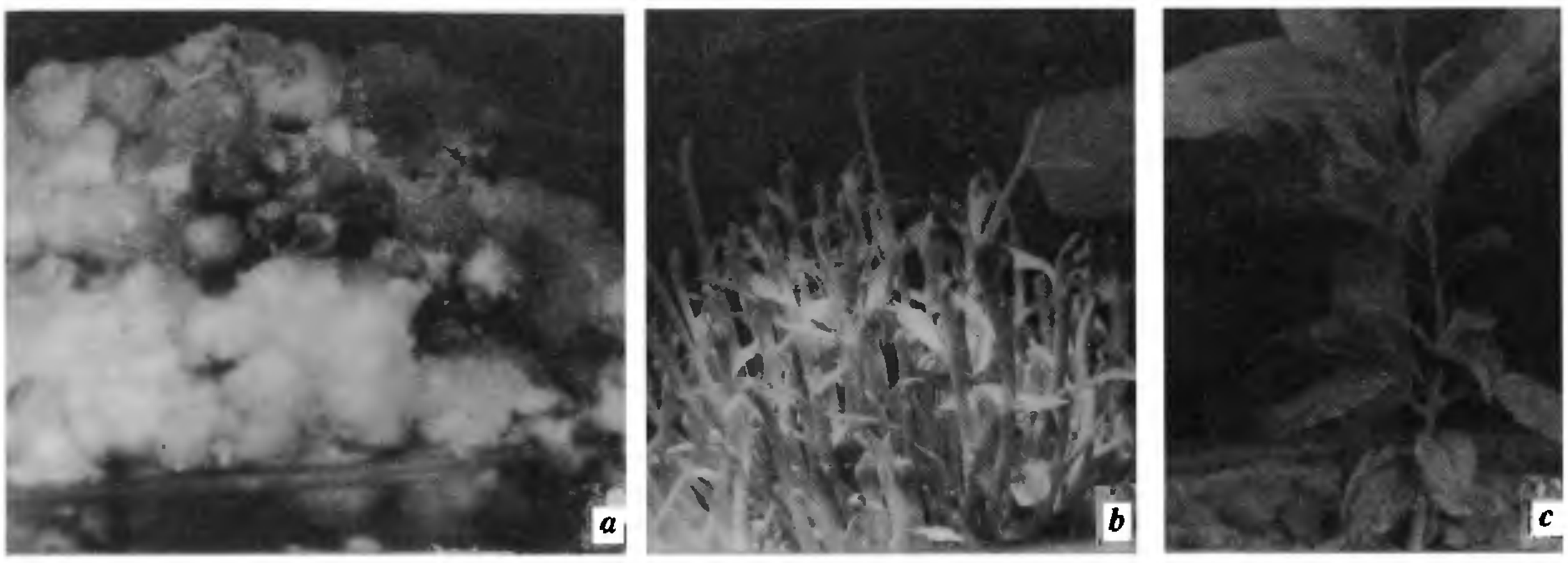
Regeneration of *Aegle marmelos* (L.) Corr. plants *in vitro* from callus cultures of embryonic tissues

Aegle marmelos is a medium, spreading deciduous spiny tree belonging to the family Rutaceae. It is supposed to be originated in the Indo-Burma subcontinent[1]. Various parts, including fruits, possess medicinal properties and have been extensively used in ayurvedic and folk medicine[2,3]. Since *A. marmelos* is an outbreeding crop and cross-pollination leads to highly heterozygous population[4], propagation through seeds is not encouraged. Singh[5] was first to culture *A. marmelos* but regeneration of plants from nucellar tissue was not possible. Arya *et al.*[4] described callus formation and some organogenesis from cotyledon and hypocotyl cultures. Recently, regeneration of multiple shoots through organogenesis was achieved from seedling leaves[6,7], nucellus[8] and cotyledons[9]. This communication presents high-frequency plant regeneration system using callus cultures from zygotic embryos of *A. marmelos*.

Mature seeds from ripe fruits and immature seeds from unripe green fruits were collected and surface-sterilized with 0.1% $HgCl_2$. Seeds were decoated and embryo axes excluding cotyledons were excised and cultured. All cultures were maintained in a growth room under 16 h photoperiod at 26°C. All media were solidified with 7 g/l agar (BDH, high gel strength) and media pH values were adjusted to 5.7 ± 0.1 prior to autoclaving. The basic culture medium was Murashige and Skoog (MS) salts[10] variously supplemented with naphthalene acetic acid (NAA), 2,4-D, IAA, IBA, BA and kinetin. Different concentrations of auxins in the range of 2.5–10 µM were used for callus induction. Plant regeneration was achieved by transferring pieces of callus tissue (fresh weight 100–150 mg) on to the MS medium with different combinations of auxins and cytokinins. Rooting was induced in IBA supplemented (5 µM) MS medium. After rooting, the leafy shoots were washed and transferred to pots containing soil mixed with compost (2:1).

Different concentrations 2.5–10 µM of IAA, NAA and 2,4-D were tried to induce callus and it was observed that 2,4-D in general was more efficient for the induction of callus and induced as much as 80% callus from both mature and immature embryo axes at a concentration of 10 µM. However, addition of a lower concentration (0.5 µM) of BA together with 10 µM 2,4-D induced 100% callus formation in all explants obtained from mature and immature seeds. The 6-week-old hard, green and compact irregularly shaped calli (Figure 1 *a*) induced on 10 µM 2,4-D and 0.5 µM BA were used to study morphogenesis. Such calli obtained from each explant were cut into small pieces (100–150 mg fresh weight) and were transferred to fresh medium. Both BA and kinetin at various concentrations were capable of inducing differentiation from undifferentiated calli (Table 1). Of the two cytokinins, kinetin was better than BA and of the auxins IAA were more efficient than NAA. Thus, the best results for shoot formation were obtained using kinetin at 5–10 µM and IAA at 0.5 µM. In all cases both mature- and immature-embryo-axes-derived calli were capable of producing multiple shoots. However, regeneration frequency and number of shoots per callus were much higher than mature embryo axes.

Proliferating shoots attained a height of 2–3 cm within 8 weeks of culture (Figure 1 *b*). The regenerated shoots were transferred to previously established rooting medium (MS + 5 µM IBA)[9] for adventitious root induction. The rooted shoots reached up to 62% within 4–5 weeks of culture. Regenerated shoots were transferred to pots containing soil mixed with compost. During the first one week, the potted plantlets were covered with glass



Figures 1 *a–c*. Plant regeneration from callus cultures in *A. marmelos*. ***a***, Six-week-old hard green and compact irregular shaped callus induced on MS + 10 µM 2,4-D + 0.5 µM BA. ***b***, Development of multiple shoots from subcultured immature-embryo-axis-derived callus on MS + 5 µM kinetin + 0.5 µM IAA six weeks in culture. ***c***, A potted plant after four months of transfer.

Table 1. Morphogenetic responses of 6-week-old mature- and immature-axes-derived calli of *A marmelos* to various concentrations of cytokinins and auxins. Each treatment consisted of two replications with 12–18 explants per replication

		Immature embryo axes		Mature embryo axes	
Concentration of cytokinin (μM)	Concentration of auxin (μM)	Percentage calli with shoots	Mean number of shoots per callus ± SE	Percentage calli with shoots	Mean number of shoots per callus ± SE
BA 2 5	IAA 0 5	40.1	38 7 ± 2,5	35 0	17 2 ± 1 8
5 0	0 5	50 3	48 7 ± 1 9	40 2	20 3 ± 2 0
10 0	0 5	54 0	41 2 ± 2 9	42 8	28 2 ± 2 2
25 0	0 5	50 8	29 6 ± 2.4	38 5	21 6 ± 2 3
BA 2 5	NAA 0 5	36 4	15 2 ± 1 2	25 6	8 2 ± 0.9
5 0	0 5	40 3	24 2 ± 2 7	30 2	14 2 ± 2 4
10 0	0 5	44 0	20 8 ± 2 6	30 8	13 6 ± 2.0
25 0	0 5	40 9	18 6 ± 1 8	28 9	10 2 ± 2 1
Kinetin 2 5	IAA 0 5	54.2	24 6 ± 2.0	41.2	19 6 ± 1 9
5 0	0 5	94 2	56 2 ± 2 1	49 6	26 2 ± 1 8
10 0	0.5	78 5	44 8 ± 2 4	52.5	30 4 ± 1.7
25 0	0 5	60 4	34 6 ± 2 1	44.2	20 8 ± 3 0
Kinetin 2 5	NAA 0.5	50 4	18 6 ± 1 4	35.2	8.2 ± 1.4
5 0	0 5	58 9	30 9 ± 1 8	40 1	14 2 ± 2 6
10 0	0 5	64 0	30 3 ± 3.0	36 0	14 8 ± 2 1
25 0	0 5	64 2	28 0 ± 2 9	37 2	11.4 ± 1 9

beakers to provide high humidity. Transplantation success was 55%. Plantlets were subsequently transferred to larger pots (Figure 1 *c*) and gradually acclimatized to outdoor conditions.

The results presented here indicate that *in vitro* regeneration of complete plantlets is possible using callus cultures from embryo axes of *A. marmelos*. The physiological state of the explant plays an important role in the regeneration of plants. Both immature and mature embryo-axes-derived calli were capable of producing plants. However, while the regeneration frequency of immature-embryo-derived calli was 34.4–94.2%, the morphogenetic capacity of the mature-embryo-axes-derived calli was lower (25.6–52.5%). The immature embryos are known to be a good source for initiating callus which possesses high regenerative capacity, as has been shown in peach[11], oak and linden[12]. The procedure described here may be suited to use in transformation studies and for mass propagation of *A. marmelos* if the frequency of off-types is low.

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In vitro axillary bud break and multiple shoot production in *Acacia auriculiformis* by tissue culture technique

Acacia auriculiformis A Cunn. Ex. Benth is a species native to Australia, Indonesia and Papua New Guinea. It has been an important exotic in many tropical countries for more than half a century. The species is currently planted for paper pulp industry[1], fuel wood, erosion control, shade and ornamental purposes. It is a leguminous, nitrogen-fixing tree of the subfamily Mimosoideae. Mass multiplication of elite and rare trees is of paramount importance[2]. Though *Acacia auriculiformis* is propagated by seeds, poor seed germination, around 20–25% has been recorded[3]. So, research is needed on large-scale multiplication of elite clones. The tissue culture technique can be used as a promising tool in overcoming this problem. Much less work has been carried out in this regard in Acacia species. A few workers have reported plantlet formation at seedling level through cotyledonous callus, shoot tip callus and axillary buds[4–6]. But no work has been reported on plant regeneration in mature Acacia trees. The present study reports on *in vitro* development of nodal shoots from axillary buds and multiplication of these nodal shoots into plantlets from 20-year-old Acacia trees

Small tender branches (10–15 cm length, with 6–7 nodal segments) with