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IN VITRO ROOTING OF MICROSHOOTS OF TEA (*CAMELLIA SINENSIS* L.)

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The effects of two types of auxin (IBA and NAA), an auxin pre-treatment and the physical form of the culture medium on in vitro rooting of microshoots of tea (*Camellia sinensis* L.) was studied. The incorporation of auxin in the rooting medium did not bring about the formation of roots but caused basal callus formation. Microshoots were successfully induced to root with a pre-treatment of IBA (50mg/l dip for 3hrs) followed by culture of shoots in auxin-free half-strength MS liquid medium with continuous agitation. Shake cultures accelerated the emergence of root and increased the number and the length of the roots compared to static cultures. An initial dark period of 7 or 14 days had no significant effect on root initiation, over cultures exposed to a continuous 12hr photoperiod.

Abbreviations: IBA – Indole-3-butyric acid: **MS** – Murashige and Skoog, (1962) medium: **NAA** - α naphthaleneacetic acid.

INTRODUCTION

The success of micropropagation depends very much on the ability of rooting the propagated shoots. *In vitro* rooting can be induced in many herbaceous plant species quite easily (Bajaj, 1991). In some plant species, shoots are readily rooted even on the shoot multiplication medium or following transfer to growth regulator free medium (George, 1996). However, in many instances, the culture conditions used to produce shoots do not favour root formation, especially in woody species where in vitro rooting is most difficult to achieve (Hu & Wang, 1983). Like many other woody species rooting in vitro is difficult to achieve in most species of *Camellia* (Samartin et al., 1986).

Although some success in *in vitro* rooting have been reported for *Camellia sinensis*, china type, (Jha & Sen, 1991; Nakamura, 1991; Jha & Sen, 1992), this has not been studied in detail or reported for tea varieties cultivated in Sri Lanka, except the studies by Seneviratne *et al.*, (1988) and Sarathchandra *et al.*, (1997). In the study by Seneviratne *et al.*, (1988) however, rooting efficiency in relation to auxin treatment has not been worked out and it was reported that with higher IBA concentrations (1,2 and 3 mg/l) callus formation occurred at the basal end of the micro cutting. In the study by Sarathchandra *et al.*, (1997), root initiation occurred along with a prominent callus formation at the base of the microshoot. This kind of roots are difficult to acclimatised as a vascular connection between the shoot and root are incomplete (George, 1996).

The aim of this study, therefore, is to find the optimum requirements for improved root production *in vitro* by minimising callus growth by studying the various factors related to culture medium, culture environment as well as auxin pre-treatment.

MATERIALS AND METHODS

Shoot cultures were initiated *in vitro* from stem nodal explants taken from plants raised in glasshouse at approximately 25 °C temperature with 16hr photoperiod using supplementary lighting when necessary. These plants were raised from open pollinated seeds obtained from Assam type clone, TRI 2025. *In vitro* cultures were established and multiplied on MS medium containing 2 mg/l BAP and 0.5 mg/l IBA to obtain axillary shoots, as previously described (Gunasekare, 1997). A separate shoot elongation phase preceded rooting and individual shoots (2 cm long) were maintained in growth regulator free MS medium for 3-4 weeks before *in vitro* rooting. Single microshoots, 3-4 cm long, were the initial explants used in all rooting experiments.

Glass jars filled with either liquid or solid [0.7% (w/v) agar] medium were used. Shoots in liquid medium were supported on Heller's rafts (Whatman Grade 4, 3µm filter papers, Whatman International Ltd. Kent). Except when stated otherwise, liquid cultures were continuously shaken at 30 rpm on a gyratory shaker. Cultures were incubated at 25±2°C either in the light (12 h photoperiod) or with an initial continuous dark period as mentioned in each individual experiment.

After 35 days, following measurements were recorded: Percentage of shoots initiating roots (with more than 2 mm long roots), number of roots per rooted shoot and average root length (mm), percentage of explants initiating roots, based on visual root emergence, was also recorded at 4 day intervals. Each experiment was consisted of 10-12 replicates (each replicate consisting of single jar with single shoot) and each experiment was repeated at least once.

Effect of two types of auxin on rooting

Microshoots were cultured on solidified half-strength MS medium without growth regulators or with NAA or IBA at concentrations of 0.1 and 0.5 mg/l to compare the effect of two auxins as well as the auxin concentration on root induction and development.

Physical form of the culture medium in the continuous presence of auxin

Effect of the physical form of the medium on rooting was investigated by culturing microshoots on both liquid and solid media containing half-strength MS supplemented with the auxins mentioned above.

Effect of an IBA pre-treatment

The basal end of the microshoots was dipped in 50 mg/l and 100mg/l IBA in half-strength MS medium for 3 hrs. Shoots were supported with Sorbarods (Sorbarod System, 20x15, Gilbert House, Kent) during auxin pre-treatment. After the pre-treatment, shoots were cultured on half-strength MS liquid medium. As a control, basal ends of shoots, which were dipped in half-strength MS medium without auxin for 3hrs, were also cultured in the same rooting medium for comparison.

Effect of the physical form of the medium for the culture of pre-treated shoots

The best IBA pre-treatment selected from the previous experiment (i.e. 50 mg/l IBA dip for 3 hrs) was used. Pre-treated shoots were cultured in either solid or liquid growth regulator free half-strength MS medium to compare root development in relation to the physical form of the rooting medium.

Effect of static and shake culture system

The best IBA pre-treatment selected was used and shoots were cultured on liquid medium. One set of cultures was kept static while the other set was kept on a shaker (30 rpm).

Effect of an initial dark period

To determine whether there is any effect of an initial dark period on rooting, immediately following IBA pre-treatment (50 mg/l dip for 3 hrs), shoots were subjected to an initial dark period of either 7 days or 14 days after which the cultures were transferred to standard light conditions (12 hr photoperiod). One set of cultured shoots were maintained throughout the experimental period at 12 hr photoperiod after the IBA treatment and this was used as a control. All shoots were cultured in liquid half strength MS medium free from growth regulators following pre-treatment.

RESULTS

Effect of two types of auxins on rooting

Shoots on auxin (NAA or IBA at 0.1 and 0.5 mg/l) supplemented medium or growth regulator free medium did not initiate roots. Instead, callus formed at the base of the microshoots when NAA or IBA was added to the medium. However, the degree of callus formation was less in shoots on IBA supplemented medium than in the corresponding concentration of NAA containing medium (Table 1).

Table 1. Response of tea microshoots to NAA and IBA incorporated rooting medium.

Growth regulators (mg/l)	% rooting	Callus formation at the base of the microshoot
Growth regulator free	0	--
IBA 0.1	0	+
0.5	0	+ +
NAA 0.1	0	+ +
0.5	0	+ + +

-- no callus; + indicates the intensity of callus; Data were recorded after 35 days culture

Physical form of the culture medium in the continuous presence of auxin

Root initiation along with the formation of some callus were observed only in liquid medium containing 0.5 mg/l IBA in 28% of the explant (Fig.1). These roots were very small (less than 2 mm long) and did not elongate further, even after 35 days culture.

Effect of IBA pre-treatment

The highest percentage of explants with roots, number of roots per explant and root length after 35 days shoots cultured on half-strength MS medium was found when shoots were pre-treated with IBA at 50 mg/l. Roots that developed in shoots pre-treated with 100 mg/l IBA were thicker than those pre-treated with 50 mg/l IBA (Fig. 2). After 2 months of culture shoots pre-treated with 50 mg/l for 3 hrs had a well developed root system (Fig. 3).

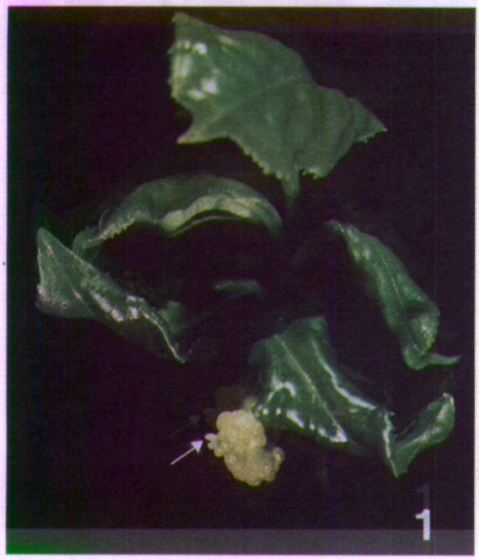

Fig 1. Prominent basal callus formation together with some initial stage of root for mation(arrow) in microshoots cultured on half-strength MS medium supple mented with 0.5 mg/l IBA, 40 days after culture.

Fig. 2. Roots developed in microshoots pre-treated with two levels of IBA; left- 100 mg/l IBA; right – 50 mg/l IBA (after 45 days culture).

Fig. 3. An *in vitro* rooted plantlet after 2 months in rooting medium.

Although there was no significant difference in the number of roots per shoot among shoots pre-treated with the two concentrations of IBA, root length was significantly higher ($p<0.05$) in 50 mg/l treated shoots than in 100 mg/l . However, the percentage of shoots that developed roots was higher in 50 mg/l IBA (80%) than in 100 mg/l IBA (63.3%). Untreated shoots (control) did not initiate any roots throughout the period of observation (Table 2).

Root length increased steadily in 50 mg/l IBA treated shoots from 25 up to 45 days culture. However, in the 100 mg/l IBA treated shoots the increase in root length was always less and only a slight increase was observed between 35 and 45 days. A mean root length of 19.6mm compared to 8.2mm was recorded in 50 and 100 mg/l respectively when measured after 45 days culture. A similar pattern was observed in respect to root number. However, only a small increase in the number of roots per shoot was observed even in the shoots treated with 50 mg/l IBA between 35 and 45 days culture (Fig. 4).

Table 2. Effect of IBA pre-treatment on rooting of microshoots (after 35days culture).

Treatment	Percentage rooting	Mean root length S.E. ± (mm)	Number of roots per rooted shoot (mean ± S.E)
Control	00.0	--	--
50mg/l IBA	80.0	14.7± 1.8a	4.4± 0.9
100mg/l IBA	63.3	7.9± 1.4b	2.6± 0.4
		$P < 0.05$	NS

-- no roots; NS = Treatment means are not significantly different at $p< 0.05$
Means followed by different letters are significantly different at $p < 0.05$. Mean values of three individual experiments with 10 replicates per treatment in each experiment.

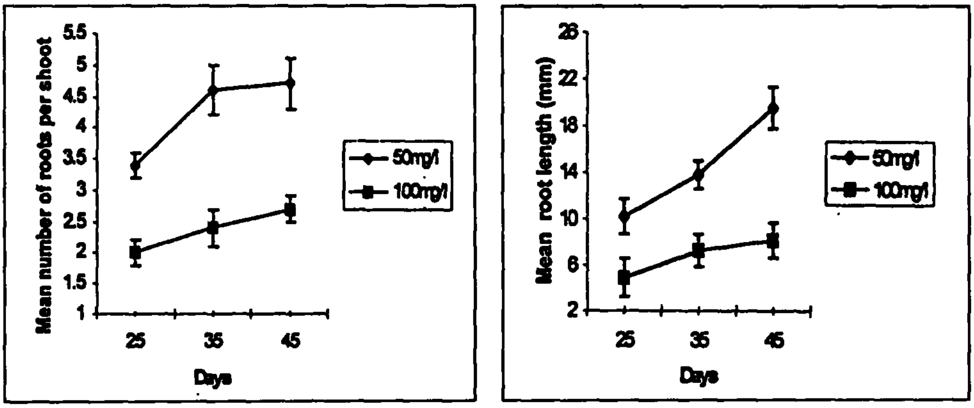

Figure 4. (a) Mean number of roots and (b) length of roots developed in shoot pre-treated with two IBA concentrations for 3 hrs
Shoots without IBA treatment (control) had no roots. X- axis represents the number of days in rooting medium following IBA pre-treatment.

Effect of the physical form of the medium for the culture of pre-treated shoots

Roots were initiated in 33.3% and 40% in 50 and 100 mg/l IBA treated shoots respectively but they did not elongate further in solid medium. Shoots in liquid medium developed roots and the rooting percentages, root length and the number of roots are given in Table 3.

Table 3. Effect of the physical form of the culture medium on root development of shoots pre-treated with various concentrations of IBA.

IBA treatment (mg/l)	Type of culture medium	Percentage rooting	No: of roots per shoot ± S.E.	Root length (mm) ± S.E.
50	Liquid	73.3	17.2 ± 1.6	4.2 ± 0.9
50	Solid	33.3	--	--
100	Solid	40.0	--	--

--not recorded as roots formed were less than 2mm in length. Data were recorded after 35 days of culture. Data based on mean values of two individual experiments with 15 replicates per treatment in each experiment.

Effect of static and shake culture system

Shake culture gave better results compared to static culture and percentage rooting was 82% and 50.2% respectively. The length and the number of roots in the former were significantly ($p<0.05$) higher than in the latter (Table 4).

Table 4. Root development in pre-treated shoots (50 mg/l IBA) in static and shake cultures

Treatment	Percentage rootin	Number of roots per gshoot (mean ± S.E.)	Root length (mm) (mean ± S.E.)
Shake	82.0	4.6 ± 0.5a	16.5 ± 1.4^{a}
Static	50.2	2.5 ± 0.5b	9.8 ± 1.5^{b}
		$P < 0.05$	$P < 0.05$

Effect of an initial dark period

No significant differences were observed in each of the parameters recorded (% rooting, length and number of roots per responding culture) among the shoots incubated in light or in the dark for an initial period of 7 and 14 days (Table 5).

Data as recorded after 35 days of culture. Means followed by different letters in each column are significantly different at $p < 0.05$. Mean values of 3 experiments each consisting of 10 replicates per treatment.

Table 5. Effect of an initial dark period on rooting of shoots after IBA pretreatment

Dark period (days)	% rooting	Number of roots per shoot (mean ± S.E.)	Root length (mm) (mean ± S.E.)
0 (Control)	82.3	4.2 ± 0.7	17.4 ± 2.1
7	84.4	4.8 ± 0.9	15.2 ± 1.4
14	78.9	3.4 ± 1.0	18.8 ± 1.6

Data were recorded after 35 days culture. Mean values of 2 individual experiments with 15 replicates per treatment in each experiment.

DISCUSSION

Shoots did not form roots in the continuous presence of IBA or NAA (0.1 mg or 0.5 mg/l) in the rooting medium regardless of the type of culture matrix (liquid or solid) used. Instead, callus formation occurred on the microshoots. The presence of auxin, even at low levels, over a long period may cause callus formation (Lane & McDougald, 1982). Furthermore, formation of callus at the shoot base may inhibit normal root development (Lane, 1979). For the initiation of root primordia just after wounding of cutting, a supply of auxin is critical (James & Thurbon, 1979). The low level of IBA used at 0.1 or 0.5 mg/l may have resulted in a limited uptake that did not meet the auxin requirement during the critical period when root induction takes place.

As compared to the presence of auxin in the rooting medium, shoots treated with a high concentration of IBA (50 or 100 mg/l for 3hrs) followed by culture on growth regulator-free liquid medium, formed roots to a varying degree depending on the IBA concentration. This showed that the application of auxin at much higher concentrations, for a short duration, was necessary for root formation. In certain woody and non-woody plant species, there is a need for a constant supply of auxin throughout the initiation phase (Jarvis, 1986). High concentrations of auxin, applied even for a shorter period (even as short as 30 min.), may ensure an adequate supply of auxin to initiate cell division and control organisation of the root primordium (Jarvis, 1986; James, 1983).

Although 100 mg/l IBA treated shoots formed roots, their elongation was reduced significantly compared to the low level tested. The process of root formation can be separated into two phases, initiation and root elongation/ growth (James & Thurbon, 1979). It is often found that root induction and initiation requires a higher level of auxin but higher concentrations are inhibitory for further development (Jarvis, 1986; George, 1996). For root initiation and growth may depend on the concentration as well as the duration of auxin treatment. However, the effect of different periods of duration of IBA pre-treatment was not undertaken in the present study. Of the two IBA concentrations tested (with equal exposure time) in this study, 50 mg/l is better than 100 mg/l with 3 hr exposure for *in vitro* rooting of tea.

Although microshoots pre-treated with the best IBA level when cultured on agar medium did produce roots, they did not elongate further compared to those which cultured on liquid medium. The low oxygen availability in shoots cultured in the former may be one of the reasons for low rooting in agar. Liquid medium may also allow a better distribution of nutrients than a semi-solid medium. It is also likely that the uptake of minerals is comparatively lower in shoots cultured in agar solidified medium compared to those in liquid medium, which may be partly responsible for the different responses in rooting. The mineral nutrition of the shoot has been found to be one of the determinants in the formation of adventitious roots (Haissig, 1986).

A problem more often faced with *in vitro* cultures of tree species is the accumulation of inhibitory substances in the growth medium (Hu & Wang, 1983). The diffusion rate of molecules, including toxic metabolic wastes released by the explant, through agar may be lower than in liquid medium. Therefore, accumulation of toxic substances released by the explant near the base of the shoot, which is the potential site of root formation, would be less in liquid medium than in agar medium.

Shake cultures accelerated the emergence of roots compared to static cultures. In addition, a significant reduction in the number and length of roots was observed in static cultures. This may be due to the lack of aeration in static liquid medium. Shaking of liquid medium may facilitate aeration and it is known that for normal physiological activities, such as root growth in higher plants, there is a need for an adequate supply of oxygen. The promotive effect of oxygen on rooting of cuttings *in vivo (ex vitro)* has been demonstrated in many cases, even though the stage in which rooting is more favoured by oxygen concentration is not clear (Haissig, 1986). In addition, the diffusion efficiency of oxygen through the medium to the tissue is important for the oxygen availability to the tissue (Gislerod, 1982). And this efficiency may be higher in agitated cultures than in static cultures.

It has been reported that in some plant species, such as peach (Hammerschlag et al., 1987), apple (Zimmerman & Fordham, 1985) and *C. japonica* (Samartin et al., 1986), *in vitro* rooting is influenced by an initial dark period. In the present study, however, an initial dark treatment (7 or 14 days duration) did not have any significant effect on rooting. In this study where IBA was used as a rooting stimulus, is more stable than IAA, which is photolabile even at low light intensities (Jacobsen, 1983). Therefore, when IBA is used as an auxin source there may not be any beneficial effect by a dark treatment.

Microshoots used in this study was from plants raised from seeds and thus, not true-to type. Therefore, it is recommended to use clonal material to test the reproducibility and consistency of this protocol.

Ex vitro rooting was believed to be the preferred method for woody plants, in which secondary thickening is important. On the other hand, some researches found that micro cuttings of some other woody plant species rooted *in vitro* have required a shorter period of acclimatisation and have produced plantlets of better quality than cuttings of the same kind rooted *extra vitrum* (George, 1996). Further work is in progress to optimise the conditions for acclimatisation of *in vitro* rooted plantlets on various soil mixtures as well as non-soil substrates to obtain field plantable *in vitro* propagated tea plants.

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