Plant Cell Reports (1992) 11:101–104

Plant Cell Reports

Micropropagation of an elite Darjeeling tea clone

Timirbaran Jha and S.K. Sen

Plant Molecular & Cellular Genetics, Bose Institute P-1/12, C.I.T. Scheme VII-M, Calcutta 700 054, India

Received August 20, 1991/Revised version received January 3, 1992 – Communicated by G.C. Phillips

ABSTRACT

Shoot cultures of Camellia sinensis (L.) O. Kuntz var. T-78, an elite Darjeeling tea clone, were established from cotyledonary nodes and shoot tips of germinated seedlings as well as from nodal explants of field grown plants. Shoot multiplication rate ranged from 4x in nodal explants to 35x in cotyledonary nodes after 18 weeks of culture. Rooting was achieved in 80-90% micro-shoots by either placing them on an inductive medium for 10 d and then transferring shoots to hormone-free medium, or by treating micro-shoots with a chronic dose of IBA (500 mg/l) for 30-40 min. Rooted plants were established in soil under glasshouse condition at 60% frequency after hardening phase of 4-6 weeks. The regenerated plants show a constant chromosome number of 2n=30 and are morphologically true to type. This procedure can be applied for conservation and utilisation of an elite clone of Darjeeling tea.

INTRODUCTION

Tea is an important commercial crop. Darjeeling tea is valued for its quality in the world beverage market. It, unlike the Assam type, is characterised by its flavour, which is acclaimed worldwide. The genetic variability for flavour and quality is rich in and around the Darjeeling district. The maintenance, multiplication and utilisation of these germplasms for higher production is desirable.

Tissue culture studies in tea have been mainly reported from Taiwan, Japan and Sri Lanka (Wu et al., 1981; Kato, 1986, 1989; Arulpragasam and Latiff, 1986). There are few reports on tissue culture studies on tea varieties cultivated in India. Induction of callus in pollen was reported by Raina and Iyer (1974). Raina and Iyer (1983) also reported callus production in a Chinese hybrid. Subsequently, Phukan and Mitra (1984) reported shoot bud regeneration from nodal callus, but no rooting was reported. Banerjee and Agarwal (1990) recently reported in vitro rooting in C. sinensis var. TV_1. To our knowledge, there are no reports on in vitro propagation of Darjeeling tea. The present communication describes the in vitro culture of Darjeeling tea for propagation of an elite Darjeeling tea clone from the planters' field.

Offprint requests to: T. Jha

MATERIALS AND METHODS

In vitro cultures were established from mother bushes of Camellia sinensis (L.) O. Kuntz (Chinese type) variety T-78, growing in a nursery of Goomtee Tea Garden, Darjeeling, West Bengal. Twigs were collected during different seasons of the year. Nodal stem explants and shoot tips were surface sterilized in 0.1% mercuric chloride for 25 min and then rinsed several times in sterilized distilled water.

Green and mature seed capsules were collected during July to December from the mother bushes. Seeds were surface sterilized in 0.2% mercuric chloride for 45 min, washed five times with sterilized distilled water, dipped in 70% alcohol and flamed. Seeds germinated in full strength Murashige and Skoog's (1962) basal medium (MS) within 8 weeks. Two types of explants were excised from germinated seedlings, shoot-tips (0.5 cm long) and cotyledonary nodes. Explants were cultured on half-strength MS medium supplemented with 3% sucrose, thiamine-HCl (1 mg/l), nicotinic acid (1 mg/l), pyridoxine-HCl (0.5 mg/l), folic acid (0.4 mg/l), biotin (0.5 mg/l), riboflavin (0.05 mg/l), calcium pantothenate (1 mg/l), p-aminobenzoic acid (0.2 mg/l) with or without N_6-benzyladenine (BA, 0.5-10 mg/l), indole-3-butyric acid (IBA, 0.1-500 mg/l), kinetin (0.5-10 mg/l), coconut milk (CM, 5-15% v/v) and casein hydrolysate (enzymatic, 500-1000 mg/l). The pH of the medium was adjusted to 5.6 ± 0.2 before autoclaving. IBA (100-500 mg/l) used for root induction was filter sterilized. The medium was gelled with 8 g/l agar and sterilized for 15 min at 1.05 kg/cm^2 pressure. Thirty cultures per treatment were grown at 25 ± 2°C under 16 h light period of 3000 lux.

Regenerated shoots with well developed roots were transferred to a mixture of soil and peat (1:1) and maintained under high relative humidity (80-90%) for the first four weeks and then established in soil under glasshouse condition.

For chromosome counts root-tips of in vitro raised plants were pretreated in 0.05% colchicine for 3 h at 12-14°C and fixed in Carnoy's fixative overnight. Root-tips were stained and squashed using aceto-orcein. Chromosome counts were made from root-tips of 20 in vitro raised plants.

RESULTS AND DISCUSSION

Shoot proliferation in nodal stem explants :

Nodal stem explants obtained from the mother bush suffered heavily due to contamination and often it was quite difficult to obtain contamination-free cultures. However, the aseptic cultures developed axillary buds within 4 weeks in medium containing half-strength MS inorganics with CM (10% v/v), casein hydrolysage (1000 mg/l), BA (5 mg/l) and IBA (0.1 mg/l). The explants produced a little callus at the base, and each axillary bud elongated (2.45 ± 0.33 cm) and produced 4-5 new axillary buds within 18 weeks (Fig. 1) in this medium. Each axillary bud could then be recultured to proliferate following the same method. The use of kinetin (0.5 - 10 mg/l) in the medium failed to induce any response. Increase or decrease in levels of BA (1, 2, 10 mg/l) did not improve axillary bud development in nodal explants (Table 1). Shoot-tips (0.5-1 cm) collected from mother bushes could not be grown in any cultural conditions tried.

Induction of multiple shoots from shoot-tips and cotyledonary nodes :

Capsules collected from the mother bush during July to November showed differences in response with respect to seed germination, and axillary bud development in cotyledonary node explants. Seed germination from green capsules collected during July-August was 100%, and 80-90% of cotyledonary nodes from such seedlings showed axillary bud development in culture. On the other hand, only 30-40% seeds germinated when capsules were collected during September to November, and 30-40% of cotyledonary nodes from such seedlings showed axillary bud development in culture.

Two types of explants were used from germinated seedlings : Cotyledonary nodes and shoot-tips (0.2-0.5 cms) excised from two month old germinated seedlings. Shoot-tips were cultured for induction of multiple shoots using half-strength MS medium containing BA (1-10 mg/l) alone or in combination with IBA (0.1-0.5 mg/l), CM (10% v/v) and casein hydrolysate (500-1000 mg/l). No response was obtained in hormone free basal medium. BA (1 mg/l) in combination with IBA (0.1 mg/l), CM (10%) and casein hydrolysate (1000 mg/l) produced the largest number of shoots (8.45 ± 0.35) after 18 weeks (Fig. 2, Table 1). Increasing the level of BA (2, 5, 10 mg/l) or IBA (0.2 mg/l) did not yield a comparable response. Shoot cultures were maintained in this medium and subcultured every 6 weeks.

Cotyledonary node explants were cultured for induction of multiple shoots using half-strength MS medium and BA, IBA, CM and casein hydrolysate. Cotyledonary nodes did not show any response in the media found best for shoot proliferation in nodal stem explants obtained from mother bushes or in shoot-tip explants obtained from germinated seedlings (Table 1). A high level of BA is required to induce multiple shoots in cotyledonary node explants. While multiple shoots could be induced in presence of BA (10 mg/l) and IBA (2 mg/l), addition of CM (10%) and casein hydrolysate (1000 mg/l) increased the number of shoots per explant and enhanced the growth of the shoots (Table 1). It was concluded that half-strength MS medium in combination with BA (10 mg/l), IBA (2 mg/l), CM (10%) and casein hydrolysate (1000 mg/l) produced the optimal number of shoots (35.12 ± 0.18) and better growth (2.54 ± 0.44 cm) in 18 weeks. This multiplication rate was maintained for over two years by repeated excision and reculture of elongated shoots using the same medium (Figs. 3 & 4).

Direct organogenesis in hypocotyl explants :

Hypocotyl segments from one month old germinated seedlings were cultured on half-strength MS medium supplemented with BA (10 mg/l), IBA (0.5 mg/l) and CM (10%), and shoot bud induction was observed directly from 5-10% of explants. The shoot buds developed into the two leaf stage but then ceased further growth.

Rooting of micro-shoots and establishment in soil :

Micro-shoots (2-2.5 cm long) produced from the various explants were excised and placed on MS medium (full strength or half strength) supplemented with various concentrations of IBA for different periods of time for induction of roots. No rooting was induced in unsupplemented MS medium (full or half strength) or medium supplemented with 1-4 mg/l of IBA. When micro-shoots were treated for 10 d on MS medium with 100 mg/l IBA, 80-90% of them produced roots on transfer to hormone-free full strength MS medium. It was also noted that when the base of micro-shoots were soaked for 30-40 min in 100 mg/l of filter-sterilized IBA, only 20-30% of shoots produced roots; while treatment with 500 mg/l of filter sterilized IBA for 30-40 min induced roots in 80-90% of the micro-shoots. Rooted shoots were transferred to half-strength MS medium for 6-8 weeks for growth of shoots and roots (Fig. 5).

The rooted plants were further transferred to one-fourth strength MS medium for 4 weeks, and plants with well developed root systems were transferred to pots containing a mixture of peat and soil (1:1) and hardened in the glasshouse. Nearly 60% of the plants survived (Fig. 6) and are morphologically similar to the donor plants. Cytological analysis of parental and 20 randomly selected *in vitro* raised plants revealed a chromosome number of 2n=30, indicating a stable nature.

The present experiments demonstrate a procedure for propagation of an elite Darjeeling tea clone from nodal stem explants, seedling shoot-tips, and most significantly from cotyledonary node explants. A large number of shoots were regenerated using cotyledonary node explants in an elite Darjeeling variety of *C. sinensis*, which has not been reported earlier. Cotyledonary node tissue has been found to be useful for regeneration in large seeded legumes (Wright *et al.*, 1986; McClean and Grafton, 1989), but cotyledonary nodes have not been used as an explant source for *in vitro* propagation in any variety of tea earlier.

Differences in genotypic responses to root initiation in tea varieties have been reported (Seneviratne *et al.*, 1988). IBA is the most commonly used auxin for root induction in *in vitro* shoots in tea, but the concentration and mode of treatment of IBA differed in different varieties (Kato, 1985; Seneviratne, 1988; Banerjee and Agarwal, 1990). In the present study, pretreatment of shoots with 500 mg/l of filter sterilized IBA for 30-40 min induced roots in 80-90% of cultures within 4 weeks. Regenerated plants have been found to be stable with respect to morphology and cytology and are similar to parental plants. The present procedure offers an

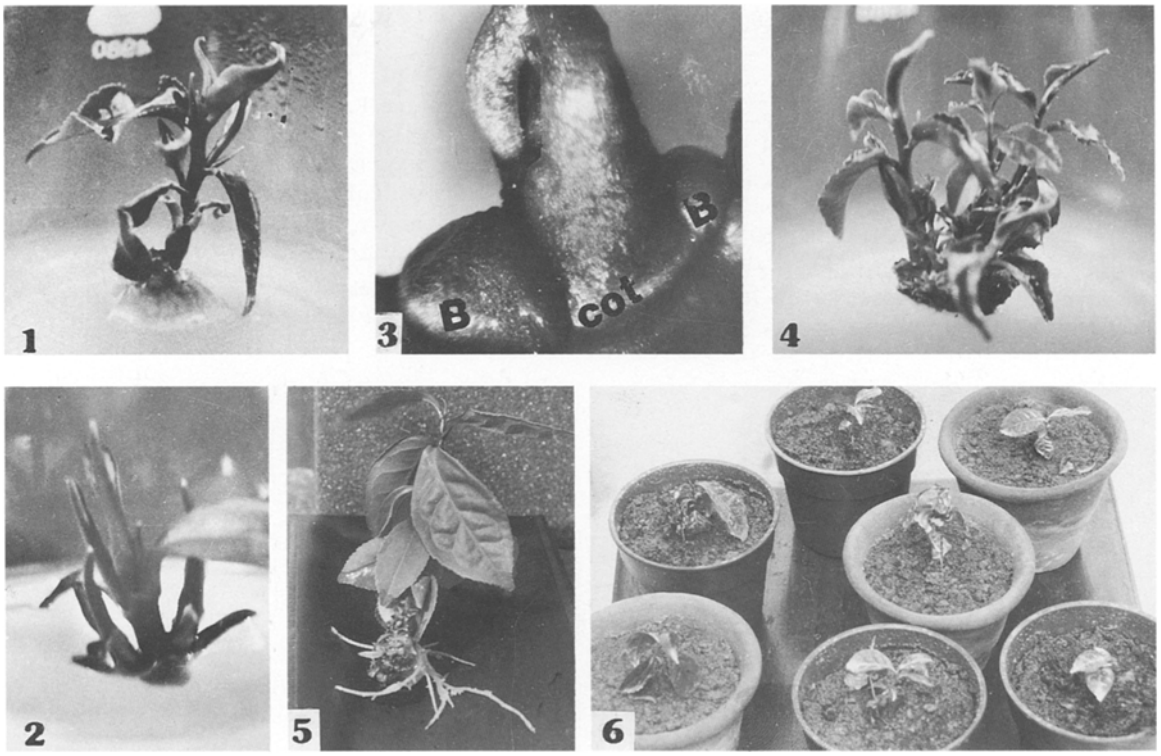

Fig. 1 - Production of 4-5 fresh axillary bud from single nodal explants (X0.5). **Fig. 2** - Proliferation and multiplication of shoot-tip explant. **Fig. 3** - Induction of new buds from cotyledonary node (Cot = Cotyledon, B = new buds X30). **Fig. 4** - Multiple elongated shoots induced from cotyledonary node. **Fig. 5** - Rooted shoot (X0.5). **Fig. 6** - One month old potted plants (X0.5).

Table 1. Influence of growth regulators on number and height of micro-shoots produced from three types of explants in C. cinensis var. T-78 after 18 weeks of culture.

Treatment Half-strength MS medium +				Mean number* of micro-shoots per			Mean height* of micro-shoots (in cm)
BA (mg/l)	IBA (mg/l)	CM (% V/V)	Casein hydrolysate (mg/l)	Seedling shoot-tip explant	Cotyledonary node explant	Nodal stem explant	
0	0	0	0	1±0	-	-	-
1	0.1	0	0	1±0	-	-	-
1	0.1	10	1000	8.45±0.35	-	-	1.81±0.28
2	0.1	10	1000	6.31±0.27	-	-	1.60±0.24
5	0.1	0	0	1±0	-	2.0±0	0.26±0.11
5	0.1	10	1000	1±0	-	4.4±0.56	2.45±0.33
10	2	0	0	1±0	14.28±0.16	-	2.12±0.45
10	2	10	1000	1±0	35.12±0.18	-	2.54±0.44

* Mean number of 30 replicates ± S.E.
- = No response

effective strategy for the conservation of an elite variety of Darjeeling tea.

ACKNOWLEDGEMENT

TJ gratefully acknowledges financial assistance from D.S.T., Govt. of India and Goomtee Tea, Darjeeling for plant materials.

REFERENCES

Arulpragasam PV, Latiff R (1986) S L J Tea Sci 55 : 44-47

Banerjee M, Agarwal B (1990) Ind J Exp Biol 28 : 936-939

Kato M (1986) Japan J Breed 36 : 31-38

Kato M (1989) In : Biotechnology in Agriculture & Forestry. Bajaj YPS (ed), Springer-Verlag - Berlin - Heidelberg Vol 7, p 82-98

Murashige T, Skoog F (1962) Physiol Plant 15 : 473-497

McClean P, Grafton KF (1989) Plant Sci 60 : 117-122

Nakamura Y (1988) Proceedings Int Sym Tea Experimental Station, Taiwan : 63-74

Phukun M, Mitra GC (1984) Current Sci 53 : 874-876

Raina SK, Iyer RD (1974) Ind J Genet 34A : 283-286

Raina SK, Iyer RD (1983) J of Plantation Crop (Suppl) : 63-67

Seneviratine P, Latiff R, Arulpragasam PV (1988) S L J Tea Sci 57 : 161-169

Vieitez MA, Barciela J (1990) Plant Cell Tissue Organ Cult 21 : 267-274

Wu C, Huang T, Chen W, Chen S (1981) Proc COSTED Symp, Singapore : 110-112

Wright MS, Koehler SM, Hinchee MA, Carnes MG (1986) Plant Cell Reports 5 : 150-154