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In vitro clonal propagation of tea (*Camellia sinensis* (L.) O. Kuntze)

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Abstract

A system for *in vitro* clonal propagation has been develcped in tea plants. Shoots obtained from primary explants were induced from terminal buds and axillary buds of mature field-grown plants. Cultures were initiated from both types of explants on Murashige and Skoog (MS) medium supplemented with 10% coconut milk (CM), 200 mg l^{-1} of yeast extract (YE), 1.4 μM indoleacetic acid (IAA) and 17.8 μM benzyladenine (BA). The shoot tips were multiplied on 1/2 strength MS medium containing 10% CM, 2.9 μM IAA and 17.8 μM BA. The larger shoots were separated after multiplication and rooted on 1/2 MS medium supplemented with 11.4 μM ascorbic acid and 34.5 μM indolebutyric acid (IBA). A pretreatment of the plants with an aqueous solution of 493 μM IBA greatly increased the frequency of rooting. More than 60% of the rooted plants have been transferred to soil successfully.

Abbreviations: BA – benzyladenine, IAA – indoleacetic acid, IBA – indolebutyric acid, YE – yeast extract, CM – coconut milk, MS – Murashige and Skoog medium (1962)

Introduction

Tea is the single most important cash crop of India. Like many other woody species, it has traditionally been propagated vegetatively because it is highly heterozygous and seedlings are too variable for commercial use. A suitable micropropagation system would eliminate many of the seasonal difficulties associated with the rooting of stem cuttings that is currently the main method of propagation.

There are a number of reports on the micropropagation of tea (Arulpragassam & Latiff 1986; Arulpragassam et al. 1988; Kato 1985, 1986; Seneviratne et al. 1988) and remarkable differences in response have been reported for different genotypes (Samartin 1989). No work has so far been done on micropropagation of an elite Indian tea cultivar. Thus, the aim of this work was to develop a micropropagation system for the cultivar TV_1, one of the most important commercial cultivars grown in Darjeeling.

Materials and methods

Explant

Explants were terminal bud and nodal stem segments (both 0.7–1 cm in size) from the first 3–4 nodes of actively growing shoots from field-grown plants of cv TV_1 in Darjeeling district. The explants were washed in running tap water for 1 h followed by 5% Teepol (British Drug House) for 5 min, disinfested with 0.1% mercuric chloride (W/V) for 15–18 min and washed with sterile distilled water 5 times to remove the sterilant before use.

Initiation of culutre (Stage I)

The medium for initiation of culture (M_1) consisted of the mineral nutrients of Murashige & Skoog (1962) along with the following amendments: thiamine hydrochloride 1 μM, pyridoxine hydrochloride 1.5 μM, nicotinic acid 12.2 μM, folic acid 0.7 μM, riboflavin 0.13 μM, calcium pantothenate 2 μM and biotin 0.2 μM, CM 10% (V/V), YE 200 mg l^{-1} and sucrose 30 g l^{-1}. Various growth regulators were used including IAA, IBA, BA and kinetin.

Multiplication of shoot-tips (Stage II)

Shoot-tips (about 1–1.5 cm in length) obtained from primary explants were used for multiplication of shoots. The media for multiplication (M_2) consisted of 1/2 strength MS mineral nutrients together with similar additives and growth regulators described in M_1 excepting yeast extract.

Rooting of shoots (Stage III)

The shoots (3–4 cm in size) were transferred to rooting medium (M_3) consisting of 1/2 strength MS mineral nutrients along with modified MS vitamin (Kato 1985), ascorbic acid 11.4 μM, calcium pantothenate 2 μM, biotin 0.4 μM, sucrose 40 g l^{-1} and various concentrations of NAA, IAA and IBA.

Cultural conditions

Unless otherwise stated, all media were adjusted to pH 5.6 with 1 N KOH or 1 N HCl as required, solidified with 0.9% agar (Hi Media) and sterilized at 121°C under 103 kPa pressure for 15 min. Liquid cultures, with or without filter paper bridges, were also tried. Primary explants were placed in culture tubes (15 cm × 2.5 cm diameter) each containing 20 ml of solid media and capped with a cotton plug. Later, shoots and whole plants were transferred to 250 ml Erlenmeyer flask or bottles, each containing 80 ml of solid or liquid medium and capped with a cotton plug or aluminium foil respectively. All cultures were maintained at 25 ± 1°C, 55–60% relative humidity, under Philips fluorescent day light tubes emitting about 60 μmol $m^{-2}s^{-1}$, with a 16-h photoperiod and uninterrupted dark period. Subcultures were made after 30–45 days as required. Each experiment consisted of 10 replicates and successful results were confirmed after repeating at least 3 times. Evaluation was made on the basis of nature of response, frequency of response and time taken for the same.

Results

Contamination has been reported to be a major difficulty in establishing shoot cultures of tea (Arulpragassam & Latiff 1986), but with the above mentioned technique only 5–10% of the primary explants were contaminated.

Stage I

Initiation of growth of primary explants depended mainly on cytokinin used. Kinetin could initiate growth but could not sustain it. When various concentrations of kinetin ranging from 4.44 μM–44.4 μM were used, explants elongated but did not multiply. Moreover explant growth ceased despite regular subculture. A combination of IAA and BA was found to be best for both growth of the explant and multiple shoot formation. Initiation of growth of both terminal and axillary buds was best obtained on M_1 along with 1.4 μM IAA and 17.8 μM BA (Table 1). It started within 10–15 days of inoculation regardless of the type of explant and consisted of a little elongation of the explant and proliferation of leaves and very little multiplication in some cases showing a maximum of 2 shoots/explant (Fig. 1).

Stage II

When the shoots were 1–1.5 cm long 30–35 days after inoculation, they were transferred to M_2 medium containing 2.9 μM IAA and 17.8 μM BA (Table 2) (M_2A). After 30–35 days of transferring the shoots to M_2A, an average of 8–10 shoots/explant was obtained (Fig. 2). These

Table 1. Effect of growth regulators on shoot development *in vitro* from terminal and axillary bud on MS medium supplemented with YE (200 mg l^{-1}) and CM (10%) after 25 days (results are mean of 30 replicates ± S.E.)

Concentrations (μM)		Frequency of explant response (%)		No. of shoots per explant	
IAA	BA	Terminal bud	Axillary bud	Terminal bud	Axillary bud
1.4	4.4	20 ± 0.4	20 ± 0.9	1 ± 0.04	1 ± 0.06
1.4	8.9	27 ± 0.9	23 ± 0.8	1 ± 0.05	1 ± 0.06
1.4	13.3	40 ± 0.8	43 ± 0.8	2 ± 0.06	1 ± 0.07
1.4	17.8	60 ± 0.7	60 ± 0.8	3 ± 0.05	2 ± 0.03
2.9	4.4	23 ± 0.4	23 ± 0.7	1 ± 0.05	1 ± 0.07
2.9	8.9	30 ± 0.8	27 ± 0.4	1 ± 0.06	1 ± 0.07
2.9	13.3	33 ± 0.6	37 ± 0.7	2 ± 0.05	1 ± 0.06
2.9	17.8	47 ± 0.8	40 ± 0.6	2 ± 0.03	2 ± 0.04

Table 2. Effect of growth regulators on multiple shoot formation from terminal and axillary buds after 30 days of transferring them on 1/2 strength MS medium supplemented with 10% CM (results are mean of 30 replicates ± S.E.)

Concentrations (μM)		No. of shoot-tips per explant	
IAA	BA	Terminal	Axillary
1.4	4.4	3 ± 0.19	3 ± 0.08
1.4	8.9	3 ± 0.04	3 ± 0.04
1.4	13.3	4 ± 0.06	3 ± 0.04
1.4	17.8	5 ± 0.05	6 ± 0.06
2.9	4.4	3 ± 0.08	2 ± 0.06
2.9	8.9	4 ± 0.03	2 ± 0.07
2.9	13.3	6 ± 0.11	4 ± 0.07
2.9	17.8	10 ± 0.24	8 ± 0.21

shoots (1–1.5 cm long), when transferred to M_2 medium with 1.4 μM IAA and 4.45 μM BA, started elongating showing profuse vegetative growth. After another 30–35 days (i.e. 90–105 days after inoculation of primary explant) when the larger shoots were excised and the stock was subcultured to M_2A again, a new crop of shoots was obtained. The stock retained shoot proliferation capacity for 30 months after which this capacity started declining.

Providing the shoots are regularly removed, one initial explant gives rise to 10 well-developed shoots with good rooting capacity in each successive subculture. Nevertheless, the total number of buds and shoots obtained at various subcultures was much higher depending on whether the explant was subcultured as a whole or divided into small parts. Removal of shoots and division of the stock stimulated proliferation of more buds.

Stage III

Ninety to 105 days after first inoculation, shoots about 3–4 cm long were transferred to rooting medium M_3 on which rooting was best initiated with 34.5 μM of IBA. Rooting started within 30 days. Dipping the cut ends of the shoots in an aqueous solution of 493 μM IBA for 30 min before inoculation increased the frequency of rooting and decreased the time taken for initiation. Immediately after rooting started, the plants were transferred to 1/2 strength MS medium without growth regulators where profuse growth of both root and shoot system was observed (Fig. 3).

Stage IV

After 30 days in 1/2 strength MS basal medium the rooted plants were transferred to a sterile mixture of peat and soil (1:1) in bottles, irrigated with sterile water and kept aseptically under the same cultural conditions described earlier. After 30 days, they were transferred to a similar mixture in pots and kept in a greenhouse (temperature 25 ± 1°C and humidity 85–90%). The frequency of survival was 60% or more (Fig. 4).

Discussion

Regeneration of whole plants of tea has earlier been reported from cotyledons (Kato 1986; Arulpragassam et al. 1988) and callus (Wu et al. 1981; Kato 1985). The only work on an Indian

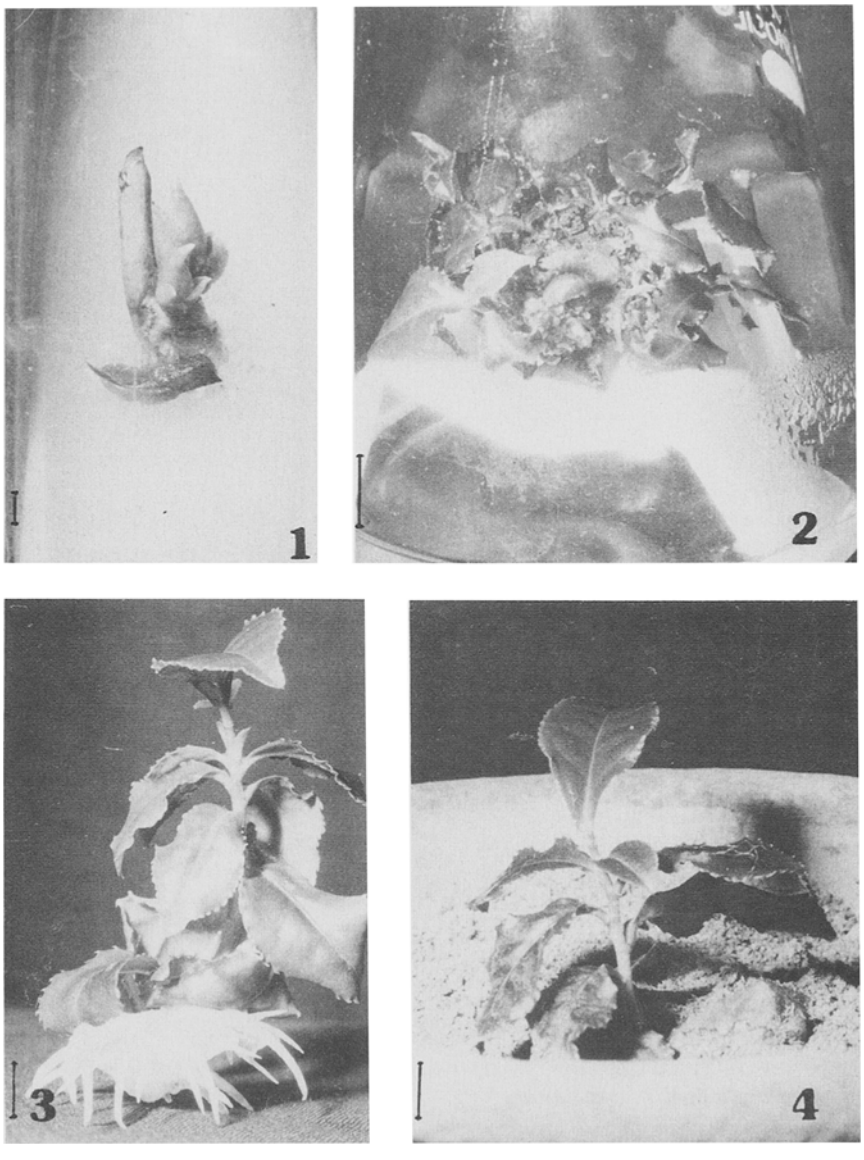

Fig. 1. Proliferation of shoots from axillary bud 30 days after inoculation.

Fig. 2. Development of multiple shoots from a single shoot-tip obtained from primary explant of tea.

Fig. 3. A whole plant of tea showing well-developed root and shoot system.

Fig. 4. A plantlet 60 days after potting. Bar = 1 cm.

variety of tea has been carried out by Phukan & Mitra (1984) but they could not induce whole plants from shoot-tip or nodal explants. Sarwar (1985) also tried to induce whole plants from similar explants without success. However, there is only one report of induction of shoots from shoot-tips (Arulpragassam & Latiff 1986) and rooting of proliferated shoots thereafter (Seneviratne et al. 1988). But, in contrast to their reports, contamination as well as browning of explants due to excretion of phenolic compounds was completely eliminated following our procedure. Also an 8–10 fold multiplication of shoots was observed in contrast to 2–4 fold

multiplication by them (Arulpragassam & Latiff 1986). Unlike earlier reports (Kato 1985; Seneviratne et al. 1988) dipping of the plants in IBA solution before inoculation on rooting medium was found to be the single most important factor for the induction of rooting. Hardening of the micropropagated plants as well as transferring them to field was found to be the most difficult of the four stages of micropropagation.

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