estimate free energy of folding ($\Delta G^L(H_2O)$) in solution and can be used to calculate the relative populations of the different states as illustrated earlier, in the case of α-LA and DT.

Previous studies have shown that ANS has maximum fluorescence emission when bound to molten globule states of proteins[1,3]. However, a quantitative model that could explain the ANS fluorescence data was not attempted before. Our present analysis suggests that simple binding studies with hydrophobic fluorescent probes can provide insight into the apparently complicated problem of protein folding. It should be noted that although the analysis presented here deals with only three state transitions, it can be extended to higher order transitions also, provided the probe is capable of detecting the intermediate states.

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ACKNOWLEDGEMENT. This research was supported by a grant-in-aid from DST, New Delhi.

Received 13 July 2000; accepted 6 November 2000

In vitro propagation of *Ziziphus mauritiana* cultivar Umran by shoot tip and nodal multiplication

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Shoot tip cultures of field-grown *Ziziphus mauritiana* cultivar Umran proliferated rapidly *in vitro* on MS medium containing 100 mg/l glutamine, 125 mg/l myo-inositol and 0.1 mg/l benzyladenine (BA). Multiple shoots were obtained from the shoot tip and stem nodal explants cultured on MS medium supplemented with 0.01–0.1 mg/l BA. Isolated shoot tips elongated in MS medium and produced plantlets with 6–7 nodes within 3 weeks time. A 7-fold multiplication was achieved in MS medium without any growth regulators. Isolated plantlets were rooted in MS medium containing 10 mg/l indolebutyric acid (IBA). Rooted plantlets were planted in sterile soil mix and gradually acclimatized to the greenhouse environmental conditions. Hardened plantlets were maintained in the greenhouse for the field transplantation.

SEVERAL tree species have been mass propagated by tissue culture technology, mainly fruits and ornamental trees[1–7]. While the tissue culture technology has been developed for the mass propagation of several fruit tree species, several other tree species are lagging behind due to their recalcitrant nature to *in vitro* techniques.

Ziziphus is a multipurpose tree species widely grown for its fruits, fodder, fuel, medicinal and ornamental purposes. It belongs to the botanical family Rhamnaceae and is generally propagated by seeds. Several cultivars have been selected among the seedling populations for their superior fruit quality. Seed propagation method is generally not used since it is heterozygous in nature. *Ziziphus mauritiana* cultivar Umran is an improved Indian cultivar vegetatively propagated by grafting or air layering methods.

Recently, grafted plants of cultivar Umran were brought from India and introduced in Kuwait. Since the conventional vegetative methods have little success in mass propagation of this cultivar producing large-sized fruits, we explored the possibility of propagating via tissue culture technology. *In vitro* propagation method on *Z. mauritiana* and two of its cultivars has been reported earlier[8–13]. As the *in vitro* responses of this species are cultivar dependent[8], the reported methodology may not be applicable to the cultivar Umran. Hence we carried out experiments to develop *in vitro* techniques for mass production of this cultivar. We are reporting here our findings on mass propagation of *Z. mauritiana* cultivar Umran for the mass production of planting stock, to enhance the introduction of this cultivar in Kuwait.

Shoot tip cuttings of *Z. mauritiana* cultivar Umran were collected from a healthy and well-established fruit-yielding mature tree growing in the Kuwait Institute for Scientific Research (KISR) campus, Kuwait

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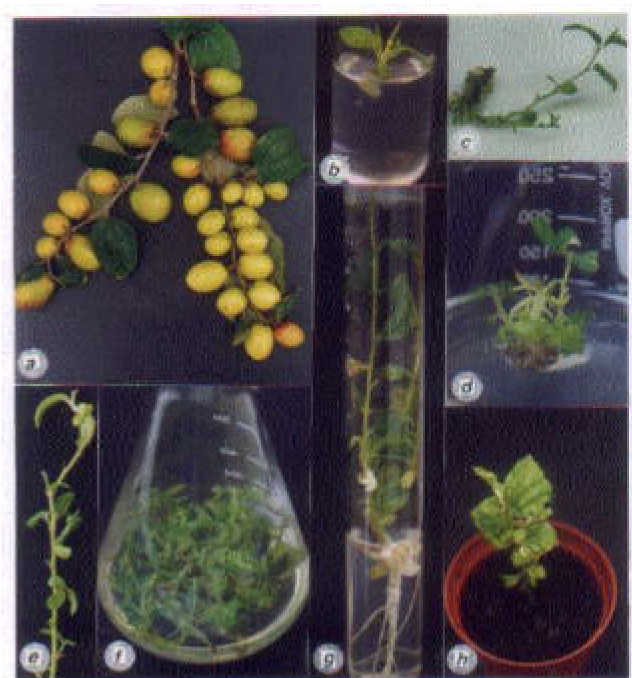

Figure 1. *Z. mauritiana* cultivar Umran. ***a***, Fruiting branch (0.25 ×); ***b***, Shoot tip initiation in culture (1 ×); ***c***, *In vitro* plantlet showing axillary bud initiation (1 ×); ***d***, Nodal explant showing multiple shoot buds (1 ×); ***e***, Plantlet showing axillary shoot buds (1 ×); ***f***, Shoot multiplication (0.5 ×); ***g***, Rooted plantlet (1 ×); ***h***, Acclimatized plantlet (0.5 ×).

(Figure 1 *a*). The shoot tips were excised from cleaned cuttings and surface sterilized with 20% commercial Chlorox solution containing 1.05% sodium hypochlorite and a drop of tween 20 for 15 min. The shoot tips rinsed in sterile distilled water were dipped in 0.1% mercuric chloride solution for 3 min, followed by thorough rinsing in sterile distilled water. The plant materials were then dipped in 70% ethanol for one second and rinsed in sterile distilled water. MS salts with 100 mg/l glutamine and 125 mg/l myo-inositol were used as basal medium for all experiments. Shoot tip explants devoid of leaf primordia were placed on the MS[14] medium with different concentrations of BA (obtained from Sigma) in 25 × 150 mm culture tubes. The pH of the medium was adjusted to 5.6 prior to autoclaving. All the cultures were incubated under 1000 lux light intensity provided by white fluorescent lamps for 16 h photo period at 25 ± 1°C. For each treatment, 40 replicates were made and the experiment was repeated twice. The explants were subcultured once in 15 days. After 45 days, the shoot tips and nodal segments were isolated from the *in vitro* plantlets obtained from the primary cultures and transferred to fresh medium for further multiplication. The isolated plantlets measuring 4–6 cm in length and with 4–5 nodes were planted on MS medium containing different concentrations (0.1–100 mg/l) of IBA and NAA for rooting experiment. Rooted plantlets were removed from the medium, washed in running water and treated with 0.5% Benlate solution prior to planting in soil mix. The fungicide-treated plantlets were planted in autoclaved soil mix containing sand, peat moss and humus (1:1:1) for acclimatization. The plantlets were hardened at normal culture room temperature for 25 days and then transferred to the greenhouse.

After two weeks of culture the shoot tip explants, responded to the MS medium without any growth regulators (Figure 1 *b*) and with different concentrations of BA (Table 1). All the shoot tip explants showed callusing at the cut end (Figure 1 *b–d*) touching the medium in all the treatments. Callus growth and proliferation was 100% in the medium containing 1 mg/l BA. Initially callus developed at the cut end and latter on the entire stem and the leaf primordia callused. The shoot buds which developed from the explants after 20 days in this medium were highly vitrified. At low concentrations of BA (0.01–0.1 mg/l), callus developed at the cut end and the shoot tip elongated with axillary branching. The nodal segments produced multiple shoot buds in the medium containing 0.01–0.1 mg/l BA. The plantlets obtained from the treatments, when placed horizontally on the surface of the hormone free-medium, produced several axillary shoot buds (Figure 1 *c*). The isolated shoot tips elongated and reached an average height of 7 cm with 5–6 nodes after 20 days (Figure 1 *e*) in hormone-free MS culture medium. Some of the plantelts produced 2–3 axillary branches in the same medium. Single nodal explants produced multiple shoot buds (Figure 1 *d*) after 15 days in the medium with 0.01 mg/l BA, while in the hormone-free-medium each node produced a single shoot bud. The shoot length of the plantlets reached up to 5 cm in the control and decreased from 4.8 to 3.6 cm when the BA concentration in the medium increased from 0.01 to 1 mg/l after 30 days (Table 1). At higher concentrations of BA up to 1 mg/l, the stem nodal segments callused and covered the axillary bud meristem completely within 20 days time.

Table 1. Effect of BA concentration on shoot tip growth response of *Z. mauritiana* cultivar Umran after 30 days

Medium	BA conc. (mg/l)	Mean shoot length (cm)	% branching	No. of nodes (average)
MS salts + 100 mg/l Glutamine and	0	5 ± 0	11	7
125 mg/l myo- inositol	0.01	4.8 ± 1.9	44	7
	0.1	4.2 ± 1.6	67	6
	1.0	3.6 ± 2.8	89	5

(±) Standard error; data from 40 replicates; experiment was repeated twice.

All the shoot buds isolated from the primary cultures, when transferred to the hormone-free MS medium, elongated into plantlets with 6–7 nodes (Figure 1 *e*). The nodal segments and shoot tips isolated from the primary cultures when subcultured regularly, i.e. once in 15–20 days in the hormone-free medium showed continuous growth and multiplication (Figure 1 *f*). Plantlets kept in the culture medium for longer duration without subculture, showed shedding of leaves and hardening of stem. All the plantlets maintained in cultures for more than three months without subculture failed to produce any adventitious roots when transferred to the rooting medium. One-month-old plantlets with 4–6 nodes, when transferred to the rooting medium containing 10 mg/l IBA, produced adventitious roots (Figure 1 *g*) after 30 days of culture. It was observed that only 30% of the shoots produced adventitious roots in the rooting medium with 10 mg/l IBA. All the plantlets survived during the acclimatization stage. Acclimatized plantlets were maintained in the greenhouse for field transfer. Hardened plantlets (Figure 1 *h*) showed normal growth and development.

In vitro studies on *Z. mauritiana* are limited to certain cultivars[8–13]. Goyal and Arya[8] found different growth responses from two different cultivars cultured in MS culture medium containing growth hormones. In our present investigation on the cultivar Umran, we obtained 7-fold clonal multiplication in hormone-free MS medium through shoot tip and nodal multiplication (Figure 1 *f*) of plantlets obtained from the primary cultures. The medium containing growth hormones suppressed shoot growth and induced callusing in this cultivar. However, at lower concentrations (0.001–0.01 mg/l BA), shoot tips elongated into a single shoot and produced 2–3 axillary branches. Nodal segments produced multiple shoot buds in this medium containing low BA concentration. When the present results on cultivar Umran were compared with the previous studies on cultivars Seb and Cola[8], it was seen that each cultivar needs a separate culture medium with or without growth regulators.

The conditioning of the stock plants has been very important for the establishment of shoot tips and nodal explants of some species[5,6]. In papaya, guava, jackfruit and in the present study, *in vitro* establishment is dependent on active vegetative growth season[2,3,5]. All the shoot tip explants collected during the summer season failed to grow on MS medium with or without cytokinin however, they produced callus in the medium containing cytokinin. After 10–12 subcultures in hormone-free MS medium, the shoot tip explants started growing. However, the plantlets were miniature in size, with small-scale leaves and short internodes. The explants collected during the spring season responded within 10 days to the hormone-free culture medium and produced healthy plantlets with normal green leaves.

The rooting response of cultivar Umran was also found to be different when compared with the earlier reports on *Z. mauritiana* cultivars, Seb and Cola. Three-month-old plantlets in culture totally failed to produce adventitious roots in all the treatments. However, one-month-old plantlets with 5–6 nodes produced adventitious roots in MS medium containing 10 mg/l IBA. Adventitious roots were initiated in 30% of the cultures after 30 days in the rooting medium. Prolonged culture of plantlets in the rooting medium induced callus at the basal region. Callusing was avoided by transferring the plantlets to the hormone-free medium immediately after the root initiation. Better root growth response was obtained in hormone-free medium. Further refinement of rooting medium on this cultivar is being carried out in our laboratory.

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Received 9 June 2000; revised accepted 28 October 2000