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PLANT REGENERATION FROM ISOLATED PROTOPLASTS OF MAHUA (*MADHUCA LATIFOLIA* MACB)

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Protoplasts were successfully isolated from young leaf tissue of selected1-3 yrs old naturally grown plants of *Madhuca latifolia* using a combination of three enzymes cellulase (3-4%) hemicellulase (1.5-2%) and pectinase(1.5-2%) after maceration in 13% mannitol. The incubation time required for the maximum release of protoplasts was on gyratory shaker for 3hrs in diffused light at a temperature of 25±2°C and RH 65±10%. The isolated protoplasts were intact with different densities in different media.The protoplasts were purified by filtration (through multilayer of muslin cloth) followed by centrifugation in mannitol and finally by treatment with sucrose (20-80%). Protoplast were cultured on MS basal media and incubated. Formation of cell masses were noted after 3-4 days in controlled environmental condition.

Keywords: Cellulase; Hemicellulase; *Madhuca latifolia*; Mahua; Pectinase; Protoplast.

Introduction

Protoplasts have been regarded as totipotent cells well suited for fundamental studies on one hand and genetic manipulations on the other [1]. Since the first demonstration of enzymatic degradation of plant cell wall considerable progress has been made concerning regeneration from protoplasts of monocotyledons and dicotyledons species in general[2] and in woody plant species in particular[3-6]. Mahua (*Madhuca latifolia* Macb) is a large deciduous tree well distributed in central India. It is an important Minor Forest Produce (MFP) species having multifarious uses[7]. It plays an important role in Tribal economy of Gujrat, Maharashtra, U.P. and M.P. The productivity of Mahua is insufficient to satisfy expected future demands. The long life cycle and large size of tree prevents breeding experiments on a large scale. *In vitro* regeneration of this species has been accomplished from cotyledons[8], which are available for only a limited period (May and June) in the year. Besides they remain viable, for even shorter period. As a result both propagation and regeneration of the species cannot fulfill the goal. Therefore, it is necessary to regenerate the entire plant from isolated protoplast. In order to explore the potential of protoplast technology for the improvement of woody spices an efficient and reproducible methods for protoplast isolation and of purification must first be established. The present paper reports the development of protoplast isolation and purification schedule and establishment of culture technique.

Materials and Methods

Fresh leaves from 1-3 year old plants growing in natural habitats were used as explants for protoplast isolation. Leaves were surface sterilized by first dipping them into ethanol (2 minutes) and then in 0.1% (w/v) mercuric chloride solution (10 minutes) followed by thorough wash with sterile distilled water. The detached leaves were macerated in 13% (w/v) mannitol and kept for 1hr in this solution before incubation[9].

Isolation of protoplasts: Macerated leaf tissue (1-2g) was used for the experiment. It was rather difficult to remove lower epidermis from the excised leaves it was scratched with scalpel to break epidermal and cuticular surfaces[9] and the leaf sliced in to small pieces.

To assess the optimal concentration of enzymes required, the tissue was incubated in various concentrations and combinations of enzymes (0.5, 1, 1.5, 2, 2.5, 3, and 4%)[10]. For this purpose cellulase (CDH, India), Hemicellulase (Sigma, USA) and pectinase (CDH India) were used alone and in combination of two and three. The enzyme solutions were prepared in mannitol (13% w/v) and the pH of the media was set at 5.6.The sliced segments were inoculated into sterile incubation media and were incubated on a gyratory shaker (120-140 rpm) for 1hr in light at 25±2° C, 65± 10% RH and later by keeping tissue stationary in enzyme mixture[11] for different durations of time.

Purification of protoplast : After sufficient incubation, the resulting suspension containing protoplasts was filtered through multilayer of muslin cloths to separate protoplasts from undigested plant debris (Figs. 1-2) and later transferred to centrifuge tubes and centrifuged at 300rpm for 5min, to remove the enzyme solution and concentrate the protoplast by pelleting technique[12]. This process was repeated three times. For a final cleaning, mannitol was replaced by 20-80% (w/v) sucrose solution and centrifuged at 300 rpm for 5 min using this procedure the cleaned protoplasts (Figs. 3-5) floated and the debris settled. The floating protoplasts, concentrated as a dark band at the

top layer, were pipetted out with a pasteur pipette and resuspended in 13% mannitol.

Determination of density of protoplast : Density of protoplast was determined by haemocytometer. A double chamber haemocytometer was used for this purpose[13] .The number of protoplasts were counted in one triple lined square (= n)

Total yield =n x5x10 ^{3} x10 (vol.in ml)

=5n x10 ^{4}.

Culture of isolated protoplasts : For establishment of protoplast culture on plant tissue culture medium plating in soft agar technique[14] was employed. The protoplasts were suspended in MS basal medium supplemented with BA (1mg/l) and NAA (1mg/l). Two ml of protoplast suspension was dispensed in petriplate using a sterile pipette followed by 4 ml of the molten agar medium. The mixture was mixed thoroughly and allowed 30minutes for the agar to set. Petri plates were sealed with parafilm and stored inverted in the culture room. The dishes were provided with 16-hrs illumination of 1000 lux at 25±2 ^{0}C and 65±10% RH. The observations were recorded on alternate days.

Result and Discussion

In the present study the protoplasts were isolated from leaf tissue after 5hr to overnight incubation in varying concentrations of cellulase, hemicellulase and pectinase solution in 13% mannitol. Leaf tissue is the most commonly used source of protoplasts[15] in many species[15-17]. Mannitol which is preferred for protoplast isolation from mesophyll tissue[15]was used as plasmolyticum. Plasmolysis in 13% mannitol solution prior to the enzyme treatment was employed as it helped to improve the isolation procedure[18-19]. Different concentrations of enzyme (0.5-4%) [10] alone and in combination of two or three were screened simultaneously.

The protoplasts yields, obtained following overnight treatment of leaf tissue with varying concentration of enzyme, are presented in Table 1. Treatment of leaf –tissue with 3% cellulase, 1.5-2% hemicellulase and 1.5-2% pectinase provided the highest yield as counted using haemocytometer. The differential response of leaves and enzyme concentration[20] in cucumber leaves required lower cellulase and higher concentration of macrozymes. Minimum time of incubation required in the species was 5hr although the maximum yields were obtained after overnight incubation. This incubation period varied according to species such as 1hr in *corn*[21] and 20hr in *Ulmus*[22].

Protoplast preparations obtained by enzyme isolation contained considerable amounts of cellular debris, vascular elements, cell wall, tissue fragments, membranes, nuclei, plastids etc. Therefore; the protoplasts were purified by a combination of filtration, centrifugation and washing. Debris was removed by filtration through muslin cloth and by centrifugation at moderate low speed as in most instances[21].

The protoplasts of various sizes (0.3-1.2μ) were observed. Very few protoplasts were vacuolated. Approximately 1.5 gram of material was enough to liberate a reasonable number of protoplasts. Freshly isolated protoplasts were spherical with prominent nuclei.

Protoplasts have a maximum as well as a minimum plating density for growth[23]. The optimal plating density for *Madhuca latifolia* was about 1.5x10^{4} protoplasts/ml, which was obtained after treatment with 3-4% cellulase, 1.5-2% hemicellulase and 1.5-2 pectinase. At this plating density on basal MS medium using droplet culture and plating in soft agar techniques, the mini calli were formed after 10-12 days of incubation. These types of minicalli were reported in *Cucumis sativas*[20]. No regeneration occurred at other densities.

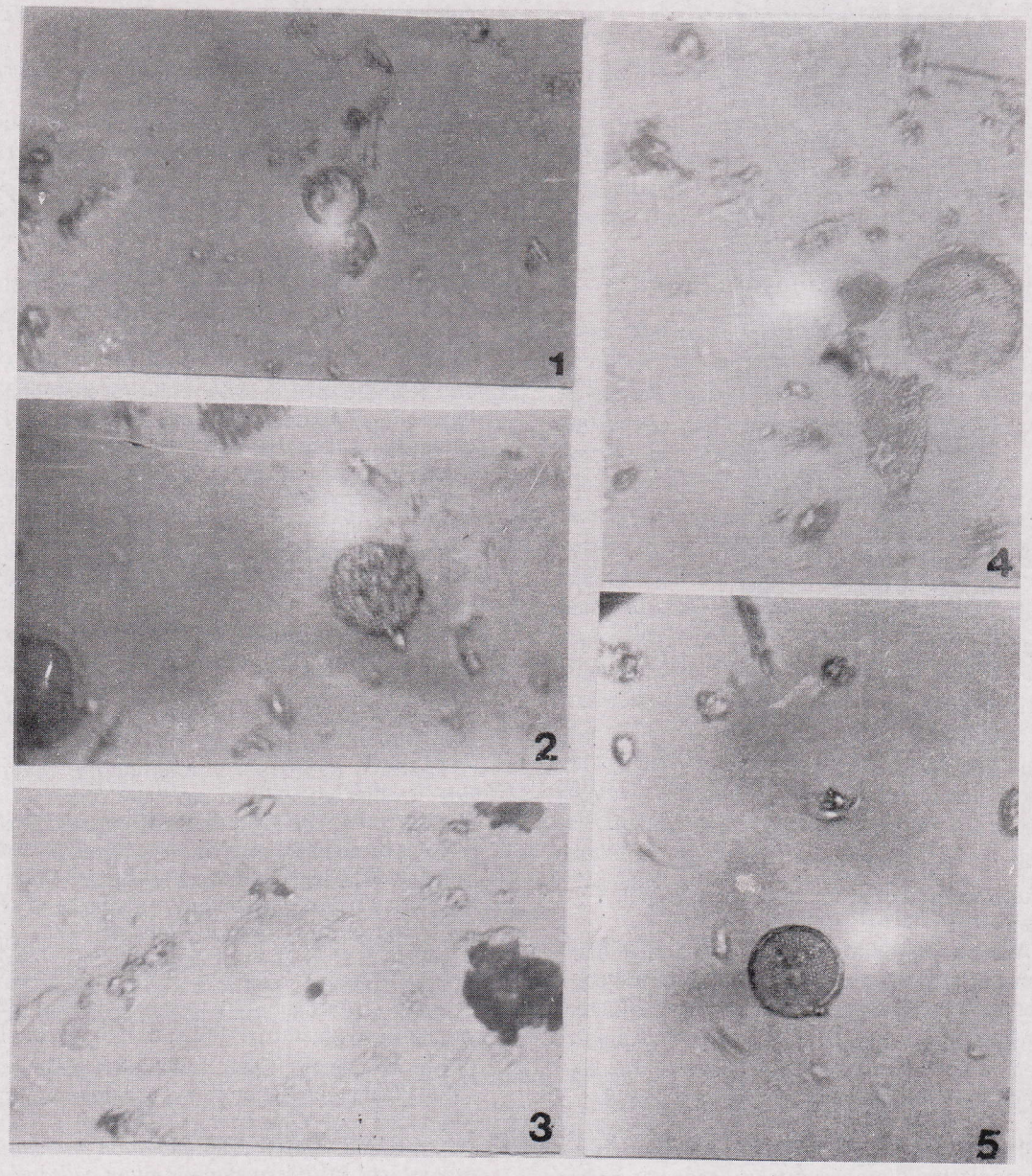
This work has shown that many factors such as age, physiological condition of the tissue[9], procedural details along with time of digestion, composition of digestive media, choice of plasmolyticum, filtration, centrifugation at low speed and handling protoplast during isolation may be critical for isolation of protoplasts[24].

Several hormone combinations favoured vigorous growth of calli (Figs. 6,7). On MS+BA(1mg/l) +NAA (1mg/l), small green patches were observed in 40% cultures. On transfer of such differentiating protoclones the small green patches developed into complete plantlets (Fig. 8). It is concluded that although a large population of living protoplasts can be isolated from *Madhuca*

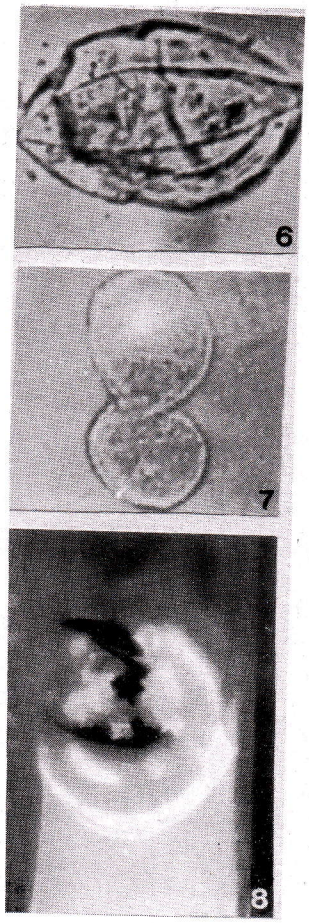
Table 1. Yield of protoplast (x10^{4} protoplast/ml) from leaves of *Madhuca latifolia.*

	C (%) / Conc.	0.0	0.5	1.0	1.5	2.0	2.5	3.0	4.0
H+P (%)	0.0+0.0	-	-	-	-	-	-	-	-
	0.5+0.5	-	-	-	-	-	-	-	-
	1.0+1.0	-	-	-	0.31	0.35	0.50	1.36	1.25
	1.5+1.5	-	-	-	0.39	0.46	0.81	1.58	1.55
	2.0+2.0	-	-	-	0.43	0.83	0.83	1.56	1.54

C-Cellulase, H-Hemicellulase, P-Pectinase



Figs.1-5. Isolation of protoplasts from leaves of *M. latifolia*.
1,2. Freshly isolated protoplast of *M. latifolia*.(x1000); 3,4,5. Cleaned protoplasts of *M. latifolia*.(x1000)



Figs. 6-8. Regeneration of protoplasts into plants in *M. latifolia.*
6. Dividing protoplast(x1000); 7. Multicellular colony derived from protoplast (x1000); 8. Plant differentiation from protoclones cultured a month after transfer.

latifolia more work is required on this species for the establishment of more efficient regeneration protocol.

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