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COMMERCIALIZATION OF CLONAL MICROPROPAGATION OF SUPERIOR TEA GENOTYPES USING TISSUE CULTURE TECHNOLOGY

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EXECUTIVE SUMMARY

Commercialization of Clonal Micropropagation of Superior Tea Genotypes using Tissue Culture Technology

Tea (*Camellia sinensis L.*) is an important estate crop cultivated in Indonesia, India, China, Kenya and Sri Lanka. Most of the existing tea plantations are over 50 years old, with current productivity far below optimum. Thus, their is an urgent need for rejuvenation through adoption of superior tea clones. Conventional vegetative propagation of tea using single leaf-bud cuttings is considered technically inefficient for large-scale production of superior planting materials. An alternative method of rapid clonal propagation by tissue culture technology was being developed in this project. Two experimental approaches have been used to achieve this objective, namely plant multiplication through enhancement of axillary branching *in vitro* and via somatic embryogenesis.

In vitro proliferation of tea shoot was achieved by culturing sterilized single leaf-bud cuttings of clone TRI 2025 in woody plant (WP) basal media supplemented with 6-benzylaminopurine (BAP) or thidiazuron (TDZ), both in combination with indole-3-acetic acid (IAA). Shoot multiplication at 4.1-fold increase after every 6 weeks was attained in media supplemented with 10 uM BAP. Higher multiplication rate at 5.4-fold increase could be achieved in a similar medium provided with 5 ug/l TDZ. Micropropagated shoots were recultured in WP media containing either 2-10 uM GA_3 or 5-10 uM IAA to promote uniform shoot elongation prior to rooting treatment. Rhizogenesis was induced by subjecting the tea shoots first to 3-5 mg/l NAA for 7 days in the dark, followed by transfer of the shoots to an auxin-free medium in the light. Under such treatment, about 69-83% of the shoots rooted.

Plantlet regeneration via somaticembryogenesis has been accomplished in seed cotyledon cultures. Callus formation occured at a high frequency in all cultures treated with 10-20 mg/l 2,4-D. Embryogenic calli were observed after 6 weeks in modified MS basal media supplemented with 20 mg/l 2,4-D. About 30% of the embryogenic calli developed into somatic embryos. Plantlet regeneration was achieved after subculturing to a regeneration medium without 2,4-D.

Compact and globular calli were obtained from *in vitro* leaf explants cultured in MS basal media containing 2-20 mg/l putrescine. While the globular calli showed an organized growth pattern, no structures were found to resemble any of the stages in somatic embryogenesis.

Due to the diffilculties in inducing somatic embryogenesis from leaf calli, an attempt has been made using *in vitro* shoot tips and axillary buds as alternative sources of explants. Compact and nodular calli were formed from both types of explants in B5 basal media supplemented with 2 mg/l IBA alone or in combination with 3 mg/l BAP a frequency of 20% and 50% respectively. Histological observations of the nodular calli indicated evidence of early stages of somatic embryogenesis. Although the frequency of embryogenesis was very low, it was a significant observation as no known reports had been cited in the literature on the embryogenesis in mature tea tissues.

In light of the aforementioned accomplishments, it can be concluded that the primary objectives of this research projects have been achieved.

Collaboration between IBRIEC and Plantek has been benefial in that the exchange of scientific and technical information has been useful to both parties. Through this project, the R & D capabilities of both institutions have been strengthened.

RESEARCH OBJECTIVE

Tea (*Camellia sinensis* L.), being a natural outbreeder, produces seedling progenies of high heterogeneity. Thus, a method of vegetative propagation is highly desirable to maintain in selected genotypes the important characters such as yield potential, disease resistance and manufacturable leaf quality. Conventionally, tea is vegetatively propagated by single leaf-bud cuttings. However, this method is inefficient for mass production of planting materials for replanting.

In light of such limitation, an alternative method for rapid propagation by tissue culture technology is sought. In vitro propagation of tea plants has been generally accomplished through shoot regeneration from nodal explants (Phukan & Mitra, 1984; Arulpragasm & Latief, 1986). In this research project, however, our experimental approach to tea micropropagation involved (i) shoot multiplication from axillary buds excised from their nodal explants, and (ii) plant regeneration from leaf callus culture through somatic embryogenesis. Since previous attempts have not been made to

study the potential of such a system, this would be a novel and innovative approach for tea crop improvement.

METHODS AND RESULTS

1. Shoot Multiplication via Enhancement of Axillary Branching

1.1. Disinfection of Plant Materials

Axillary buds from softwood cuttings of tea clone TRI 2025 were obtained from tea shrubs grown in the plantation, and explanted for culture. In order to avoid a high contamination rate, a sterilization protocol was developed for surface disinfection of the field-derived plant materials. Optimal decontamination of nodal explants was achieved, following a sequence of disinfection steps using 0.5% calcium hypochlorite (15 min), 0.2% benlate (15 min), 0.2% tetracycliin (20 min) and 0.1% mercuric chloride (10 min). All these steps were carried out in a laminar flow cabinet under partial vacuum.

Applying the disinfection method as described above, nearly 60% of the buds cultured in vitro were visibly free from contaminants (Table 1). Bacterial and fungal contaminants accounted respectively for 14% and 9% of culture losses, while the remaining 17% of the losses was due to severe tissue browning.

Table 1. Degree of contamination (%) of cultured axillary buds of tea clone TRI 2025 derived from field-grown materials

Disinfection methods	Contaminated culture		Axenic culture	
	Fungal	Bacterial	Discolored	Viable
Conventional	12.4	53.5	32.0	2.1
Improved	7.6	12.8	8.6	71.0

Observations were recorded after 4 weeks of culture.
Values represent averages from 5 experiments.

1.2. Shoot Proliferation in Excised Axillary Buds

Experiments were conducted to determine the type and concentrations of plant growth regulators needed for initial bud break and subsequent proliferation. Two types of cytokinin, namely benzyl-adenine-purine (BAP) in concentrations of 1, 5, 10 and 20 uM and thidiazuron (TDZ) at 1, 5, 10 and 20 ug/l were selected for incorporation into the basal medium (WP) suitable for woody plants (Lloyd & McCown, 1981). The effect of doubling the concentrations of macro nutrients of WP medium alone or in conjunction with 40 mg/l adenine sulfate AS was also studied.

Sterilized axillary buds excised from different nodal positions were cultured to examine whether bud position (age) had any effect on bud break and subsequent proliferation.

In preliminary studies, it was noted that the response of bud culture to BAP appeared to be somewhat affected by bud position (age). While buds from the sixth node consistently gave slightly higher rates of shoot proliferation, this effect disappeared through subcultures. Thus, no distinction was made with regard to the position of axillary buds used in subsequent experiments.

Bud break was observed to be quite uniform, occurring 1-2 weeks after inoculation (Fig. 1a). Enhancement of axillary branching, leading to the formation of multiple shoots was observed after another 3-4 weeks (Fig. 1b). Table 2 shows the response of excised axillary buds *in vitro* to BAP in standard WP media and in media with the concentrations of macronutrients doubled.

The percentage of responsive bud cultures ranged between 35 to 100%, giving a shoot mutiplication rate of 1.4 - to 5.4-fold increase. Standard WP media appeared to be adequate for shoot multiplication, even though slighly higher rates could be attained by doubling the concentrations of macro nutrients in conjunction with adenine sulfate. Addition of TDZ at 5 ug/l substantially improved shoot multiplication in comparison with BAP treatments. High rates of shoot multiplication were maintained through regular re-culturing of in vitro-derived tea shootlets in WP media with similar supplements.

Table 2. The effect of growth regulators on shoot proliferation in excised axillarybudsof teaclone TRI2025 6 weeks after culture initiation

Treatment no.	Growth regulator (uM)	Nutrient addenda	% Proliferating culture	Shoot multi-plication rate
	BAP			
1	1	standard	100	2.6
2	5	WP macro-	85	3.5
3	10	nutrients	95	4.1
4	20		100	3.6
	BAP			
5	1	WP macro-	60	2.2
6	5	nutrients	85	4.4
7	10	doubled	95	3.6
8	20		95	3.0
	BAP			
9	1	WP macro-	35	1.4
10	5	nutrients	80	3.9
11	10	doubled &	100	4.5
12	20	40 mg/l AS	95	4.5
	TDZ			
13	5	WP macro-	100	5.4
14	10	nutrients	95	3.6
15	50	doubled	75	4.1
16	100		70	3.5

Each treatment received 5 uM IAA
Values represent averages from 20 replicates.

1.3. Promotion of Shootlet Elongation

Prior to induction of rooting, shootlets were cultured singly or in clusters in WP media provided with either GA_3 (2-10 uM) or IAA (2-10 uM) to promote uniform shoot elongation. The promotive effect of IAA on shoot elongation of micropropagated tea was much greater than that of GA_3 (Table 3). However, callusing at the basal ends of shoots in media provided with IAA would not be beneficial for producing a good root system.

Table 3. The effect of GA_3 and IAA on elongation of micropropagated tea shoots after 6 weeks of culture

Treatment No.	Plant growth regulator (uM)		Average lenght of shoots (nm)		% Increament
	GA_3	IAA	Initial	Final	
1	2	0	8.3	10.5	26.5
2	5	0	9.9	12.6	27.3
3	10	0	10.1	12.1	19.8
4	0	5	10.4	19.2	84.6
5	0	10	10.1	24.9	146.5

Note : - All treatments in WP media provided with 2 uM BAP
- Callusing at basal ends of shoots in treatment 4 & 5
- Values represent averages from 20 replicates.

1.4. Induction of Rhizogenesis

Rhizogenesis was induced by culturing the shootlets in darkness for 1-2 weeks in WP media provided with 1-5 mg/l NAA and 1.5-3.0% sucrose. Root initiation was achieved about 10 days after the transfer of shootlets to an auxin-free medium in the light. An optimum rooting response (69-83%) was achieved after exposure for 7 days to 3 mg/l NAA (Table 4 & Fig. 1c).

Tabel 4. Rooting percentage of tea shootlets as effected by NAA concentration, exposure time and suucrose concentration 6 weeks after culture.

Exposure time (days)	Sucrose (g/l)	NAA (mg/l)		
		1	2	3
3	15	0	0	0
	30	0	0	0
7	15	36	83	47
	30	23	69	73
14	15	20	75	50
	30	20	50	25

Values represent averages from 20 replicates

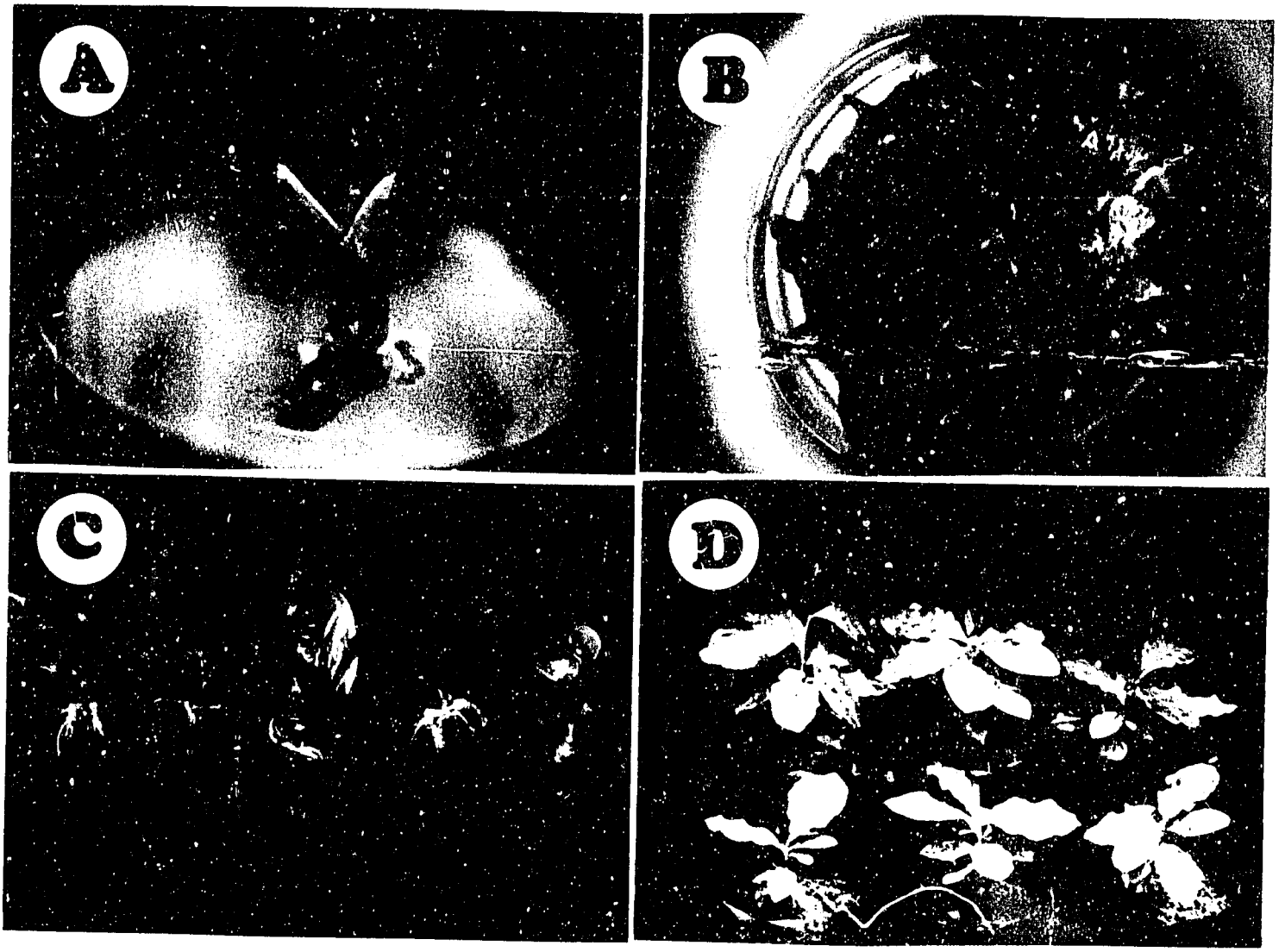

Fig. 1. Micropropagation of tea: (a) Excised axillary bud 4 weeks after culture; (b) Clusters of tea shootlets; (c) Rooted shootlets; (d) In vitro tea plants established in soil

1.5. Establishment of Plantlets Ex Vitro

Micropropagated tea shoots with sufficiently developed root systems were transfered to a soil-sand-compost (2:1:1) mixture and acclimatized to ambient conditions. The tea plantlets were placed under plastic cover and kept in an air-conditioned room with a daylength of 12 hours and temperature regime of 26-28 C for 3-4 weeks prior to their transfer to the glasshouse condition. After another 3-4 weeks in the shaded glasshouse, the plastic cover was removed. The survival rate of tea transplants was less than 30% (Fig. 1d).

2. Plant Regeneration through Somatic Embryogenesis

The embryogenic potentials of calli derived from different tissue sources were studied in explants of de-embryonated cotyledons from mature seeds, leaf laminar tissues, shoot tips and axillary buds derived from *in vitro* culture. For callus initiation and induction of somatic embryos, a combination of auxin (2,4-D, IBA, NAA, IAA) and cytokinins (BAP, Kinetin) was studied in MS basal medium. The effect of other adjuvants (putrescine) was also studied in this regard.

2.1. Embryogenic Callus Induction in Cotyledonary Explants

De-embryonated cotyledons from seeds of TRI 2025 were cultured in MS basal media containing 2,4-D at varying concentrations (20, 40, 60 and 80 mg/l), 0.1% casein hydrolysate, 0.1% activated charcoal and 4% sucrose. The cultures were incubated in darkness at 26 ± 2°C. Nodular and embryogenic calli started to form three weeks after inoculation (Fig 2a). About 30% of the cultures underwent somatic embryogenesis in media containing 20 mg/l 2,4-D (Fig. 2b). Higher levels of 2,4-D resulted in excessive root formation and necrosis.

Cotyledon-derived somatic embryos were germinated in MS basal media containing varying combinations of phytohormones. After 8 weeks in culture, 75% of the somatic embryos germinated in media containing 1 mg/l BAP and 0.5 mg/l GA_3 and 2% sucrose (Fig. 2c). Rooting of germinated somatic embryos was induced in MS basal media containing 8-10 mg/l IBA. The concentrations of sulfate, halides, phosphates, boric, acid and molybdenum were reduced to one-half. After 4 weeks, the shoots were transferred to an IBA-free medium containing 3 mg/l GA_3 and 0.5% activated charcoal. It was observed that, although the level of root induction was high, the shoots remained

stunted and were not suitable for hardening for field planting. At any rate, the experiments using cotyledon explants instead of leaf from mature tea plants was discouraged by BOSTID as this was not in line with the objective of the Project.

2.2. Embryogenic Callus Induction from Leaf Explants

Attemptsto induce somatic embryogenesis in tea were preceeded by induction of calli from explants. Leaves (1st & 2nd) of TRI 2025 were obtained from stock plants in the nursery and surface-sterilized in 10% chlorox for 15 minutes and rinsed thrice in sterile distilled water. Leaf strips measuring 2 mm wide were cultured in MS basal media in the dark at 26 ± 2°C.

The combined effects of auxins (2,4-D or NAA at 1 or 5 mg/l) and cytokinins (BAP, Kin, 2iP and BPA at 1 mg/l each) on the induction of leaf calli was studied. The first leaf responded more readily than the second (more mature) leaf to callus induction. Callus formation from the leaf explants was observed in all treatments at high frequencies. All calli formed were compact, hard, white or light-green in colour. There was, however, no evidence for somatic embryogenesis, or organogenesis, even after several subculturing.

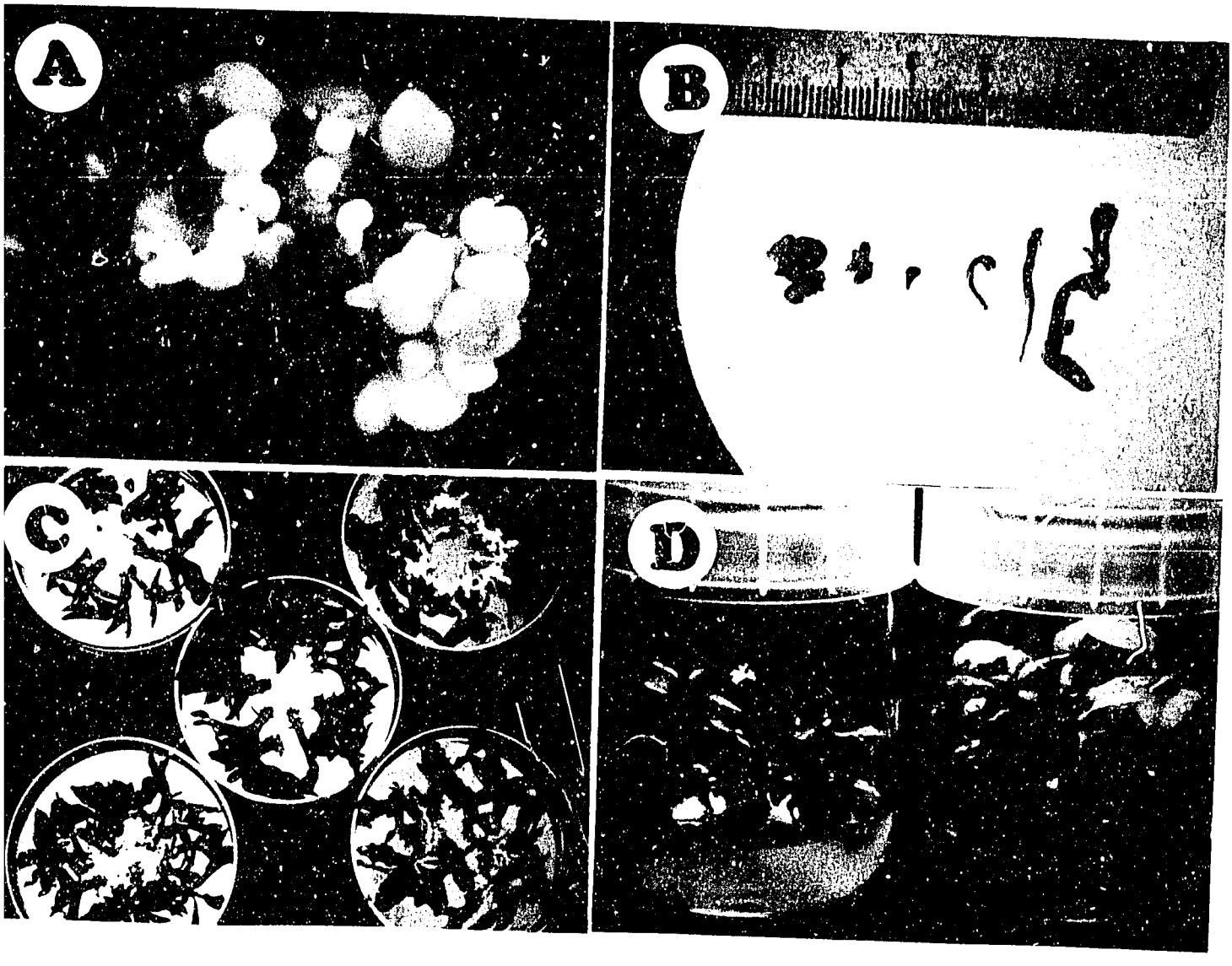

Fig. 2. Somatic embryogenesis and plant regeneration in tea : (a) Nodular and embryogenic calli from cotyledonary explants; (b) Stages of somatic embryogenesis and plant regeneration from cotyledonary callus; (c) Plantlets regenerated from cotyledonary callus.

In another series of experiments, the non-embryogenic calli from leaf explants were subcultured to MS basal media with varying strengths of nitrates (0, 0.5, 1, 1.5 and 2.0 times). As shown in Table 5, MS nitrates did not affect callus growth and regeneration in either somatic embryogenesis,shoot or root formation. However, leaf calli subcultured to MS basal media containing putrescine (2-20 mg/l) gave rise to organized structures resembling somatic embryos in nearly all treatments (Table 6). Root and shoot organogenesis were observed in certain treatments.

Table 5 : The effect of nitrate strength on TRI 2025 tea leaf callus growth and morphogensis

Strength of MS nitrates *	Shoot Formation %	Root Formation %	Somatic Embryogenesis %	Callus Growth %
0	0	5.0	0	50.0
0.5	0	0	0	40.0
1.0	5.0	0	0	55.0
1.5	0	0	0	50.0
2.0	0	0	5.0	45.0

* MS nitrates : The concentration of ammonium nitrate and potassium nitrate as listed in the Murashige & Skoog's (1962) culture media.

Table 6 : The effect of putrescine on TRI 2025 tea leaf callus growth and morphogenesis

Concentration of Putrescine (mg/l)	Shoot Formation %	Root Formation %	Somatic Embryogenesis %	Callus Growth %
0	0	5.0	0	45.0
0.1	5.0	10.0	10.0	25.0
0.5	0	10.0	0	55.0
1.0	0	0	5.0	35.0
2.0	0	0	15.0	30.0
5.0	5.0	20.0	20.0	30.0
10.0	0	0	10.0	50.0
20.0	0	10.0	20.0	30.0

2.3. Embryogenic Callus Induction from *In Vitro* Axillary Bud and Shoot Tips

Shoot tips and axillary buds from in vitro cultures of TRI 2025 were cultured onto B5 basal medium containing combinations of IBA (0, 0.5, 1 & 2 mg/l) and BAP (0, 3, 15 & 30 mg/l), and 2% sucrose at pH 5.7. The cultures consisting of 10 replicates for each treatment were incubated in the growth room with 16-hour photoperiod at an average light intensity of 10,000 lux. Another set of replicates were incubated in the dark.

After two months, white compact nodular calli were formed from shoot tips and axillary buds in media containing 2 mg/l IBA at a frequency of 20 and 50% respectively. In media containing both IBA at 2 mg/l and BAP at 3 mg/l, the frequency of callus formation was 30% for shoot tip culture and 20% for axillary bud culture. The calli appeared to have features resembling somatic embryos.

2.4. Evidence of Somatic Embryogenesis in Shoot Tip and AxillaryBud Calli

Representative calli from shoot tip and axillary bud cultures showing signs of somatic embryogenesis were sent to the Botany Department of the University of Singapore for microtome sectioning. The thin layers were stained using (i) Safranin and Fast Green, and (ii) Hematoxylin and Erythrosine.

All the stained slides were viewed under a microscope with magnifications ranging from 40X to 1,000X and photographs taken. Observations of the thin-layer preparations showed globular calli with compact cellular organizations as well as dense cytoplasm that resemble proembryos (Fig. 3a). These proembryos also gave rise to heart-shaped embryos as shown in Fig. 3b & 3c. It was also observed that meristematic cell clumps arising from within the vacuolated tissues (Fig. 3d). It is possible that these meristematic tissues would give rise to plantlets directly.

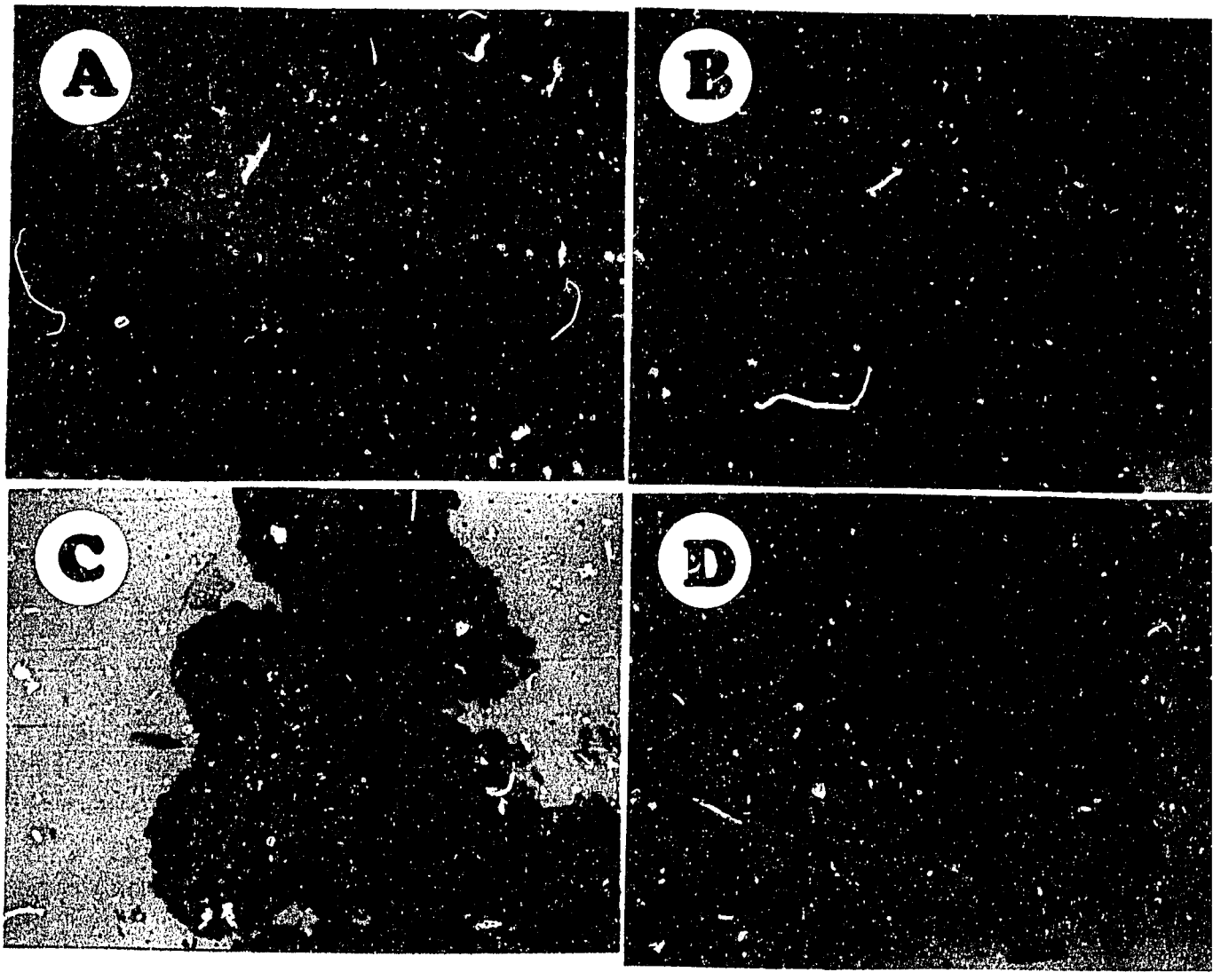

Fig. 3. Development of somatic embryos in tea callus derived from in vitro shoot tips: (a) Globular and cytoplasmically dense calli; (b) Heart-shaped embryo with dense cytoplasm; (c) Heart-shaped embryo showing indentation; (d) Meristematic mass of tissue arising directly from highly vacuolated surrounding cells.

IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

This Tea Research Project will have a major impact on the development of a commercially applicable micropropagation system for tea. Production of tea propagules through enhanced proliferation of axillary buds in vitro would provide an alternative and efficient method of vegetative propagation for the tea industry in Indonesia. This aim has been achieved using clone TRI 2025, even though further work may be needed to improve the weaning success of the plantlets for ex vitro establishment.

Somatic embryogenesis in tea using in vitro shoot tip and axillary bud materials has also been achieved, even though the frequency of occurrence was low. This is the first known case of successful induction of somatic embryogenesis, and is expected to have a major impact on future research strategies in tea improvement which rely on an efficient plant regeneration system. The results obtained will certainly be beneficial to IBRIEC in pursuing further along this line to develop a complete system, viz. from the obtainment of somatic embryos through germination and plantlet development.

These two methods of plant regeneration which have been developed at the laboratory scale will be scaled up for commercial application at IBRIEC.

Through this Project, IBRIEC has gained a good perspective and experience in developing a model commercial micropropagation system for tree crops. The acquisition of selected laboratory equipments for micropropagation work through this Project has enhanced its applied research capability. Similarly, Plantek has obtained a better understanding into the somatic embryogenesis of woody plants. This Project has contributed much to the knowledge in developing a somatic embryogenesis system potential for commercial micropropagation and crop improvement.

PROJECT ACTIVITIES/OUTPUTS

1. In May, 1990, Drs. Fried, Litz and Griesbach of the Tissue Culture Networking Committee of BOSTID paid a visit to IBRIEC and Plantek. Progress on project implementation covering shoot multiplication and somatic embryogenesis was reviewed at each institute organization.
2. In May/June, 1990, Mr. Ridwan, a laboratory assistant to the Tea Project at IBRIEC was trained for 2 weeks at Plantek on the basics of tea micropropagation via enhancement of axillary buds. The training was intended to provide the trainee with an alternative perspective on the micropropagation technique developed at Plantek. This was in accordance with the terms of the M.O.A. between IBRIEC and Plantek.
3. In July, 1990, the PI (Dr. Tahardi) attendedthe Networking/ Workshop Meeting at Davis, California, and made a presentation on the research accomplishment to date on the micropropagation of tea both at Plantek and IBRIEC.
4. At the request of Plantek, experimental plant materials in the form of softwood cuttings and seeds of tea clone TRI 2025 and proliferating shoot cultures were sent in March and May, 1991. Technical advice in disinfection of field-derived plant materials was provided. At the same time, Plantek advised IBRIEC on the rooting of micropropagated shoots.
5. In March, 1992, the Co-PI (Ms. Wendy Shu of Plantek) visited IBRIEC. Samples of tea plantlets derived from cotyledon cultures were given to IBRIEC for acclimatization and field planting. The visit also included discussions on the research programs of both parties, and the presentation of the Project's results at the Networking/Workshop Meeting organized by BOSTID. TRI 2025 seeds were collected during the visit to Pasir Sarongge Tea Experimental Station and brought back to Plantek.
6. In April, 1992, a presentation of the Tea Project was given at the Networking/Workshop Meeting in Costa Rica. Due to unforseen circumstances, Ms. Wendy Shu (Co-PI) was unable to enter Costa Rica because of visa problems. Fortunately Dr. Tahardi (PI) was able to present the tea research paper at the Workshop. 7. In September, 1992, Dr. Tahardi visited Plantek to discuss future experimental plans. A visit to the Botany Department of the University of Singapore was arranged for Dr. Tahardi to view the hitological thin-sections of tea embryogenic calli.

8. Results on the *in vitro* regeneration of tea from excised axillary buds have been published in the Annual Report 1990 of the Bogor Research Institute for Estate Crops and also presented as a poster at the VIIIth International Congress of Plant Tissue and Cell Culture, Firenze, Italy.
9. In May, 1993, Drs. Ben Bergman and Nancy Johnson (AAAS Fellows) representing the Office of Research, USAID, Washington, D.C. visited IBRIEC. The visit included a technical briefing on the Tea Project, and a short visit to the Experimental Garden.
10. Ms. Jacintha Ramachandra (Co-PI) for the Tea Project resigned from Plantek on June 30, 1990. Plantek appointed Ms. Wendy Shu to take over the Project activities on June 25, 1990 untilher resignation on May 7, 1992. Plantek then appointed Mr. Sim Mong Shing to the Project to ensure continuity.

PROJECT PRODUCTIVITY

1. Development of improved laboratory protocols for plant multiplication
   a. Micropropagation of tea through shoot proliferation of excised axillary buds has been established.
   b. Evidence for somatic embryogenesis from callus culture of *in vitro* axillary buds and shoot tips has been achieved albeit at low frequency. Due to the lack of time, it was not possible to achieve regeneration of somatic embryos.
2. Establishment of procedures for *in vitro* or *ex vitro* rooting, acclimatization and transplanting of tea propagules.
   a. Micropropagated shoot derived from axillary buds have been rooted *in vitro*.
   b. Somatic embryos derived from both sources of explants have been hardened and transplanted.
3. Exchange of scientific and technical details on tea micropropagation between IBRIEC and Plantek.
   a. Scientific and technical exchanges have been frequent and beneficial.

FUTURE WORK

While tea micropropagation through enhancement of axillary buds has been achieved, the rate of acclimatization and survival percentage of tea transplants *ex vitro* needs to be improved, if the technique is to become commercially effective. Furthermore, the possibility of rooting *ex vitro* of micropropagated shoots and their simultaneous hardening should be examined in order to reduce the time frame for tea micropropagation.

Evidence of somatic embryogenesis using axillary buds and shoot tips is very encouraging. It was not possible, however, within the Project's time frame to repeat the experiment on a larger scale. This leaves room for future research. The experiments can be repeated and refinements made to induction higher frequencies of somatic embryogenesis.

Once somatic embryogenesis can be achieved, research on regeneration will follow. When the system of inductionand regeneration of somatic embryos has been completely achieved, acclimatization and field trials can be conducted. Perhaps future research can be on cryopreservation and encapsulation of tea somatic embryos.

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