 dichlorophenoxy acetic acid), BAP (Benzyl amino-purine) and TDZ (Thidiazuron) were used for callus induction. All the cultures were maintained at temperature of 25±2°C and 16-h photoperiod with irradiance of 30 µmol m^{-1} (fluorescent tubes). The response of cultures was recorded after 4 weeks.

III. RESULTS

After 2-3 weeks of culture, multiple shoot tips were observed from the *in vitro* Germinated seedling explants(F 1-a,1-c , 1-d and 1e). The maximum proliferation of shoot buds occurred on medium containing 1 mg l^{-1} Kn or 1 mg l^{-1} BAP with 0.5 mg l^{-1} NAA. Among the tested cytokinins Kn proved to be the most effective **(Table 1)**.

Rooting followed by elongation of shoot buds were observed in the MS medium containing 1 mg l^{-1} or 2 mg l^{-1} IAA, IBA and NAA or 1 mg l^{-1} IBA (Fig. 1-f). The best concentration for producing highest number of root is found in 2 mg l^{-1} NAA or 1 mg l^{-1}

IBA. However NAA proved to be the best growth regulator for rooting of *Microcos paniculata L* **(Table-2)**.

In the next set of experiment, callus was induced from the shoot explants by using BAP, 2,4 D and TDZ (Fig. 1b). The callus was formed on MS Media supplemented with 1-2mgl-1 2,4D alone or in combination with 0.5mgl-1 BAP and 1-2mgl-1 TDZ. In BAP alone there is no response in callus formation. Best callusing was observed in 2mgl 1 TDZ (Table 3). Callus formation increases with increasing concentration of 2,4 D. We can observe good response of callusing in a concentration 1-2mgl-1. 2, 4 D in combination 0.5 mgl-1 BAP.

Table 1: Effect of growth regulators on multiple shoot bud induction from shoot-tip explants of *Microcos paniculata* L after four week's culture. Means ± SE, n=10. Means followed by the same letters are not significantly different.

Sl. No.	BAP(mgl^{-1})	Kin(mgl^{-1})	NAA	Response(%)	Shoot number
1	0.25	-	-	35.5	1.5 ± 0.19^{c}
2	0.50	-	-	83.3	2.1 ± 0.25^{de}
3	1	-	-	100	3.4 ± 0.18^{cd}
4	2	-	-	66.6	2.5 ± 0.44^{de}
5	0.25	-	0.5	33.3	1.3 ± 0.21^{e}
6	0.50	-	0.5	100	3.8 ± 0.26^{b}
7	2.0	-	0.5	100	5.3 ± 0.28^{a}
8	2.0	-	0.5	83.3	2.3 ± 0.38^{bcde}
9	0.25	0.5	-	35.3	1.3 ± 0.21^{e}
10	0.50	0.5	-	50.0	2.0 ± 0.23^{de}
11	1.0	0.5	-	33.3	1.3 ± 0.19^{c}
12	2.0	0.5	-	100	3.4 ± 0.28^{bcd}
13	-	0.25	-	35.3	1.7 ± 0.20^{e}
14	-	0.5	-	83.3	2.7 ± 0.30^{bcde}
15	-	1	-	100	5.5 ± 0.31^{a}
16	-	2	-	66.6	2.5 ± 0.46^{bcde}

Table 2: Effect of auxins on rooting and elongation of shoot buds derived from shoot tip explants of *Microcos paniculata* L. Means ± SE, n=6 Means followed by the same letters are not significantly different at $p<0.1$

Sl. No	1AA(mgl^{-1})	1BA(mgl^{-1})	NAA(mgl^{-1})	Rooting(%)	Shoot length	Leaf no.
1	1	-	-	70	3.4 ± 0.34^{bc}	4.7 ± 0.35^{bc}
2	2	-	-	40	2.0 ± 0.49^{bc}	4.3 ± 0.51^{ab}
3	-	1	-	80	3.7 ± 0.45^{ab}	6.0 ± 0.42^{bc}
4	-	2	-	60	3.2 ± 0.37^{a}	5.4 ± 0.25^{a}
5	-	-	1	40	1.5 ± 0.32^{c}	3.2 ± 0.29^{d}
6	-	-	2	10	1.2 ± 0.19^{b}	3.0 ± 0.21^{d}

Table 3: Effect of BAP, 2,4 D and Thidiazuron(TDZ) on Callus induction from the shoot explants of *Microcos paniculata L* after 16 weeks of inoculation.

BAP(mgl^{-1})	2,4D(mgl^{-1})	TDZ(mgl^{-1})	Response(%)	Callusing
0.25	-	-	N-R	-
0.5	-	-	NR	-
-	1	-	70	PC
-	2	-	95	GC
-	-	1	100	GC
-	-	2	100	BC
0.5	1	-	100	GC
0.5	2	-	100	GC

NR= No Response; PC= Poor callusing; GC=Good callusing; BC=Best callusing

Four week old rooted plantlets were carefully removed from the flask and gently washed under running water to remove adhering pieces of gelled medium. These were then transplanted to perforated plastic cups containing pre-sterilised sand: soil (5:1) and maintained in a 50% shaded net house for hardening. The plants hardened here within 15 days and these were indicated by the emergence of new apical leaves. The polythene bags were finally removed and the plants were transferred to bigger earthen pts containing soil or to the field condition.

IV. DISCUSSION & CONCLUSION

Till now, overall of 70 compounds have been isolated and reported from leaves, fruits, barks and roots extracts of *Microcos paniculata L* (Yang et al 2010; Hu et al 2012, Bandana et al 2000), including 30 flavonoids,10 triterpenoids, 3 steroids, 11 alkaloids, 9 organic acids, and 7 other compounds.

Most copious components of *M.paniculata L* leaves were flavonoids and the content of flavonoids in leaves, stems, fruits of *M.paniculata L* were 16.94%, 5.15% and 1.52%, correspondingly (Bi et al 2006).

The potential mechanisms of the polyphenol-enriched fraction from *Microcos paniculata L* leaves on oxidative stress and acetaminophen (APAP)-induced hepatotoxicity was assessed (Wu *et al* 2017).

The effect of *M. paniculata L* leaves on isoprenaline (ISO) induced acute myocardial ischemia (AMI) rats was examined (Chen *et al* 2016). The toxicity against brine shrimp nauplii of *M. paniculata L* extract were measured (Rahman *et al* 2011 and Aziz *et al* 2013, 2015).In the study, it was disguised that alkaloids in *M. paniculata L* may have 58.2% ± 9.2% cytotoxic activity against certain cells, but only single dose (10 $\mu mol \cdot L^{-1}$) was used in nicotinic receptor antagonistic activity study. Dai *et al* 2010 stated the digestion sponsoring activity of *M. paniculata L* leaves in rats for 7 days. Aziz *et al* 2014 investigated the antidiarrheal action of *M. paniculata L* fruits. Moreover, methanol and chloroform extracts from *M. paniculata L* barks, fruits and roots as well as and microcosamine A and microcosamine B displayed the potential larvicidal activity against the 4^{th} instar *Culex quinquefasciatus* mosquito (Feng 2015).

Induction of axillary shoots by decapitation of *in vitro* generated plantlets was reported in many plant species (Hyde and Phillips 1996,;Haripyaree *et al*. 2011).So far there is no report of micropropagation of *Microcos paniculata L.* We used the seeds of this species to take the shoot tip explants. The most preferred are seed and bud wood (Novaro L ,1981).

In most of the fruits for multiple shoot bud induction, BAP, Kn and NAA were used. (Paudyal and Haq, 2000). We used to test the shoot bud induction and found to be effective. In rooting also we used IAA, IBA and NAA. NAA is found to be the best growth regulator for rooting of *Microcus paniculata*.L

For callus induction, Thidiazuron (TDZ) was found tremendous effect in callus formation.

From the findings of tissue culture it can be concluded that plant growth regulators play an important role in the callusing, shoot tip induction and rooting in the cultures of *Microcos paniculata L*. Therefore concerted effort is required to develop a reproducible protocol for *in vitro* propagation of these plants to facilitate its effective utilization in the production of healthy planting materials. It will help in the conservation of germplasm for further generation.

The induction of multiple shoot buds from the shoot tip explants provide a novel protocol for propagation of *Microcos paniculata L* in tissue culture. It results in the regeneration of a large number of plantlets from single explants. These techniques, therefore, presents an efficient system for germplasm conservation and mass multiplication of this important fruit plant as compared to propagation by seed.

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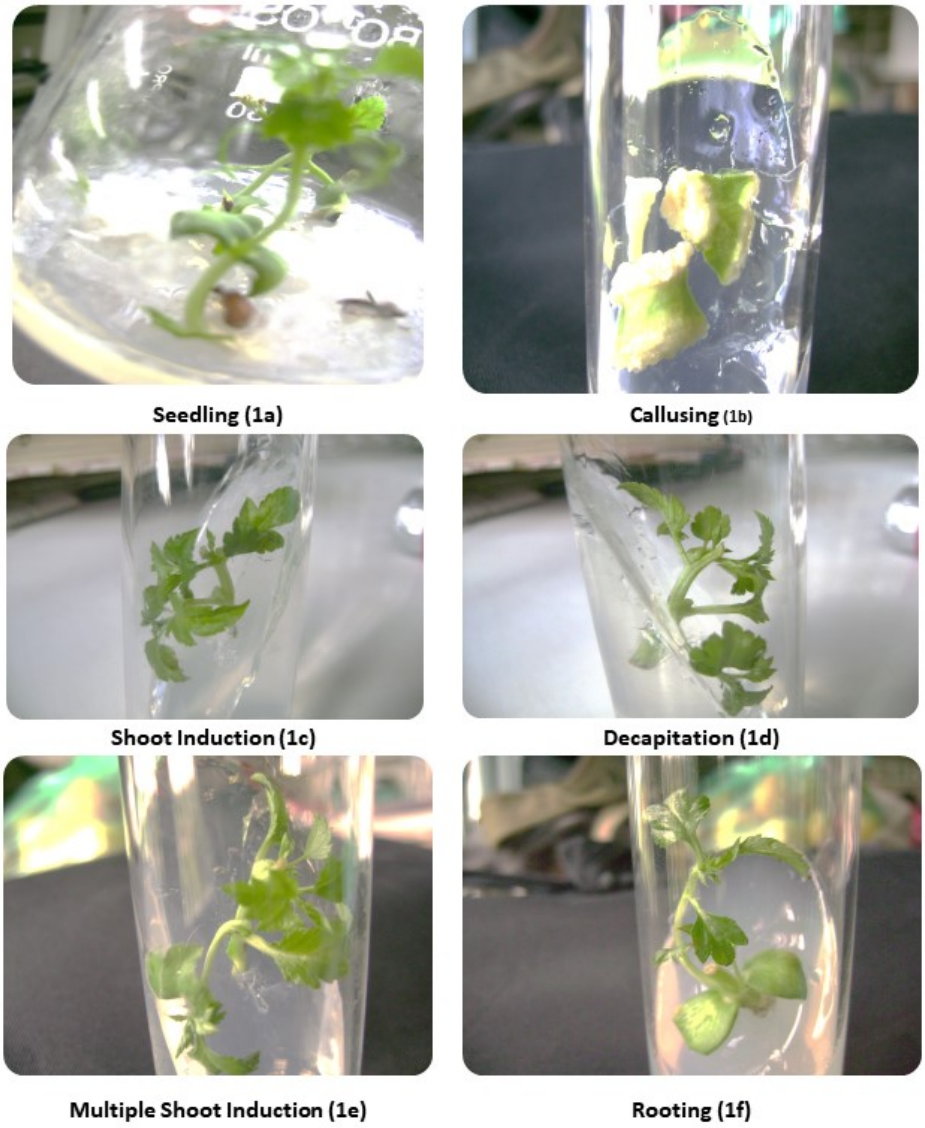

In vitro Propagation of *Microcos paniculata* L