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In Vitro Plant Regeneration of *Morus indica* L. cv. V1 Using Leaf Explant

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ABSTRACT

Adventitious bud induction and plantlet regeneration were studied in a popular mulberry variety, V1 using leaf as an explant. Fully expanded leaf explants were cultured on Murashige and Skoog's (MS) medium supplemented with thidiazuron (TDZ) (0.5 - 4.0 mg/l), 6-benzylaminopurine (BAP) (0.5 - 2.0 mg/l), indole acetic acid (IAA) (2.0 mg/l), gibberlic acid (GA_3) (1.0 - 2.0 mg/l) silver nitrate ($AgNO_3$) (2.0 mg/l) and different carbon sources such as sucrose, fructose and glucose (10% - 30%) either individually or in combination to induce adventitious buds and regeneration. The highest percentage (63%) of adventitious bud formation and regeneration (68%) was achieved in the medium containing MS with TDZ (1.0 mg/l), IAA (2.0 mg/l) and $AgNO_3$ (2.0 mg/l). For subsequent regeneration and shoot elongation the MS medium having BAP (1.0 mg/l), GA3 (2.0 mg/l) and $AgNO_3$ (2.0 mg/l) was found to be suitable. Amongst the carbon sources tested, the most suitable carbon source was found to be sucrose (3%) followed by fructose (2%) for adventitious bud formation. Excised *in vitro* shoots were rooted (60% - 80%) in half strength MS medium supplemented with indole-3-butyric acid (1.0 mg/l). The well rooted plantlets were hardened in soil + sand + farm yard manure (FYM) mixture with a success rate of 70% - 90%. Since *in vitro* regeneration is highly genotype-dependent in mulberry, the standardized protocol can be effectively used for further improvement of this leading genotype using biotechnological approaches.

Keywords: *In Vitro* Regeneration; Adventitious Bud; Thidiazuron; Mulberry cv. V1; Carbon Source

1. Introduction

Mulberry (*Morus* spp.) is a woody perennial tree of importance to the sericulture industry as mulberry leaf is the sole food for the silkworm (*Bombyx mori* L.) larvae. Owing to its long juvenile period and heterozygosity [1], improvement of specific characters through conventional breeding is cumbersome and time consuming. Therefore targeted manipulation of elite genotypes through incorporation of specific genes encoding desired traits using modern biotechnological methods offers a new opportunity for crop improvement. An efficient *in vitro* regeneration procedure is pre-requisite for transgenic approach in any crops. Information on development and standardization of *in vitro* regeneration protocols in promising mulberry genotypes is limited, although there are reports in a few genotypes. Studies have been made in mulberry to examine the impact of various growth regulators on *in vitro* organogenesis and plant regeneration by using different explants viz. leaf, internodal segment, hypocotyls and cotyledons [2-12]. However, the shoot differentiation from callus is confined only to a few genotypes and repeatability of protocols developed was not assured due to the recalcitrant nature of the plant. In this study, we made an attempt to develop and standardize *in vitro* regeneration protocol in a widely cultivated mulberry variety, V1 using leaf explants. The variety is highly popular due to its economic characters under irrigated conditions and the foliage is suitable for both young and late age bivoltine silkworm rearing. The major emphasis was given to investigate the effect of Thidiazuron (TDZ), a substituted phenyl urea and different carbon sources in inducing adventitious buds and efficient regeneration in V1.

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2. Materials and Methods

2.1. Explant Preparation and Surface Sterilization

Fully expanded leaf from the top 2nd - 5th position was collected from actively growing shoots of (six months old) mulberry variety, V1. The explants were thoroughly washed in running tap water for 1 hour, followed by immersion in a 1% (v/v) liquid detergent (Labolene, Qualigenes, India) for 4 - 5 minutes and again washed thoroughly in running tap water to remove the traces of the detergent. Surface sterilization was done by treating the explants with 0.1% (v/v) $HgCl_2$ (Qualigens, India) for 3 - 4 minutes and rinsing in sterile distilled water to remove traces of $HgCl_2$.

2.2. Induction of Adventitious Shoot Buds and Plant Regeneration

The surface sterilized leaf explants were cultured on Murashige and Skoog's (1962) [13] supplemented with thidiazuron (TDZ) (0.5 - 4.0 mg/l), indole acetic acid (IAA) (2.0 mg/l), silver nitrate ($AgNO_3$) (2.0 mg/l) either added singly or in combination. As a carbon source, sucrose (1% - 3%) was added singly or a mixture of sucrose, fructose and glucose in the proportion of 1:1. The pH of the medium was adjusted to 5.8 before gelling with agar-agar (0.8%, Himedia, India). The induced shoot buds were transferred to various shoot induction media supplemented with 6-benzylaminopurine (BAP) (0.5 - 2.0 mg/l), gibberlic acid (GA_3) (1.0 - 2.0 mg/l) and silver nitrate ($AgNO_3$) (1.0 - 2.0 mg/l). All the cultures were maintained at 25˚C ± 2˚C under 16/8 h (day/night) photoperiod with light provided by cool, white fluorescent tubes (Phillips, TL 40 W/54) at a light intensity of 150 - 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$.

2.3. Rooting and Hardening of Plantlets

Regenerated *in vitro* shoots of 3 - 4 cm long were clipped off and transferred to half strength MS media fortified with NAA or IBA (1.0 mg/l) to induce roots. The well rooted plantlets were transplanted to small earthen pots filled with potting mixture containing garden soil, sand and FYM (2:1:1 ratio) for hardening and establishment under controlled condition. The transparent plastic cups were inverted over the potted plantlets to maintain high humidity and three weeks after hardening, the plantlets were transplanted to field.

2.4. Data Recording

The data on adventitious bud formation and subsequent regeneration were recorded after 4 and 8 weeks of cultures respectively. The percentage of rooting and root length were recorded after 20 days and 30 days of subculture respectively on rooting medium. Each value of data represented the mean (±SE or SD) of 24 cultures per treatment.

3. Results and Discussion

In vitro regeneration in some perennial plants like mulberry is regulated by several factors and regeneration efficiency is highly genotype-dependent. Similar to other species, the morphogenic response of mulberry leaf explants was influenced by concentrations and combinations of the phytohormones and carbon source supplemented in the medium. First sign of leaf expansion, swelling at the midrib region and basal cut ends with slight callusing were noticed after 10 - 15 days in all the media tested. After 30 days, nodule-like structures were formed on the midrib region and basal cut ends. These structures later turned into the shoot buds (**Figures 1(a)** and **(b)**) and subsequently into the shoots, after 45 - 55 days (**Figure 1(c)**). The adventitious bud formation was maximum at the basal cut ends compared to the midrib region. Of the different adventitious bud induction media tested, TDZ (1.0 mg/l), IAA (2.0 mg/l), $AgNO_3$ (2.0 mg/l) and sucrose (3%) resulted in maximum response of adventitious bud formation (63%, **Table 1**). The substituted phenyl urea, TDZ, has been shown to be an effective regulator of *in vitro* morphogenesis of many dicot plants especially in woody perennials, influencing callusing, adventitious bud formation, shoot regeneration, somatic embryogenesis and protoplast division [10,14,15]. Similar to other studies, in this study, addition of TDZ had beneficial effects in inducing bud regeneration. The regeneration ability of the adventitious buds transferred to different regeneration medium was significantly higher (68%, **Table 1**) in BAP (1.0 mg/l), GA_3 (2.0 mg/l) and $AgNO_3$ (2.0 mg/l) containing medium (**Figure 1(c)**). Similar types of findings were also reported in earlier studies in mulberry [10,15,16].

Carbon source is one of the very important components of the nutrient media. In the present investigation, different concentrations of sucrose (2% and 3%) and those in combination with fructose and glucose were tested for adventitious bud formation. Among the carbon sources tested, the most suitable carbon source was found to be sucrose (3%) followed by fructose (2%, **Table 1**). As reported earlier, sucrose seems to be the best source of carbon for *in vitro* regeneration [2,3,10,15,16] of mulberry variety, V1.

In vitro regenerated shoots were rooted successfully with rooting per cent between 60 - 80 and the mean root length ranged from 3.58 - 5.20 cm on 1/2 MS supplemented with indole butyric acid (1.0 mg/l) medium after 30 days (**Figure 1(d)**, **Table 2**). Well-rooted plantlets were hardened with a success rate of 70% - 90% (**Figure 1(e)** and **(f)**). Similar observations were reported in *M.*

Table 1. Response of leaf explants cultures of V1 mulberry genotype. Sugar (%): A. Sucrose (3%), B. Sucrose (2%), C. Fructose (3%), D. Fructose (2%); E. Sucrose (3%), F. Glucose (2%), G. Sucrose + Fructose (1:1), H. Sucrose + Glucose (1:1).

Medium (mg/l)	Sugar (%)	Adventitious bud induction (%) (±SE)	Regeneration (%) (±SE)
1. MS + TDZ (0.5) + IAA (2.0) + $AgNO_3$ (2.0)	A	10.0 ± 5.77(16.35)	1.67 ± 1.67
	B	23.3 ± 10.93(27.78)	13.33 ± 1.68
	C	13.3 ± 1.68(21.34)	3.33 ± 1.67
	D	36.67 ± 6.67(37.14)	23.33 ± 3.33
	E	13.33 ± 1.68(21.34)	21.67 ± 3.33
	F	18.33 ± 3.33(25.19)	5.00 ± 1.67
	G	41.67 ± 3.33(40.18)	0.00 ± 0.00
	H	38.33 ± 3.33(38.22)	0.00 ± 0.00
2. MS + TDZ (1.0) + IAA (2.0) + $AgNO_3$ (2.0)	A	63.33 ± 1.67(52.74)	68.33 ± 4.41
	B	51.67 ± 3.33((45.96)	60.00 ± 2.89
	C	26.67 ± 4.41(30.95)	18.33 ± 1.67
	D	23.33 ± 1.67(28.86)	13.33 ± 1.67
	E	28.33 ± 8.82(31.64)	51.67 ± 3.33
	F	28.33 ± 3.33(32.09)	21.67 ± 1.67
	G	15.00 ± 1.33(22.77)	3.33 ± 1.67
	H	23.33 ± 1.68(28.86)	30.00 ± 2.89
3. MS + TDZ (2.0) + IAA (2.0) + $AgNO_3$ (2.0)	A	16.67 ± 4.47(23.74)	5.00 ± 2.89
	B	11.67 ± 1.67(19.86)	0.00 ± 0.00
	C	8.33 ± 4.11(15.09)	5.00 ± 5.00
	D	61.67 ± 1.67(51.76)	31.67 ± 6.67
	E	48.33 ± 4.41(44.03)	18.33 ± 3.33
	F	31.67 ± 6.67(34.04)	10.00 ± 1.67
	G	16.67 ± 4.41(23.74)	5.00 ± 2.89
	H	41.67 ± 6.00(40.11)	28.33 ± 3.33
4. MS + TDZ (4.0) + IAA (2.0) + $AgNO_3$ (2.0)	A	28.33 ± 8.82(31.64)	5.00 ± 2.89
	B	51.67 ± 1.67(45.96)	11.67 ± 1.67
	C	20.00 ± 2.89(26.45)	16.67 ± 8.33
	D	20.00 ± 5.00(26.15)	3.33 ± 3.33
	E	33.33 ± 1.67(35.25)	13.33 ± 1.67
	F	31.68 ± 6.67(34.04)	8.33 ± 1.67
	G	25.00 ± 10.00(29.23)	10.00 ± 5.00
	H	41.68 ± 3.33(40.17)	20.00 ± 3.33
CD at 5%	Medium	4.99	3.15
	Sugar	7.06	4.46
	Medium × Sugar	9.85	8.98

laeviagata [17]; *M. indica* var. C176 and C776 [18] and other mulberry varieties [15,16].

The results generated in this study provided a reliable and high frequency regeneration protocol with high reproducibility. The standardized protocol could suitably be used for large scale *in vitro* propagation and genetic transformation.

Table 2. Effect of different auxins on root induction.

Medium (1/2 MS + mg/l)	Percentage of rooting	Mean root length (cm) (±SD)
IBA (0.5)	60.00	3.58 ± 0.83
IBA (1.0)	80.00	5.20 ± 0.90
NAA (0.5)	55.00	1.16 ± 0.30
NAA (1.0)	35.00	1.20 ± 0.47

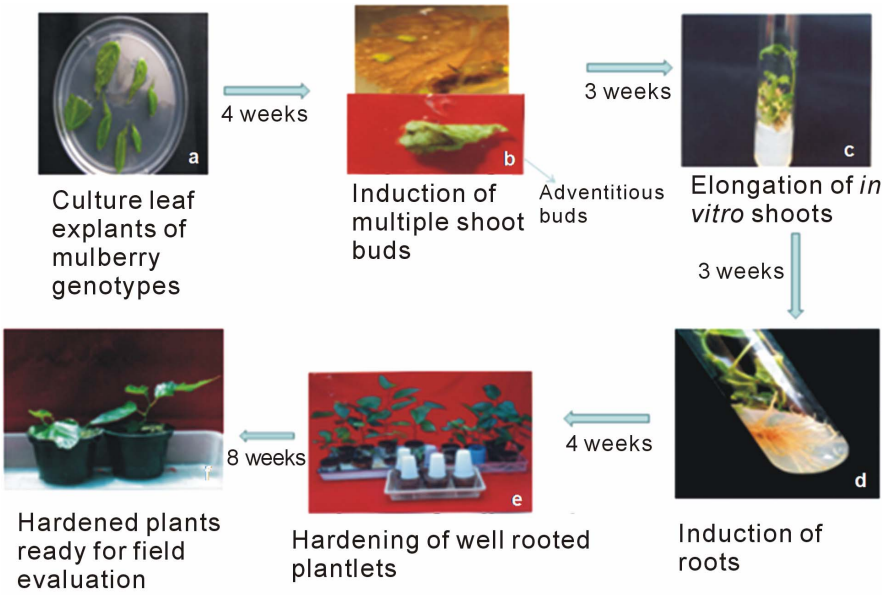

Figure 1. Protocol for plantlet regeneration from leaf explants in V1 mulberry variety.

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