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SYNSEED PRODUCTION FOR STORAGE AND CONSERVATION OF *OCHRADENUS BACCATUS* DELILE

FAHAD AL-QURAINY, MOHAMMAD NADEEM*, SALIM KHAN, SALEH ALANSI, MOHAMED TARROUM AND ABDULHAFED AL-AMERI

Department of Botany and Microbiology, College of Science, King Saud University, Riyadh-11451, KSA
**Corresponding author's e-mail: mohammadnadeem911@hotmail.com*

Abstract

Ochradenus baccatus is a medicinal plant of high value, spread over sandy and stony places of Kingdom of Saudi Arabia and most of the desert regions of Egypt. This species contains several flavanoids and specific constituents which are important as these have already been effectively used in lowering cholesterol in the blood of rats and high inhibition potential of the malarial parasite (*Plasmodium falciparum)*. Synseeds were produced from stem segments and apical bud of *O. baccatus* growing *In vitro*. Two sets of synseeds were produced, one non-dried and the other dried under running laminar air-flow for 30 min. Regeneration and regrowth were evaluated for 16 weeks storage under various temperatures (4, 8 and 12ºC). The maximum frequency of conversion into plantlets was achieved on the MS medium containing 1.0 µM BA in encapsulated nodal segments stored at 4ºC. Rooting in these shoots was induced by the pulse treatment of 100 µM IBA for 10 days, and the rooted shoots were transferred on the MS medium devoid of any PGR. Fair percent rooting occurred after one week of transfer on the MS medium. Plantlets were successfully established. No phenotypic variations were observed between the synseed originated plants with mother plant. Genetic stability of synseed grown plants and mother plant was evaluated by inter-simple sequence repeat (ISSR) marker. The mother plant as well as regenerated plants from synseed resulted in a monomorphic banding pattern developed from ISSR markers confirming genetic stability among the clones. This protocol will help multiply and conserve the plant as well as for short-term storage of germplasm for commercial use and exchange.

Introduction

Ochradenus baccatus is a very important medicinal plant, which grows in the wilds of Kingdon of Saudi Arabia, deserts of Egypt and adjoining countries (Tackholm, 1974; Abd El-Wahab *et al*., 2008, Al-Fredan 2010). It has been reported that the active constituents of this species are effective in controlling blood cholesterol in rats as well as it is a potent growth inhibitor of the malarial parasite *Plasmodium falciparum* (Sathiyamoorthy *et al.,* 1999). A number of compounds and some unique flavonoids have been isolated from the leaves (Barakat *et al.,* 1991). Since the plant is medicinally very important, its demand has increased manifold in the recent past. Due to this and coupled with various anthropogenic factors, the natural populationof this is depleting. Therefore, it would be appropriate to search for alternate methods of propagation and conservation of this plant. Although a few reports of mass multiplication are available through seeds as well as biotechnological approaches (Nadeem *et al*., 2012 and Al-Qurainy *et al.,* 2013). Barakat *et al*., (1991) have isolated a number of compounds including some novel flavonoids from the leaves of this plant. An unique chemical known as glucosinolate was isolated from *O. bacaatus* (Blank *et al.,* 2012). Isothiocynate was also found in the plant which is formed due to the hydrolytic activity of myrisinase (Blank *et al.,* 2012). However, the use of the extract of *O. baccatus* in folk medicine is already well documented (Nawash & Al-Horani, 2011). The plant has antibacterial and antimalarial activities (Abutbul *et al.,* 2005, Sathiyamoorthy *et al.,* 1999) as well as nematicidal activity (Oka *et al.,* 2013). The glucosinolates are related to nematicidal activity which is well known in the case of *O. baccatus*. Therefore, *O. baccatus* can be planted as a cover plant/green manure to control root-knot nematodes (Oka *et al.,* 2013). The demand of *O. baccatus* has increased tremendously during the last few decrades. So, there is a need to develop and adopt different strategies for its multiplication and propagation. To supplement this, plant tissue culture can play an important role in rapid multiplication for large scale from a small source of plant material (Edson *et al*., 1997). However, plant regeneration is essential for genetic transformation to improve yield and quality traits such as drought resistance, heat registance, longer self-life, nutritional quality and acidic soil adaptation (Lal & Lal, 1993). Thus, this technique after proper optimization can provide a platform for conserving germplasm of prime importance as well as threatened, endangered and rare plants (Arora & Bhojwani, 1989).

In vitro strategies have been applied for management and conservation of the plant species whoes genetic and natural resources are depleting at an alarming speed. Encapsulation and *In vitro* storage of gametophytic and meristematic tissues are considered as a cost saving and easy adoptable method of conservation of germplasm (Redenbaugh, 1990; Dodds, 1991; Danso & Ford-Lloyd, 2003). Synseed is an alternative for suitable germplsm exchange and storage for short- and long-term. Furthermore, this can also be used in easy distribution and exchange of important germplasm (Rao *et al.,* 1998; Naik & Chand, 2006). Encapsulated plant parts in synseed can be stored for long time for conservation of germplasm. This provides an opportunity of regeneratin whenever required. Our present investigation highlights the production of synseed from *In vitro* grown shoots, their storage conditions (specifically storage temperature) for a certain period of time and the regenerability of the synseeds. Furthermore, the molecular markers based techniques such as RAPD, ISSR and AFLP have been used to elucidate genetic diversity and phylogenetic relatedness in numerous taxa.

Materials and Methods

In vitro culture of *O. baccatus* was established using stem segments collected from mature plants growing in a natural habitat in Riyadh, Saudi Arabia. Axenic shoot tips and node portions having axillary buds (5-8 mm) were cut from the *O. baccatus* plant growing *In vitro*. These explants were encapsulated by mixing them in 3% sodium alginate solution and dropped in 100 mM $CaCl_2$ solution with the help of a wide mouth pipette so that each drop encapsulated one explant to form the beads. Complete polymerization occurred when the beads were left in $CaCl_2$ solution for 30 min. Then the resulting beads were washed with sterilized water three times. Now the synseeds were divided into two sets; one set of about 100 synseeds were stored in a plastic jar, sealed with parafilm and kept in a refrigerator at 4ºC. Another set of 100 synseeds were dried under a running laminar hood for 30 min. Similarly, this set was also stored in a refrigerator.

The synseeds obtained were subjected to the MS medium without any PGRs and kept at 4, 12 and 20°C in a culture room. Production of synseeds was performed under sterilized conditions. After an interval for two weeks, the synseeds were taken into culture conditions of a growth room on the MS medium supplemented with 1.0 µM BAP and 3.0% sucrose. Synseed germination was counted on percent basis at the time of shoot emergence from synseed. The regenerated shoots were exposed to 100 µM IBA for root induction for 10 days. Rooted plantlets were kept in a growth chamber prior to hardening. The hardened plants were transplanted in plastic pots containing FYM and garden soil (sterilized). Initially, the plants were waterd on daily basis for three days and afterwards at regular interval. All experiments were repeated thrice and each treatment consisted of 30 synseeds. Data were analysed by the Systat programme, and ANOVA and mean comparisons were done at 5% LSD.

ISSR markers were used to study the clonal fidelity of the plants raised from synseeds (clones). This was carried out by taking 10 hardened plants raised from synseed (stored at 4°C) chosen randomly from the population and compared with the mother plant. Total genomic DNA of the mother plant and *In vitro* raised clones was extracted from young leaf tissues by using the modified cetyltrimethyl ammonium bromide (CTAB) method (Khan *et al.,* 2007). The quantity and quality of total DNA was verified by spectrophotometry (Nanodrop spectrophotometer, 8000). The PCR bead (GE Healthcare) was used for the amplification of genomic DNA which contained 25 ng/2 µL total DNA, 2 µL 10-mer oligo-deoxynucleotide ISSR (Macrogen, Korea) primer and rest of deionized water. The PCR amplification was performed using a Thermal cycler (Techne, UK). For ISSR analysis, amplification conditions were used as initial DNA denaturation at 94°C for 5 min followed by 40 cycles at 94°C for 1 min, 45.2°C for 1 min, and 72°C for 2 min. Final cycle at72°C for 7 min was also performed. The amplified products were electrophoresed on 1.2% agarose gel and photographed in Geldoc (Syngene, USA).

Results and Discussion

Encapsulated nodal segments and shoot tips of *O. baccatus* in culture resulted in regrowth and shoot proliferation. The stem segments devoid of any axillary bud could not regrow and multiplied. Synseeds resumed growth within 7-10 days after being taken out from storage conditions (Fig. 1A) to *In vitro* culture conditions for all types of synseeds and non-encapsulated stem segments stored at 4ºC. The synseeds and stem segments showed regrowth under stored conditions at 8 and 12ºC. Therefore, further storage carried out at 4ºC was suitable for causing dormancy. After 2 weeks, synseeds exhibited high regrowth in all the sets and similar high regrowth percentage (80-86%) was observed during two weeks of storage (Fig. 1B). Storing of the synseeds at 4ºC up to two weeks has not resulted in any significant difference in regrowth (Table 1). Whereas encapsulated segments stored at 12°C or above were not storable after 2 weeks due to sporadic shoot formation at higher temperatures. A similar response of synseeds was observed during storage of *Jaccaranda mimosaefolia* (Maruyama *et al.,* 1997) and cassava (Danso & Ford-Lloyd, 2003). This was ascribed to early onset of metabolism in synseeds which restricted them for furthers storage. Therefore, this phenomenon will help in smooth germplasm transfer beause it can resume growth at one's convenience *In vitro* (Danso & Ford-Lloyd, 2003). The storage at 4°C has increased the storage capacity up to 120 days, and beyond this time of storage a limited regrowth was achieved to form shoots. Moderate (60.5±11.5%) to high (76.6±6.6%) regrowth was seen during 30-45 days of storage (Table 2). A steep decline in percent synseed regrowth of non-encapsulated (10.0%), dry- encapsulated (40.0%) and non-dry-encapsulated (43.3%) observed, which was ascribed to the loss of viability. This may have happened due to loss of moisture and oxygen present in synseeds. The present investigation shows that the synseeds stored at 4 °C and/or above freezing temperature retained higher potential of storability and regrowth of *O. baccatus* synseeds as reported in other plants such as pineapple (Soneji *et al.,* 2002). The decrease in synseed regrowth capacity due to longer period of storage was also evident in *Dalbergia sissoo* (Chand & Singh, 2004). The loss of regeneration potential of shoots may have occurred due to long storage, respiration stress and insufficient hydration (Faisal *et al.,* 2012). Rooting was induced in the shoots by treating them with 100 µM IBA for 10 days, then the shoots were transferred to the MS medium lacking any PGR. Considerable rooting occurred after one week of transfer on the MS medium (Fig 1C). The plantlets originated from synseed showed good growth of shoots and roots in the plastic pots (Fig. 1D). After two months of acclimatization and hardening under cotrolled conditions in a greenhouse, plants were transferred to natural conditions within the glasshouse which survived well after when they were transferred to the field. Synthetic seed technology has brought a big leap in the area of plant tissue culture and seed science. This technology has been successfully applied in the longer storage of planting material, seed germination and viability as well as stored vegetative propagules can be used in conventional multiplication programmes (Table 3). The sodium alginate covering in a proper concentration acts as a protective covering for somatic embryos (Malabadi & Van

Staden, 2005), shoot tips/nodal segments (Danso & Ford-Lloyd, 2003; Faisal *et al.,* 2012), axillary buds (Refouvelet *et al.,* 1998), micro-plants/cuttings (Tsvetkov & Hausman, 2005). The roots conserving the regeneration capacity (Brischia *et al.,* 2002) are regarded as synthetic or artificial seeds. As mentioned above and elsewhere the somatic embryogenesis is not reported in all plant species capable of regeneration under tissue culture. Therefore, it is desirable to encapsulate *In vitro* originated tissues and organs particularly shoot tips and nodal segments as synseeds. Regrowth of *O. baccatus* synseeds stored for various time periods showed stable regeneration and plant production. This study has resulted in the development of an effective *In vitro* conservation of *O. baccatus* germplasm through shoot tips encapsulation. Synseeds were optimally stored up to 120 days with fairly high regrowth. The synseed technology has attained a widespread acceptability and use of this technology in storage, exchange and mass propagation of plant species, especially rare, endangered and important medicinal plants.

Regenerated plantlets of *O. baccatus* were tested for clonal fidelity using 20 ISSR primers (Table 4). Out of 20 primers, 10 gave weak ISSR banding profile which was not further used in testing the genetic fidelity. Ten primers gave reproducible and sharp bands. All loci produced were monomorphic. The maximum number of loci was amplified by the primer OP-13 **(**Fig. 2). Polymorphism was not detected in the mother plant and cloned plantlets produced by synseeds. It was clear that in production of synseed, the mutation was not created and thus the clones produced from the synseeds were free from any insertion and deletion mutations. The genetic stability in re-grown shoots from artificial seeds of *Bacopa monnieri, Rauvolfia serpentina* and *Cineraria maritime* was confirmed by using the ISSR and RAPD (Srivastava *et al.,* 2009, Faisal *et al.,* 2012, Muthiah *et al.,* 2013). A genetically stable rooting protocol for *Celastrus paniculatus*, a medicinal plant, was also developed (Phulwaria *et al.,* 2013).

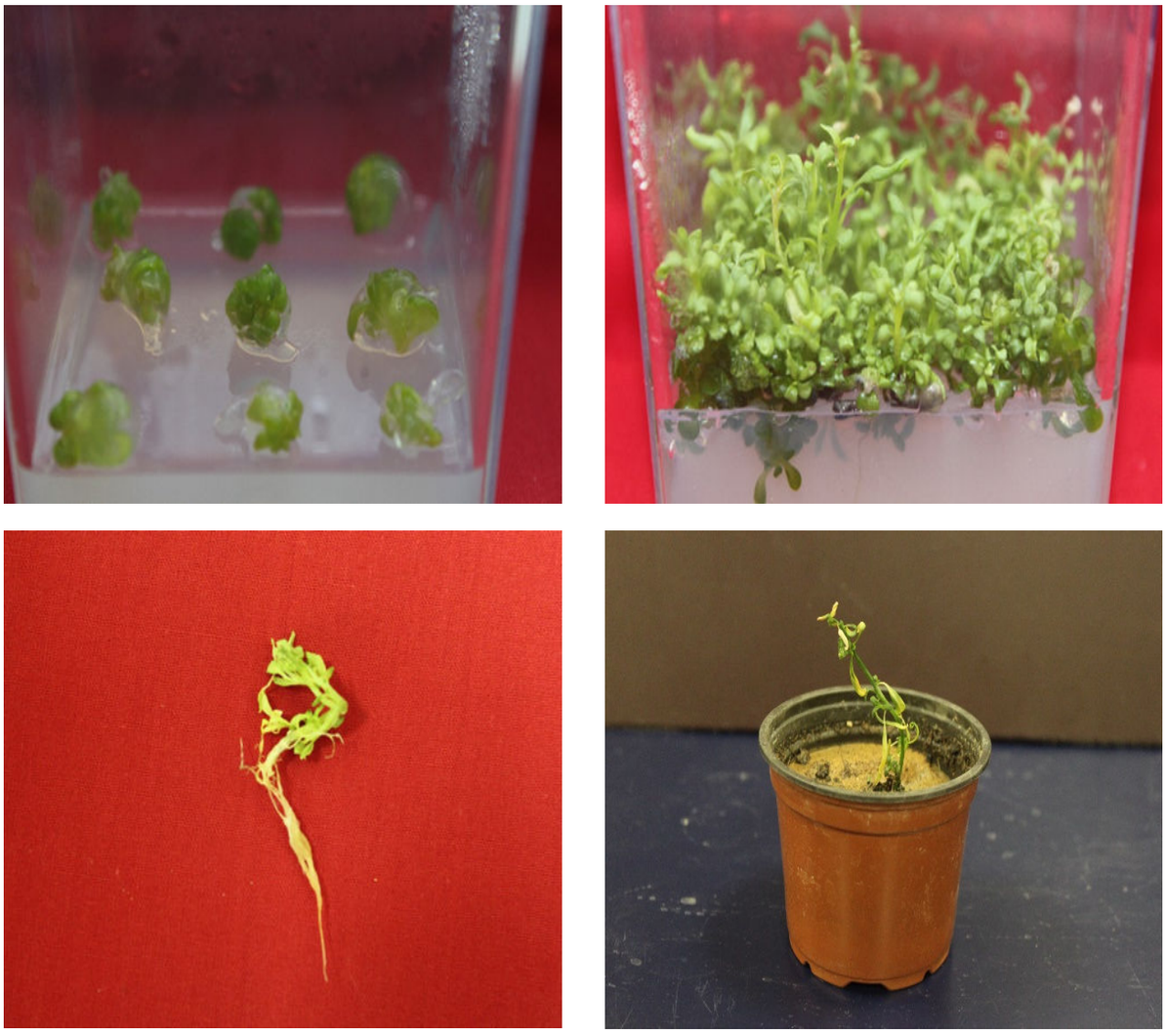

Fig. 1. A: Synseeds of *O. baccatus* on the MS medium containing 1.0 µM BA. B: Shoot regeneration from synseeds. C: Individual shoot rooted on the MS medium containing 100 µM IBA pulse treatment for 10 days. D: Acclimatized and hardened plant ready for field transfer.

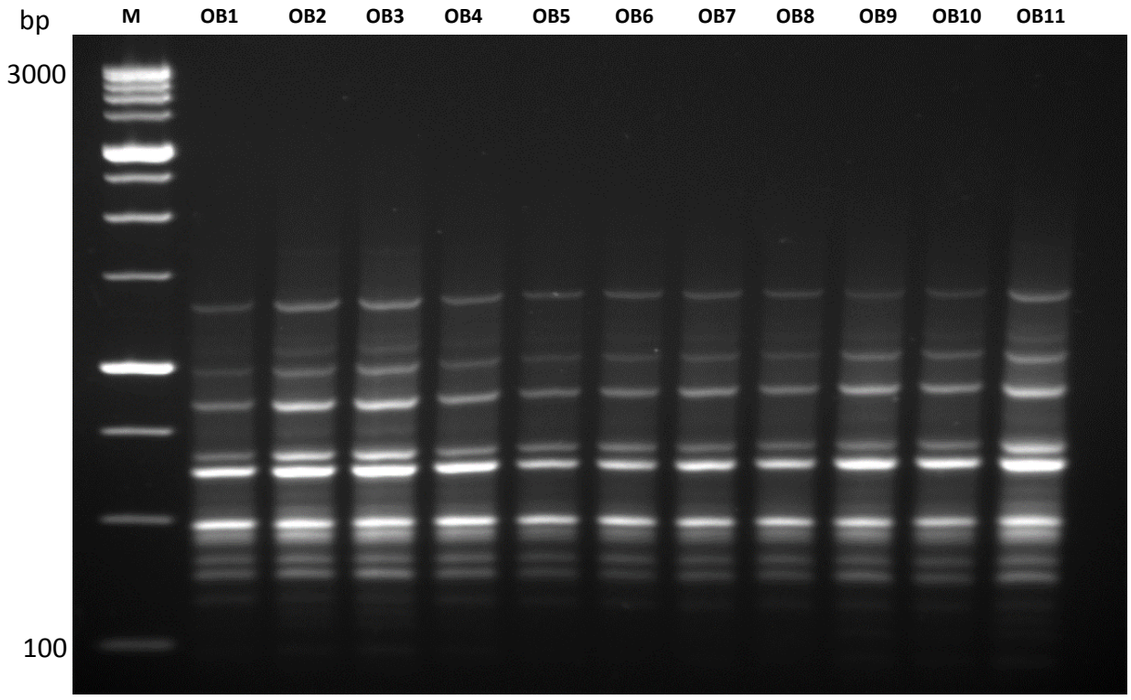

Fig. 2. ISSR profile produced by the primer OP-13.

Table 1. Synseed conversion on the MS medium supplemented with 1.0 µM BA after storage at 4ºC in a refrigerator at various time intervals.

S. No.	Days	Conversion frequency ±SD		
		Non-encapsulated	Dry	Non-dry
1.	15	80.0 ± 5.7	80.0 ± 5.7	86.6 ± 3.3
2.	30	70.0 ± 10.0	73.3 ± 3.3	80.0 ± 5.7
3.	45	40.0 ± 5.7	60.0 ± 11.5	76.6 ± 6.6
4.	60	13.3 ± 3.3	40.0 ± 10.0	50.0 ± 5.7
5.	120	10.0 ± 0.0	40.0 ± 11.5	43.3 ± 3.3

Values are means of three replicates ± SE

Table 2. Percent rooting in shoots derived from synseed regeneration on the MS medium containing 100µM IBA treated for 10 days.

S.No.	Days	Rooting ± SD		
		Non-encapsulated	Non-dry	Dry
1	15	80.0 ± 5.7	83.3 ± 3.3	80.0 ± 10.0
2	30	70.0 ± 15.2	76.6 ± 6.6	70.0 ± 5.7
3	45	-	76.6 ± 8.87	73.3 ± 6.6
4	60	-	73.3 ± 12.0	73.3 ± 8.8
5	120	-	70.0 ± 5.7	70.0 ± 5.7

Values are means of three replicates ± SE

Table 3. Percent survival of plantlets developed from synseed in garden soil after acclimatization.

S.No.	Days	Survival ± SD		
		Non-encapsulated	Dry	Non-dry
1	15	73.3 ± 6.6	80.0 ± 5.7	76.6 ± 8.8
2	30	66.6 ± 3.3	76.6 ± 8.8	66.6 ± 6.6
3	45	-	73.3 ± 3.3	60.0 ± 5.7
4	60	-	63.3 ± 8.8	63.3 ± 3.3
5	120	-	38.6 ± 16.5	60.0 ± 11.5

Values are means of three replicates ± SE

Table 4. List of primers used in the generation of ISSR profile.

OP-1	AGTCAGTCAGTCAGTC
OP-2	ACTCACTCACTCACTC
OP-3	GAGAGAGAGAGAGAGAA
OP-4	CACACACACACACACAT
OP-5	TCT CTC TCT CTC TCT CA
OP-6	TCT CTC TCT CTC TCT CG
OP-7	AGAGAGAGAGAGAGAGYC
OP-8	ACA CAC ACA CAC ACA CT
OP-9	CTTCACTTCACTTCA
OP-10	GGAGAGGAGAGGAGA
OP-11	AGAGAGAGAGAGAGAGT
OP-12	GAGAGAGAGAGAGAGAC
OP-13	TATATATATATATATAA
OP-14	GAGAGAGAGAGAGAGAC
OP-15	CACACACACACAGT
OP-16	GTGTGTGTGTGTGG
OP-17	GAGAGAGAGAGACC
OP-18	GAGGAGGAGGC
OP-19	CTCCTCCTCGC
OP-20	TCT CTC TCT CTC TCT CA

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