Original Research Report

Chapter 6

EFFICIENT ROOTING OF MICROSHOOTS OF TEA – A CRUCIAL PREREQUISITE FOR SUCCESSFUL HARDENING AND FIELD ESTABLISHMENT OF MICROPROPAGATED PLANTS

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ABSTRACT

The application of tissue culture techniques for rapid and clonal mass propagation and improvement of tea is well known. While several laboratories have successfully developed *in vitro* propagation protocols, the use of this technology for mass multiplication of tea at commercial level is still limited largely due to difficulty in the quality of rooting of microshoots, and problems associated with subsequent hardening and field establishment. The present study reports a reproducible protocol for 100% rooting of *in vitro* propagated tea microshoots using a two-step method. This involves culture of microshoots on IBA (indole-3-butyric acid; 175.0 μM) containing Murashige and Skoog (1962; MS) medium for 10 days followed by transfer to 1/3 strength MS medium without any plant growth regulator. Besides the concentration of IBA, the period of exposure of microshoots to IBA containing medium (prior to transfer to PGR-free medium) also played a significant role in rooting. In addition, the culture temperature was found to be quite important; for maximum rooting response the optimum temperature range was 25-30°C.

Abbreviations: BAP: 6-benzylaminopurine, IBA: indole-3-butyric acid, IAA: indole-3-acetic acid, MS: Murashige and Skoog (1962), NAA: α-naphthaleneacetic acid, PGR: plant growth regulator.

Key words: *Camellia sinensis, IBA, in vitro rooting, microshoots, tea.*

Introduction

The application of tissue and organ culture for multiplication of elite tea clones has been advocated (Palni *et al.*, 1991, 1999). Tissue and cell culture technology along with advancements in the field of genetic engineering are likely to make a major impact on the industry in the areas of rapid and mass clonal propagation of selected clones, in disease elimination and development of stress (biotic and abotic) tolerant clones, production of pure breeding lines (through haploid technology), germplasm storage and exchange, interspecific and intergeneric hybridisation, development of polyploids and mutants, and for the overall improvement in quality and yield of tea (Jain and Newton, 1990). While several laboratories have successfully developed *in vitro* propagation protocols, the use of this technology for mass multiplication of tea is limited largely due to difficulty in rooting of microshoots and subsequent hardening and field establishment.

The use of auxin for induction of adventitious rooting is well known. In fact, auxins are the only substances that noticeably induce and increase root formation following external application, as long as the time of application and the amount applied are suitable (Moncousin, 1991). In culture, microshoots are

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small, delicate and extremely succulent and in active state of growth, and can be easily "over" treated with auxins. Therefore, selection of appropriate root promoting substance(s), and development of a reproducible protocol for efficient rooting of such delicate shoots requires careful experimentation (McCown, 1988).

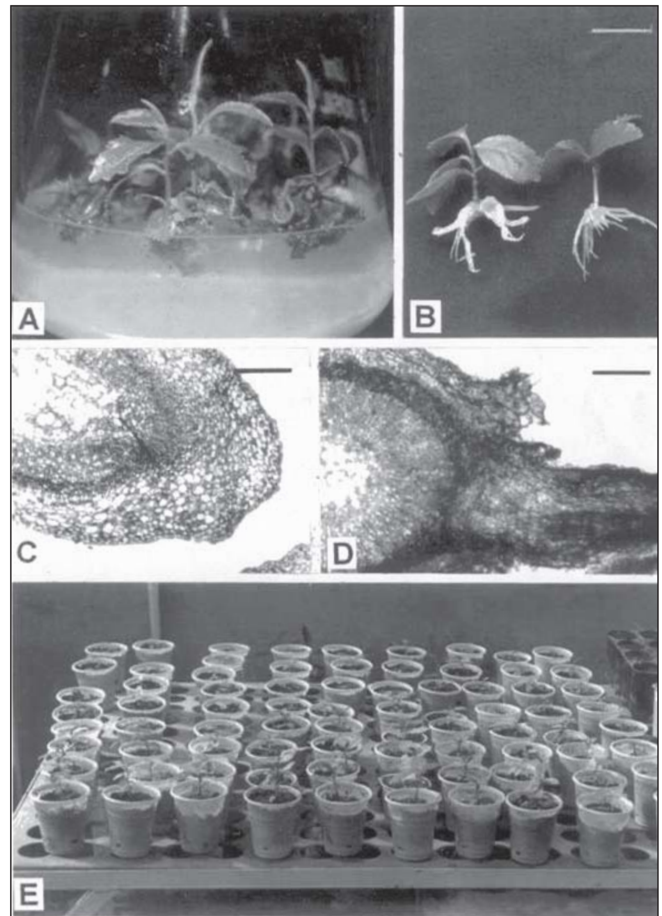
Rooting of tea microshoots has been reported by several workers (Samartin, 1986; Banerjee and Agarwal, 1990; Phukan and Mitra, 1984; Nakamura, 1991; Jha and Sen, 1992; Bag *et al.*, 1997, 2001). The concentration of auxins used and the response obtained reportedly vary. Nakamura (1991) and Jha and Sen (1992) used low concentration of IBA (less than 2 mg l^{-1}), while others have shown direct planting of auxin treated shoots in peat/soil mixture (Sharma *et al.* 1999). Rooting and subsequent acclimatisation of rooted shoots were significantly affected by the concentration of macronutrients in the medium, and good results were obtained when ½ or ¼ strength Murashigue and Skoog (1962; MS) medium was used (Nakamura, 1991; Bag *et al.*, 2001).

In view of these considerations, experiments were carried out to assess the effect of IBA concentration, duration of exposure to IBA before transfer to PGR-free medium, and the incubation temperature on rooting of tea microshoots for improving the rooting efficiency as well as the quality of roots formed; complementary histological studies were also carried out.

Materials and Methods

Microshoots of tea [*Camellia sinensis* L. (O) Kuntze] were obtained following the culture of immature cotyledons leading to somatic embryogenesis, as described earlier (Bag *et al.*, 1997). Microshoots formed by *in vitro* germination of somatic embryos were excised, multiplied (Fig. 1A) and maintained on MS medium containing 5.0 μM BAP and 1.0 μM NAA and solidified with phytagel (Sigma, 0.2%, w/v). After 30-35 days of transfer to the above multiplication medium, shoots of 25-30 mm height

Fig. 1: In vitro rooting and hardening of tissue culture raised tea plants.



A. Profuse shoot multiplication on MS medium containing 5.0 μM BAP and 1.0 μM NAA.
B. Adventitious root formation in microshoots kept on PGR-free medium after transfer from IBA containing medium (175.0 μM; 10 days incubation; bar = 20 mm).
C. Anatomical details at the base of the stem (microshoot) at the time of formation of root primordia in the pericycle 2 days after transfer to plant growth regulator free medium (bar = 250 μm).
D. Root emergence pericycle 4 days after transfer to plant growth regulator free medium (bar = 250 μm).
E. A batch of tissue culture raised tea plants being maintained under green house conditions, following biological hardening and transfer to soil (temperature 25ºC ±5ºC; light 15% of ambient, RH 70%).

were harvested and used for the rooting experiments.

The microshoots were cultured on 1/3 strength MS medium, supplemented with various concentrations of IBA (0-300.0 μM) for 10 days; treated shoots were then transferred to PGR-free basal MS medium.

Unless otherwise stated all culture media were gelled with phytagel (0.2%) and incubated at 25±1°C under cool white fluorescent lights (16 h photoperiod, 42.0 μmol $m^{-2}s^{-1}$ light intensity). Each treatment consisted of 4 shoots per flask (in triplicate) and per cent rooting, number of roots formed per shoot and length of the longest root were recorded following 8 weeks culture on PGR-free medium (plus initial 10 days on IBA containing medium). The data were also subjected to statistical analysis (Snedecor and Cochran, 1968).

The effect of different periods (0-20 days, in steps of two days) of exposure to IBA (175.0 μM) supplemented medium was also assessed. The effect of temperature (5-50°C, in steps of 5°C) was also examined using growth cabinets, without changing other culture conditions and photoperiod.

Anatomical Studies

Samples were fixed in FAA (formaldehyde, acetic acid, 50% ethanol; 1:1:18, v/v) for seven days, dehydrated in t-butyl alcohol series and embedded in paraffin (60ºC melting point, Qualigens Fine Chemicals, India). Transverse serial sections (10 μm thick) were cut using a rotary microtome, and double stained with safranin and fast-green following de-waxing in graded xylene-ethanol solutions, and finally mounted in DPX (Qualigens Fine Chemicals, India).

Results

Exogenous application of IBA

The effect of IBA concentration on *in vitro* rooting of microshoots of tea (Fig. 1A) is depicted in Table 1. Rooting was not observed in untreated (control) microshoots. Root formation was also not detected during the first 10 days when the microshoots were

Table 1: Effect of IBA concentration on *in vitro* rooting of microshoots of tea

Concentration of IBA (μM)	% Survival	% Basal Callusing	% Rooting	Average no. of roots per shoot	Average length of the longest root (mm)
0.0	100.0	0.0	0.0	0.0	0.0
10.0	100.0	83.3	0.0	0.0	0.0
25.0	100.0	66.7	16.7	1.3	1.9
50.0	100.0	58.3	41.7	3.1	4.0
75.0	100.0	50.0	58.3	3.1	5.8
100.0	100.0	41.7	66.7	4.3	6.4
125.0	100.0	16.7	75.0	6.0	9.5
150.0	100.0	0.0	100.0	9.2	16.0
175.0	100.0	0.0	100.0	12.5	8.4
200.0	91.7	0.0	100.0	14.0	8.3
250.0	91.7	0.0	100.0	15.8	8.1
300.0	83.3	0.0	100.0	16.7	6.6
LSD (p=0.05)	12.16	19.82	22.21	3.0	4.2

Each treatment consisted of 4 shoots per flask (in triplicate) and the values are an average of 12 explants. Cultures were grown on 1/3 MS medium containing 50.0 mM sucrose (w/v) and phytagel (0.2%, w/v). Following 10 days of incubation on IBA (different concentrations) containing medium, these microshoots were transferred to PGR-free medium. The average length of shoots at transfer was approx. 25 mm. Results were recorded after 66 days from the date of initiation of rooting experiments at 25°C in the presence of light (16h/day).

exposed to medium containing different concentrations of IBA. However, rooting was noticed when IBA treated shoots were transferred to PGR-free medium (Table 1). Depending on the IBA concentration used, significant differences were observed. Best results were obtained following treatment with 150.0 or 175.0 µM IBA which resulted in 100% rooting with 100% survival of shoots, and >9.0 roots per rooted shoot (Fig. 1B). Second best response was obtained with 125.0 µM IBA treatment that resulted in 75% rooting, 100% survival of shoots, and an average of 6.0 roots per rooted shoot. In general, higher concentrations also resulted in 100% rooting but the per cent survival was of shoots was reduced to 83-92% (Table 1).

Thus for effective rooting, the above mentioned two step procedure gave best and highly reproducible results. It may be mentioned that continuous culture of microshoots, even up to 60 days on root induction medium supplemented with a low concentration of IBA, did not cause root formation (results not shown). Higher concentrations of IBA resulted in the formation of protuberances and abnormal roots from the entire surface of the stem portion of microshoots in direct contact with the medium, rather than from the shoot base.

Root emergence was noticed within 4-5 days of transfer to PGR-free medium following the treatment with 175.0 µM IBA; 70-80% rooting occurred within 5-6 days of transfer and the value increased to almost 100% within 10-12 days.

The effect of the duration of incubation in IBA containing medium on *in vitro* rooting of microshoots is summarized in Table 2. When the shoots were shifted to 1/3 strength MS basal medium, following 2 days of incubation with IBA, 8.3% rooting occurred. Per cent rooting, number of roots per rooted shoot and the length of the longest root formed were found to gradually increase up to 10 days of treatment (Table 2). The maximum rooting success of 100% was achieved within 10 days of treatment. Increase in the period of exposure to IBA containing medium (12-16-days) did not have a significant effect on the rooting behaviour, but some increase in the average number of roots formed was noticed (Table 2). In all

Table 2: Effect of the period of exposure to 175* µM *IBA containing medium on in vitro rooting of microshoots of tea

Incubation period with IBA (days)	% Survival	% Callusing	% Rooting	Average no. of roots per shoot	Average length of the longest root (mm)
2	100.0	0.0	8.3	0.3	0.7
4	100.0	0.0	25.0	1.2	1.8
6	100.0	0.0	33.3	2.2	2.8
8	100.0	0.0	78.3	5.5	9.1
10	100.0	0.0	100.0	12.5	8.4
12	100.0	0.0	100.0	14.9	7.8
14	100.0	0.0	100.0	18.3	7.3
16	100.0	0.0	100.0	20.7	6.8
18	91.7	0.0	91.7	21.3	6.1
20	83.3	0.0	83.3	21.6	5.8
LSD (p=0.05)	11.0	0.0	17.4	4.7	3.5

Each treatment consisted of 4 shoots per flask (in triplicate) and the values are an average of 12 explants. Cultures were grown on 1/3 MS medium containing 50.0 mM sucrose (w/v) and phytagel (0.2%, w/v). Following incubation in 175 µM IBA (different time periods) containing medium these microshoots were transferred to PGR-free medium. The average length of shoots at transfer was approx. 25 mm. Results were recorded after 66 days from the date of initiation of rooting experiments at 25°C in the presence of light (16h/day).

the treatments (2-16 days) the survival was also 100%. Further increase in the duration of treatment (18-20 days), however, resulted in reduced per cent survival.

During the course of continuous culture of microshoots on IBA containing medium, swelling of epidermal tissues on the stem surface was noticed after 14-16 days onwards. The basal portion of about 50% of the shoots was found to be swollen within 16-18 days. Different concentrations of IBA (10.0 to 300.0 µM) in the induction medium caused varied response (Table 1). The best rooting response (100.0%), without basal callusing, was obtained following 150.0 and 175.0 µM IBA treatment. The application of higher concentrations of IBA (200.0-300.0 µM) also showed 100% rooting and generally enhanced the number of roots formed, but reduced the root length; in such cases shoot tip "burning" was also seen in due course of time.

Incubation temperature

Temperature was found to be yet another important physical factor influencing *in vitro* rooting of tea microshoots. A temperature range of 5-50°C was assessed in respect of rooting efficacy. It was observed that at temperatures of 15°C and below or 35°C and above, the overall rooting of tea microshoots was significantly suppressed. No rooting of shoots was observed at of 10 °C and below. Similarly, rooting as well as the survival of microshoots were adversely affected at 35°C and above. Only 75% shoots survived at 35°C with only 33% of those shoots being rooted. Shoots incubated at 40-50°C did not survive. Optimum temperature for 100% rooting was found to be between 25 and 30°C. In this temperature range, 11-12 roots per rooted shoot, with a root length of up to 24-26 mm were formed.

Histological studies

The basal portion of microshoots exhibited almost similar anatomical details prior to and after 2 days incubation on IBA containing medium. As the incubation period gradually increased periclinal/anticlinal cell divisions were observed in a few cells of the pericycle (Fig. 1C). This resulted in the formation of meristematic pockets as root primordia beneath the endodermis; these primordia began to grow, and finally pushed through the endodermis, cortex and epidermis when microshoots were transferred to PGR-free medium. The first root primordia were clearly visible about 4-5 days after transfer to PGR-free medium (Fig. 1D); some of these had started to differentiate into roots and had extended out from the cortex.

After 45 days in the root elongation medium the plantlets with well developed root system were successfully transferred to soil following a step of biological hardening using bacterial inoculation (Pandey *et al.*, 2000, 2002) with over 90% survival (Fig. 1E).

Discussion

The overall success of any micropropagation technology depends upon efficient shoot proliferation, formation of a well developed root system in micropropagated shoots, successful acclimatization of plantlets and final establishment in the field (Palni *et al.*, 1999; Sharma *et al.*, 1999; Bag *et al.*, 2001). Most earlier studies on micropropagation of tea have focused on shoot multiplication and formation of tea plantlets (Samartin, 1986; Banerjee and Agarwal, 1990; Phukan and Mitra, 1984; Nakamura, 1991; Jha and Sen, 1992; Bag *et al.*, 1997, 2001), but detailed and systematic information on rooting and hardening is often lacking (Samartin, 1989; Sharma *et al.*, 1999; Pandey *et al.*, 2000, 2002; Bag *et al.*,

2001). The results of this investigation clearly establish that IBA (175.0 µM) is quite effective in stimulation of rooting of *in vitro* propagated tea microshoots and can be used for mass scale propagation. It is well known that auxins are the primary class of plant growth substances involved in the process of rooting (Hartman *et al.*, 2002). For instance, an accumulation of auxin at the bases of microcuttings acts as a triggering factor for root induction but this substance also disturbs root growth (Berthon *et al.*, 1989; Eliasson *et al.*, 1989). The mechanism of auxin regulation of rooting is, however, still not clear (Baraldi *et al.*, 1995). The efficacy of various auxins (NAA, IBA and IAA) used in earlier experiments was found to vary in respect of rooting of tea shoots (Bag *et al.*, 2001). The effect of IBA on rooting of tea shoots was also variable; rooting was found to be significantly affected by its concentration in the medium as well as by the duration of exposure of shoots to IBA containing medium. 175.0 µM IBA gave best rooting response and while the higher concentrations of IBA also resulted in 100% rooting and more number of total roots formed, the length of roots formed was considerably reduced. Higher levels of IBA in the root induction medium and over exposure (in terms of prolonged period of treatment) also resulted in subsequent shoot tip burning of otherwise well rooted plants. In addition, temperature between 25-30°C considerably improved rooting.

The present study demonstrates that the overall rooting success, number of roots formed per rooted shoot and the root length are affected by the concentration of IBA in the medium as well as the incubation period. The culture temperature also plays an important role. The study clearly showed that given a proper stimulus, rhizogenesis can be effectively completed, without the continued presence of auxin in the medium. In conclusion, the study presents a practical method for inducing *in vitro* rooting in micropropagated shoots of tea, and offers a method which should be useful for commercial production of tissue culture raised tea plants. In addition, the use of previously described method of biological hardening (Pandey *et al.*, 2000, 2002) should go a long way in overcoming the frequently encountered problem of mortality of tissue culture raised tea plants during lab to land transfer.

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