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RESEARCH ARTICLE

High frequency multiple shoot induction and *in vitro* regeneration of mulberry (*Morus indica* L. cv. S-1635)

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Abbreviations:
BAP- 6-Benzyl amino purine,
IAA-Indole acetic acid,
IBA-Indole butyric acid,
TDZ-Thidiazuron (N-phenyl N' 1,2,3-thidiazol-5-yl urea),
NAA- Naphthalene acetic acid,
GA – Gibberellic acid
MS- Murashige and Skoog

Abstract

A simple, high frequency and reproducible protocol for multiple shoot induction and *in vitro* regeneration from nodal explants for commercially exploited mulberry cultivar S-1635 have been developed. Explants collected from the field grown plants were cultured on MS medium supplemented with different concentration/combination(s) of phytohormones. Presence of TDZ (9.08µM) in the shoot induction medium showed pronounced increment on the number of multiple shoots (6.22±0.70) produced. Rapid bud breaking with respect to duration was achieved in MS media supplemented with BAP (4.44 µM) and the maximum mean shoot length (6.02 ±0.6 cm) was obtained with combination of BAP (4.44 µM) and GA (1.44 µM). Highest number of roots (17.0±1.67 no. plantlet^{-1}) with maximum mean length of root (6.3cm) was inducted after 30 days of inoculation in IBA (9.8 µM) fortified half MS media. The regenerated micro plantlets after 60 days of indoor acclimatization were successfully established in the soil under field conditions without visible morphological variation. The protocol may provide a suitable platform to generate clones of triploid commercial mulberry cultivar S1635 round the year in a significantly shorter period with less involvement of land mass and manpower and for utilization in advanced breeding program.

Introduction

Mulberry is a deciduous woody perennial, belonging to the genus *Morus* spp.; Family: Moraceae, Order: Utricales. It is cultivated largely in Asian countries for its foliage and considered as the major sole food for silkworm (*Bombyx mori*). Mulberry is a heterozygous, cross-pollinated plant with relatively long juvenility due to its tree nature. In India, different mulberry varieties suitable for different agroclimatic zones have been developed. Among the valuable mulberry genotypes adapted for the Eastern and North Eastern zones of India, S1635 is the most recommended one. It is a selection of open pollinated hybrid of CSRS-2 of mulberry, *Morus indica*, triploid in nature and average yield potential being 43mt/ha/year.

Mulberry is conventionally propagated through stem cutting clones and rarely by graftings. Propagation via cutting is highly restricted to certain months of the year and is labour intensive. In view of crop improvement through biotechnological approaches, attempts have been made to standardize *in vitro* regeneration protocols in different mulberry varieties. In vitro shoot regeneration in mulberry is greatly dependent on the genotype, type of explant and combination of growth regulator used in the culture media (Bhau and Wakhlu 2001; Tewary *et al*., 1996). Using different explants such as axillary bud (Jain *et al*., 1990; Vijayan *et al*., 2000), hypocotyl and cotyledon (Bhatnagar *et al*., 2001), shoot tip and nodal segment (Yadav *et al*., 1990; Vijaya Chitra and Padmaja, 1999) and leaf (Kapur *et al*., 2001) *in vitro* regeneration has been attempted with various degrees of success.

Owing to the triploid nature of the mulberry cultivar S1635, exploitation in the breeding program is quite difficult. In order to facilitate the development of plant biotechnology based cultivar improvement for this crop, considerable effort has to be devoted in optimizing efficient *in vitro* regeneration protocols. Establishment of a rapid and throughput protocol for

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micropropagation becomes indispensible to induce genetic transformation or for utilization in advanced breeding program. Further, the huge land and labour requirement and seasonal dependence for the conventional clonal propagation methods may needs to be minimized. Such problem(s) can be overcome by using the applications of tissue culture technology. The present study was carried out to study the high frequency multiple shoot induction and *in vitro* regeneration from nodal explants of mulberry (*Morus indica* L. cv. S1635).

Material and Methods

Plant material and explant preparation:

Nodal explants were excised from semi-lignaceous shoots (35-40 days after pruning) of mulberry variety S1635 grown in the mulberry garden of CSR&TI, Berhampore. The leaves were removed and the nodal region measuring about 2-3cm each containing an axillary bud from the shoot was excised and used as explant for the present study. The explants were washed in running water for 2-3 times and again washed with mild detergent and rinsed thoroughly with distilled water.

Sterilization:

The explants were then surface sterilized by keeping them in 70% ethanol for 5 minutes. After washing 2-3 times with water, they were treated with 0.1% (w/v) of systemic fungicide, Bavistin for 10 minutes. Subsequently, the explants were washed and kept in 0.1% (w/v) of $HgCl_2$ for 10 minutes in an orbital shaker at 100 rpm. Finally the explants were serially washed 2-3 times with sterile double distilled water to remove traces of sterilizing chemicals before inoculation.

Inoculation and Incubation:

Inoculation was carried out in a sterile laminar airflow hood chamber. The terminal regions of the surface sterilized nodal explants were trimmed off, and the explants with single axillary bud were cultured in culture medium containing MS (Murashige and Skoog, 1962) salts and vitamins, supplemented with sucrose (3%w/v agar), (0.8 %w/v) along with varied concentration/combination(s) of cytokinin and auxin for multiple shoot induction and subsequent elongation. The pH of the media was adjusted to 5.6 - 5.8 prior to sterilization at 15 lbs cm^{-1} for 15 minutes. The explants were cultured singly in 150mm X 25mm culture tubes each containing 15 ml of culture medium and fitted with cotton plugs. The inoculated explants were maintained in the culture room at a temperature of 25±2˚C and a relative humidity 55-65% under four cool white fluorescent tubes with a photoperiod of 16 /8hours (day/ night) at a light intensity of 60µmol m^{-2} s^{-1}.

Healthy *in vitro* derived plantlets (3-5 cm long) from the sprouted axillary buds were separated individually and transferred to half-strength MS basal medium with 1.5 % (w/v) sucrose, 0.8% (w/v) agar supplemented individually with two different concentrations of IBA, IAA and NAA and 0.05% (w/v) activated charcoal for *in vitro* rooting. The rooted plantlets (5-7 cm long) were washed in sterile distilled water to remove the traces of medium and transplanted to container (150 x 60 mm) filled with autoclaved mixture of garden soil, farm yard manure and sand (1:1:2) and were kept at 25± 2°C under 16 h photoperiod. After five weeks, the plants were transferred to pots containing garden soil and grown in greenhouse conditions.

Data collection:

Observations on the number of days taken for bud breaking, number of multiple shoots, mean shoot length and leaves per culture vessel were recorded at 30 days after inoculation in the shoot induction medium. Data on number of days taken for root initiation, number of roots per shoot, root length and nature of roots were collected from the *in vitro* shootlets after 30 days of culturing in the rooting media.

Experimental design and data analysis:

Experiments were set up in completely randomized design and repeated thrice with ten explants per replicate. Data were subjected to Analysis of Variance (ANOVA) for testing the differences among the different media. The least significant difference (LSD) was determined in appropriate cases. The photographic illustrations were also given wherever necessary.

Result and Discussion

Among the different combinations of growth hormone tested (Table 1) TDZ was found to be most effective in multiple shoot induction. Thidiazuron is one among the most active cytokinin-like substances for woody plant tissue culture (Huetteman and Preece 1993) facilitating efficient micropropagation of many recalcitrant woody species. The average number of shoots formed per explant varied significantly between the different treatments of BAP and TDZ. However there is no significant difference within the BAP and TDZ treatments. Presence of TDZ (2.27 - 9.08 µM) in the shoot induction medium produced multiple shoots (4.5– 6.2 no. per explant (Fig 2)) while the treatments of BAP (2.22- 8.88 µM) produced significantly less multiple shoots (3.0 -3.89 no. per explant) at 30 days of culturing. However,

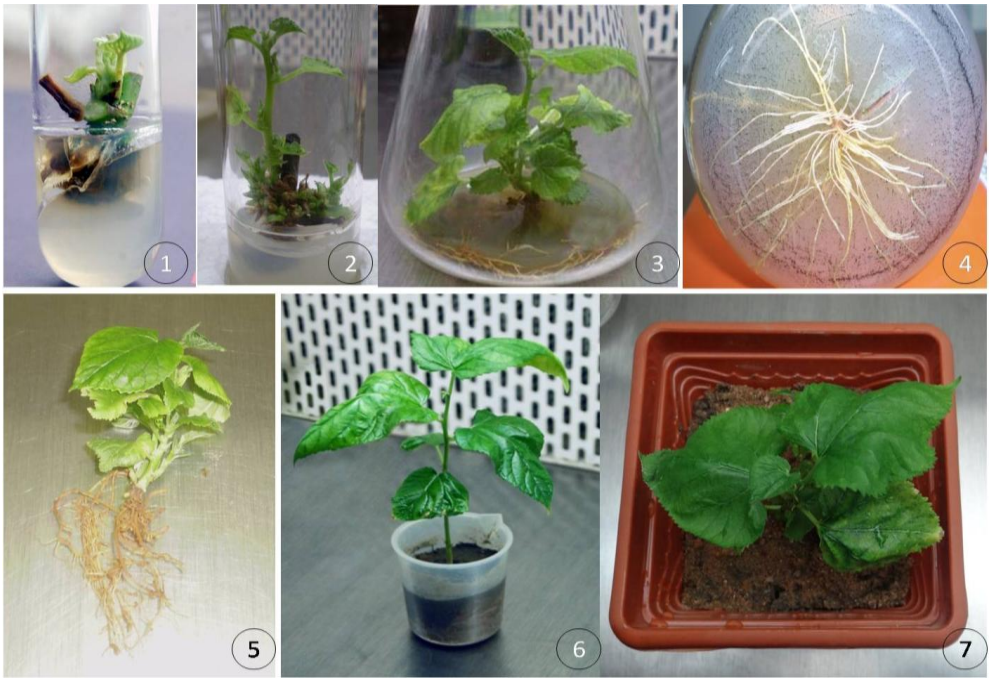

Fig. 1-7. Plant regeneration of *Morus indica* L. cv. S-1635 via direct shoot bud differentiation from nodal explants

1. Shoot bud initiation
2. Multiple shoot induction on MS media supplemented with TDZ 2.27 µM
3. Root initiation on half MS media supplemented with IBA 9.8 µM
4. Root proliferation obtained after 30 days of inoculation in rooting media
5. Rooted *in vitro* raised plantlet ready for acclimatization
6. Hardening (garden soil, farmyard manure and sand, 1:1:2).
7. Established plantlet in the pot five weeks after transfer.

TDZ at concentrations higher than 2.27 µM, instead of direct organogenesis, induced callus tissues and shootlets induced from nodal segment on TDZ-containing media did not elongate further and resulted in a rosette of shoots. This effect may be due to high cytokinin activity as reported by Ahmad *et al*. (2006). Hence, optimization of TDZ concentration to obtain best results on multiple shoot induction was attempted by scoring the callus formation. Callus tissues hindered the normal growth of the plantlets by diverting the available nutrients in the media. Maximum proliferation of callus tissues were recorded in the basal portions of the explants inoculated in MS media with 9.08 µM TDZ.

The results on the duration of bud breaking (Table 1; Fig. 1) of the inoculated nodal explants revealed rapid response in MS media with 4.44 µM BAP. The maximum shoot length (6.02 ± 0.63 cm) was achieved in MS media with 4.44µM of 6 BAP and 1.44µM GA in 30 days of culturing. This gave an indication that sub culturing of the multiple shoots in TDZ-free medium and presence of GA may promote good shoot proliferation and elongation. This two-step regeneration strategy has been reported earlier by Sajeevan, *et al* (2011). There is no significant difference noticed in the average number of leaves produced between the treatments after 30 days of culture.

The plantlets were induced roots in half strength MS medium enriched with auxins, IAA, IBA or NAA at two different concentrations along with 0.05% activated charcoal (Table 2). Root initiation was observed within 7-11 days of culturing in MS media fortified with IBA. The maximum rooting (91%) i.e., (number of roots 17.0±1.67, and mean length of root 6.27±0.74cm) per plantlet was noted on MS medium supplemented with 9.8µM IBA (Fig.4) after 30 days of culturing in rooting media. The presence of IBA in the rooting media induced medium thick and spreading roots, while short thick and short slender roots were the characterestics of roots induced by NAA and IAA respectively. IBA is clearly more effective in promoting root induction than IAA and NAA (Table 2; Fig. 5). Anuradha and Pullaiah (1992) reported that NAA was a more effective rooting agent for *M. alba*. However IBA was reported better for root induction by Sajeevan *et al*, (2011) and Jain *et al* (1990). The *in vitro* root growth parameters

were found optimum at 4.9-9.8 µM IBA. The *in vitro* rooted micro-shoots were acclimatized successfully (Fig. 6). The transplanted plantlets established well in pots and showed no apparent difference in phenotype (Fig. 7). The regenerated micro plantlets were successfully established in the soil under field conditions after 60 days of indoor acclimatization.

TABLE 1: Effect of BAP or TDZ alone or in combination with GA on multiple shoot formation and *in vitro* shoot growth parameters from nodal explants of mulberry var. S1635.

MS Media with 3% (w/v) sucrose,0.8%(w/v) agar & PGR in µM			Duration for initiation of bud breaking response (days)#	Callus formation at the basal portion of explant	Shoot morphology (per nodal explant)*		
					Multiple shoots (no)#	Mean shoot length (cm)#	Leaves (no)#
BAP	TDZ	GA					
2.22			4.4±0.31	-	3.44±0.38	3.07±0.39	3.22±0.38
4.44			3.9±0.41	-	3.56±0.44	3.47±0.43	5.11±0.58
6.66			5.9±0.46	-	3.89±0.50	3.49±0.37	5.22±0.63
8.88			7.3±0.87	-	3.11±0.53	3.96±0.51	5.44±0.69
2.22		1.44	5.9± 0.57	+	3.22±0.46	5.33±0.34	5.50±0.37
4.44		1.44	6.9± 0.81	+	3.00 ±0.47	6.02±0.63	5.22±0.63
	2.27		5.7±0.26	+	5.11±0.60	4.41±0.46	5.67±0.62
	4.54		5.1±0.50	++	5.13±0.81	4.24± 0.60	6.00±0.90
	6.81		5.6± 0.45	++	5.33±0.57	4.49±0.48	6.33±0.70
	9.08		6.1± 0.50	+++	6.22±0.70	4.20±0.47	6.67±0.80
	2.27	1.44	7.1±0.85	+	4.56±0.48	4.00±0.44	6.56±0.77
	4.54	1.44	9.2 ±0.89	+	5.00±0.75	4.53±0.62	5.50±0.78
$LSD_{(p=0.05)}$			1.74	-	1.52	1.55	NS

Each value represents mean (± standard error) from 3 replicates having ten explants per replicate.
*Observation taken after 30days of inoculation in respective media
Rating of amount of callus: – No callus; + Small amount of callus;
++ Moderate amount of callus; +++ Large amount of callus

Table 2: Effect of different auxins on *in vitro* rooting of mulberry variety S 1635.

Media Half MS with activated charcoal (0.05% w/v)		% response #	Duration for root initiation # (days)¤.	Root morphology (per plantlet)*		
				Roots (no)#	Mean root length (cm)#	Nature of the roots
PGR	µM					
IBA	4.9	88.33±7.26	9.00±0.47 (7-10)	15.33±1.47	5.45±1.47	Medium thick and spreading roots
	9.8	91.67±4.41	8.67±0.51 (7-11)	17.0±1.67	6.27±0.74	
NAA	5.37	68.00±3.61	12.17±0.50 (10-14)	6.33±0.61	3.32±0.38	Short and thick roots
	10.74	75.33±5.49	12.33 ±0.84 (10-15)	5.67±0.61	3.17±0.35	
IAA	5.71	54.67±2.91	10.83±0.72 (7-12)	6.17±0.44	2.57±0.35	Short and slender roots
	11.42	67.33±1.33	12.50 ±1.31 (8-18)	7.17±0.86	3.03±0.40	
$LSD_{(p=0.05)}$		14.10	2.47	3.32	1.47	

¤Data given in parenthesis is range of duration for root initiation
Each value represents mean (± standard error) from 3 replicates having ten explants per replicate.
*Observation taken after 30 days of inoculation in respective rooting media

Conclusion

In the present study, TDZ was found to be a better option as compared to other amino purine cytokinins as far as multiple shoot induction was concerned. But the concentration of TDZ was optimized to 2.27 µM as the higher concentrations induced the callus formation which hampered the *in vitro* shoot growth parameters and did not give rise to significantly higher no of multiple shoots. A rapid, simple and comparatively efficient and reproducible direct shoot regeneration system for mulberry cultivar S1635 has been worked out utilizing nodal explants. The regeneration and rooting protocols can be applied to increase the efficiency of transformation methods using nodal parts as explant source. The protocol may provide a suitable platform to generate clones of triploid commercial mulberry cultivar S1635 round-the-year in a significantly shorter period with less involvement of land mass and manpower.

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References

Ahmad N., Siddique I., Anis M. (2006). Improved plant regeneration in *Capsicum annum* L. from nodal segments. *Biologia Plantarum*, 50 (4): 701-704.

Anuradha M and Pullaiah T (1992). Micropropagation of mulberry (*Morus alba* L.). *Annali Di Botanica* 15 : 35-41.

Bhatnagar S, Kapur A, Khurana P (2001). TDZ-mediated differentiation in commercially valuable Indian mulberry, *Morus indica* cultivars K2 and DD. *Plant Biotech*. 18: 61 - 65.

Bhau B.S. and Wakhlu A.K., (2001). Effect of genotype, explant source and growth regulators on organogenesis in *Morus alba* L. *Plant Cell Tissue and Organ Culture*, 66: 25-29.

Chitra. D.S.V and Padmaja G., (1999). Clonal propagation of mulberry (*Morus indica* L. cultivar M-5) through *in vitro* culture of nodal explants. *Scientia Horticulturae*, 80: 289−298.

Huetteman, C.A. and J.E. Preece. (1993). Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell Tissue and Organ Culture*,33: 105-119.

Jain AK, Dandin SB, Sengupta K (1990). *In vitro* propagation through axillary bud multiplication in different mulberry genotypes. *Plant Cell Rep.* 8: 737 – 740.

Kapur A, Bhatnagar S, Khurana P (2001). Efficient regeneration from mature leaf explants of Indian mulberry via organogenesis. *Sericologia,* 41(2): 207 – 214.

Murashige T and Skoog F (1962). A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiol. Plant* 15: 473 – 497.

Sajeevan R. S., Jeba Singh, S., Nataraja K N and Shivanna M. B. (2011). An efficient *in vitro* protocol for multiple shoot induction in mulberry, *Morus alba* L variety V1. *International Research Journal of Plant Science* 2(8) : 254-261.

Tewary PK, Raghunath MK, Venkateswarlu M, Sarkar A (1996). Genotypic differences in response to *in vitro* shoot development of mulberry (*Morus* spp.). *Indian Journal of Sericulture,* 35: 104 – 106.

Vijaya Chitra DS and Padmaja G (1999). Clonal propagation of mulberry (*Morus indica* L. cultivar M-5) through *in vitro* culture of nodal explants. *Scientia Horticulturae,* 80: 289 – 298.

Vijayan K, Chakraborti SP and Roy BN (2000). Plant regeneration from leaf explants of mulberry: influence of sugar, genotype and 6- benzyladenine. *Ind. J. Exp. Biol.* 38: 504 – 508.

Yadav, U. Lal, M.and Jaiswal, V. S. (1990). Micropropagation of *M. nigra* L. from Shoot tip and Nodal Explants of Mature Trees. *Scientia Horticulturae*, 44: 61-67.