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In vitro clonal propagation of *Balanites aegyptiaca* (L.) Del

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Abstract In vitro propagation technique of *Balanites aegyptiaca*, a multipurpose woody tree was studied. Nodal segments including axillary bud from mature tree were used as an explant and their morphogenetic potential was tested on MS media with various concentrations (2.5–15.0 μM) of 6-benzyladenine (BA), Kinetin, and Thidiazuron alone or in combination with different concentrations (0.5–2.5 μM) of α-naphthalene acetic acid (NAA). Nodal segments showed axillary bud proliferation in almost all media tried. MS medium containing 12.5 μM BA alone was effective for inducing multiple shoots (5.0 ± 0.22) with an average shoot length (3.7 ± 0.26 cm) in 67% of cultures. A better shoot differentiation and elongation was achieved in a combined treatment of BA (12.5 μM) and NAA (1.0 μM). Half strength MS medium supplemented with Indole-3-butyric acid (IBA) gave the best result for rooting. The maximum frequency of root formation (68%), number of roots (5.3 ± 0.32) and root length (4.1 ± 0.38 cm) was obtained on half strength MS medium containing 1.0 μM IBA. The regenerated plantlets were potted and acclimatized successfully in a growth chamber and then moved to the greenhouse.

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Abbreviation

BA	6-Benzyladenine
IBA	Indole-3-butyric acid
Kin	6-Furfurylaminopurine
MS	Murashige and Skoog medium
NAA	α-Naphthalene acetic acid
TDZ	Thidiazuron

Introduction

Balanites aegyptiaca L. (Del.) (Balanitaceae), commonly known as Desert Date, is a spiny, evergreen xerophytic tree found in Tropical and Northern Africa, Syria, West Asia, Sudan, Egypt, neighbouring parts of East and West Africa particularly Senegal, Nigeria, Arabia and Burma. It is distributed throughout drier parts of India like Rajasthan (Bhandari 1995). It is well adapted to different agroclimatic regions characterized by arid and semi arid climatic features (Von Maydell 1984). The plant is drought hardy, fire resistant and ecologically very flexible with excellent persistence.

Medicinally, it is stated to possess anti-leishmanial (El-Tahir et al. 1998), anti-molluscidal, anti-fertility, anti-bacterial, antifungal, and antitumor properties (Corbiere et al. 2003). Fruits, kernel oil, leaves, resin, roots, bark, shoots, and seeds are used traditionally to

treat a wide range of diseases like whooping cough, sleeping sickness, guinea worm disease, skin disorders, etc. Its potential utilization lies in the extraction of a secondary metabolite, diosgenin (a saponin) which is a starting material for partial synthesis of oral contraceptives, sex hormones, and other steroids (Zenk 1978; Chapagain and Wiesman 2005).

Unscruplous and unscientific management practices coupled with limited cultivation and insufficient attempts for its replenishment, the wild stock of this useful plant species has been markedly depleted. *Balanites* is propagated through seeds, root suckers and vegetative cuttings but these methods are not efficient in producing sufficient number of elite planting stocks due to poor seed germination frequency, age dependent root suckers production and very low survival rate of vegetative cuttings during transport and plantation. Alternative propagation methods would be beneficial in large scale multiplication, improvement and conservation of its elite clones.

The use of biotechnology on trees has opened up new possibilities for rapid mass multiplication of existing stocks of germplasm, as well as conservation of medicinally important plants/plant parts (Bajaj 1986; Haissig et al. 1987; Gupta and Agrawal 1992; Anis et al. 2005; Husain et al. 2007, 2008). In general, woody taxa are difficult to regenerate under in vitro conditions. Some success, however, has been achieved in a few woody tree species (Giri et al. 2004). There are few earlier reports on regeneration of *B. aegyptiaca* through various explants which yielded a low number of shoots (Ndoye et al. 2003; Gour et al. 2005, 2007). Earlier, we reported regeneration of *Balanites aegyptiaca* from seedling derived explants (Siddique and Anis 2009). Since seeds are units of recombination, plants developed from them differ genotypically and therefore the advantage of an elite genotype to be retained can be achieved only through regeneration from explants derived from an adult plant. Clonal fidelity is a major consideration in commercial micropropagation using in vitro culture methods. Micropropagation of tree species offers a rapid means of producing clonal planting stock for forestation programmes, woody biomass production and conservation. In the present study, we have chosen axillary shoot bud regeneration as an alternative method to achieve a higher rate of shoot multiplication and establishment of complete plantlets from excised nodal explants obtained from a mature tree of *Balanites aegyptiaca*.

Materials and methods

Plant material and source of explant

Healthy, small twigs (3–4 cm) of *B. aegyptiaca* were collected from 20 years old healthy mature tree growing at Arid Forest Research Institute (AFRI) campus Jodhpur, India. The twigs were washed under running tap water for half an hour, followed by treatment with 1% (w/v) Bavistin, a fungicide, for 30 min. After that twigs were immersed in 5% (v/v) Teepol solution, a detergent, for 15 min, and finally washed in running tap water for 20 min. Subsequently, the twigs were surface disinfected with 0.1% (w/v) mercuric chloride solution for 8 min. To remove any trace of sterilant, the twigs were washed with at least five changes of sterile distilled water. Shoot segments (1–1.5 cm) containing single node were excised from the surface sterilized materials and used as explants.

Culture media and culture conditions

Murashige and Skoog (MS, 1962) basal medium supplemented with 3% (w/v) sucrose and 1% (w/v) agar was used during the study. The pH of the medium was adjusted to 5.8 with 1N NaOH or HCl prior to autoclaving. The media were dispensed in 25 mm × 150 mm test tubes (Borosil, India), each containing 20 ml of medium and cotton plugs (double-layered muslin cloth stuffed with non-adsorbent cotton) were used as closures. Glasswares, culture media and instruments were sterilized by autoclaving at 121°C for 15 min. The cultures were incubated at 25 ± 2°C in a growth chamber with an irradiance of 50 μmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes (40 W, Philips) for 16 h photoperiod.

Shoot induction and multiplication

Nodal segments were placed on MS medium augmented with various cytokinins [6-benzyladenine (BA), Kinetin (Kin), and Thidiazuron (TDZ)] at different concentrations (2.5, 5.0, 10.0, 12.5, and

15.0 μM), either singly or in combination with α-naphthalene acetic acid (NAA) (0.5, 1.0, and 2.5 μM). The microshoots regenerated from these nodal explants on different media were subcultured onto the same medium after 4 weeks. The frequency of explant producing shoots, number of shoots per explant and shoot length were recorded after 4 weeks of culture.

In vitro rooting

For rooting of in vitro proliferated shoots, individual shoots (3–4 cm) were isolated and cultured on MS basal medium alone or supplemented with different concentrations of NAA or IBA (0.1, 0.5, 1.0, 2.0, and 5.0 μM). Data were recorded on percentage of rooting, mean number of roots per shoot and root length after 4 weeks of culture.

Acclimatization

Plantlets with well developed roots were washed gently under running tap water and transferred to plastic pots containing soilrite and placed under diffuse light (16:8 h photoperiod) conditions. Potted plantlets were covered with a transparent polythene membrane to ensure high humidity and watered once in a week with ½ MS salt solution. Polythene membranes were opened after 2 weeks in order to acclimatize plants to field conditions. After 4 weeks, acclimatized plants were transferred to pots containing garden soil and maintained in a greenhouse under normal day length conditions.

Data analysis

Ten explants were used in each treatment and all experiments were repeated thrice. The data of different treatments was quantified and subjected to statistical analysis using One Way Analysis of Variance (ANOVA) and pair wise means compared using Duncan's Multiple Range Test procedure ($P < 0.05$).

Result and discussion

The nodal explants failed to respond morphogenetically to a growth regulator free MS medium. In contrast, on media containing cytokinins, an enlargement and subsequent break of axillary buds was

Table 1 Effect of cytokinins on shoot regeneration from mature explants of *Balanites aegyptiaca* after 6 weeks of culture

Plant growth regulators (μM)			% Regeneration	No. of shoots/explant	Shoot length (cm)
BA	Kin	TDZ			
0.0	0.0	0.0	00	0.0 ± 0.00^{i}	0.0 ± 0.00^{h}
2.5			55	1.9 ± 0.11^{fg}	1.1 ± 0.15^{g}
5.0			60	3.2 ± 0.17^{d}	2.2 ± 0.11^{de}
10.0			64	4.0 ± 0.23^{b}	3.0 ± 0.17^{bc}
12.5			67	5.0 ± 0.22^{a}	3.7 ± 0.26^{a}
15.0			59	3.9 ± 0.26^{bc}	2.6 ± 0.23^{cd}
	2.5		47	1.4 ± 0.20^{gh}	1.3 ± 0.18^{fg}
	5.0		52	2.3 ± 0.18^{f}	1.8 ± 0.20^{ef}
	10.0		57	3.3 ± 0.14^{cd}	3.1 ± 0.23^{bc}
	12.5		63	4.2 ± 0.26^{b}	3.4 ± 0.27^{ab}
	15.0		54	2.4 ± 0.20^{ef}	2.7 ± 0.14^{cd}
		2.5	53	3.0 ± 0.19^{de}	1.3 ± 0.27^{fg}
		5.0	60	3.9 ± 0.22^{bc}	2.7 ± 0.17^{cd}
		10.0	56	3.1 ± 0.20^{d}	2.2 ± 0.11^{de}
		12.5	50	2.0 ± 0.17^{fg}	1.9 ± 0.14^{e}
		15.0	47	1.0 ± 0.66^{h}	1.2 ± 0.10^{g}

Values represent means ± SE. Means followed by the same letter within columns are not significantly different ($P = 0.05$) using Duncan's multiple range test

observed in all media tested within 2 weeks of explant inoculation. The percentage response of explants for shoot induction, shoot number and shoot length varied according to the type and concentration of cytokinins used (Table 1). The bud breaking and shoot induction in cultures of nodal explants, therefore appears to be the function of cytokinins. The axillary buds of all explants sprouted and then developed to shoots with two to four pair of leaves at 3 weeks of culture. Multiple shoots were observed after 6 weeks of culture when cultured onto the fresh medium (Table 1). Increasing the concentration of cytokinins beyond the optimal level decreased the percentage of responding cultures as well as number of shoots.

The best response of shoot proliferation and growth was observed on MS medium containing 12.5 μM BA which produced 5.0 ± 0.22 shoots with an average shoot length (3.7 ± 0.26 cm) in 67% of cultures (Table 1) (Fig. 1a). The nodal explants cultured on this medium showed their first response by initial enlargement of the nodes with axillary buds followed by bud break after 2 weeks of inoculation. In another 2 weeks, the shoot buds got elongated and developed into healthy shoots. The stimulating effect of BA on bud break and multiple shoot formation has been reported earlier for several medicinal woody plant species viz., *Acacia tortilis* subsp. *raddiana* (Sane et al. 2001), *Acacia koa* (Skolmen and Mapes 1976), *Leucaena leucocephala* (Dhawan and Bhojwani 1985), *Bupleurum kaoi* (Chen et al. 2006), *Syzygium alternifolium* (Sha Valli Khan et al. 1997), *Pterocarpus marsupium* (Chand and Singh 2004a, b; Anis et al. 2005) and *Celastrus paniculatus* Willd. (Rao and Purohit 2006). Reduction in the number of shoots generated from nodal explants at BA concentration higher than the optimum level was also reported for several woody plants (Lakshamanan et al. 1992; Pattnaik and Chand 1997; Chandaramu et al. 2003; Amin and Jaiswal 1993; Sahoo and Chand 1998) where higher concentration of BA resulted in complete suppression of bud break. Although this condition may not to be valid for all tree species, at least it can be suggested that high level of BA is not appropriate for culture of a number of tropical species such as *Boswellia serrata* (Purohit et al. 1995), *Pterocarpus marsupium* (Chand and Singh 2004a, b) and *Holarrhena antidysenterica* (Kumar et al. 2005).

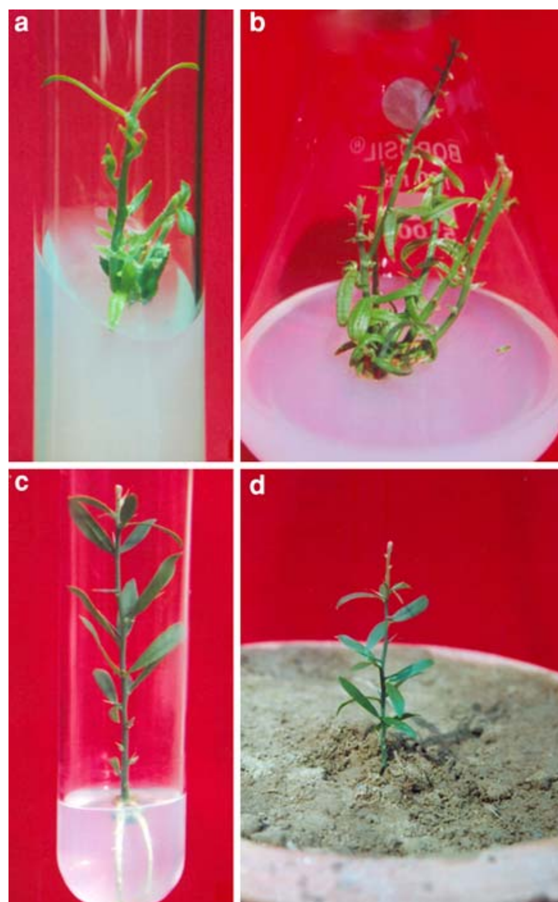

Fig. 1 In vitro clonal propagation of *B. aegyptiaca*. **a** Induction of shoot buds on MS + BA (12.5 μM). **b** Proliferation and elongation of shoots on MS + BA (12.5 μM) + NAA (1.0 μM). **c** Rooted plantlet. **d** An acclimatized plant

Indirect regeneration from cotyledon derived calli (Gour et al. 2007) and direct regeneration from axillary bud (Ndoye et al. 2003) are available but that cannot be used for large scale production of elite plants. However, better shoot growth response observed in present study could be due to number of factors such as physiological status of the mother plant, difference in culture conditions, size of cultured explant, size of culture vessels and photoperiod etc. Like the current study, no separate elongation was required by shoots regenerated from seedling-derived explants of *Balanites aegyptiaca* which were cultured under same conditions (Siddique and Anis 2009).

TDZ was found to be least effective of all the cytokinins tested either singly or in combination with

NAA. Although multiple shoots were induced on MS medium containing TDZ at an optimal concentration of 5.0 μM, the shoots failed to elongate and were often fasciated (Table 1). The formation of stunted shoots or the fasciation of the shoots on TDZ supplemented medium has been reported in several plant species such as *Pyrus malus* (Van Nieuwkerk et al. 1986), *Rhododendron* (Preece and Imel 1991) and *Dalbergia sissoo* (Pradhan et al. 1998). The inhibition of shoot elongation may be due to the high cytokinin activity of TDZ whereas the presence of a phenyl group in TDZ may be possible cause of shoot bud fasciation (Huetteman and Preece 1993).

The synergistic influence of auxins with cytokinin was evident when combination of optimal concentration of each cytokinin with different concentrations of NAA (0.5, 1.0, and 2.5 μM) were tested (Table 2). Addition of NAA stimulated production of new shoots with well developed leaves during subculture in BA supplemented media (Table 2). Among all cytokinins–auxin combinations, the maximum number of shoots (7.7 ± 0.40) and shoot length (4.4 ± 0.20 cm) per explants were obtained at 12.5 μM BA with 1.0 μM NAA (Fig. 1b). This was considered to be the optimal growth regulator combination for maximum shoot regeneration in *Balanites aegyptiaca*. Results obtained here showed consistency with other studies where BA and NAA promoted the proliferation and multiplication of shoots in number of plants (Mercier et al. 1992; Luis et al. 1999; Yang et al. 1993; Caboni et al. 2002; Koroch et al. 2003; Shailendra et al. 2005; Ahmad and Anis 2007; Mallikarjuna and Rajendrudu 2008).

Rooting of microshoots

Shoots regenerated from nodal explants failed to produce roots on full strength MS basal medium. Only one root was formed on half strength MS basal medium (Table 3). Addition of auxins in half strength MS medium facilitated better rhizogenesis in vitro. Half strength MS medium fortified with IBA was found superior to NAA with respect to the induction of roots (Table 3). The maximum frequency of root formation (68%), number of roots (5.3 ± 0.32) and root length (4.1 ± 0.38 cm) was obtained in half strength MS medium containing 1.0 μM IBA. The roots were thick with secondary root hairs, which help in establishing the plantlets in the soil. Optimum rooting response using IBA has been reported in several tree species including *Syzygium alternifolium* (Sha Valli Khan et al. 1997), *Azadirachta indica* (Eeswara et al. 1998), *Melia azedarach* (Thakur et al. 1998), *Anacardium occidentale* (Bogetti et al. 1999), *Dendrocalamus asper* (Arya et al. 1999), *Ziziphus spina-christi* (Sudhersan and Hussain 2003) and *Zyziphus jujuba* (Hossain et al. 2003).

Acclimatization

The microshoots were rooted on the half strength MS medium supplemented with IBA (1.0 μM) (Fig. 1c). The root system consisted of one to four main root

Table 2 Effect of optimal concentration of cytokinins with different concentration of NAA on shoot multiplication after 8 weeks of culture

Plant growth regulators (μM)				No. of shoots/explant	Shoot length (cm)
BA	Kin	TDZ	NAA		
12.5			0.5	5.6 ± 0.32[c]	4.0 ± 0.17[ab]
12.5			1.0	7.7 ± 0.40[a]	4.4 ± 0.20[a]
12.5			2.5	4.4 ± 0.32[a]	3.4 ± 0.24[bcd]
	12.5		0.5	4.2 ± 0.34[d]	3.2 ± 0.17[cd]
	12.5		1.0	6.8 ± 0.20[b]	4.1 ± 0.26[ab]
	12.5		2.5	4.0 ± 0.17[d]	3.5 ± 0.20[bcd]
		5.0	0.5	3.8 ± 0.23[de]	3.0 ± 0.26[cd]
		5.0	1.0	5.4 ± 0.20[c]	3.7 ± 0.23[bc]
		5.0	2.5	3.3 ± 0.1[e]	2.8 ± 0.20[d]

Values represent means ± SE. Means followed by the same letter within columns are not significantly different ($P = 0.05$) using Duncan's multiple range test

Table 3 Influence of different auxins on root formation in *Balanites aegyptiaca* after 4 weeks of culture

Treatments	% Rooting	Mean no. of roots/shoots	Mean root length (cm)
MS	00	0.00 ± 0.00^{g}	0.00 ± 0.00^{h}
1/2MS	20	1.00 ± 0.09^{f}	0.50 ± 0.12^{g}
1/2MS + IBA (0.1 μM)	43	1.33 ± 0.37^{e}	1.10 ± 0.32^{ef}
1/2MS + IBA (0.5 μM)	45	2.83 ± 0.40^{cd}	1.76 ± 0.31^{def}
1/2MS + IBA (1.0 μM)	68	5.30 ± 0.32^{a}	4.16 ± 0.38^{a}
1/2MS + IBA (2.0 μM)	56	3.70 ± 0.23^{c}	3.00 ± 0.17^{ab}
1/2MS + IBA (5.0 μM)	53	2.13 ± 0.29^{de}	2.30 ± 0.32^{abc}
1/2MS + NAA (0.1 μM)	40	1.20 ± 0.32^{e}	0.90 ± 0.20^{f}
1/2MS + NAA (0.5 μM)	43	3.13 ± 0.31^{cd}	2.13 ± 0.29^{cde}
1/2MS + NAA (1.0 μM)	62	4.66 ± 0.40^{ab}	3.36 ± 0.21^{ab}
1/2MS + NAA (2.0 μM)	55	3.96 ± 0.48^{bc}	2.66 ± 0.63^{abc}
1/2MS + NAA (5.0 μM)	48	2.12 ± 0.35^{de}	1.60 ± 0.34^{def}

Values represents means ± SE. Means followed by the same letters within columns are not significantly different ($P = 0.05$) using Duncan's multiple range test

with lateral ones. Plantlets with fully expanded leaves and well developed roots were successfully transferred to soilrite and hardened off in a growth chamber for 4 weeks. The percentage survival of the plantlets after transfer to garden soil was 70% and did not show any detectable variation in morphological or growth characteristics when compared with their respective donor plants (Fig. 1d) and about 60% plants were survived from greenhouse to field conditions.

Conclusions

Success work in establishing plant regeneration by tissue culture and acclimatization of regenerated plantlets to soil in woody species is a pre-requisite for biotechnological approaches to plant micropropagation and improvement. The experiment proved that plantlet formation from nodal segment of a mature tree of *Balanites aegyptiaca* is feasible. The method will provide wide possibility for conservation and an effective way of propagation of other tropical tree species.

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