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Clonal in vitro multiplication of grey mangrove and assessment of genetic fidelity using single primer amplification reaction (SPAR) methods

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An efficient protocol for clonal multiplication of an important mangrove, *Avicennia marina*, was developed through in vitro culture of nodal segments obtained from a mature plant. The nodal explant induced multiple shoots when cultured on the Murashige and Skoog (MS) basal medium supplemented with varying concentrations and combinations of 6-benzyladenine (BA) and α-naphthalene acetic acid. The highest response in terms of per cent regeneration (73%), average number of shoots/explant (3.25 ± 0.25) and maximum shoot length (5.2 ± 0.27 cm) was obtained on the MS medium supplemented with BA 5.0 μmol/L + NAA 1.0 μmol/L + 3 g/L activated charcoal after 8 weeks of culturing. The regenerated shoots were rooted well in the MS medium supplemented with 1.0 μmol/L indole-3-butyric acid with an average of 2.9 ± 0.24 roots per microshoot. The rooted plantlets were successfully transferred to pots containing normal garden soil with 70% survival rate. The genetic stability of the regenerated plants was evaluated using single primer amplification reaction (SPAR) methods viz., random amplified polymorphic DNA, directed amplification of minisatellite DNA and intersimple sequence repeat polymorphism. The SPAR analysis revealed monomorphic banding patterns in all in vitro regenerated plantlets of *A. marina* and similar with that of the mother tree confirming their genetic uniformity and clonal fidelity.

**Keywords:** micropropagation; tissue culture; genetic stability; RAPD marker; DAMD marker; ISSR marker

Introduction

Mangroves, the characteristic intertidal plant formations of sheltered tropical and subtropical shorelines, are usually referred to as ‘coastal woodlands’, ‘mangals’, ‘tidal forests’ or ‘mangrove forests’. [1,2] *Avicennia marina* (Forssk.) Vierh. (family: Avicenniaceae), commonly known as grey mangrove or white mangrove, is the most widespread mangrove distributed throughout the tropical and subtropical regions of the world. It is one of many mangroves found in the arid regions of the coastal Arabian Peninsula as well as on both sides of the Red Sea in Egypt, Eritrea, Yemen Sudan and Saudi Arabia. The mangrove habitat is threatened throughout its range mainly because of the increasing human activities at coastal areas, and there has been a 21% assessed decline in mangrove areas since 1980 (International Union for Conservation of Nature, IUCN).[3] Nowadays, *A. marina* are more at risk from coastal development for tourism and other land use purposes and extraction at the extremes of their distribution as well as the climate changes due to global warming.

Biotechnology, offering new tools for sustainable development and utilization of natural resources, has become increasingly imperative worldwide. The application of biotechnological approaches to plants would also benefit from the development of tissue culture systems for in vitro development and regeneration of rare and endangered species.[4,5] Tissue culture techniques could be a cost-effective means of bulk production of elite planting material throughout the year without any seasonal constraints. It may also play an essential role in clonal mass multiplication and ex situ preservation of germplasm that is rare, endangered or on the edge of extinction. An important limitation of the in vitro technique is the development of somaclonal variation among regenerated plantlets, which may affect the quality and quantity of micropropagated plants and, in turn, obstruct the ex situ conservation programme.

Confirmation of genetic uniformity using polymerase chain reaction (PCR)-based profiling methods is a common practice nowadays and has also found application in studies of varietal identification, phylogenetic analysis and genetic diversity.[4] A group of such methods using a single primer in the PCR is collectively described as single primer amplification reaction (SPAR) methods and includes random amplified polymorphic DNA (RAPD) developed by Williams et al. [6] and Welsh and...
McClelland [7], directed amplification of minisatellite DNA (DAMD) developed by Heath et al. [8] and inter-simple sequence repeat (ISSR) polymorphism developed by Zietkiewicz et al. [9]. These methods are robust, rapid and have proven useful in the diversity studies permitting precise and versatile analysis of genetic stability. The SPAR techniques have been employed extensively in characterization of micropropagated plantlets.[10–16]

Earlier, a study was conducted by Al-Bahrany and Al-Khayri [17] to obtain in vitro plantlets of A. marina, but there is still no report on the molecular characterization of micropropagated plants. However, the developed method is meager and gave a low number of shoots. This study aimed to develop an efficient method for in vitro clonal propagation and ex situ conservation of A. marina. Furthermore, to the best of our knowledge, the genetic stability of the in vitro regenerated A. marina plants was assessed for the first time by SPAR methods (RAPD, DAMD and ISSR).

Materials and methods

Explants source and disinfection

The plant materials were collected from a healthy field plant at Al-Mawassam (16°25′6.66″N/42°45′55.52″E), Jazan, Saudi Arabia. Nodal segments were excised from collected materials and used as explants. The explants were thoroughly washed under running tap water for 30 min, followed by soaking with 5% (v/v) liquid detergent solution for 5 min. After repeated washes with sterile distilled water, the explants were surface sterilized with 0.1% (w/v) mercuric chloride solution for 5 min. After five washes with sterile distilled water, the explants were finally cut into 0.5–0.8 cm-sized pieces and aseptically cultured on the Murashige and Skoog (MS) medium.[18]

Nutrient media and culture conditions

The basic nutrient medium consisted of MS salts and vitamins containing 3% (m/v) sucrose, 0.8% (m/v) agar and 3% (m/v) activated charcoal. Growth regulators and their combinations were added to the medium, as specified below. The pH of the medium was adjusted to 5.8 with 1 mol/L NaOH or HCl. The culture vials containing the media were steam sterilized in an autoclave at 121 °C at 1.06 kg/cm² for 20 min. All the cultures were maintained at 24 ± 2 °C under a 16/8 h photoperiod with a photosynthetic photon flux density of 50 μmol/(m² s²) provided by cool white fluorescent lamps (Philips, Poland).

Shoot initiation and multiplication

In this study, experiments were carried out on multiple shoot induction and proliferation from nodal explants of A. marina. Sterilized nodal segment explants were cultured on the MS medium supplemented with various concentrations and combinations of 6-benzyladenine (BA; 0.5, 2.5, 5.0 or 10.0 μmol/L) and α-naphthalene acetic acid (NAA; 1.0 μmol/L). Data for shoot formation and proliferation were recorded after eight weeks of culturing. MS medium without any growth regulators was used as a control.

In vitro rooting and establishment of plantlets

For rooting, elongated healthy shoots measuring about 4–5 cm in length were excised from shoot clumps and transferred into the MS medium supplemented with indole-3-butyric acid (IBA) at different concentrations (0.5, 1.0, 1.5 or 2 μmol/L) as described in Table 1. The percentage of root formation, root numbers and root length were recorded four weeks after transfer. Regenerated plantlets with well-developed shoots and roots were transferred to pots containing sterile potting soil (Planta Guard) after thorough washing in running tap water. The plants were covered with transparent plastic bags to ensure high humidity and maintained under diffuse light conditions (16/8 h photoperiod). The potted plants were watered every three days with half-strength MS salt solution for two weeks. After one month, the successfully acclimatized plantlets were transferred to pots containing normal garden soil and maintained in glasshouse under normal conditions.

Genomic DNA extraction

Genomic DNA was extracted from 500 mg of young leaves of micropropagated plantlets and the donor plant, using the modified cetyltrimethyl ammonium bromide method.[19] Purified total DNA was quantified and its quality verified by spectrophotometry (6705 UV/vis spectrophotometer, Jenway, UK), and each sample was diluted to 25 ng/μL in sterile Milli-Q water and stored at 4 °C.

Table 1. Effect of IBA on root formation from in vitro raised shoots of A. marina in the MS medium after 4 weeks of culturing.

| IBA (μmol/L) | Rooting (%) | Mean number of roots/shoot | Mean shoot length (cm) |
|-------------|-------------|---------------------------|-----------------------|
| 0.0         | 0.0         | 0.0d                       | 0.0c                  |
| 0.5         | 47          | 2.1 ± 0.11b                | 3.1 ± 0.27c           |
| 1.0         | 71          | 2.9 ± 0.24a               | 3.8 ± 0.30a           |
| 1.5         | 62          | 2.0 ± 0.23b               | 3.5 ± 0.18bc          |
| 2.0         | 45          | 1.4 ± 0.15a               | 2.5 ± 0.15d           |

Note: Data represent means ± standard error of the means (SEM). Means followed by the same letter within columns are not significantly different (P > 0.05) based on Tukey’s test.
**PCR amplification and analysis of SPAR**

Regenerated plantlets were tested for genetic stability, using 20 RAPD (Kit-B), 4 DAMD and 12 ISSR primers (Gene Link, New York, USA) for their unambiguous and reproducible banding patterns. PCR amplification was performed in a volume of 20 μL reaction mixture containing 1× PCR buffer, 25 ng sample DNA, 2.0 mmol/L MgCl₂, 200 μmol/L of deoxyribonucleoside triphosphates (dNTPs) mix, 5 pmol primer (RAPD, DAMD or ISSR) and 2.5 U Taq DNA polymerase (Fermentas, GmbH, Germany). The amplifications were carried out in a thermocycler (T100™ Thermal Cycler, Bio-Rad, USA). PCR reactions were performed with initial DNA denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C (30 s), primer annealing at 35 °C –58 °C (Tables 2–4) for 45 s, primer extension at 72 °C for 90 s and final extension at 72 °C for 7 min. PCR products obtained from RAPD, DAMD and ISSR markers were resolved in a 1.5% agarose gel for 2 h in 1 × TBE (Tris–borate–EDTA) buffer, stained with ethidium bromide, and photographed using a Gel Documentation System (G-Box, Syngene, UK).

**Data analysis**

All the experiments were repeated thrice with a minimum of 10 explants per treatment. The data were analysed statistically using SPSS Ver. 20 (SPSS Inc., Chicago, USA) and the significance of differences among the means was evaluated using Tukey’s test at \( P < 0.05 \) probability level. The data presented in figures and tables are mean values from three repeated experiments, with standard error of the means (±SEM).

**Results and discussion**

Nodal segments containing axillary buds have the potential to develop into complete plantlets. In natural conditions, these buds remain inactive for a specific period depending on the growth pattern of the plant. However, by tissue culture, enhanced axillary branching can be achieved for clonal mass multiplication of plants by nodal segment culture on a nutrient medium augmented with suitable plant growth regulators.

**Clonal in vitro multiplication of A. marina**

In this study, a protocol for direct regeneration of *A. marina* from nodal segments explants was formulated by testing plant growth regulators either singly or in different combination on the MS medium. Nodal segments explants of *A. marina* cultured on the plant-growth-regulators-free MS medium or medium supplemented with cytokinin alone did not show any morphogenetic response and failed to develop shoots even after eight weeks of culture.

Multiple shoots formations were achieved from nodal explants on the MS medium supplemented with BA in combination with NAA (Figures 1 and 2). Among the various concentrations and combinations of BA and NAA, the highest shoot regeneration frequency (73%), number of shoots (3.25 ± 0.25) and maximum shoot length (5.2 ± 0.27 cm) were recorded on the MS medium supplemented 5.0 μmol/L BA, 1.0 μmol/L NAA and 3 g/L activated charcoal. A decrease in the number of shoots was observed by increasing the BA concentration beyond 5 μmol/L (Figure 2).

Similarly, the synergism of BA in combination with NAA has been well documented in several plant species, such as *Rauvolfia tetraphylla*, *Tylophora indica*, *Enicostemma littorale* [21] and *Metabrassia ovalifolia* [22].

![Table 2. RAPD primers used to evaluate the extent of genetic fidelity of micropropagated *Avicennia marina* plantlets.](image)

| Sl no. | Primer | Primer sequence \((S’–3)\) | \(T_a (°C)\) | Number of bands |
|--------|--------|-----------------------------|--------------|----------------|
| (1)    | OPB-01 | GTTTCGCTCC | 35           | 7              |
| (2)    | OPB-02 | TGATCCCCGG | 35           | 7              |
| (3)    | OPB-03 | CATCCCCCTG | 35           | 6              |
| (4)    | OPB-04 | GGACTGGAGT | 35           | 10             |
| (5)    | OPB-05 | TGGCGCCTTC | 35           | 8              |
| (6)    | OPB-06 | TGCTCTGCCC | 35           | 10             |
| (7)    | OPB-07 | GTGACGCAG | 35           | 11             |
| (8)    | OPB-08 | GTCCACACGG | 35           | 4              |
| (9)    | OPB-09 | TGGGGGACTC | 35           | 11             |
| (10)   | OPB-10 | CTGCTGGGAC | 35           | 3              |
| (11)   | OPB-11 | GTAGACCCGT | 35           | 10             |
| (12)   | OPB-12 | CCTGACCAGA | 35           | 6              |
| (13)   | OPB-13 | TTCCCCCCTG | 35           | 4              |
| (14)   | OPB-14 | TCCGCTCGG | 35           | 5              |
| (15)   | OPB-15 | GGAAGGTGTT | 35           | 5              |
| (16)   | OPB-16 | TTTCGCCCGGA | 35  | 4              |
| (17)   | OPB-17 | AGGGAACGAG | 35           | 7              |
| (18)   | OPB-18 | CCAACAGCAGT | 35      | 6              |
| (19)   | OPB-19 | ACCCGCCGAAG | 35     | 5              |
| (20)   | OPB-20 | GGACCCCTAC | 35           | 4              |

Note: \( T_a \), annealing temperature.
In agreement with these findings, the present report also demonstrates the positive modification of shoot induction efficiency achieved by using a low concentration of NAA in combination with a high concentration of BA. This differential morphogenetic response may be due to apical dominance. Apical dominance is governed by the ratio of auxin and cytokinin and is very well documented to be caused by the action of basipetally transported auxin from the apex and its consequent inhibition of axillary bud growth.

It is well known that cytokinin regulates auxin levels and vice versa. Similarly, the effectiveness of BA and NAA on in vitro axillary shoot multiplication has also been observed in Excoecaria agallocha as described by Arumugam and Panneerselvam.

For rooting, 4–5 cm long in vitro regenerated shoots were transferred to the MS medium augmented with different concentrations of IBA (Table 1). The frequency of rooting varied from 45% to 71%, depending on the concentration of IBA used. The highest frequency of root formation (71%), number of roots (2.9 ± 0.24) and maximum average root length (3.8 ± 0.30) was recorded at 1.0 µmol/L of IBA after 4 weeks of culturing. The efficacy of IBA in root induction has been reported in Rauvolfia serpentina, Mentha arvensis, Sansevieria cylindrica and in Cyamopsis tetragonoloba. Plantlets having 4–5 fully expanded leaves and well-developed roots were

![Figure 1](image1.png)

**Figure 1.** Effect of 6-benzyladenine (BA) on shoot regeneration (%) of A. marina in the MS medium augmented with NAA (1.0 µmol/L) + AC (3.0 g/L). Data represent means ± SEM. Values followed by the same letter within response variables are not significantly different (P < 0.05) based on Tukey’s test.

![Figure 2](image2.png)

**Figure 2.** Effect of 6-benzyladenine (BA) on in vitro shoot multiplication and shoot length of A. marina in the MS medium augmented with NAA (1.0 µmol/L) + AC (3.0 g/L). Data represent means ± SEM. Values followed by the same letter within response variables are not significantly different (P < 0.05) based on Tukey’s test.

### Table 4. ISSR primers used to evaluate the extent of genetic fidelity of micropropagated Avicennia marina plantlets.

| Sl no. | Primer | Primer sequence (5’–3’) | T<sub>a</sub>(°C) | Number of bands |
|--------|--------|--------------------------|------------------|-----------------|
| (1)    | UBC-811| GAG AGA GAG AGA GAG AC   | 49               | 9               |
| (2)    | UBC-825| ACA CAC ACA CAC ACA CT   | 46               | 12              |
| (3)    | UBC-827| ACA CAC ACA CAC ACA CG   | 50               | 11              |
| (4)    | UBC-834| AGA GAG AGA GAG AGA GYT  | 50               | 11              |
| (5)    | UBC-841| GAG AGA GAG AGA GAG AYC  | 50               | 18              |
| (6)    | UBC-855| ACA CAC ACA CAC ACA CYT  | 50               | 8               |
| (7)    | UBC-866| CTC CTC CTC CTC CTC CTC | 55               | 14              |
| (8)    | UBC-868| GAA GAA GAA GAA GAA GAA | 46               | 10              |
| (9)    | UBC-880| GGG TGG GGT GGG GTG      | 50               | 18              |
| (10)   | UBC-889| DBD ACA CAC ACA CAC AC   | 46               | 5               |
| (11)   | UBC-891| HVH TGT GTG TGT GTG TG   | 46               | 7               |
| (12)   | UBC-900| ACT TCC CCA CAG GTT AAC ACA | 58            | 0               |

Note: T<sub>a</sub> annealing temperature.
successfully hardened off in the growth room in pots containing sterile soilrite mixture and covered with transparent polybags to maintain high humidity. After four weeks, the plantlets were transferred to pots containing normal garden soil with a 70% survival rate.

**Genetic fidelity of regenerated plants**

Genetic uniformity of regenerated plants is of key importance in the micropropagation of any plant species. The SPAR methods, viz. RAPD, DAMD and ISSR, were used in order to assess the genetic fidelity of micropropagated *A. marina* plantlets and compare them with the mother plants. Twenty RAPD primers were screened and yielded clear, reproducible bands. The number of bands varied from 3 to 11 with an average of 6.65 bands per primer (Table 2). Four DAMD primers were tested to evaluate the micropropagated plantlets of *A. marina*, and three of them yielded clear, reproducible bands with a total of 38 bands and an average of 9.5 bands per primer (Table 3). For ISSR analysis, 12 primers were used to screen the regenerated plantlets. Eleven primers gave clear and scorable bands and the number of bands for each primer ranged from 5 to 18, with an average of 10.25 bands per primer (Table 4). The results of this molecular study revealed that all the primers used in this study showed a monomorphic pattern in all the sample plants, confirming the genetic uniformity of the regenerated plantlets and that no variation was induced during *in vitro* propagation of *A. marina* (Figures 3–5). The findings of this investigation corroborate several earlier reports on genetic analysis of regenerated plantlets in *Mentha arvensis*,[16] *Aegle marmelos*,[27] *Bambusa balcooa*,[28] *Gerbera jamesonii* [29] and *Gloriosa superba* [30], using SPAR analysis.

**Conclusions**

The results from this study demonstrated that the proposed protocol for clonal *in vitro* multiplication of *A. marina* is effective and showed a definite advantage over the earlier study. For the first time, to the best of our knowledge, the regenerated plants were genetically assessed by SPAR (RAPD, DAMD and ISSR) methods, and it was found that these plants were genetically stable under *in vitro* conditions. The developed protocol could be helpful to explore the possibility of preserving the genetic stability of the selections and *ex situ* conservation of this important plant.

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Disclosures statement
No potential conflict of interest is reported by the authors.

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