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Induction of hairy roots in tea (*Camellia sinensis* L.) using *Agrobacterium rhizogenes*

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***Agrobacterium rhizogenes* strain A4-mediated 'hairy roots' have been induced for the first time in leaves of *in vitro*-grown seedlings of *Camellia sinensis*. The hairy roots originated at the proximal end of the young leaves after 17 ± 2 days of bacterial inoculation. These hairy roots were cultured in hormone-free MS medium, and MS supplemented with 1 mg/l NAA. Root biomass production was better in auxin-containing medium.**

AGROBACTERIUM RHIZOGENES is the causal agent of the hairy root disease and is characterized by its ability to cause root proliferation at the site of infection of the susceptible hosts. Virulence of this species is dependent on the root-inducing Ri-plasmid a portion of which, called 'T-DNA', is transferred and integrated into the host plant DNA (refs. 1, 2). Susceptibility towards *A. rhizogenes* infection varies among different plant families and also with the bacterial strains[3]. Such hairy roots are characterized by their ability to grow profusely on a hormone-free culture medium under *in vitro* condition in a large number of plant species[4]. The major consideration behind induction and *in vitro* culture of *A. rhizogenes*-mediated hairy roots has so far been to device an alternate strategy for the production of commercially useful secondary metabolites of plant origin which are known to be synthesized and accumulated in roots[5,6]. In a few recent studies, however, the root-inducing capacity of Ri-plasmid has also been gainfully employed in vegetative cloning of some otherwise difficult to root, recalcitrant woody plant species such as almond, olive, *Actinidia deliciosa* and apple[7–11]. The approach was found to work satisfactorily both in species where self-rooted cuttings or efficient root-stocks were required.

Camellia sinensis (family Theaceae) is an important cash crop of India. Like other woody perennials, the rooting efficiency of the stem cutting of tea is low[12]. This, coupled with slow vegetative growth rates of cutting, poses a serious limitation in the early introduction of high-yielding clones of tea for their commercial plantation and cultivation. Cell culture-based micropropagation approaches such as axillary or apical bud proliferation, induction of somatic embryogenesis and multiple shoot cultures are being tried in many laboratories to overcome this problem[13–17]. While establishment and *in vitro* proliferation of microshoots is now a routine in tea tissue cultures[6,17], rooting in these shoots and regeneration frequency of complete plantlets is still very low[18]. Based on this information, it was thought logical to explore the possibility of improving

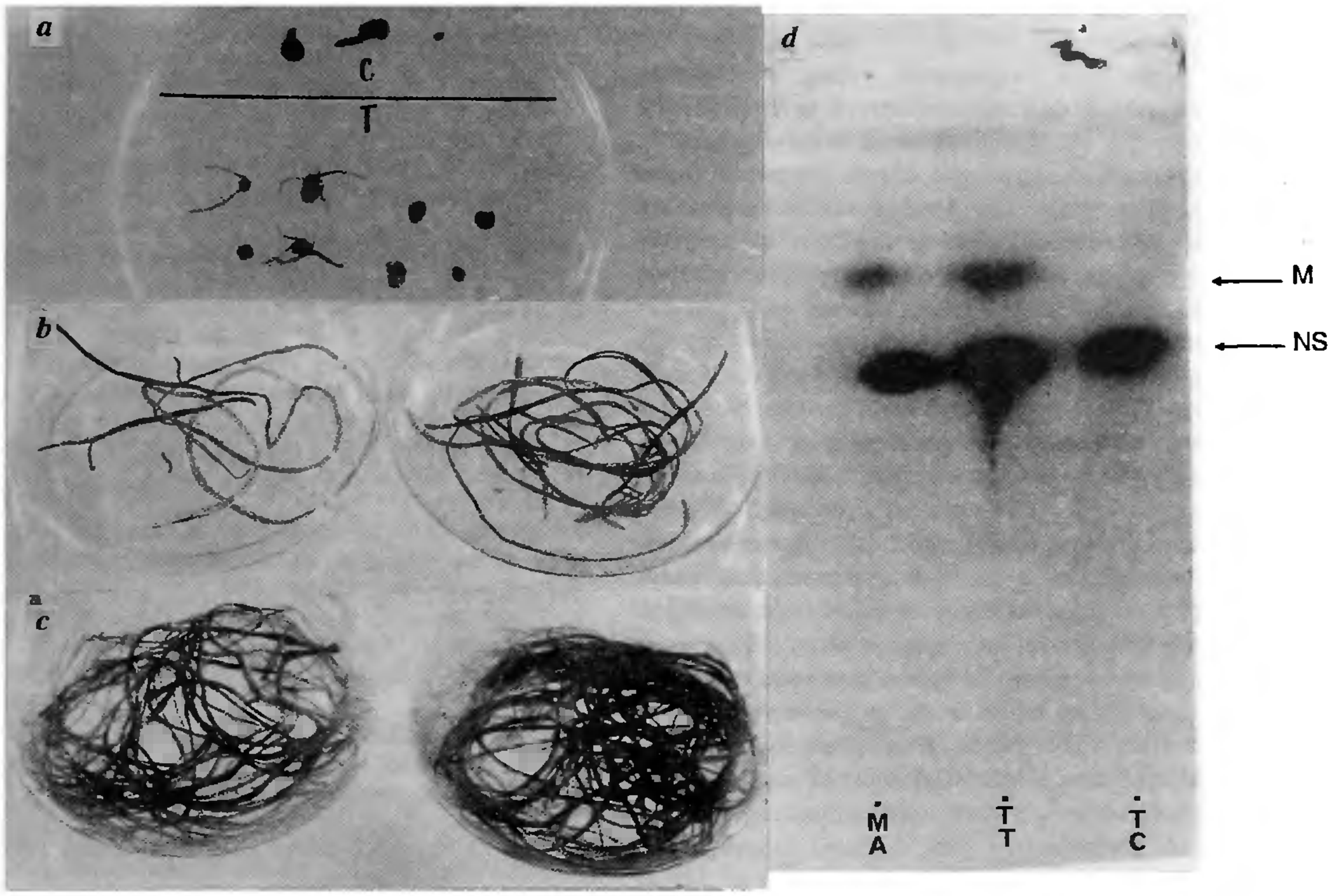

Figure 1 *a–d*. *a*, Transformed roots arising from the cut ends of young tea explants after 17 days of bacterial inoculation [C, nontransformed (control) explants; T, transformed explants]; *b*, *c*, Biomass produced after 35 days of culture in MS medium: (*b*) without any hormone, (*c*) supplemented with 1 mg/l NAA; *d*, Detection of mannopine in the extract of hairy roots of *C. sinensis* by paper electrophoresis. Lane TC, nontransformed (control) roots; TT, transformed roots; MA, standard mannopine; M, Mannopine; NS, neutral sugar.

upon the poor rooting ability of stem cutting and *in vitro* regenerated microshoots of tea via Ri-mediated transformation.

As a first step towards this approach the susceptibility of *C. sinensis* to *A. rhizogenes* infection was tested using a virulent mannopine positive strain A4 and standard leaf disc method of cocultivation. Here we report the efforts initiated and encouraging results obtained in this direction.

A. rhizogenes strain A4 (supplied by D. P. S. Verma, Biotechnology Centre, Ohio State University, USA) was regularly subcultured on solid YMB medium[19]. For transformation studies, bacteria were grown in liquid YMB medium for 48 h on a rotary shaker (90 rpm) at 25 ± 2°C in dark. The bacterial population was approximately 10^8 cells/ml as measured by OD at 660 nm.

Seeds of *C. sinensis* were obtained from the tea plantation at Indian Institute of Petroleum (IIP), Dehradun. Seed coat was removed and cotyledons with embryo were surface sterilized in 0.1% $HgCl_2$ for 3 min followed by thorough washing with sterile distilled water. Seeds were germinated in petri plates on a hormone-free MS medium. Leaves of 35-day-old seedlings were used as the explant source for transformation.

The leaves were excised from *in vitro* grown-seedlings and the cut ends were dipped in bacterial suspension for 2 min, and were blotted dry on a sterile filter paper. The explant were cocultivated with bacteria for 2 days on solid basal MS medium, as well as on solid MS medium supplemented with 1 mg NAA in dark at 25 ± 2°C. The cultures were transferred to basal MS medium without hormone containing 1000 µg/ml Cephalexin (Ceff 250, Lupin Laboratories) for two successive culture passages of 15 days long to get rid of the bacteria and finally the roots were grown in antibiotic-free medium. For the opine analysis, transformed and nontransformed control roots were homogenized with water and 0.5 ml of 0.1N HCl. After centrifugation at 12,000 g for 5 min, the supernatant was separated and spotted on Whatman No. 3 MM chromatography paper along with the standard mannopine (Sigma). The paper was then subjected to electrophoresis (10 V/cm). Formic acid : acetic acid : water (1 : 3 : 11, v/v/v) buffer was used. After drying, the electrochromatogram

was stained with silver nitrate as described by Petit *et al.*[20].

In the preliminary experiments, young incompletely expanded leaves from node numbers 3 to 5 (from the apex) of 35-day-old seedlings were tried for genetic transformation. Fully expanded leaves were not found suitable for cocultivation because of excessive leaching of the phenolics. Cocultivation in the hormone-free MS medium led to only callus formation at the proximal cut end of the leaves. The emergence of roots did not occur even after callusing. However, when the explants were cocultured on the MS medium in the presence of 1 mg/l NAA, 2–5 roots per explants were observed after 17 ± 2 days of bacterial inoculation (Figure 1 *a*). Emergence of hairy roots was observed in about 40% of the plated leaf explants (25 explants were used in three different experiments, out of which 10 responded positively). These results indicate that presence of auxin during cocultivation plays a critical role for the induction of hairy roots in *C. sinensis*. Similar observations have already been made in case of carrot, pea and tobacco[3,21]. Emerging roots were white at the beginning but under continuous light they turned green. For root biomass production, both liquid MS basal and MS supplemented with 1 mg/l NAA media were used. Branching was not observed in hormone-free MS medium although the elongation occurred to some extent. Addition of 1 mg/l NAA in the medium showed marked difference in branching pattern as well as in the growth rate (Figure 1 *b, c*). The biomass production was approximately 3 times higher (i.e. 1.34 ± 0.655 g) in auxin-supplemented medium as compared to basal MS medium (i.e. 0.42 ± 0.177 g) after 35 days of culture. Presence of mannopine in the hairy roots and its absence in nontransformed control roots, as evident from paper electrophoresis (Figure 1 *d*), confirmed the transformed nature of roots.

The results summarized above clearly indicate that *A. rhizogenes*-mediated genetic transformation in *C. sinensis* is a possibility and fast-growing hairy root lines can be induced in tea. Based on these preliminary findings efforts are now under way to produce hairy roots in vegetative stem cuttings and *in vitro*-regenerated microshoots of tea so that an efficient mass propagation system can be deviced for this difficult-to-root cash crop of India.

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A study on mechanism of phyllody disease resistance in *Sesamum alatum* Thonn.

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Phyllody disease is a highly destructive mycoplasma disease of *Sesamum* transmitted by leafhopper. Resistance to the disease has been recently reported in *Sesamum alatum* and hybrids between *S. alatum* and *S. indicum* were produced. Screening of the F_2 generation under controlled condition using insect vector showed that the resistance may be controlled by a single recessive gene. Screening of F_3 generation under natural condition using infector-row-technique could not confirm the single recessive gene inheritance of phyllody resistance. Further screening by grafting method of inoculation required information on survival rate of seedlings after grafting and mechanism of resistance present in *S. alatum*. Experimental results showed 40% survival rate after grafting. Graft inoculation of mycoplasma-like organisms (MLOs) followed