Journal of Genetic Engineering and Biotechnology (2017) 15, 249–256

HOSTED BY

ELSEVIER

Academy of Scientific Research & Technology and National Research Center, Egypt

Journal of Genetic Engineering and Biotechnology

www.elsevier.com/locate/jgeb

ORIGINAL ARTICLE

Improved micropropagation and *in vitro* fruiting of *Morus indica* L. (K-2 cultivar)

Gaurab Gogoi [a,*], **Prodeep K. Borua** [a], **Jameel M. Al-Khayri** [b]

[a] *Department of Life Sciences, School of Science and Engineering, Dibrugarh University, Dibrugarh 786004, Assam, India*
[b] *Department of Agricultural Biotechnology, College of Agriculture and Food Sciences, King Faisal University, Al-Hassa 31982, Saudi Arabia*

Received 27 October 2016; revised 31 January 2017; accepted 19 February 2017
Available online 17 March 2017

KEYWORDS

Commercial;
Micropropagation;
Indian mulberry;
Nodal explant;
Tissue culture;
2-4,D;
Two-phase

Abstract A rapid economically viable micropropagation protocol has been developed in the present work for *Morus indica* L. (K-2 cultivar) utilizing the readily available nodal explants. Explants were established on different plant growth regulators (PGRs) either individually or in combinations. MS medium containing 1 mg L^{-1} Kinetin (Kin) showed the best shoot multiplication with 4.8 ± 0.23 cm average shoot length and 6.5 ± 0.03 number of internodes. Regenerated shoots were elongated in MS medium supplemented with 1.5 mg L^{-1} gibberellic acid (GA_3). Elongated shoots cultured in full-strength MS medium supplemented with 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) for one week and then cultured in half-strength MS proved to be more effective in rooting compared to other PGRs in significantly shorter duration. Micropropagated plants transferred to soil fortified with the quarter-strength of MS salts along with humidity regulation process showed 89% survival frequency. *In vitro* flowering in the regenerated shoots was also observed in the MS medium supplemented with (1.5 mg L^{-1}) Kin and carbon source replaced by commercial sugar cubes. This method can be effectively used for *in vitro* culture of *M. indica* in commercial scale owing to its enhanced quality and reduced time frame.

1. Introduction

Morus indica L., commonly known as Indian mulberry, is a woody, perennial plant belonging to the family Moraceae. The cultivated varieties are mostly diploid and monoecious, which flower during August and the fruit set starts in early September. *M. indica* is considered as an economically important plant, which is cultivated commercially for its edible fruit and its foliage as the primary feed of silkworm (*Bombyx mori* L.). Anthocyanins derived from this plant are a natural food colorant and fabric-tanning agent [1]. Moreover, different clinical trials have established the presence of therapeutic agents showing hypoglycemic, hypolipidemic, diuretic and hypotensive properties [2,3]. The presences of different bioactive compounds such as quercetin, rutin, and isoquercetin were reported in the mulberry plant [4]. This plant is currently under

* Corresponding author at: Plant Tissue Culture Laboratory, Department of Life Sciences, School of Science and Engineering, Dibrugarh University, Dibrugarh 786004, Assam, India.
E-mail address: gaurab_gogoi@rediffmail.com (G. Gogoi).

Peer review under responsibility of National Research Center, Egypt.

http://dx.doi.org/10.1016/j.jgeb.2017.02.005
1687-157X © 2017 Production and hosting by Elsevier B.V. on behalf of Academy of Scientific Research & Technology.

various pharmacognostic examinations to explore the mechanism of action in different traditional medicines in which various parts of the plant are the major component.

Conventionally, the propagation of Indian mulberry is mainly limited to cuttings, as the seeds are mostly heterozygous due to cross pollination. However, there is a major concern over the difficulty in rooting through cuttings [5]. Furthermore, the successful rooting through conventional cutting depends on the genotype, environmental conditions and physiological state of the donor plant [6]. Under the intensive care and standardizing the limiting factor, only less than 40% success rate is obtained in the final transplantation stage [7]. This shows the exigency for an alternative more efficient method for the clonal propagation of this species.

Plant tissue culture technique has been successfully utilized to propagate uniform Indian mulberry platelets in mass scale while maintaining the genetic fidelity. *In vitro* propagation of *M. indica* has been reported by various workers utilizing different protocols. In the majority of the work reported, 6-benzylaminopurine (BAP) was considered effective in inducing shoots from axillary bud culture [8–10]. Lalitha et al. [11] obtained a high frequency of multiple shoots in excised nodal explants supplemented with thidiazuron (TDZ, 9.08 μM). The effectiveness of 2,4-dichlorophenoxyacetic acid (2,4-D) was found to act both as a shooting and as a rooting agent at varying concentrations in *M. indica*. Chitra and Padmaja [12] reported that 0.3 mg L^{-1} 2,4-D induced healthy shoots which subsequently induced rooting in the presence of 1 mg L^{-1} 2,4-D.

Despite various successful micropropagation reports, *in vitro* contamination by the microorganisms trapped in lenticels and scale leaves is one of the most challenging problems particularly for the field-grown explants of *M. indica* [13,14]. Another challenge is the long multistep acclimatization process [15–17] which causes economically unviable cost of production. The quality of roots of *in vitro* plantlets along with the number and status of stomata greatly influences the successful acclimatization [18].

Simultaneous development of inflorescence during elongation of the shoot by application of exogenous growth factor is a common reproductive trait in mulberry [19]. Although explant type and age, plant hormones, nutrient composition and physical factors have variable effects on *in vitro* flowering process of different species [20,21], the effect of the type and concentration of sucrose in flowering is largely advocated by previous researchers [22–24]. Mulberry under the natural condition is a dioecious plant with male and female flower occurring in different plants. However, under *in vitro* condition, modification of sex expression was reported by various researchers. Thomas [25] obtained bisexual flower in *M. alba in vitro* culture in the presence of silver nitrate; similarly, Patel et al. [26] obtained bisexual flower in *M. indica* which later self-fertilized and fruits were set.

The prime goal of the current study was to develop a protocol for the commercial *in vitro* culture of *M. indica* (K-2 cultivar) with an integrated approach to minimize the culture duration, decrease propagation cost through utilizing of low-cost options in culture medium and increase plantlet survival frequency for commercial feasibility and applicability. The current works also standardize the *in vitro* flowering and fruiting conditions for the reproducibility of the same independent of seasonal factors. The specific objectives were as follows: (1) To test the effect of different plant growth regulators in *in vitro* propagation of *M. indica*; (2) to evaluate the response of rooting in 2,4-D mediated two-phase rooting system; (3) to assess the effect of different carbon sources on *in vitro* flower and fruit induction; and (4) to examine the physiomorphological and biochemical differences between *in vitro* and *in vivo* raised plants.

2. Materials and methods

2.1. Plant material and explants disinfection

Healthy twigs of *M. indica* (K-2 cultivar) were obtained from the Botanical Garden, Department of Life Sciences, School of Science and Engineering, Dibrugarh University [94°53′37″N, 27°27′5″E]. Nodal segments, 2 cm long, were isolated with the axillary bud positioned at 0.5 from the distal and 1.5 cm from the proximal cut ends. The explants were cleaned under tap water using a soft brush followed by shaking in 2% tween-20 solution (HiMedia®, Mumbai, India) for 5 min on a rotary shaker set at 100 rpm and then rinsed in sterile distilled water three times. Cleaned explants were surface sterilized through a series of treatments consisting of 2–3% sodium hypochlorite (NaOCl), 0.1–0.15%% mercuric chloride ($HgCl_2$) and 70% ethanol (EtOH) [Merk Specialties Pvt. Ltd. Mumbai, India] as summarized in Table 1. Finally, the explants were washed in sterile double distilled water three times for 1 min each. The cut ends were then properly trimmed using a sterile scalpel and inoculated vertically in the culture media in a laminar airflow cabinet (Klenzaids Aseptic Technologies, Mumbai, India).

2.2. Medium composition and culture conditions

The basal medium of Murashige and Skoog (MS) [27] was used for all culture stages. The medium was supplemented with 2 mg L^{-1} glycine, 100 mg L^{-1} myo-inositol, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine hydrochloride, 0.10 mg L^{-1} thiamine hydrochloride (MS vitamins) in addition to 3% (w/v) sucrose and 0.8% (w/v) agar (Plant Culture Tested, HiMedia®, Mumbai, India). In a separate set of experiment sucrose was substituted with table sugar to examine its effectiveness. The pH was adjusted to 5.7 using 1 N HCl and NaOH after adding the growth regulators. The medium was sterilized by autoclaving at 121 °C in 1.1 kg cm^{-2} for 15 min. Shoot organogenesis was carried out in culture tubes (25 × 150 mm, Actira™) containing 10 mL of medium, whereas rooting was conducted in the conical flask (250 mL, Borosil, India) containing 25 mL of medium. Cotton gauze plugs were used to close the culture vessels. Throughout the micropropagation procedures, the cultures were incubated in a growth chamber set at 25 ± 2 °C, 75% relative humidity and a 16-h photoperiod of 40 μmol m^{-2} s^{-1} provided by white fluorescence lamps (15 W, Crompton Greaves Ltd., India).

2.3. Shoot multiplication and elongation

To investigate the effectiveness of plant growth regulators (PGRs) on bud break and subsequent shoot formation, sterilized explants were inoculated in MS culture medium supplemented with 6-benzylaminopurine (BAP; 0.5–2.5 mg L^{-1}),

Table 1 Surface sterilization treatments of *M. indica* nodal explants using a series of thee disinfectant solutions.

Disinfectant	Concentration and exposure duration			
	Treatment 1	Treatment 2	Treatment 3	Treatment 4
NaOCl	2%, 5 min	3%, 10 min	3%, 5 min	3%, 5 min
$HgCl_2$	0.1%, 3 min	0.1%, 10 min	0.1%, 10 min	0.15%, 7 min
EtOH	70%,1 min	70%, 1 min	70%, 1 min	70%, 1 min

Table 2 Effect of plant growth regulators added to MS medium on *in vitro* shooting of *M. indica*.

PGR (mg L^{-1})			Response (%)	Avg. leaf. number	Avg. shoot length (cm)	Internodes number
BAP	Kin	Ads				
0.5			100	5 ± 0.91^{d}	2 ± 0.01^{c}	$4 \pm 0.02\ c^{d}$
1			100	5.5 ± 0.62^{cde}	1.8 ± 0.01^{ce}	3.8 ± 0.06^{d}
1.5			60	6.75 ± 1.01^{b}	1.75 ± 0.02^{d}	4.2 ± 0.06^{c}
2			50	5 ± 0.67^{d}	1.26 ± 0.01^{e}	3.6 ± 0.10^{d}
2.5			50	5.3 ± 0.52^{d}	1.2 ± 0.07^{e}	3.33 ± 0.20^{d}
	0.5		60	3.5 ± 0.23^{f}	1.75 ± 0.63^{c}	2.75 ± 0.05^{d}
	1		100	7.85 ± 0.09^{ab}	4.8 ± 0.23^{a}	6.5 ± 0.03^{a}
	1.5		80	$5.9 \pm 0.61\ c^{d}$	3 ± 0.66^{b}	4.4 ± 0.07^{c}
	2		80	6.3 ± 0.17^{c}	3.5 ± 0.08^{b}	5.5 ± 0.07^{b}
	2.5		70	3.6 ± 0.55^{ef}	2.1 ± 0.07^{c}	3 ± 0.03
0.5	0.5		90	3.62 ± 0.69^{ef}	2.01 ± 0.11^{c}	3.11 ± 0.05^{d}
1	0.5		80	4.6 ± 0.91^{e}	2 ± 0.05^{c}	3.2 ± 0.11^{d}
1.5	0.5		60	4.25 ± 0.71^{e}	$1.87 \pm 0.12\ c^{d}$	2.25 ± 0.16^{de}
2	0.5		60	4.25 ± 0.08^{e}	1.75 ± 0.16^{c}	2.25 ± 0.13^{de}
2.5	0.5		80	3.8 ± 0.66^{f}	1.5 ± 0.19^{de}	2.1 ± 0.01^{e}
		10	60	1.22 ± 0.19 g	0.5 ± 0.17^{f}	1.1 ± 0.01^{f}
		20	70	1.11 ± 0.13 g	0.5 ± 0.18^{f}	$1 \pm 0.01\ ^{f}$
		30	60	1.02 ± 0.13 g	0.49 ± 0.09^{f}	$1 \pm 0.33\ ^{f}$
		40	80	1.33 ± 0.19 g	0.81 ± 0.11^{fe}	$1.3 \pm 0.08\ ^{f}$
	1	10	100	7.11 ± 0.11^{ab}	3.89 ± 0.21^{a}	2.33 ± 0.03^{de}
	1	20	100	6.9 ± 0.19^{b}	3.5 ± 0.16^{b}	2.1 ± 0.05^{e}
	1	30	100	4.7 ± 0.12^{e}	2.2 ± 0.17^{bc}	1.3 ± 0.08^{f}
	1	40	100	4.1 ± 0.11^{ef}	2.2 ± 0.16^{bc}	1.1 ± 0.04^{f}

Data are represented as mean ± standard error of mean (SEM). Mean followed by the same letter in each column is not significantly different at $p < 0.05$ by Tukey's multiple comparison test. Data were collected after 45 days. BAP-6-benzylaminopurine, Kin - kinetin, Ads - adenine sulfate.

kinetin (Kin; 0.5–2.5 mg L^{-1}) and adenine sulfate (Ads; 10–40 mg L^{-1}) [HiMedia®, Mumbai, India] either singly or in combinations. The tested hormonal treatments are shown in Table 2. After 30 days of incubation, individual shoots 2–3 cm height were separated and further elongated on MS medium supplemented with gibberellic acid (GA_3; 1.5 mg L^{-1}) and incubated for additional 14 days. All cultures were observed periodically for morphological and physiological changes and potential contamination. The effects of different hormonal treatments on explant response, including shoot length, number of leaves and number of internodes were recorded after 45 days of culture. Shoots were subcultured on the same medium to obtain a sufficient number of shoots for subsequent experiments.

2.4. Rooting

To test the effect of various auxins on rooting, individual shoots of 4–4.5 cm height were cultured on MS medium augmented with 0.5 mg L^{-1} indole-3-butyric acid (IBA), 0.5 mg L^{-1} 1-naphthaleneacetic acid (NAA) or 0.5 mg L^{-1} indole-3-butyric acid (IAA) in addition to 0.05% (w/v) activated charcoal (AC). Moreover, rooting of shoots was tested using a two-phase system: Micropropagated shoots were initially cultured in MS medium containing different concentrations of 2,4-D (0.1–2 mg L^{-1}) for varying culture durations (1–9 days). The shoots showing root initials were then transferred to 1/2 MS hormone-free medium for further root proliferation and development. The quantitative and qualitative characters of proliferated roots were observed after 21 days of culture in 1/2 MS hormone-free medium.

2.5. Acclimatization

Rooted plants were removed out of the culture flasks and carefully washed with tap water to remove agar residual traces. The plantlets were then potted in garden pots (round container, 500 mL) containing an autoclaved mixture of field soil, vermicompost and sand (1:1:2) and covered with polythene bags. The potted plants were maintained in the culture room

at 25 ± 2 °C and 16-h photoperiods (40 μmol m^{-2} s^{-1}). The relative humidity was initially set at 75% for 4 days, and gradually reduced to 60% using a dehumidifier (Saveer, New Delhi, India) during the subsequent 6 days. The polythene bags were removed from pots during the humidity regulation process. For the first week, the plantlets were irrigated with 1/2-strength MS medium. After 15 days of acclimatization in culture room, the plants were transferred to a green house and maintained for 3 weeks for further growth and subsequently transferred to the field. The survivability frequency and phenotypic characteristics were recorded after 4 weeks of planting in the field.

2.6. *In vitro flowering and fruiting*

Regenerated shoots were transferred to fresh MS medium supplemented with BAP at 0.5–2 mg L^{-1} and Kin at 0.5–2 mg L^{-1}. The effects of different carbon sources (PTC grade sucrose, table sugar and sugar cubes) and various concentrations (20, 30, 40, 50, 60 g L^{-1}) were also investigated to explore their effects on flower induction and subsequent fruiting. The culture conditions were the same as described above for the micropropagation protocol. To ensure availability of nutrients for a longer duration during the flower induction process, 35 mL of culture medium in culture tubes (25 × 150 mm, Actira™) was used in each treatment.

2.7. *Physiomorphological and biochemical study*

Physiomorphological and biochemical parameters were estimated in both the donor and the *in vitro* regenerated plants. Relative water content (RWC) was estimated following the method described by Smart and Bingham [28] and according to the formula: RWC (%) = [(W-DW)/(TW-DW)] × 100, where W – sample fresh weight, TW – sample turgid weight, DW – sample dry weight. Stomatal density (S_D) and stomatal index (S_I) were calculated through smear impression following microscopic count [29]. A thin smear of nail varnish was applied to the surface of the leaves and allowed to air dry followed by peeling the layer for microscopical observations. S_D = no. of stomata/cm^2; $S_I = \{S/(E + S)\} \times 100$, where S = number of stomata for unit area, E = number of epidermal cells per unit area. For chlorophyll estimation, 0.5 g fresh leaf sample was extracted in 80% acetone (25 mL) and estimated spectrophotometrically at 645 and 663 nm after 48 h dark incubation at 4 °C [30].

2.8. *Experimental design and statistical analysis*

Experiments were set up in a completely randomized design and each experiment was carried out using 25 replications per treatment. One replication corresponds to one culture vessel containing one explant. Data were expressed as mean ± standard error of the mean (SEM) and the Tukey's multiple range test (TMRT) was performed to evaluate the statistical significance difference of means among the various treatments at $p < 0.05$. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla California USA, www.graphpad.com).

3. Results and discussion

3.1. *Culture initiation and shoot multiplication*

The organogenic response of the explants was dependent upon the type and duration of the disinfection treatment. Semi-tender nodal explants having greenish protruding auxiliary buds responded most; however, prolong treatments with various surface disinfectants considerably affected the response to organogenesis. Considering the various disinfection treatments tested (Table 1), the optimum organogenic response (74.66 ± 1.45%) was obtained in response to treatment group no. 4 (Fig. 1) with minimum contamination and necrosis compared to other treatment groups. The explants collected from field-grown mulberry plants are associated heavily with epiphytic and endophytic fungi. This association depends on the plant maturity level, genotype and explant collection season [17]. Even after successful shoot development from the nodal explants, latent contamination was noted after 3–4 weeks of culture in our study. This can be managed by sub culturing the young *in vitro* shoots before contamination starts owing to the endophytic fungus as described by Vijayan et al. [14].

Shoot initiation was best achieved on MS medium fortified with different concentrations of Kin. The initial bud break started from the 7th day through the development of the apical bud which later differentiated into leaves (Fig. 3A and B). Shoot morphological characters responded differentially to various PGRs (Table 2). Maximum shoot length (4.8 ± 0.23 cm) with the highest average leaf number (7.85 ± 0.09) and internodes number (6.5 ± 0.03) were obtained in response to 1 mg L^{-1} Kin. The majority of the work reported earlier designated BAP as superior over Kin for *in vitro* shooting of mulberry [15,10]. Moreover, Kin is mostly known for its efficacy in bud breaking and rejuvenation and to induce synergetic effect with other cytokinins [31]. Although BAP was effective in shoot organogenesis, in our study, low concentrations required a longer period for bud break and higher concentrations showed fleshy leaves and stem. A significant negative effect was recorded in the combined treatment of BAP and Kin producing callogenic activities, and correlation was obtained for greater callogenic activity with increasing BAP concentration. Replacement of carbon source with

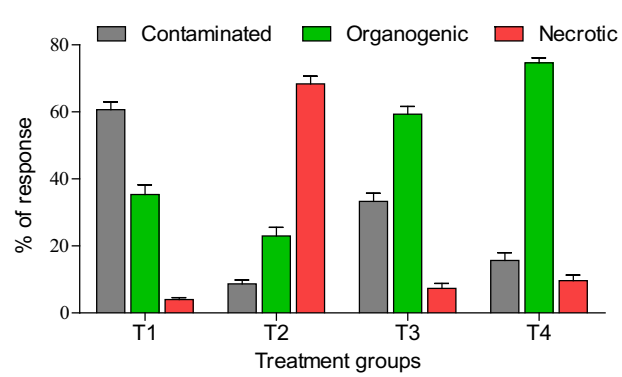

Figure 1 Physiological response to various sterilizing treatments as specified in Table 1.

table sugar (3%) shows equal effect as of sucrose, and thus sucrose can be substituted with table sugar in commercial production of *M. indica*, which can reduce the cost of chemicals considerably. Culture of microshoots in GA_3 (1.5 mg L^{-1}) fortified MS medium for two weeks showed optimum elongation which makes them suitable to introduce in the rooting medium.

3.2. Rooting

It is well established that root induction is generally affected by the factors such as medium strength, type, concentration and duration of auxin treatment and additive such as AC [32]. In our study, excised microshoots cultured in 1/2 MS medium salts devoid of activated charcoal and plant growth regulators did not show any observable rooting, even after 4 weeks of culture. However, root initiation occurred in the AC fortified media. Moreover, rooting was further enhanced by the addition of auxins. The highest number of roots per shoot (22.05 ± 0.41) was observed in a medium supplemented with 0.05% AC and 0.5 mg L^{-1} IBA (Table 3) after 45 days of culture. Low concentrations of AC (0.01–0.03%) required longer incubation period for root induction, whereas higher concentrations (>0.06%) were inhibitory. However, incorporation of only auxin in 1/2 MS medium did not show any satisfactory result. Different auxins viz. IAA, IBA, NAA (0.5–2 mg L^{-1}) were tested to explore their rooting efficiency, but none of them without AC could produce sufficient roots and root initiation took much longer period compared to the auxin treatment with AC. The results reported here are contradictory with the findings of Chitra and Padmaja [12] where AC (0.2%) was found to decrease the rooting frequency when incorporated with auxins in *M. indica* (M-5 cultivar).

Using the 2,4-D mediated two-phase rooting method resulted in a greater rate of root proliferation and reduced culture duration. The initial culture of microshoots in full strength MS medium supplemented with 2,4-D (1 mg L^{-1}) followed by culturing in 1/2 MS hormone-free medium gave more number of roots and greater root length in a considerably shorter duration as compared to the above conventional rooting method. Root initials developed through intermittent 2,4-D treatment showed a synergetic growth in 1/2 MS hormone free medium (Fig. 3C and D). Low concentration (0.1–0.7 mg L^{-1}) was ineffective to develop root initials, whereas concentration above 1.5 mg L^{-1} showed callogenic activity and lower response. Fig. 2 illustrates the response of different treatments and incubation period of 2,4-D in rooting of *M. indica*. Continuous incubation of microshoots in 2,4-D induces short and thick roots with soft texture preceded by callusing at shoot bases, suggesting that 2,4-D is only effective as rooting stimulant in pulse form for the current genotype of *M. indica*. Pascual and Marin [33] obtained a similar result in *Prunus* rootstock regeneration, where 2,4-D pulse treatment increased the regeneration rate, but the presence of 2,4-D in regeneration medium showed a negative effect. Auxin is necessary for root induction which enhances the meristematic competence of the regenerated cells, but continuous presence may sometime inhibit root proliferation [34,35]. The 2,4-D is a phenoxy auxin having an oxygen molecule in the aromatic ring and side chain, which is reported to induce callusing [36]. The treatment duration of 2,4-D in development of root initials is an essential factor for successful rooting in *in vitro* culture. The *in vitro* rooting of *M. indica* using 2,4-D is reported earlier by Chitra and Padmaja [12], by supplementing 1 mg L^{-1} 2,4-D in MS medium for 30 days. However, our study accounts considerably higher no of roots and root length in 28 days of two-phase culture (Fig. 4). Morphogenetic differences were observed in continuous [AC (0.05%) + IBA (0.5 mg L^{-1})] and intermittent 2,4-D two-phase rooting system along with differences in root number (Table 4) (Fig. 4). The rooted platelets acclimatize well under greenhouse and 89% survivability rate was observed under the natural condition and no any physical aberrations were observed. This clearly shows that

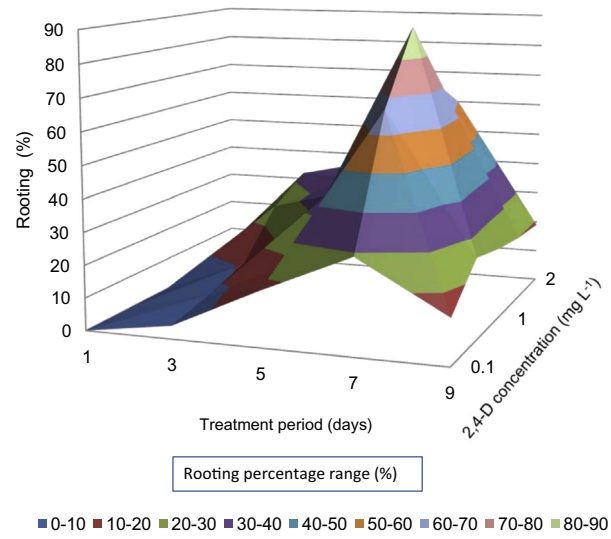

Figure 2 Rooting percentage in response to 2,4-D concentration (mg L^{-1}) and treatment duration in the two-phase rooting system of *M. indica*.

Table 3 Effect of different growth regulators added to 1/2 strength MS medium on rooting response of *M. indica*.

Treatments	Time taken for root initiation (Days)	No of roots per shoot	Length of roots (cm)
1/2 MS	NR	0	0
AC (0.05%)	13^{b}	20.85 ± 0.35^{b}	3.22 ± 0.04^{ab}
AC (0.05%) + IBA (0.5 mg L^{-1})	15^{cb}	22.05 ± 0.41^{a}	3.31 ± 0.03^{a}
AC (0.05%) + IAA (0.5 mg L^{-1})	14^{b}	21.41 ± 0.31^{b}	3.17 ± 0.15^{b}
AC (0.05%) + NAA (0.5 mg L^{-1})	17^{a}	21.11 ± 0.46^{b}	3.07 ± 0.04^{abc}

Data are represented as mean ± standard error of mean (SEM). Mean followed by the same letter in each column is not significantly different at $p < 0.05$ by Tukey's multiple comparison test. Values followed by different letters are significantly different. Data were collected after 45 days in rooting medium. NR: no rooting.

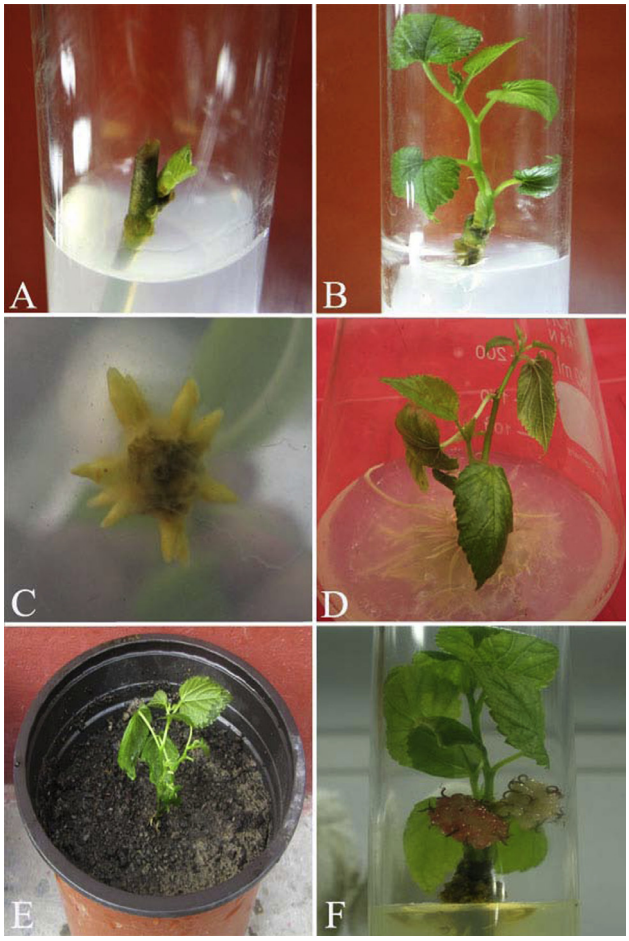

Figure 3 Auxiliary bud proliferation of *M. indica*: (A) Bud break in nodal explants cultured in MS media fortified with Kin (1 mg L^{-1}); (B) shoot elongation in the same media; (C) root initiation in MS medium with 2,4-D (1 mg L^{-1}) after 7 days of incubation; (D) root proliferation in 1/2 MS medium with 2,4-D after 21 days; (E) *in vitro* raised platelets in a potting soil during acclimatization phase; (F) *in vitro* fruiting of *M. indica*.

improved roots can be obtained through this 2,4-D mediated two-phase culture rooting system, which will significantly reduce the cost of commercial production due to its time-saving process and increased survivability rate as to the existing micropropagation protocols. Furthermore, similar to the shooting phase table sugar performed equally as of sucrose in rooting phase also and therefore, table sugar can be used in the both shooting and rooting phases of *M. indica* micropropagation.

3.3. Acclimatization

The plantlets on rooting medium developed healthy shoot and root systems after 28 days of culture in rooting phase. They were then transferred to garden pots containing autoclaved mixture of field soil, vermicompost and sand (1:1:2) and covered with polythene bags to maintain high humidity. However, after 5 days the polythene bags were removed during humidity regulation process for subsequent 10 days (Fig. 3E). Severe wilting and low survivability (40%) were observed when the plantlets were directly transferred to the greenhouse. The underdeveloped stomata and epicuticular wax result in rapid loss of water from the leaves surface and poor-root soil contact limits the water uptake, which contributes to the wilting of the plant causing mortality [37,38]. Mortality was sufficiently minimized by regulating the relative humidity in the culture room before transferred to the green house. In our study, the relative humidity was adjusted in three phase at an interval of 48 h, with reduction of 5% humidity in each phase. Stomatal density and number of open stomata are dependent factors for successful acclimatization of *in vitro* raised plants. Higher numbers of open stomata in *in vitro* condition caused severe wilting when the plantlets were brought out of the culture chamber and introduced to the natural condition. The gradual reduction of relative humidity to the green house condition in culture chamber has reduced the number of open stomata to almost 24%. Humidity condition in subepidermal cells and water potential of the ambient air have a direct influence on the stomatal aperture [39,40]. Higher leaf transpiration and stomatal conductance cause water stress and reduce the survival percentage in *ex vivo* condition [41]. Commercial utilization demands efficient technique for judicious resource management taking its quality into account [42] and this is the first report of humidity regulation process in *M. indica* to improve the survival frequency for commercial interest.

3.4. Physiomorphological and biochemical study

The comparative account of physiomorphological and biochemical parameters of the *in vitro* raised and the donor plant is illustrated in Table 5. The RWC was significantly higher in the *in vitro* raised platelets but during transplantation in *ex vivo* condition, a decrease in RWC was observed. However, with time lapse the RWC was recovered after proper acclimatization. Acetone extracted chlorophyll in the *in vitro* raised plants was found to be a bit lower than that of the donor plants. The synthesis of chlorophyll needs to be studied with varying concentration of different nutrients of culture medium and culture condition to enhance its production in further studies.

3.5. In vitro flowering and fruiting

The flowering of the *in vitro* cultured shoots was observed in our study. Repeated sub culturing in 1 mg L^{-1} Kin supplemented MS media without transferring to the rooting phase produced pistillated catkin inflorescence (on average 2 inflorescence/explants) (Fig. 3F). The induction of flowering was observed after 6 weeks of culture in 16/8-h light/dark period with a temperature of 24 ± 2 °C and relative humidity of 60–70%. Light is the major environmental factor regulating flowering through the morphological and physiological changes. Florigen (sucrose, isopentenyladenine) the flower-inducing hormone is believed to be regulated by day length and light quality by altering the photosynthetic turnover [43]. Cytokinin is a common hormone which is used in inducing flower in *in vitro* condition. The *in vitro* inflorescence induction was reported earlier in *M. australis* [10] and *M. indica* [12], where the researchers reported the induction of flower to be independent of hormonal treatment and explants collected during November – February only responded to *in vitro*

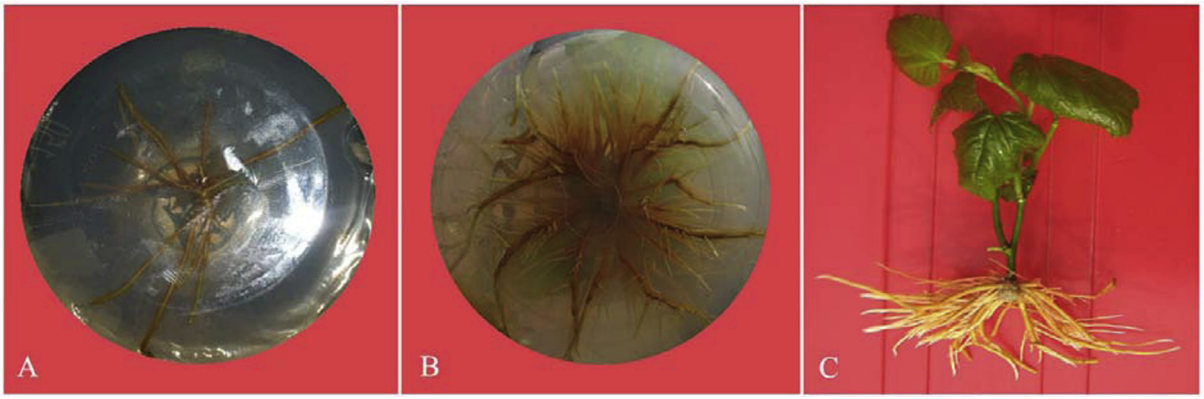

Figure 4 *In vitro* rooting response of *M. indica* in the different systems: (A) *In vitro* rooting in 1/2 MS supplemented with AC (0.05%) + IBA (0.5 mg L^{-1}) after 45 days of culture; (B, C) *in vitro* rooting through 2,4-D mediated two-phase rooting system.

Table 4 Comparison of two different rooting systems tested in *M. indica*.

Parameter	Continuous: AC (0.05%) + IBA (0.5 mg L^{-1})	Two-phase: 2,4-D (1 mg L^{-1}) followed by culture in 1/2 MS
Root number	22.05 ± 0.41	43 ± 0.74[#]
Root length	3.31 ± 0.03 cm	3.4 ± 0.10 cm[ns]
Root nature	Slender and spreading	Thick and lateral
Root hair	Absent	Present
Maturation period	45 days	28 days[#]
Survival rate	84.6 ± 0.73%	89.0 ± 0.67%[#]

[#] Indicates statistically significant difference at $p < 0.05$, ns. not significant.

Table 5 Physiomorphological and biochemical parameters.

	Donor plant	*In vitro* raised plant
Relative water content (RWC)	0.54 ± 0.03%	0.59 ± 0.05%
Stomatal density (S_D)	890.2 cm^{-2}	877.5 cm^{-2}
Stomatal index (S_I)	35.6%	33.9%
Chlorophyll a concentration	3.07 ± 0.09 mg L^{-1}	4.2 ± 0.05 mg L^{-1}
Chlorophyll b concentration	1.2 ± 0.03 mg L^{-1}	1.7 ± 0.02 mg L^{-1}

Data are represented as mean ± standard error.

flowering. In our study, Kin was only effective on produced inflorescence in the explants collected during the late summer season. *In vitro* induction of flower is also greatly influenced by the sucrose and sometimes it is the limiting factor for floral induction while other factors are essential in the later developmental period of flowers [44]. Low concentration of sucrose (2%) was ineffective in flower induction, whereas medium supplemented with 4% commercial sugar cubes as carbohydrate source yielded better results than that of plant tissue culture grade sucrose. The inflorescence size and length significantly increased when the shoots were cultured on commercial sugar cube supplemented medium. The development of inflorescence in the early stage of shoot development is reported to retard the development of shoots and they are mostly excised to restore the shoot development rate in commercial *in vitro* propagation [45], but *in vitro* sterile flower can be an important tool for the study of improvement programs and development studies of the plant.

4. Conclusion

This study has resulted in a considerably improved micropropagation approach for *M. indica* with a great potentiality toward commercial propagation. Shoot induction and optimum development in the current genotype of *M. indica* (K-2 cultivar) were found to be solely dependent upon Kin as a growth regulator in *in vitro* culture. The two-phase culture in rooting of *M. indica* reduced the culture duration and improved the quality of the roots and consequently improved the plant survivability compared to the available protocols. Strict humidity adjustment procedure is a key factor for the observed high economically viable survivability rate. This is the first report of integrated survivability and quality management through effective rooting and enhancement in culture condition in *in vitro* culture of *M. indica* and it will no doubt find immense commercial importance and future developmental studies of the plant. The *in vitro* flowering and subsequent fruiting technique developed in the present work will assist researchers interested in genetic improvement and *in vitro* breeding of mulberry.

References

[1] J.T. Matus, R. Loyola, A. Vega, J. Exp. Bot. 60 (2009) 853–867.
[2] S.M. Kelkar, V.A. Bapat, T.R. Ganapathi, G.S. Kaklig, P.S. Rao, M.R. Heble, Curr. Sci. 71 (1996) 71–72.
[3] B. Andallu, V. Suryakantham, S.B. Lakshmi, G.K. Reddy, Clin. Chim. Acta 314 (2001) 47–53.
[4] J. Zhishen, T. Mengcheng, W. Jianming, Food Chem. 64 (1999) 555–559.

[5] M. Anis, M. Faisal, S.K. Singh, Plant Tis. Cult. 13 (2003) 47–51.
[6] P. Narayan, S. Chakraborty, G.S. Rao, Indian Natl. Sci. Acad. 55 (1989) 469–472.
[7] V.A. Bapat, M. Mhatre, P.S. Rao, Plant Cell Rep. 6 (1987) 393–395.
[8] K.K. Sharma, T.A. Thorpe, Sci. Hortic. 42 (1990) 307–320.
[9] P.S. Rao, V.A. Bapat.1993. Kluwer Academic Publishers, Netherlands, pp. 317–345.
[10] S.K. Pattnaik, P.K. Chand, Plant Cell Rep. 16 (1997) 503–508.
[11] N. Lalitha, S. Kiho, R. Banerjee, S. Chattopadhyay, A.K. Saha, B.B. Bindroo, Int. J. Adv. Res. 1 (2013) 22–26.
[12] D.S. Chitra, G. Padmaja, Sci. Hortic. 80 (1999) 289–298.
[13] A. Habib, M.R. Ali, M.N. Amin, M.M. Rahman, J. Bio. Sci. 3 (2003) 1181–1187.
[14] K. Vijayan, P.J. Raju, A. Tikader, B. Saratchnadra, Emir. J. Food Agric. 26 (2014) 472–496.
[15] V. Yadav, L. Madan, V.S. Jaiswal, Sci. Hortic. 44 (1990) 61–067.
[16] S. Chattopadhyay, S.G. Doss, S. Halder, A.K. Ali, A.K. Bajpai, Afr. J. Biotech. 10 (2011) 18153–18159.
[17] K. Vijayan, A.J. Tikader, T.D. Silva, Tree For. Sci. Biotech. 5 (2011) 1–13.
[18] R. Sekeli, J.O. Abdullah, P. Namasivayam, P. Muda, B.U.K. Abu, ISRN Biotechnol. 1 (2013) 1–10, http://dx.doi.org/10.5402/2013/958945.
[19] M. Akram, F. Aftab, Pak. J. Bot. 44 (2012) 285–289.
[20] J.M. Al-Khayri, F.H. Huang, T.E. Morelock, T.A. Busharar, F. E. Lane, Hort. Sci. 26 (1991) 1422.
[21] K.S.R. Murthy, R. Kondamudi, R.P.V. Chalapathi, T. Pullaiah, J. Agric. Tec. 8 (2012) 1517–1536.
[22] K. Kanchanapoom, J. Suttinee, K. Kamnoon, Not. Bot. Hort. Agrobot. Cluj. 39 (2011) 84–87.
[23] S. Cha-um, S. Thapanee, K. Chalermpol, In Vitro Cell. Dev. Biol. Plant 48 (2012) 259–264.
[24] N.S. Rathore, R. Nisha, N.S. Shekhawat, Ind. Cr. Pro. 50 (2013) 232–236.
[25] T.D. Thomas, Plant Cell Tiss. Org. Cult. 77 (2004) 277–281.
[26] G.K. Patel, V.A. Bapat, P.S. Rao, Z. Pflanzenphysiol. 111 (1983) 465–468.
[27] T. Murashige, F. Skoog, Physiol. Plant. 15 (1962) 473–497.
[28] R.E. Smart, G.E. Bingham, Plant Physiol. 53 (1974) 258–260.
[29] Z. Xu, G. Zhou, J. Exp. Bot. 59 (2008) 3317–3325.
[30] X. Lu, H.C. Peng, Indian S. Rem. Sen. 43 (2015) 109–120, http://dx.doi.org/10.1007/s12524-014-0397-1.
[31] S. Shyamali, H. Kazumi, Advances in plant ethylene research, in: Proceedings of the 7th International Symposium on the Plant Hormone Ethylene, Springer, Netherlands, 2007, pp. 153–155.
[32] M.J. Pan, J.V. Staden, Plant Growth Reg. 26 (1998) 155–163.
[33] L. Pascual, J.A. Marin, Sci. Horticult. 106 (2005) 582–592.
[34] J.G. Torrey, Annu. Rev. Plant Physiol. 27 (1976) 435–459.
[35] E. Gürel, M.J. Wren, Ann. Bot. 75 (1995) 31–38.
[36] S. Fatima, P.K. Rana, Plant Biotechnol. Rep. 1 (2007) 141–147, http://dx.doi.org/10.1007/s11816-007-0020-9.
[37] J.M. Santamaria, W.J. Davis, C.J. Atkinson, J. Exp. Bot. 44 (1993) 99–107.
[38] G. Fila, J. Ghashghaie, J. Hoarau, G. Cornic, Physiol. Plant. 102 (1998) 411–418.
[39] O.L. Lange, R. Lösch, E.D. Schulze, L. Kappen, Planta 100 (1971) 76–86.
[40] L. Fei, W. Pamela, In Vitro Cell. Dev. Biol. Plant 51 (2015) 669–681.
[41] L.P. Pérez, Y.P. Montesinos, G.O. Justo, B.R. Raúl, R.S. Romelio, N.M. Osvaldo, C.R.E. Rene, D. Dion, G.K. Rafael, In Vitro Cell. Dev. Biol. Plant 52 (2016) 196–203.
[42] S.K. Senapati, SAJ Biotechnol. 2 (2015) 1–6.
[43] G. Bernier, C. Périlleux, Plant Biotechnol. J. 3 (2005) 3–16.
[44] N.H. Vu, P.H. Anh, D.T. Nhut, Plant Cell Tiss. Org. Cult. 87 (2006) 315–320.
[45] V. Balakrishnan, M. Ram Latha, K.C. Ravindran, J. Philip Robinson, Bot. Res. Intl. 2 (2009) 42–49.