Plant Cell Reports (1992) 10:633–636

Plant Cell Reports

Somatic embryogenesis in protoplast derived calli of cultivated jute, *Corchorus capsularis* L.

Thakurdas Saha and S. K. Sen

Plant Molecular & Cellular Genetics, Bose Institute, Centenary Building, P1/12, C.I.T. Scheme VII-M, Calcutta 700 054, India

Received March 15, 1991/Revised version received November 7, 1991 – Communicated by G. C. Phillips

ABSTRACT

Protoplasts were isolated from cotyledon, hypocotyl and mesophyll cells of Corchorus capsularis L., a major fibre crop, by one step enzyme digestion method. They were further cultured successfully on modified KM-8p (Kao and Michayluk, 1975) medium to form microcalli. The required cultural conditions could be used to achieve 34% to 78% plating efficiency, depending upon the source of protoplasts. Hypocotyl protoplasts gave the highest plating efficiency. On transfer to regeneration medium, somatic embryos developed at high frequency. The present success is a significant step forward in the development of meaningful plant cell culture methods for application in jute.

ABBREVIATIONS

B_5 - Gamborg et al., 1968; MS - Murashige and Skoog, 1962; SH - Schenk and Hildebrandt, 1972; Ad-SO_4 - Adenine sulphate; BAP - 6-benzylaminopurine; NAA - 1-naphthalene acetic acid; GA_3 - Gibberellic acid; 2,4-D - 2,4-dichlorophenoxy acetic acid; Kn - Kinetin; IBA - Indole-3-butyric acid.

INTRODUCTION

The cultivated jute species (Corchorus capsularis L. and C. olitorius L., 2n = 14, family - Tiliaceae) is an important tropical crop which produces bast fibres, second in importance only to cotton amongst the vegetable fibres. Ninety-five percent of the total world production of raw jute fibres are from India, Bangladesh, China, Thailand, Nepal and Indonesia. Considering the merits of the naturally produced fibres over the synthetic ones that are commercially available, it is necessary that plant biotechnological efforts should be directed towards improvement of the jute crop in order to make it commercially viable. The present status of this crop is far from satisfactory. One of the impediments which has inhibited progress is the lack of available genetic variability regarding adaptibility to diverse agroecological conditions, fibre quality, yield and susceptibility to several diseases and pests (IBPGR, 1988). Incompatibility for obtaining a sexual hybrid between the two cultivated species has narrowed the scope for increasing genetic variability. Reports of success in raising interspecific F_1 hybrids are in record (Islam and Rashid, 1960; Swaminathan et al., 1961; Bhaduri and Bairagi, 1969), however, the advanced progenies have not exhibited a recombinant nature. Although Islam et al. (1987) are hopeful about the future possibilities for obtaining interspecific hybrids, the fact remains that none of the earlier claimed progenies of the sexual hybrid have been seen in the breeders fields. Such failures necessitated us to undertake protoplast fusion techniques for generating somatic hybrids. Although in the past, initial success was obtained in being able to fuse protoplasts of the two jute species and develop hybrid calli (Kumar et al., 1983), plant regeneration was not possible. This was based on the contention that the interspecific hybrid calli may differentiate into plants (Austin et al., 1985; Schieder, 1982), even though the parental species did not regenerate. Hence we directed our efforts to standardise methods for differentiation of the callus cells derived from protoplasts of the individual parental species to yield regenerated plants. This communication deals with our success in being able to induce somatic embryos from protoplast derived calli of Corchorus capsularis.

MATERIALS AND METHODS

A number of genotypes amongst the elite cultivars viz., JRC 212, JRC 7447, JRC D154 and JRC 321 were initially utilised to define the basic conditions required for in vitro culture of jute cells and tissue. JRC 321 was finally selected for the present study on the basis of its response in preliminary studies. Seeds were obtained from Central Research Institute for Jute and Allied Fibres, ICAR. The surface sterilized seeds were germinated aseptically on SC medium (Table 1) at 30°C in dark. One week old seedlings were used for the isolation of protoplasts from cotyledons and hypocotyls. Also, protoplasts were isolated from leaf mesophyll cells of shoots in culture. Shoot cultures were maintained on APS medium (Table 1). Shoot tips were subcultured every 3 weeks.

Isolation of protoplasts: Cotyledons and hypocotyls were cut into small segments and treated for 1 h in 0.3 M mannitol + 13.6 mM $CaCl_2.2H_2O$. Tissues were then incubated with 1% cellulase Onozuka R10 (Yakult Honsha, Japan), 1% Driselase (Kyowa Hakko Kogyo, Japan) and 1% pectinase (Serva) in 0.6 M mannitol + 13.6 mM $CaCl_2.2H_2O$ for 15 - 17 h at 26° - 28°C in the dark while shaking (40 rpm).

Young leaves of 3 weeks old shoot cultures were used for mesophyll protoplast isolation. The lower epidermis was peeled off the leaves and the leaves were cut into small pieces (2-4 mm^2). They were then washed with 35% sea water diluted with 0.6 M mannitol, and placed in an enzyme solution of 1.5% cellulase Onozuka R10, 0.2% pectolyase Y-23 (Kikkoman Corporation, Japan), 3 mM 2-N-morpholino ethane sulfonic acid in 0.6 M mannitol + 13.6 mM $CaCl_2.2H_2O$.

Offprint requests to: S. K. Sen

TABLE 1. Culture media for C. capsularis L. tissue culture and protoplast regeneration

Media	Use	Basal medium	Modification needed over the basal medium		
SC	Seed germination	SH (Schenk and Hildebrandt, 1972)	0.2 Strength of SH inorganics		
			Vitamins	-	Minus,
			Sucrose	-	0.3 M
			Hormones	-	GA_3 - 1.4 µM
APS	Apical shoot culture	B_5 (Gamborg et al., 1968)	0.5 Strength of B_5 inorganics		
			Vitamins	-	Thiamine HCl - 1.2 µM
					Nicotinic acid - 4.1 µM
					Pyridoxine HCl - 1.2 µM
					Ca-Pantothenate - 1.1 µM
					Glycine - 26.6 µM
					Inositol - 0.56 mM
			Sucrose	-	0.04 M
			Hormones	-	IBA - 2.5 µM
					GA_3 - 1.4 µM
K12	Protoplast culture	KM-8p (Kao and Michayluk, 1975)	Vitamins	-	as in APS
			Casamino acid	-	0.25 gm/l
			Coconut milk	-	20 ml/l
			Glucose	-	0.6 M
			Sucrose	-	0.73 mM
			Hormones	-	2,4-D - 4.5 µM
					BAP - 2.2 µM
CP13	Callus proliferation	MS (Murashige and Skoog, 1962)	Vitamins	-	as in APS
			Casamino acid	-	0.4 gm/l
			Sucrose	-	0.09 M
			Hormones	-	NAA - 5.4 µM
					BAP - 2.2 µM
			Agar (Difco)	-	10 gm/l
RM36	Regeneration of somatic embryos	MS (Murashige and Skoog, 1962)	All constitutents are same as CP13 except Casamino acid minus; and hormones in the following manner		
			Hormones	-	2,4-D - 2.3 µM
					NAA - 1.3 µM
					Ad-SO_4 - 0.22 mM
			Amino acid	-	Glutamine - 2.75 mM
			or	-	Proline - 10-15 mM

The pH of the enzyme solution was adjusted to 5.6 before filter sterilization. The incubation condition was similar to the conditions required for cotyledon and hypocotyl tissue sources. The enzyme mixture containing the protoplasts was passed through a 60 µm nylon sieve to remove the undigested tissues. The suspension containing the protoplasts was further centrifuged (100 x g for 3 min) at room temperature. The pellet containing the protoplasts was resuspended in 0.6 M mannitol + 13.6 mM $CaCl_2.2H_2O$. The final purification of the protoplasts was carried out in a 0.58 M sucrose solution through centrifugation (100 x g for 10 min). The protoplasts were washed in the above osmoticum 2 - 3 times through centrifugation (100 x g for 3 min).

Culture of protoplasts: Three basal media (MS, B_5 and KM) with the organic compounds and vitamins modified from KM-8p were tested for suitability in protoplast cultures. In all the media, osmoticum as well as carbon source were maintained using 0.6 M glucose and 0.73 mM sucrose. Various combinations of 2,4-D (0-9 µM), NAA (0-10.7 µM) and BAP (0-8.8 µM) were also tested. Protoplasts were plated at various cell densities (10^3 - 5 x 10^4/ml) and incubated at 28-30°C in the dark.

Viability and cell wall regeneration of protoplasts were tested by fluorescein diacetate (FDA) tests (Widholm, 1972) and Calcofluor white tests (Nagata and Takebe, 1970), respectively. In order to induce sustained cell division, cultures were diluted with fresh medium (1 : 1) in which the osmoticum was reduced from 0.6 M glucose to 0.4 M glucose after 1 week. When small aggregates of dividing cells were obtained, they were plated on K12 medium (Table 1) containing 0.11 M glucose + 0.03 M sucrose and 0.6% Noble agar (AK12) medium. These cultures received 16/8 h light (2500 lux) and dark photoperiod at 26±1°C temperature.

Plating efficiency was measured as the percentage of cells forming microcalli developed on AK12 medium, relative to the total number of protoplasts initially plated.

The microcalli developed on AK12 medium were subcultured to CP13 medium (Table 1) for callus formation. Protoplast derived calli were transferred to different basal media supplemented with various growth regulators to induce differentiation. The cultural conditions were maintained as indicated above for the culture of plated cell colonies. The somatic embryos developed on RM36 medium (Table 1) after 3 weeks from the cell masses. They were transferred to different regeneration media including MS basal medium with half strength of major elements or Whites medium supplimented with various growth regulators including abscissic acid (ABA), sucrose concentration used at 0.09-0.18 M level.

RESULTS AND DISCUSSION

The yield of protoplasts from hypocotyl tissue was low. The hypocotyl protoplasts were more vacuolated and comparatively large in size (Table 2). However, large quantities of viable protoplasts (70-80% as judged by FDA staining) could be obtained from cotyledon (Fig. 1A) and mesophyll cells after enzymatic digestion (Table 2). The mesophyll protoplasts were more stable

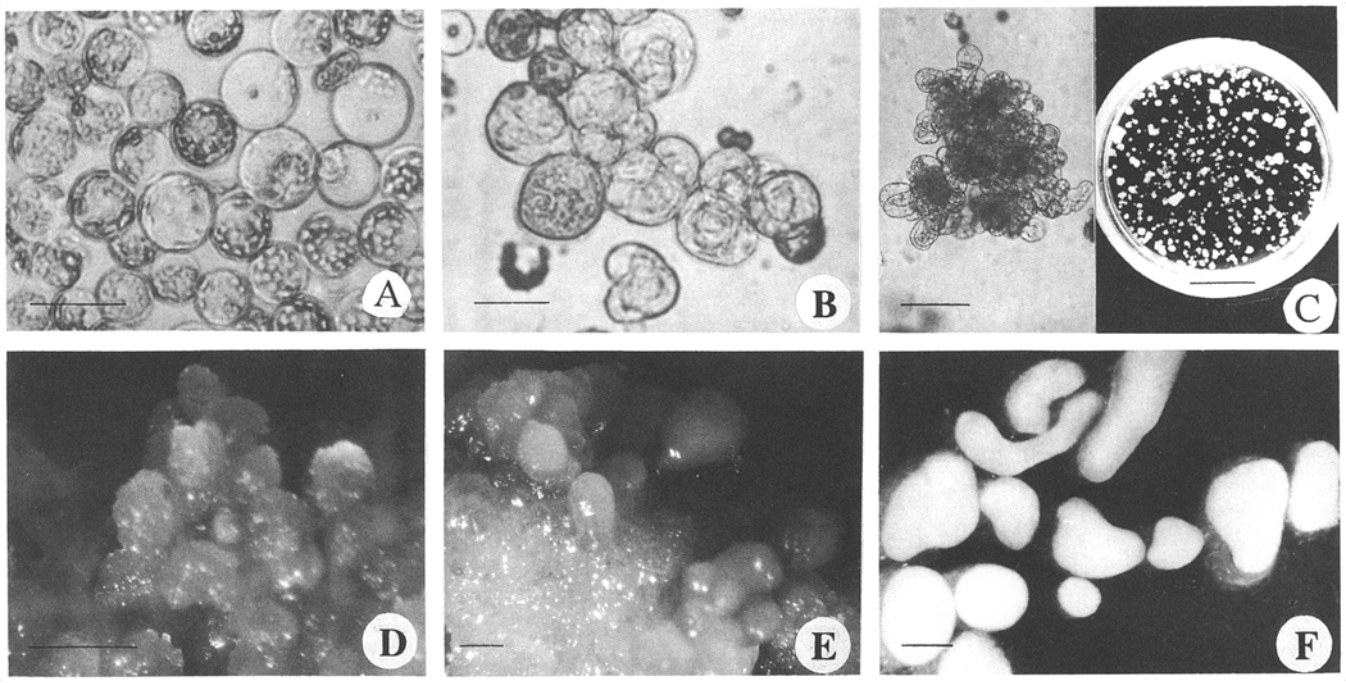

Fig. I. Somatic embryos developed from protoplasts of Corchorus capsularis L. A. Freshly isolated cotyledon protoplasts (Bar = 20 µM). B. Dividing cells derived from protoplasts (Bar = 20 µM). CI. 3-weeks old microcolony (Bar = 20 µM). CII. Small calli developed on semisolid medium (Bar = 10 mm). D. Embryogenic calli proliferation (Bar = 1 mm). E. Somatic embryos developing from embryogenic calli (Bar = 1 mm). F. Isolated somatic embryos in different stages of their development (Bar = 1 mm).

(less likely to bud) than hypocotyl and cotyledon protoplasts. Various cultural conditions were tried for culture of the isolated protoplasts. The cultural conditions necessary for growth of jute protoplasts are presented in Table 1.

The first cell divisions occurred in plated hypocotyl derived protoplasts within 48 h, whereas the first division was observed at day 4-5 in protoplasts obtained from cotyledon and mesophyll cells (Fig. IB). Within the next two days of culture, the second cell divisions took place. Of the various media tested, K12 yielded the maximum cell division responses in protoplasts isolated from different sources (Table 3). The first cell division was observed in 90% of the hypocotyl protoplasts, 72.6% of the cotyledon protoplasts and 63% of the mesophyll protoplasts. 2,4-D (4.5 µM) was found to be suitable in combination with BAP (1.1 µM - 4.4 µM) to induce protoplast division. When comparing different cytokinins, BAP proved to be superior in inducing protoplast division. Although NAA at low levels (0.3 - 0.5 µM) with 2,4-D (4.5 µM) and BAP (2.2 µM) favoured protoplast division, NAA at the 5.4 uM level discouraged protoplast division.

No significant differences in the division frequencies (Table 4) could be observed when protoplasts were cultured at the density level ranging between 10^3-10^4/ ml. When culturing of protoplasts was conducted at the density level of $5x10^4$/ml, mitotic division was not supported. Dilution of the plating density to $1x10^4$ protoplasts/ml was found to be suitable for uninhibited growth.

To induce sustained cell division, protoplast cultures needed replenishment with fresh medium on every 7th day. This was particularly important to dilute out the putative phenolic compounds produced by the cells in culture. Replenishment with a fresh medium, with lowered osmoticum, facilitated cell division. After the formation of microcolonies (Fig. 1C1) within 3 - 4 weeks, the liquid medium for culture was replaced by AK12. At this stage, plating efficiencies of mesophyll, cotyledon and hypocotyl derived protoplasts were found to be around 34%,54% and 78%, respectively.

Table 2. Yield and viability of jute protoplasts obtained from different sources

Source*	Yield/gm of tissue	Viability(%)	Size (µm)
Cotyledon	$8.2x10^6 \pm 0.08x10^6$	81.9±0.9	9.5 - 19
Hypocotyl	$1.4x10^6 \pm 0.43x10^6$	93.2±1.2	15 - 26.5
Mesophyll	$3.5x10^6 \pm 0.06x10^6$	72.1±0.7	10 - 15

*The enzyme treatments for isolation of protoplasts varied depending upon the source (see Materials and methods)

Table 3. The percentage of dividing cells obtained from jute protoplasts of various sources after 5 days of culture in different basal media supplemented with K12 organics and vitamins, unless otherwise stated. The hormone combination remained the same as K12 in all the media tested

Basal medium	Percentage of dividing cells obtained from*		
	Cotyledon protoplasts	Hypocotyl protoplasts	Mesophyll protoplasts
MS	41.9±1.2	65.3±1.7	41.1±1.4
B_5	26.3±1.3	40.8±1.2	not done
KM-8p(K12)	72.6±0.9	90.0±1.1	63.0±1.1
KM-8p+8p organics & vitamins except organic acids	73.4±1.2	86.9±1.5	61.3±1.6

*$1x10^4$ protoplasts/ml were plated to examine the effect of basal media variation

After 3 weeks, growth of small light green calli were observed (Fig. 1CII). They were then transferred to CP13 medium supplemented with 5.4 µM NAA and 2.2 µM BAP for embryogenic callus proliferation

Table 4. The percentage of dividing cells obtained from different sources of jute protoplasts at various plating densities, after 5 days of culture in K12 medium

Source	Percentage of dividing cells at various plating densities (protoplasts/ml)			
	10^3	$5x10^3$	10^4	$5x10^4$
Cotyledon	67.2±1.3	65.0±1.4	68.4±0.9	62.7±1.2
Hypocotyl	87.5±1.6	90.1±1.3	89.3±1.4	82.0±1.6
Mesophyll	58.8±1.5	60.3±1.2	62.1±1.3	59.3±1.7

(Fig. ID). Calli could not, however, be subcultured on the medium for long because they turned friable and non-embryogenic after 3-4 subcultures.

Somatic embryos (Fig. IE & IF) could be generated on differentiation medium from protoplast derived calli within 3 weeks. Various growth media were tested to determine the growth regulator requirement for embryogenesis. In these experiments, 2,4-D (0.5-9.0 µM), NAA (0.5-10.7 µM), Ad-SO_4 (0.11-0.66 mM) and Kn (5.4-21.6 µM) were used in the presence of 2.75 mM L-glutamine or 10-15 mM L-proline. Other auxins (IAA, IBA) and cytokinins (BAP, Zeatin) were also tested. When combinations of 2,4-D in the range of 2.3-4.5 µM) and 0.22-0.44 mM Ad-SO_4 were used in modified MS medium, a large number of light yellowish somatic embryos formed within 3 weeks (Table 5), while the non-embryogenic tissues turned brown. Somatic embryos could be obtained routinely in a medium where 2,4-D at the concentration of 2.3 µM was used in combination with 3.3 µM Kn. These embryos were mostly globular in their developmental stage and large in size. However, they had the tendency to form callus on transfer to fresh medium for germination. A low concentration of NAA (1.3 µM) in the presence of 2,4-D (2.3 µM) and Ad-SO_4 (0.22 mM) in the medium (RM36) improved somatic embryo formation. Up to 71% of the cultures could be induced to form somatic embryos (Table 5). No detectable difference was observed between L-glutamine and L-proline on somatic embryo formation when present in the regeneration media. On various embryo maturation media, these somatic embryos developed precocious roots and some times callus in the apical region.

Table 5. Percentage of somatic embryogenesis in callus, derived from jute protoplasts (hypocotyl and cotyledon sources) with different growth regulators in modified MS medium with 2.75 mM glutamine. Observations were taken three weeks after the callus were transferred, using 50 protoclones of uniform size per treatment

Combinations of growth regulators	Percentage of protoclones formed somatic embryos*
2,4-D(4.62 µM)+Ad-SO_4(0.44 mM)	69.1±1.3
2,4-D(2.26 µM)+Ad-SO_4(0.22 mM)	57.3±0.8
2,4-D(2.26 µM)+NAA (1.34 µM)+ Ad-SO_4(0.22 mM)	71.0±1.0
2,4-D(2.26 µM)+Kn(3.25 µM)	49.2±1.2

*Number of embryos developed from each colony ranged between 10-15

Progress in the application of plant tissue culture methodologies for jute has not yet been attained. The jute tissues and cells in culture have long been considered to be extremely recalcitrant. In spite of intensive efforts in the past by a few laboratories including ours, progress has been very difficult to attain. The bast fibre producing tissue systems in culture behaved quite differently from many of the known plant systems. The necessary cultural conditions for induction of morphogenesis in cultured tissues have till now not been possible to ascertain. We have observed infrequent plant regeneration from hypocotyl tissues either through organogenesis or via somatic embryogenesis after pretreatment of germinating seedlings with high levels of cotykinin (unpublished). This, however, turned out to be inadequate for utilisation in a genetic engineering programme. Keeping this in view, the present success in generating somatic embryos from cells derived from protoplasts is significant.

ACKNOWLEDGEMENTS

Thanks are due to UNDP/FAO (Project No. IND/017/87) for financial assistance to SKS and to CSIR, Government of India for fellowship grant to TS (Award No. 9/15/(36)/87-EMR-I).

REFERENCES

Austin S, Baer MA, Helgeson JP (1985) Transfer of resistance to potato leaf roll virus from Solanum brevidens into Solanum tuberosum by somatic fusion. Plant Sci 39: 75-82

Bhaduri PN, Bairagi P (1968) Interspecific hybridization in jute (Corchorus capsularis x C. olitorius). Sci and Cult 34: 355-357

Gamborg OL, Constabel F, Miller RA (1970) Plant cell cultures. I. Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151-158

IBPGR (1988) Annual Report of International Board of Plant Genetic Resources, FAO, Rome, pp 21

Islam AS, Rashid A (1960) First successful hybrid between the two jute yielding species, Corchorus capsularis and C. olitorius. Nature 185: 258-260

Islam AS, Imdadul Hoque M, Haque MM (1987) Prospects of utilizing interspecific hybrids in jute for commercial purpose. Phytobreedon 3(1): 26-38

Kao KN, Michayluk MR (1975) Nutritional requirements for growth of Vicia hajastana cells and protoplasts at a very low population density in liquid media. Planta 126: 105-110

Kumar PM, Das K, Sinha RR, Mukherjee P, Sen SK (1983) Interspecific somatic protoplast fusion products in cultivated jute species. In: Sen SK, Giles KL (eds) Plant Cell Culture in Crop Improvement, Plenum Press, New York, pp 237-247

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

Nagata T, Takebe I (1970) Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta 92: 301-308

Schenk RU, Hildebrandt AC (1972) Medium and techniques for growth of monocotyledonous and dicotyledonous plant cell culture. Can J Bot 50: 199-201

Schieder O (1982) Somatic hybridization : a new method for plant improvement. In: Vasil IK, Scowcroft WR, Frey KJ (eds) Plant improvement and somatic cell genetics, Academic Press, New York, London, pp 239-253

Swaminathan MS, Iyer RD, Sulbha K (1961) Morphology, cytology and breeding behaviour of hybrids between Corchorus olitorius and C. capsularis. Curr Sci 30: 67-68

Widholm JM (1972) The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Technol 47: 189-194