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Grafting micropropagated tea [*Camellia sinensis* (L.) O. Kuntze] shoots on tea seedlings – a new approach to tea propagation

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Abstract Tea microshoots excised from well-established multiple shoot cultures grown *in vitro* and 8-week-old, three- to five-leaved seedlings from a local chinery stock (Banuri-96) and UPASI-9 (from southern India) were selected as scions and root stocks, respectively, for grafting. In addition, 4-month- and 12-month-old seedlings of Banuri-96 were also used as root stocks. Cut ends of root stocks and scions were pretreated with varying concentrations of BAP and NAA for 10 min. A treatment of BAP (5 mg/l) and NAA (5 mg/l) to both scion and stocks in water renewed foliar development at a relatively early stage (40–60 days). The grafted plants were kept in hardening chambers with CO_2-enriched air. No significant difference was observed between autograft (scion and root stock of Banuri clone) and heterograft (scion of the Banuri clone and root stock of UPASI-9). Of the three types (in terms of age) of seedling-raised root stocks employed, grafts on young tea (4-month-old) performed the best (88.33%). Grafts made in early summer established relatively faster and at a high rate of success. The percentage survival of plants transferred to the field was 88.33%.

Key words Micropropagation · Grafting · *Camellia sinensis*

Abbreviations *BAP* 6-Benzylaminopurine · *FYM* Farm yard manure · *IBA* Indole-3-butyric acid · *MS* Murashige and Skoog medium (1962) · *NAA* Naphthalene acetic acid · *PGR* Plant growth regulator · *RH* Relative humidity · *IAA* Indole-3-acetic acid · GA_3 Giberellic acid

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Introduction

Tea [*Camellia sinensis* (L.) O. Kuntze] micropropagation has been widely attempted, and there are reports of successful plantlet development (Kato 1985, 1989, Arulpragasm *et al.* 1986; Palni *et al.* 1992 a, b). However, present-day technology thus far does not enable, commercial application due to problems at the root induction phase, a long gestation period between hardening and field transfer and, most importantly, the high cost factor compared to the cheaper conventional methods of propagation by single-node cuttings. If made efficient and consistent, micropropagation has merit as it would permit the introduction of few clones within a short period of time.

Micrografting has been successfully reported in a range of horticultural plants, as a means to obtain clones free of viruses and virus-like diseases and also to detect graft incompatibilities at an early stage. Some important successes are related to fruit trees like citrus (Oiyama 1992; Starrantino 1992; Obeidy and Smith 1991; Jonard *et al.* 1987), cherry (Ozzamback and Schmidt 1991), kiwifruit (Ke *et al.* 1993), pistachio (Abousalim and Mantell 1992), stone fruits (Cupidi 1992), apple (Richardson et al. 1996) and grapes (Martino 1992). Similar experiments have also been done with forest species like *Larix decidua* (Ewald and Kretzschmar 1996) and *Picea* spp. (Ponsonby and Mantell 1993). In most of these reports, *in vitro*-raised scions have directly been transplanted on *in vitro*-raised stocks under sterile conditions. Ke *et al.* (1993) reported the cleft grafting of *in vitro*-raised shoots derived from protoplast culture onto mature root stocks of kiwifruit growing under field conditions. In the study presented here, we used a similar approach in tea in which the root stocks were juvenile seedlings or only 8- to 12-week-old plants with herbaceous stems. Prevailing constraints in the micropropagation of tea led us to assess the grafting of micropropagated shoots of selected stock Banuri-96 scions (chinery hybrid) onto seedling root stock. This paper reports the first successful micrografting of *in vi-*

Table 1 Effect of BAP and/or NAA pretreatment on the success of graft unions of microshoots of chinery hybrid on roots of two different tea stocks

PGR (mg/l) used	Percentage grafted[a] plants after 100 days[b]					
	Percentage of plants surviving			Percentage of plants showing new foliar development		
	Banuri-96[c] (stock)	UPASI-9[d] (stock)	Mean	Banuri-96 (stock)	UPASI-9 (stock)	Mean
BAP (0.0)+NAA (0.0)	80.29 (93.33)	45.00 (50.00)	62.65	27.55 (21.42)	26.56 (20.00)	27.06
BAP (0.0)+NAA (5.0)	88.72 (100.00)	50.77 (60.00)	69.75	21.13 (13.33)	15.43 (10.00)	18.28
BAP (0.0)+NAA (10.0)	88.72 (100.00)	45.00 (50.00)	66.86	30.78 (26.66)	22.36 (20.00)	26.57
BAP (0.5)+NAA (0.0)	71.87 (86.66)	57.29 (70.00)	64.58	12.66 (7.69)	38.85 (40.00)	25.76
BAP (0.5)+NAA (5.0)	71.87 (86.66)	57.70 (70.00)	64.78	1.28 (0.00)	39.23 (40.00)	20.23
BAP (0.5)+NAA (10.0)	63.80 (73.33)	67.64 (80.00)	65.72	1.28 (0.00)	26.56 (20.00)	13.92
BAP (1.0)+NAA (0.0)	59.22 (73.33)	57.29 (70.00)	58.26	37.04 (36.36)	38.85 (40.00)	37.95
BAP (1.0)+NAA (5.0)	54.99 (66.66)	67.64 (80.00)	61.32	15.43 (10.00)	38.85 (40.00)	27.14
BAP (1.0)+NAA (10.0)	80.29 (93.33)	51.15 (60.00)	65.72	27.55 (21.42)	32.30 (30.00)	29.93
BAP (5.0)+NAA (0.0)	80.72 (100.00)	74.57 (90.00)	81.65	12.72 (6.66)	67.64 (80.00)	40.18
BAP (5.0)+NAA (5.0)	80.29 (93.33)	88.72 (100.00)	84.51	62.45 (78.57)	88.72 (100.00)	75.59
BAP (5.0)+NAA (10.0)	80.29 (93.33)	74.57 (90.00)	77.43	1.28 (0.00)	15.43 (10.00)	08.35
Mean	75.76	61.45		20.93	37.56	
LSD ($P=0.05$) varietal means		=10.09		LSD ($P=0.05$) varietal means		= 2.684
LSD ($P=0.05$) PGR means		=14.78		LSD ($P=0.05$) Treatment means		=10.462
LSD ($P=0.05$) for two stocks under one PGR mean		=20.91		LSD ($P=0.05$) for two stocks under one PGR mean		=14.796
LSD ($P=0.05$) for two PGR means under one stock		=20.51		LSD ($P=0.05$) for two PGR means under one stock		=20.143

[a] Fifteen graft unions were made for each PGR treatment, and observations were recorded after every 20 days for 100 days
[b] The values in the table are transformed (angular); original values are shown in parentheses
[c] A field-selected tea stock of a China hybrid
[d] A stock from southern India

tro-grown tea shoots to achieve increased vigour and simultaneous hardening of microshoots for field transfer in *Camellia sinensis.*

Materials and methods

Micropropagated shoots as scions

Multiple shoot cultures of tea [*Camellia sinensis* (L.) O. Kuntze] were established using nodal explants of an adult field-selected elite tea clone of a chinery hybrid (Banuri-96) on MS-medium supplemented with IAA and BAP as described by Sood *et al.* (1993). Axillary buds were first grown in half-strength MS-medium with 3% sucrose and after 10 days transferred to half-strength MS supplemented with BAP (2.0 mg/l), IAA and GA_3 (0.2 mg/l each) for shoot proliferation, which took 4–6 weeks. Sub-culturing on the same medium produced enough tea shoots in 8 weeks for use as scions. Each scion was a 20- to 25-mm-long microshoot with an average of four to six leaves.

Type of root stock

Tea seeds from the same chinery clone as well as from UPASI-9 (a clone from UPASI Tea Research Institute, Valparai) were collected from Tea Experimental Farms of the Institute at Palampur and placed in a moist sand medium (9:1:1, sand:soil:FYM) in pits for stratification. The seeds germinated over a period of 3–4 weeks. The semi-sprouted seeds were transferred into polysleeves containing a mixture of tea garden soil and sand (9:1) with soil pH varying between 5.3–5.5. After 8 weeks in polysleeves, seedlings with three to five leaves were used as root stocks for grafting. Four-month-old and 12-month-old seedlings of Banuri stock were also used as root stocks in order to compare the establishment of graft unions vis-a-vis young seedlings. Comparisons were made between the success rate of grafts made in winter (from the 1st week of February to the 1st week of April) and early summer (April onwards).

Method of grafting

After decapitating the hypocotyl region (Fig. 1a) of 8-week-old, 4-month-old and 12-month-old seedlings at 1.0, 1.5 and 2.0 cm above the soil, respectively, with a scalpel blade, we first applied a slanting cut so as to expose the cambium and then a matching slanting cut at the lower end of the micropropagated shoot (scion) (Fig. 1b). The decapitated end of the root stocks and matching cut ends of the scions were treated with varying concentrations of BAP and NAA for 10 min (Table 1), and untreated ones served as the control. The root stock and scion were held together at the point of matching cuts with moist cotton (Fig. 1c) wrapped in parafilm (Sigma, USA) (see Fig. 1d).

The grafted plants were kept in humid chambers or in polytunnels. The chambers were filled with water at the base of steel platforms to increase the relative humidity and enriched with CO_2 to between $20/11\times10^{-5}$ moles l^{-1} to $80/13\times10^{-7}$ moles l^{-1}. Growth in terms of emergence of new leaves was recorded at 20-day intervals over a period of 100 days. A total of 15 graft unions were made for each plant growth regulator treatment (see Table 1).

In a parallel experiment, microshoots, when excised, were treated at the cut ends with IBA (500 mg/l) for 30 min and, after washing in running tap water, transplanted in Hikko trays (Wimco, India) containing a mixture of sand:garden soils:FYM (9:1:1) with soil pH at 5.4. These shoots were also maintained in humid chambers having CO_2-enriched air as well as in polytunnels constructed with galvanised iron (GI) pipes and covered with UV-stabilised, reinforced polythene (M/s Shilpalene, Mumbai). This allowed us to com-

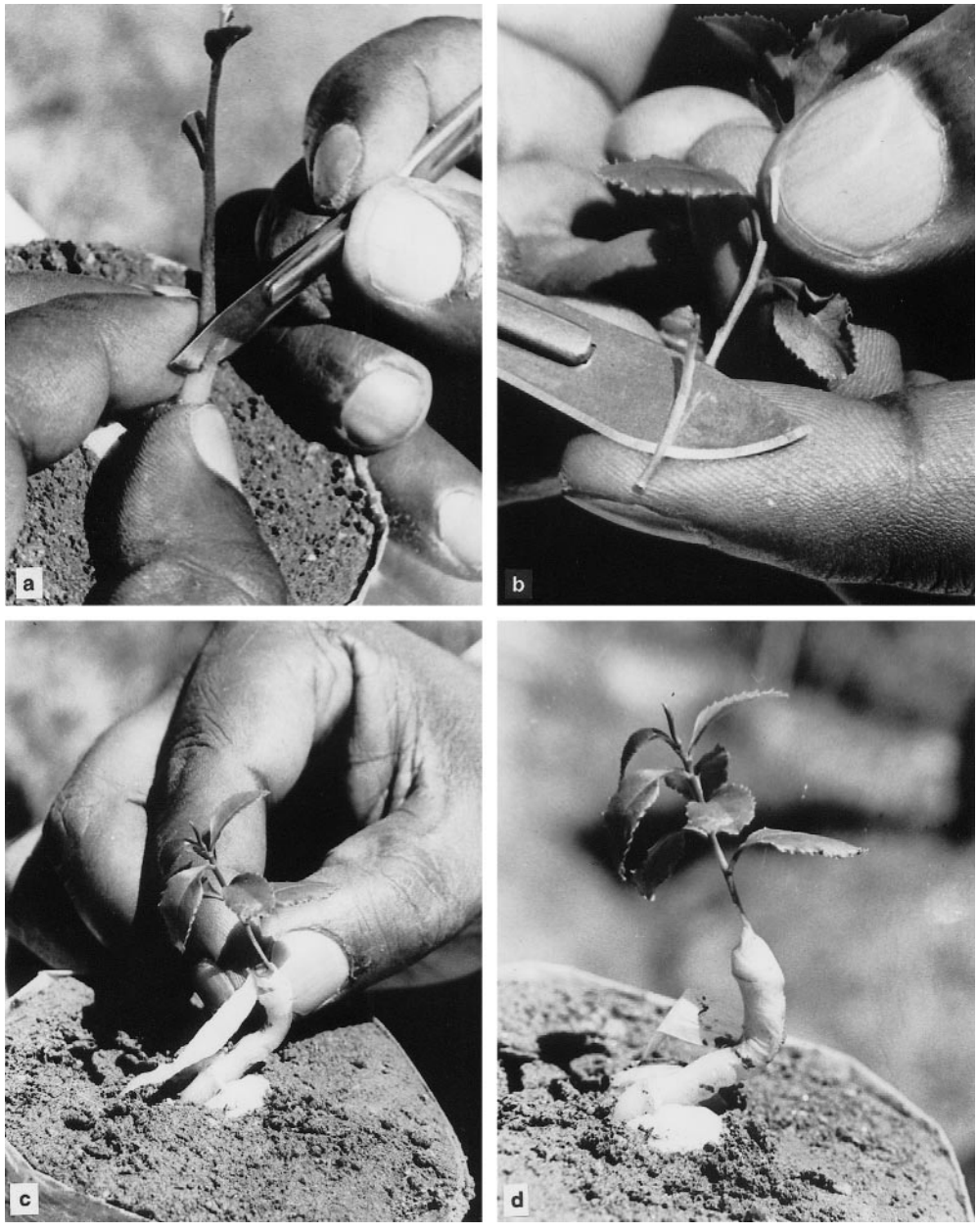

Fig. 1a–d Grafting microshoots of tea (*Camellia sinensis*). **a** Decapitation of seedling, **b** slanting cut applied to pretreated (BAP+NAA; 0.5 mg l^{-1} each; 10 min) microshoot, **c** wrapping moist cotton around graft, **d** securing graft union with parafilm

pare the growth of micrografted plants with those developed by the direct rooting in soil.

Data analysis

The relationship of percentage establishment and age of root stock was computed using the chi-square test. Data on percentage mean survival and new foliar development as affected by different PGR treatments was analysed using Split Plot Design where the root stock was the main factor.

Histological studies

For histological studies, tissue at the point of graft was fixed in FAA (formaldehyde: acetic acid: 50% ethanol, 5:5:90) and dehydrated in a *t*-butyl alcohol series. Sections (10 µm thick) were stained with safranine-fast green, and the slides were mounted in DPX [Distrene, 80-10 gm (British resin product), dibutylphthalate, 5 ml, xylene, 35 ml].

Results

Effect of PGRs on the establishment of graft unions

Experiments conducted in the early spring on the use of PGRs at the matching cut ends of the scion and root stock showed foliar development rather early, within 60–80 days after grafting. This experiment indicated the beneficial effect of the PGR treatment on early graft establishment and was confirmed through histological studies (Fig. 2c, d). The mean value of two clones showed significantly higher foliar development with the use of BAP (5.0 mg/l) and NAA (5.0 mg/l). However, in Banuri-96 combinations of media having higher NAA concentrations (5.0 and 10.0 mg/l) and a low BAP concentration (0.5 mg/l) showed

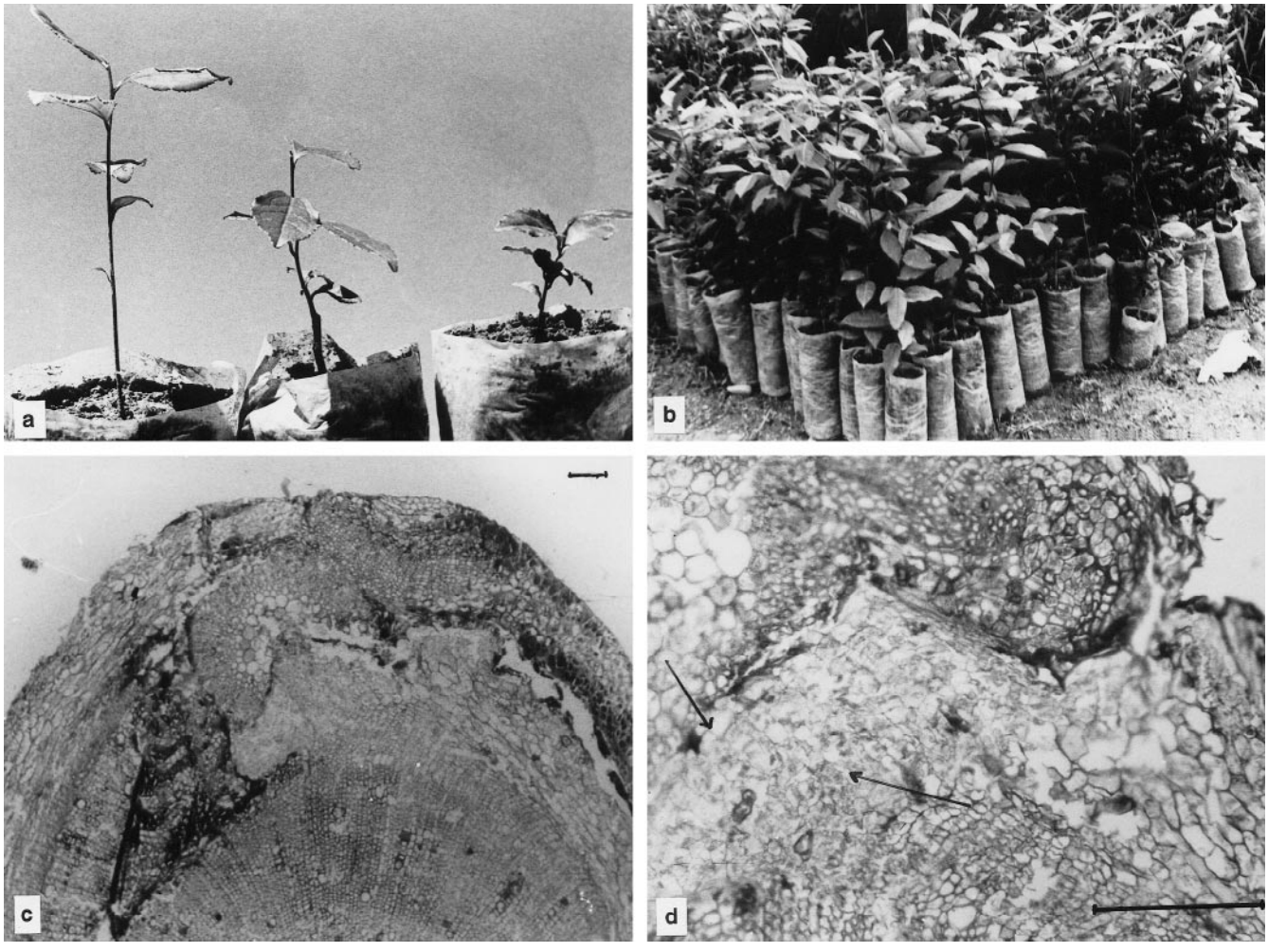

Fig. 2 **a** A comparison of growth of tea plants after 8 months, from *left* to *right*, seedling, grafted microshoot and directly rooted microshoot. **b** Graftef tea plants growing in polysleeves before transplantation. **c** Cross-section at the point of union between root stock and scion. **d** Bridge between vascular bundles of stock and scion (*arrow*). *Bar* (**c, d**): 100 μm

a deterimental effect on new foliar development (Table 1). Banuri-96 (autografts) showed a significantly higher survival rate than UPASI-9 (heterografts), indicating the importance of the compatibility of the stock and scion. However, the trend was the reverse when new foliar development was taken into consideration (Table 1). Treatment with BAP (5 mg/l) and NAA (5 mg/l) resulted in a significantly higher survival rate for UPASI-9 than for the untreated ones (control). Treatments with NAA alone (5.0 mg/l), BAP (5.0 mg/l) alone and BAP (5.0 mg/l)+NAA (10.0 mg/l) together were at par to that with BAP (5.0 mg/l)+NAA (5.0 mg/l).

In the Banuri-96 chinery clone, percentage survival was not dependent upon PGR treatment as even in the untreated graft unions (control) the mean survival percentage was the same as that of the treated ones (BAP, 5.0 mg/l+NAA, 5.0 mg/l). On the other hand, in UPASI-9 a significant response was observed when 9 different combinations of PGR (BAP, 0.5–5.0 mg/l and NAA, 5.0–10.0 mg/l) were used. Twelve-month-old root stocks achieved 72.5% plant establishment (Table 2).

In all subsequent experiments using different root stocks, the PGR combination BAP+NAA, 5 mg/l each, was employed.

Assessment of compatibility of root stock and scion

A significant difference was observed in auto- and heterografts, i.e. grafts between scion and root stocks of the Banuri clone (autograft; 88.33%) scion of the Banuri clone and root stock of UPASI-9 (heterograft; 80%) while using 4-month-old young seedlings as root stocks (see Table 2) when their establishment in soil was compared.

Effect of age of root stock and season on graft union

Upon assessing the performance of three types of root stocks, we observed that the rate of successful grafts was only dependent on the age of the root stock in the case of

Table 2 Effect of age of the root stock on the graft union with microshoots of tea

Age of root stock grown from seed	Number of grafts	Number of successful grafts	Percentage establishment
2 months			
Banuri-96	500	355	71.00
UPASI-9	250	150	60.00
4 months			
Banuri-96	180	159	88.33
UPASI-9	200	160	80.00
12 months			
Banuri-96	55	26	47.00
UPASI-9	120	87	72.50

χ^2 Cal: for Banuri-96 stocks = 41.4627; χ^2 tab, 2 *df* at 1% = 9.21
χ^2 Cal: for UPASI-9 = 21.6082; χ^2 tab, 2 *df* at 1% = 9.21

Table 3 Seasonal effect on the survival of grafted microshoots of tea (scion: local China hybrid; root stock: UPASI-9)

Season	Number of grafts	Established grafts made	Percentage establishment[a]
Winter (1st week of Jan–1st week of Feb)	200	149	74.50
Spring (2nd week Feb.–1st week of April)	200	184	92.00
Early summer (April–1st week of May)	200	167	83.50
Peak summer	200	15	7.50

χ^2 Cal: 389.46; χ^2 tab, 3 *df* at 1% = 11.345

[a] Shoots from bacterial contaminated cultures showed 47.5% establishment

Banuri-96 where percentage establishment declined significantly when the age of the root stock was 12 months (Table 2). One of the reasons for the marginally lower success rate of the 2-month-old seedlings grafts (71% and 60%) over the 4-month-old grafts (88.33% and 80%) was that most of the seedling grafts were possible only during late winter and early spring considering the nature of tea seed biology and germination behaviour. This period is generally in the domain of dormancy in tea. The best time for grafting matched with the break in dormancy of tea. This was also the time when seedling-raised root stock attain a 4-month stage of growth. Thus, in terms of root stock the matching of the 4-month growth stage of young tea and the break of dormancy concomitantly facilitates better graft establishment in a shorter period of time.

On comparing percentage establishment of grafts carried out during the winter and early summer using local China hybrid shoots (Banuri-96) as scions and UPASI-9 seedlings as root stocks, we observed that grafts made in early summer established relatively faster and with a high percentage of success. Peak summer time was not a suitable time for grafting (Table 3).

Histological studies

From histological studies it was found that 4–6 weeks were required for the graft union to take place. Light microscopy showed an initiation of callus proliferation and vascular cambium and pith 2 weeks after grafting (Fig. 2c). Cambial cells began to differentiate by 4–6 weeks and formed a bridge between the vascular bundle of scions and root stocks (Fig. 2d). New vascular elements established the continuity in the root stock and scion within 40 days. Although the graft union was established within 40 days, the normal development of the plant commenced only after 12–15 weeks of grafting.

Maintenance of grafted plants

From our earlier experiments involving direct rooting of tea microshoots, it was established that humid chambers enriched in CO_2 ($20/11\times10^{-5}$ moles l^{-1} to $80/13\times10^{-7}$ moles l^{-1}) were suitable for the rooting and hardening of tea microshoots. Tea is susceptible to misting and direct watering during hardening. On the basis of results shown above the grafted plants were maintained in CO_2-enriched humid chambers superimposed with lighting (15 μmol m^{-2} s^{-1}) for 100 days. These conditions were suitable for sustaining the development of the union and inducing growth during the establishment of the graft, i.e. for a minimum of 60 days. After this the plants could be maintained under polytunnels for 6–8 months. At the onset of spring the following year the plants became ready for field transfer (Fig. 2b). The success of plants transferred to the field, observed 100 days after field transfer, was 88.33%. To date, about 400 plants have been transferred to the field.

On comparing the rate of growth of seedling-raised tea (non-grafted) to that of grafted tea on young shoots and tea raised through direct rooting of microshoots, we observed that the health of the 12-month-old tea seedling was better than that of the young tea graft, followed by rooted tea microshoots (Fig. 2a). In the first, the new leaves that emerged after grafting were larger in size, and the plants looked relatively healthier than rooted microshoots. However, when grafted tea shoots were compared with the conventional single-node cuttings, the former showed more vigorous growth than the latter at the 1-year stage. The major advantage was the time saved. Plants of tea with grafted microshoots were transferred to the field within 10 months, whereas single-node cutting-raised plants required 1.5 years before being transferred to the field.

Discussion

Tea is a very important plantation crop in India, Kenya, Sri Lanka, China and Japan. In order to meet future challenges in relation to the growing demand for superior planting material, it is important to develop suitable methods for varietal improvement and also for the rapid propagation of

selected clones required for rejuvenating old tea plantations. In recent years, there has been a focus in the literature on the relative degree of successes in *in vitro* propagation of tea from different parts of the world. However, the problem remains, and the real challenge is that the hardening process, field transfer of the plants and extended gestation care from the laboratory to the field add to the cost of micropropagated plants.

The reported stimulation of both graft formation (Shimomura and Fuzihara 1977, 1978) and vascular tissue formation (Sachs 1981) by auxins is corroborated by our findings which show that NAA and BAP in combination (5.0 mg/l each) gave the best results. No marked effect of BAP or NAA when used alone was noticed. NAA alone at very high concentrations (10.0 mg/l) had a deleterious effect on graft establishment. Moore (1984a) outlined three phases for the development of a compatible graft: (1) cohesion of stock and scion, (2) proliferation of callus at the graft interface and (3) re-differentiation of vascular tissue across the graft interface. However, the latter phase is not absolutely essential for a successful graft as indicated by a number of reports (Herrero 1951; Muzik 1958; Moore 1984b, 1991). Moore and Walker (1983) observed that adjacent callus masses graft successfully in the absence of vascular differentiation. Incompatibility in grafts may not be synonymous with an unsuccessful graft as the reciprocal grafts have proved to be successful. In this regard, Moore (1991) has reviewed the subject quite exhaustively.

We have demonstrated the possibility of successfully grafting micropropagated shoots of tea of selected China hybrid clones onto young and 1-year-old rootstock of Stock Banuri-96 and UPASI-9. It should now be possible to establish tissue culture-raised tea shoots of desired clones on more vigorously growing root stocks with a view to improve tea quality and vigour. The technique significantly reduces the gestation time from *in vitro* to field transplantation. This method could also be used to facilitate adaptation of tailored teas to new environments.

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