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Plant regeneration from leaf explants of mulberry : Influence of sugar, genotype and 6-benzyladenine

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A protocol for plant regeneration from leaf explants was developed for tropical mulberry varieties. Effect of sugars, 6-benzyladenine and genotype on shoot regeneration was studied. Highest percentage of shoot regeneration (80±6) was obtained with genotype S799 on medium containing glucose and 8.9 µM 6-benzyladenine. Genotypes Mandalaya and MIHP, having thicker leaves with waxy cuticle, showed poorer regeneration ability than S799 and Sujanpur-5, which have thinner leaves and cuticle. Histological studies revealed that shoots regenerated from sub-epidermal cells.

Mulberry (*Morus* spp.) is a fast growing tree of importance to the sericulture industry as the larvae of the silkworm (*Bombyx mori* L) feed on its leaves. Since cross pollination is the rule rather than the exception, the plant is highly heterozygous[1]. Owing to its long juvenile period and high heterozygosity, improvement of specific characters through conventional breeding is cumbersome. However, incorporation of specific genes encoding desired traits through modern biotechnological methods offers a new avenue for its improvement.

Efficient *in vitro* regeneration procedures are required to obtain transgenic plants. Mulberry has been regenerated via., internodal callus[2,3], leaves[4], immature embryo[5], cotyledons[6] and from anthers[7]. Although regeneration from leaves has been reported, attempts to develop transgenic plants by the use of *Agrobacterium tumefacience* has failed due to the nondevelopment of plantlets from the transformed leaf buds[8]. Further, most of the regeneration work from leaf explants dealt with temperate mulberry varieties which are nonadaptive in tropical environments. Hence, to obtain transgenic plants of tropical genotypes requires the development of efficient protocols for the regeneration of plantlets from leaf explants. In the present report, a procedure for the regeneration of plants from young leaves of tropical mulberry genotypes and the influence of sugars, BA and genotype on regeneration have been described.

Materials and Methods

Sprouting axillary buds, collected from one year old mulberry plants, were surface sterilized with 0.1% mercuric chloride for 10 min and washed several times with sterile distilled water . The youngest leaves (2-5 mm long) were excised from the axillary buds and cultured on MS medium[9] containing various concentrations (4.4, 8.9, 13.3 µ*M*) of 6-benzyladenine (BA) and sucrose, fructose or glucose. For all experiments the growth regulators were added before autoclaving at 120°C for 20 min and the *p*H was adjusted to 5.7 ± 0.1 . The medium was solidified with 6 g l^{-1} agar (Hi-media, India). Explants were placed with abaxial surface down, on the regeneration madium. Leaf explants (20) were plated in each replication and were placed randomly in the rack. Cultures were maintained at 25° ± 1°C and exposed to a 16 hr photoperiod and PAR of 75 µmol s^{-1} m^{-2} provided by cool white fluorescent lamps. Adventitious shoots were rooted by growing them in MS medium containing 2.6 µM NAA. One month after root elongation, individual plantlets were transferred to an autoclaved mixture of sand, soil and vermiculite (1:1:3) in pots kept, covered with polythene bags to maintain a high relative humidity, at 25°C. After one month of growth plants were transferred to the field.

Data were analysed using Duncan's multiple range test (DMRT) for significance and the standard error was calculated using standard methods.

Leaf explants were fixed at 10 days intervals, till well developed shoots had formed, in formaline-acetic- alcohol mixture (1:1:18) overnight for histological studies. These samples were then dehydrated by passing through alcoholic grades and were finally embedded in paraffin (56°C melting

point). Sections were prepared with a rotary microtome and stained with Delafield's Haematoxylin, counter stained with 1% methylene blue and mounted with euparol.

Results and Discussion

During the first two weeks, the leaf explants expanded in all sugars, more so in glucose and fructose. In glucose, expansion of leaves was maximum and were very dark compared to those in other two media. After 3-4 weeks, nodule like structures were formed on the leaf surface, predominantly on the veins, and within 10-15 days these turned into shoot buds and subsequently into shoots after 45-60 days (Fig. 2). However, bud formation at the cut end of leaf was faster (6-7 days) compared to that on the leaf surface. Well developed shoots (1-2 cm) were obtained within 60-70 days (Fig. 3). Regeneration frequency varied from 1.67±0.51 to 15.00±2.88 % with 1-3 shoots per explant in sucrose (Table 1). In fructose, regeneration frequency varied from 6.67±1.66 to 23.33±4.11% with 1-6 buds per explant. In glucose, the frequency of regeneration was very high , showing a range of 5.00±2.88 to 80.00±5.77 with 1-12 buds per explant indicating the superiority of glucose over the other two carbohydrate sources. Though sucrose has been the carbohydrate chosen in most of the reports on micropropagation of woody species[4] , Saito and Katagiri[10] reported superiority of fructose over sucrose in induction of adventitious shoots in the leaves of temperate mulberry varieties. Results in this experiment while agreeing with these early findings, further revealed the superiority of glucose over the other two sugars (Fig. 1). Similar results were obtained by Welander *et al.*[11] in *Alnus* species and by Romano *et al*[12] in Cork Oak.

Concentration of BA was found to be a major factor determining the regeneration capacity in leaves. The optimum concentration varied among varieties;

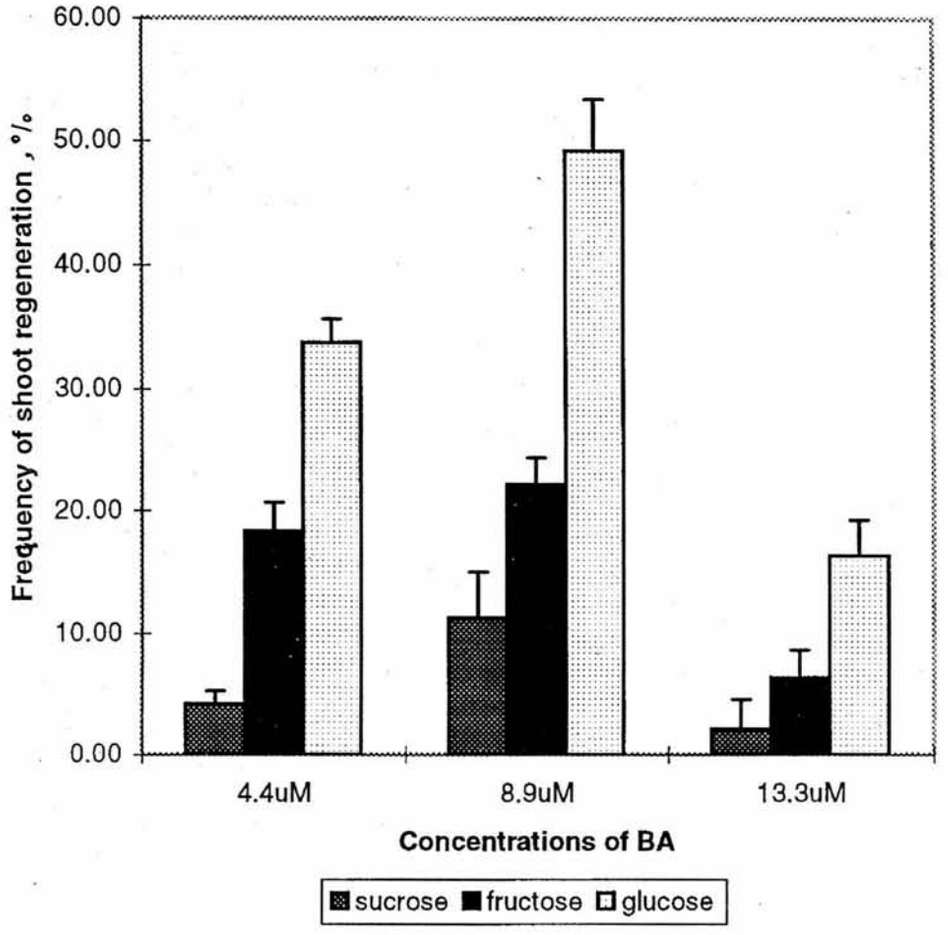

Fig. 1—Effect of sugar and 6-benzyladenine on shoot regeneration from leaf explants in mulberry (average of data from 4 genotypes).

Table 1—Effect of genotype, sugar and concentrations of BA on shoot regeneration from leaf explants in mulberry

[Valus are mean ± SE of 3 observations]

Variety	Sugar	Concentration of BA (μm)	Explants responded (%)	Number of buds/ explant
S799	Sucrose	4.4	1.67 ± 0.51a	1.00 ± 0.00a
S1	Fructose	8.9	10.00 ± 2.88b	1.11 ± 0.10a
Sujanpur-5	Glucose	13.3	3.33 ± 1.66a	1.00 ± 0.00a
MIHP	Sucrose	4.4	6.67 ± 1.66a	1.00 ± 0.00a
	Fructose	8.9	26.67 ± 3.33c	1.28 ± 0.14a
	Glucose	13.3	6.67 ± 1.66a	1.00 ± 0.00a
	Sucrose	4.4	25.00 ± 2.88b	2.58 ± 0.28a
	Fructose	8.9	80.00 ± 5.77e	2.90 ± 0.30a
	Glucose	13.3	33.33 ± 6.00c	1.37 ± 0.36a
	Sucrose	4.4	5.00 ± 2.88a	1.00 ± 0.00a
	Fructose	8.9	15.00 ± 2.88b	1.53 ± 0.37a
	Glucose	13.3	0.00 ± 0.00a	0.00 ± 0.00a
		4.4	18.33 ± 4.41b	1.22 ± 0.11a
		8.9	21.67 ± 4.41b	1.50 ± 0.09a
		13.3	6.67 ± 1.66a	1.00 ± 0.00a
		4.4	20.00 ± 2.88b	1.63 ± 0.16a
		8.9	43.33 ± 10.14c	2.42 ± 0.64a
		13.3	18.33 ± 6.00b	1.06 ± 0.05a
		4.4	8.33 ± 1.66a	1.00 ± 0.00a
		8.9	3.33 ± 1.66a	1.00 ± 0.00a
		13.3	0.00 ± 0.00a	0.00 ± 0.00a
		4.4	30.00 ± 2.88c	1.56 ± 0.42a
		8.9	16.67 ± 1.66b	2.11 ± 0.48a
		13.3	0.00 ± 0.00a	0.00 ± 0.00a
		4.4	68.33 ± 4.41d	2.40 ± 0.48a
		8.9	31.67 ± 7.26c	1.24 ± 0.14a
		13.3	5.00 ± 2.88a	1.56 ± 0.42a
		4.4	1.67 ± 1.66a	1.00 ± 0.00a
		8.9	16.67 ± 6.66b	1.00 ± 0.00a
		13.3	5.00 ± 2.88a	1.00 ± 0.00a
		4.4	18.33 ± 4.41b	1.20 ± 0.20a
		8.9	23.33 ± 4.41b	1.44 ± 0.29a
		13.3	11.67 ± 14.88b	1.44±0.290a
			21.67 ± 1.66b	1.25 ± 0.22a
			41.67 ± 10.93c	1.95 ± 0.41a
			8.33 ± 3.33a	1.78 ± 0.46a

Means in the same column with same letters are not significantly different at P=0.01

Table 2—Leaf anatomical characteristics of 4 mulberry genotypes and frequency of shoot regeneration from leaf explants in glucose containing MS medium

[Valus are mean ± SE of 10 observations]

Genotypes	Thickness of upper epidermal cuticle (μm)	Thickness of lower epidermal cuticle (μm)	Total leafblade thickness (μm)	Frequency of shoot regeneration (%)
S799	1.8 ± 0.16a	1.3 ± 0.09a	89.2 ± 2.12a	80.0 ± 3.6a
Sujanpur-5	2.4 ± 0.21a	1.3 ± 0.09a	102.8 ± 0.66b	68.3 ± 2.4b
MIHP	3.5 ± 0.22b	1.3 ± 0.09a	124. 3± 1.55c	41.7 ± 5.9c
Mandalaya	3.8 ± 0.24b	1.7 ± 1.81a	149.8 ± 0.82d	43.3 ± 5.5c

Means in the same column with same letters are not significantly different at P=0.05

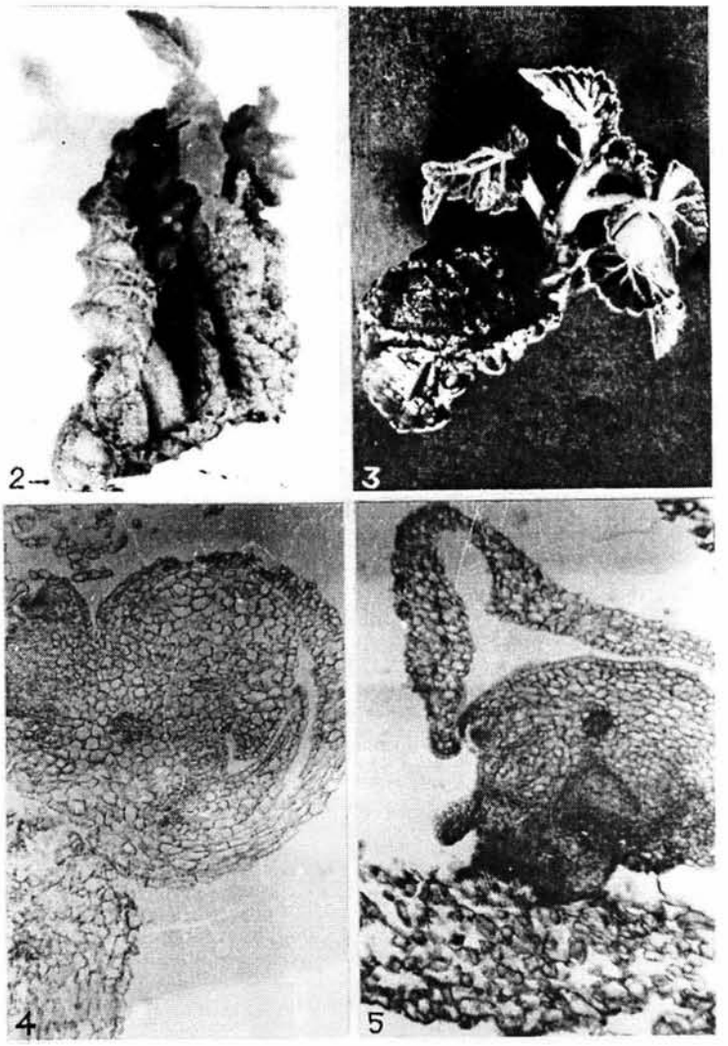

Fig. 2—Mulberry leaf with multiple shoot buds (×3); Fig. 3—Mulberry leaf with well developed shoot (×1.5); Fig. 4—Transverse section of leaf showing organisation of bud meristem and leaf primordia in a pear shaped bud (×245), Fig. 5—A mature bud with short stalk and leafy structure (×245).

8.9 μM BA was found suitable for S799, S1 and MIHP. In Sujanpur-5 the best result was observed with 4.4 μM BA. Narayan *et al.*[2] found 8. 9 μM BA as optimum for shoot regeneration from internodal callus in mulberry. This study confirms the suitability of 8.9 μM BA for shoot regeneration in three mulberry varieties.

A distinct genotypic effect for shoot differentiation was observed in this study as S799 and Sujanpur-5 showed a very high frequency of shoot differentiation (80±5.77 % and 68±4.41 % respectively) compared to the other genotypes. Leaves of S799 and Sujanpur-5 expanded and wrinkled before nodule like structures appeared on the leaf surface. No such changes were observed in the other two genotypes. A higher number of nodule like structures and buds were observed in S799 and Sujanpur-5 and the shoot buds appeared nearly one week earlier than in the other two genotypes. Anatomical studies of fresh leaves revealed structural differences between these genotypes (Table. 2). Genotypes S799 and Sujanpur-5 have thinner cuticle and leaf blade than the other two genotypes. This difference in the cuticular thickness together with the waxy deposits on the cell walls points to a possibility that the cuticle plays a role in the regeneration ability of leaves in mulberry. A heavier leaf cuticle may act as a barrier to the expansion of epidermal cells as evidenced from the poor leaf expansion and nodulation noticed in the cuticular leaves.

Histological examination of leaves (Figs. 4 and 5) revealed that cells just below the epidermis undergo periclinal divisions resulting in the formation of densely stained, highly cytoplasmic, meristematic cells as reported earlier[13]. Such active meristematic cells after repeated division gave protrusions, forming compact nodule like structures which further developed into shoot buds. Buds in the early stages of development looked like pear shaped structures with a small stalk that served to draw nutrients from leaf explants. Each bud on further elongation produced leafy shoot.

These results, thus, provide a reliable and fast regeneration protocol with high reproducibility, that could suitably be used in genetic transformation studies. Glucose was found as the best carbon source for getting higher rate of regeneration in all the genotypes. The higher frequency of shoot regeneration from less cuticular leaves offers an added advantage to such genotypes for *in vitro* genetic manipulation in mulberry.

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