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Shoot regeneration from *Agrobacterium tumefaciens*-induced tumors of a tropical timber tree, *Fagraea fragrans*

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Summary. Agrobacterium tumefaciens A208 with nopaline plasmid pTiT37 was used to obtain stem tumors on plantlets of *Fagraea fragrans* grown in vitro. Bacterial elimination and tissue proliferation were simultaneously achieved by growing tumors on cefatoxime medium. After some tissue growth the shoots regenerated. An examination of these showed the presence of nopaline, indicating genetic transformation by T-DNA.
Key words. Agrobacterium tumefaciens; nopaline plasmid; tropical timber tree; *Fagraea fragrans;* genetic transformation.

The bacterium, *Agrobacterium tumefaciens*, transfers a particular DNA segment, the T-DNA, from its large Ti plasmid into the cells of host plants. The T-DNA is integrated into the plant genome. This system is a natural example of genetic transformation. It is suggested that it might be applicable for tree improvement in certain cases. If the transformations were found to be beneficial in such modified tree tissues, they could be used for many practical purposes[8,12]. Genetic transformation of woody tree tissues was reported in poplar[11] and in loblolly pine[12]. But in both these cases, only the genetically transformed tissues were obtained, and there was no shoot or root regeneration from them. Here, we describe the regeneration of shoots from *Agrobacterium tumefaciens*-induced tumors of a tropical timber tree. As far as we know this is the first report on shoot regeneration from such tumors of a tree species.

Fagraea fragrans Roxb. (local name – Tembusu; family – Loganiaceae) is a slow growing indigenous species that attains a height of 50–60 m when mature and yields[9] heavy hardwood timber of commercial value. Multiple shoots of *F. fragrans* were initiated and maintained in in vitro culture according to the methods described earlier[10]. The shoots, approximately 4–5 cm tall with 8–10 leaves, were subcultured onto Murashige and Skoog (MS) medium[2] supplemented with sucrose (2%) and solidified with gelrite (0.2%) but without the addition of any plant growth regulators. pH value was adjusted to 5.8 before the gelrite was added, then the medium was autoclaved for 20 min at 122 °C. Two shoots were cultured in each container with 50 ml of the medium. All the cultures were incubated under white fluorescent light 136 uEm^{2}s^2, 16-h photoperiod, at 26 ± 2 °C.

Roots developed in three or four weeks after subculturing and the plantlets were ready for use in further experiments. Both the nodal and internodal regions were punctured with a sharp needle and simultaneously the bacteria were carefully smeared onto the wounded surface without touching any other parts of the plant. The bacteria were obtained from 3-day-old agar cultures of *A. tumefaciens* A208 containing nopaline plasmid pTiT37. Three to four weeks after inoculation with *A. tumefaciens*, tumors developed on the inoculated sites of *F. fragrans* plantlets. These tumors grew rapidly and were creamy yellow in color, reaching 0.4–0.6 cm in diameter seven weeks after inoculation (fig. 1).

They were detached from the stems and cultured on MS medium supplemented and sterilised with cefotaxime (0.5 g/l) in the first three passages. This was to eliminate the bacteria from the growing tissue system. After obtaining some good growth the callus cultures developed from tumors were transferred to MS medium with no antibiotics. The tissues grew vigorously on the fresh media and turned green (fig. 2). To induce shoot regeneration some of the tissues were transferred to MS + benzylamino purine (BA) medium (0.5, 1.0 and 5.0 mg/l). There was no shoot development in any of them. On the other hand, the tissues maintained on growth regulator-free medium grew well for another 6–9 months and spontaneous bud formation was observed in many of the cultures. Some of these buds developed into shoots (figs 3 and 4). Others dedifferentiated into callus again after subculturing onto fresh medium. Individual regenerated shoots cultured on MS medium did not produce any roots and again they produced callus at basal ends.

Both the axenic callus (about 100 µg) and leaf tissues (50–100 µg) from spontaneously regenerated shoots were examined for the presence of nopaline. They were incubated overnight in MS liquid medium supplemented with sucrose (2%) and arginine (0.1 M). The tissues were then blotted dry on a piece of filter paper and macerated in an Eppendorf tube by using glass rod. After centrifugation at 15,000 × g for 2 min, 20-µl aliquots of the supernatant were applied to Whatman 3-mm paper for electrophoresis at 400 V in formic

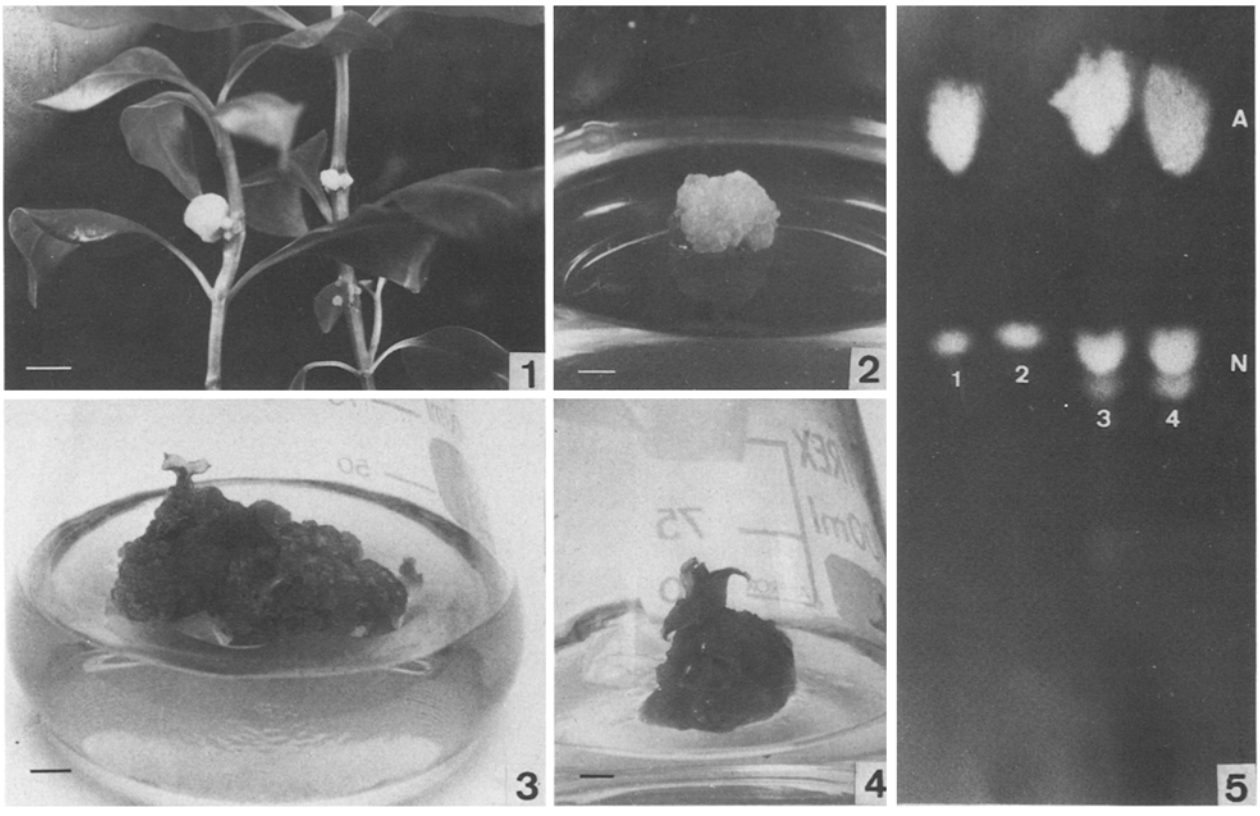

Figure 1. Tumors formed on nodal and internodal regions of *F. fragrans* plantlets seven weeks after inoculation with *A. tumefaciens* A208.

Figure 2. Axenic tumor growing on phytohormone-free MS medium.

Figures 3 and 4. Shoot regeneration on green tumor tissues. Bar: 0.5 cm in all cases.

Figure 5. Detection of nopaline in the extract of tumor and leaf tissues from regenerated shoot. A: Arginine, N: Nopaline. Lane 1: arginine + nopaline standard; lane 2: nopaline marker; lane 3: leaf extract from regenerated shoot; lane 4: axenic tumour in culture nine months after bacterial inoculation.

acid/acetic acid/water (5:15:80) following the prescribed method[5]. The paper was dried in a hot air steam after electrophoresis and sprayed with a mixture of 0.02% phenanthrenequinone in ethanol and 10% NaOH in 60% ethanol. The extracts of both the callus and leaf tissues were found to be nopaline positive (fig. 5). The extracts from the non-transformed tissues, which were used as control, lacked nopaline. The results show that *A. tumefaciens* is able to induce tumor formation on in vitro culture plantlets of *F. fragrans* although this species is not a recognised host of *A. tumefaciens*[4]. The tumor tissue grows well in plant growth regulator-free medium in a manner similar to poplar tissues[7]. The presence of nopaline in both callus and leaf tissues of regenerated shoots indicates that *F. fragrans* cells were transformed by *A. tumefaciens*, just like those in tobacco[6] and *Brassia juncea*[9]. *F. fragrans* tumor tissues tranformed by *A. tumefaciens* A208 regenerate shoots readily. *A. tumefaciens* A208 containing nopaline plasmid pTiT37 is a wild type culture. Attempts are made to induce rooting from shoots regenerated from tumor tissues as observed in tobacco[3,6] and mustard[9]. Further research is now directed towards micrografting the transformed shoots into normal tissue culture derived plantlets.

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