Efficient rooting and biological hardening of tissue culture raised tea (*Camellia sinensis* (L.) O. Kuntze) plants.

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Summary

Application of tissue culture (TC) technology for mass propagation of tea [*Camellia sinensis* (L.) O. Kuntze] is still limited on account of (i) difficulty encountered in rooting of some clones, and (ii) hardening and field establishment of TC raised tea plants. This presentation will focus on attempts made to overcome these difficulties. A two step method has been developed (culture of microshoots in ½ MS medium containing 25.0 μM NAA or 175.0 μM IBA for 10 days, followed by transfer to plant growth regulator (PGR)-free medium for two weeks) to obtain cent per cent rooting of even difficult-to-root clones. The strength of MS medium, period of exposure in auxin containing medium, sucrose concentration, presence/ absence of gelling agents, pH, temperature and light intensity were also found to have a significant effect on rooting. Use of phytagel was found to be superior over agar. Morphological, histological and biochemical (soluble proteins, protein profile, peroxidase activity, etc.) changes during the initiation and elongation of roots were also studied upto 18 days (10 days in auxin medium followed by 8 days on PGR-free medium). To overcome high mortality encountered during lab to land transfer, physical and biological methods of hardening have been tried. In particular bacterial strains isolated from the rhizosphere of established tea bushes have been found to be effective agents for hardening of TC raised tea plants.

Key words: Tea, *Camellia sinensis*, *in vitro* rooting, biological hardening

Introduction

The potential of tissue and organ culture for rapid clonal propagation of elite tea clones and the use of genetic engineering methods for bringing about improvement in tea quality and production are well recognized, and many laboratories have successfully developed *in vitro* propagation for tea including induction of somatic embryogenesis (Palni *et al.*, 1999 and references therein). 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. Application of tissue culture (TC) technology for mass propagation of tea is still limited on account of (i) difficulty encountered in rooting of some clones, and (ii) hardening and field establishment of TC raised plants.

Several factors, other than auxins, have been reported to effect *in vitro* rooting (Moncousin, 1991 and references therein); these include mineral nutrients, carbon source, pH of the medium, gelling agents, light intensity/ duration and temperature. TC raised plants experience high mortality, following lab to land transfer. Apart from various abiotic causes, one major probable cause of high mortality of such "aseptically" raised plants is their sudden exposure (particularly the root system) to microbial communities, including the minor and major pathogens, present in the soil (Pandey *et al.*, 2000). Keeping above facts in mind, the present study was carried out to develop efficient rooting of *in vitro* propagated microshoots and for successful field establishment of TC raised tea plants.

Materials and methods

Plant material

Microshoots of tea [*Camellia sinensis* (L.) O. Kuntze] were obtained using immature cotyledons or nodal cuttings as explants (Bag *et al.*, 1997). Shoots were multiplied (Fig. 1A) and maintained on MS (Murashige and Skoog 1962) medium gelled with agar (0.8%, w/v) and containing 5.0

μM BAP plus 1.0 μM IBA. After 30-35 days of transfer to multiplication medium, individual shoots (25-30 mm height) were harvested and used in the rooting experiments.

Based on the preliminary experiments, tea microshoots were cultured on ½ MS medium supplemented with various concentrations of plant growth regulators (PGRs), namely IBA, NAA, and IAA (0-300 μM) for 10 days. These were then shifted to PGR-free basal medium; in another experiment microshoots were continuously cultured on auxin containing medium. After standardizing the optimal concentration of PGR, strength of MS (MS, ½ MS, ⅓ MS, ¼ MS, ⅕ MS, $^1/_6$ MS), incubation period of microshoots on auxin containing (0-20 days, at two days interval) root induction medium, sucrose concentration (0-400 mM), pH (2-12) of the medium, gelling agent [agar (0.8%), phytagel (0.2%) or liquid medium], temperature (5-50 °C), presence or absence of light and its intensity (0-100 μmol $m^{-2}s^{-1}$) were also varied to observe their effect with a view to improve the overall rooting conditions of micrpropagated tea shoots. Histological studies, peroxidase activity and changes in proteins during rooting were also studied in the basal portion of microshoots following transfer to root inducing conditions.

Biological hardening

Four bacterial isolates, *Bacillus subtilis* and *Bacillus* sp. (associates of established tea rhizosphere), *Pseudomonas corrugata* 1 and *P. corrugata* 2 (associates of young tea rhizosphere) were used as test inoculants. The bacteria were selected on the basis of strong antifungal activity against several fungi including pathogens of tea (Pandey *et al.*, 1997).

In three different seasons (rainy, winter and summer) separate inoculation experiments (control soil without bacterial inoculation, soil inoculated with *Bacillus subtilis*, *Bacillus* sp., *Pseudomonas corrugata* 1, or *P. corrugata* 2) were carried out with TC raised tea plants during transfer to soil. The soil (~175 g per plastic pot) was inoculated by adding 1 ml of bacterial broth containing 10^4 to 10^5 cells ml^{-1}. TC raised tea plants were then transplanted (one per pot) in control (uninoculated) as well as inoculated pots. Observations on survival, microbial activity and growth performance of control and inoculated plants were recorded one year after transfer to soil.

Results and discussion

The study demonstrated that the effect of auxin treatment on rooting of microshoots varied with the concentration and the type of auxin used. The root formation was not observed in untreated (control) shoots or when the microshoots were cultured continuously on medium containing low concentration of auxin. For efficient rooting of microshoots, a two-step procedure was followed which resulted in nearly cent per cent rooting efficiency and the method was highly reproducible. In this the shoots were exposed to auxin for 10 days and then transferred to auxin free medium. Rooting was not observed during the first ten days when the shoots were grown on auxin supplemented medium; root emergence was, however, noticed within 3-5 days of transfer to PGR-free medium. Among the auxins tested, treatment with NAA was better in comparison to IBA and IAA (Table 1). When IBA, IAA or NAA were applied at different concentrations, significant differences in adventitious root formation were observed. Incubation of microshoots on medium containing 25.0 μM NAA or 175.0 μM IBA for 10 days, followed by transfer to auxin-free ⅓ MS medium resulted in cent per cent rooting (Fig. 1B) whereas 50.0 μM IAA induced 92% rooting. Besides auxins, several other factors significantly influenced rooting response. Gelling agent used in the medium also influenced rooting performance of microshoots significantly. When agar (0.8, w/v) was used as a gelling agent, the overall rooting was lower in comparison to phytagel (0.2%, w/v). Liquid medium was also found to favour good root formation. Temperature was found to be yet another physical factor influencing *in vitro* rooting of microshots. Both lower (15 °C or below) or higher (35 °C or above) temperatures significantly suppressed the overall rooting of tea microshoots. Optimum temperature for best rooting response was found to be 25-30°C. Amongst various strengths of MS medium tested, ½, ⅓ and ¼ MS were found to promote upto 100% rooting. In terms of overall performance ⅓ MS gave best response and the period of exposure of microshoots to auxin containing medium for 10 days, followed by transfer to PGR-free medium was most suitable. Amongst various concentrations tried (0-400 mM), 50 mM sucrose was found to result in best rooting response. While rooting could take place in a wide pH range (4.0 to 9.0),

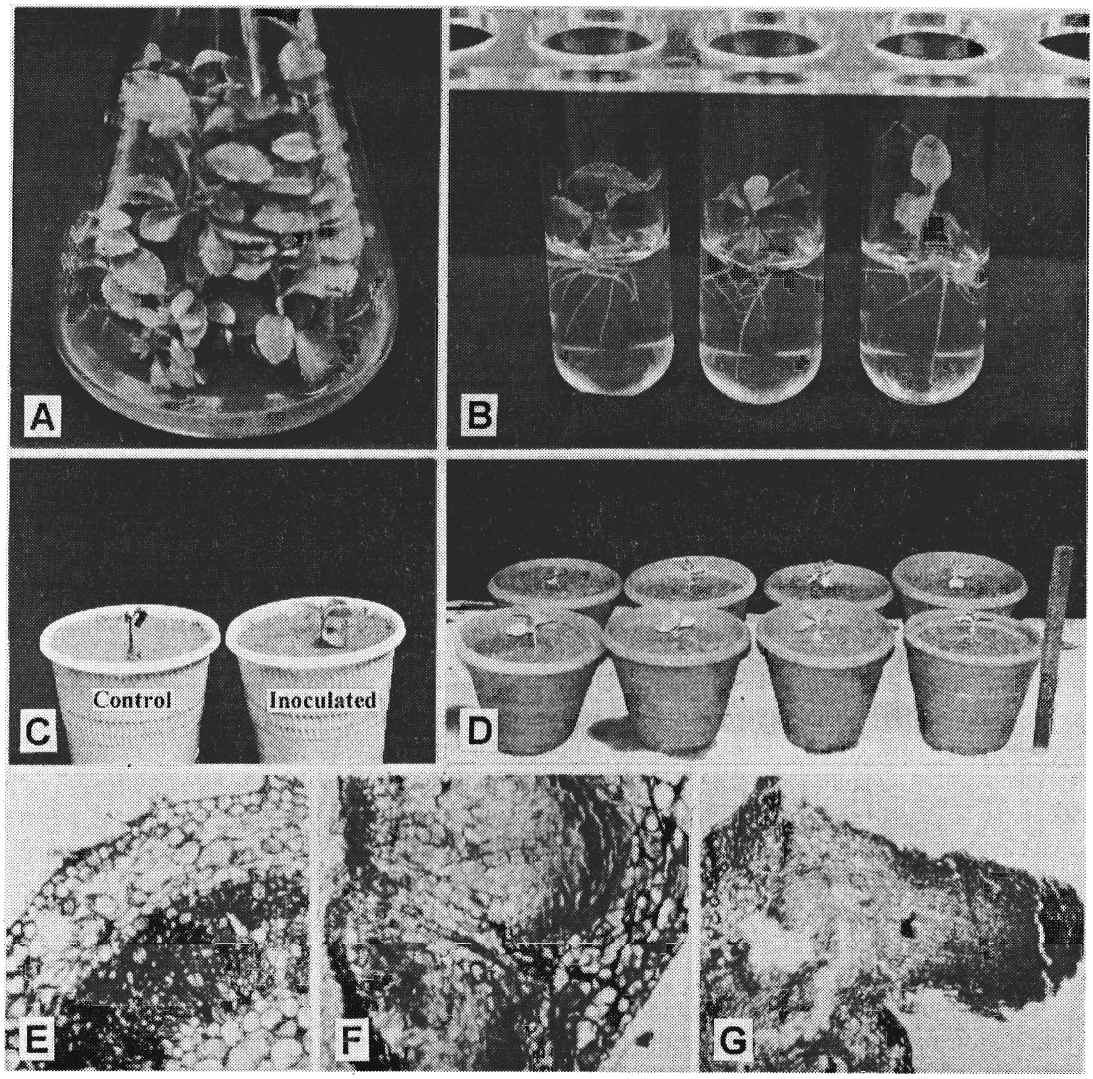

Fig. 1: *In vitro* rooting and biological hardening of TC raised tea plant.

A. Profuse shoot multiplication in culture. B. Adventitious root formation in microshoots kept on PGR-free medium after transfer from NAA containing medium (10 days incubation). C. Representative TC raised tea plants: left - wilted plant (control) and, right - healthy plant (inoculated). D. TC raised tea plants following biological hardening, some months after transfer to soil. E. Anatomical details at the base of stem (microshoot) at the time of excision (day 0). F. Formation of root primordia in the pericycle. G. Root emergence.

the recommended pH for best overall rooting is 5.5 or 6.0. Rooting response was also examined in relation to light intensity (0, 5, 12, 20, 30, 40, 50, 75, 100 μmol m^{-2} s^{-1}) and it was strongly affected with increase in intensity. Best response in terms of number of roots per shoot and length of the roots was obtained when the light intensity was kept around 40 μmol m^{-2} s^{-1} for 16 h followed by 8 h dark period.

The histological observations of the basal portions of microshoots (freshly excised; Fig. 1E) showed that 2 days after transfer to PGR-free medium following auxin treatment, periclinal/ anticlinal cell divisions occurred in a few cells of pericycle. These gradually formed meristematic pockets and developed as root primordia beneath endodermis (Fig. 1F); these finally pushed through endodermis, cortex and epidermis. The first root primordia were observed about 3-5 days after transfer to PGR-free medium (Fig. 1G). Peroxidase activity in the basal part of microshoots increased during incubation on NAA (25.0 μM) containing medium and highest values were recorded after 18 days. The marked increase in peroxidase

Table 1: Comparison of best results on *in vitro* rooting of microshoots of tea following treatment with IAA, IBA or NAA for 10 days and then transfer to PGR-free medium.

PGR	Concentration (μM)	% Survival ± SE	% Rooting ± SE	Average no. of roots per shoot ± SE	Average length of the longest root (mm) ± SE
IAA	50.0	100.0±0.0	91.7±6.8	11.6±1.3	24.0±3.2
IBA	175.0	100.0±0.0	100.0±0.0	12.5±1.0	8.4±0.7
NAA	25.0	100.0±0.0	100.0±0.0	11.4±0.3	26.3±1.3

Each treatment consisted of 4 shoots (in triplicate) and the values are an average of 12 explants. Cultures were grown on ⅓ MS medium containing 50.0 mM sucrose and phytagel (0.2%, w/v). The average length of shoots at transfer to rooting medium was approx. 25 mm. Results were recorded after incubation for 8 weeks in the presence of light (16 h/day).

activity was recorded after transfer of microshoots to PGR-free medium. Total soluble proteins increased during the initial 10 days of incubation on NAA containing medium (25.0 μM) and then decreased in both cases whether the microshoots were kept on NAA supplemented medium or on PGR-free medium.

The bacterial inoculations were effective in improving the survival of tissue culture raised tea plants (Fig. 1C & D), after transfer to suitably inoculated soil in all three seasons. The lowest survival was, however, observed during summer (36% in control plants, and upto 88% in inoculated plants). Similarly, during rainy and winter seasons, 50 and 52% survival was recorded in control plants; this could be improved to 100 and 96% due to bacterial inoculations, respectively. *B. subtilis* and *P. corrugata* 2 were found to be most effective. Bacterial inoculations were also found to positively influence plant growth, e.g., shoot length and leaf number in most cases.

With the current knowledge on various aspects of tissue culture and the availability of modern techniques, it is expected that future progress in mass propagation of elite tea clones using micropropagation will be both rapid and spectacular, and that this technology would be applied in the field at an industrial level.

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