An improved in vitro encapsulation protocol, biochemical analysis and genetic integrity using DNA based molecular markers in regenerated plants of Withania somnifera L

Nigar Fatima a, c, Naseem Ahmad a, Mohammad Anis a, b, *, Iqbal Ahmad c

a Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh 202 002, India
b Department of Plant Production, College of Food and Agriculture Sciences, King Saud University, Riyadh, Saudi Arabia
c Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh 202 002, India

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Non-embryogenic, synthetic seeds were produced by encapsulating nodal segments (containing axillary buds) of Withania somnifera L. in calcium alginate hydrogel containing Murashige and Skoog (MS) medium. A 3% sodium-alginate with 100 mM calcium chloride found to be the optimum concentration for the production of uniform synthetic seeds. Effect of different treatments (M1–M5), i.e. MS medium containing different concentrations of cytokinins (0.5, 1.0, 2.5, 5.0 & 10.0 μM) along with optimal level of auxins NAA (0.5 μM) on in vitro morphogenic response of synthetic seeds was evaluated. The maximum frequency (86.2%) of conversion of encapsulated heads into plantlets was achieved on MS (M3) medium containing 6-benzyladenine, BA (2.5 μM) and α-naphthalene acetic acid. NAA (0.5 μM) after 4 weeks of culture. Rooting in plantlets was achieved on 1/2 MS + NAA (0.5 μM). Plantlets obtained from stored synthetic seeds were hardened, acclimatized and established in field, where they grew well without any detectable malformation. Significant enhancement in the pigment contents (chlorophyll, carotenoids and net photosynthetic rates) with an increase in acclimatization days may be attributed to chlorophyll biosynthesis. Activities of antioxidant enzymes i.e. superoxide dismutase, catalase and peroxidase activity were significantly increased which suggests their preventive role in membrane oxidation and damage to biological molecules. Also, an enhanced level of lipid peroxidation, as indicated by MDA content, a sensitive diagnostic index of oxidative injury clearly indicating its positive determining role in combating oxidative stress during acclimatization of plantlets. The generated RAPD and ISSR profiles from regenerated plantlets with mother plant were monomorphic which confirms the genetic stability among the clones. This synthetic seed technology could possibly pave the way for the conservation, short-term storage, germplasm exchange with potential storability and limited quarantine restrictions.

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1. Introduction

Withania somnifera L. (Dunal) (Solanaceae) commonly referred as Indian ginseng constitute important ingredient in many formulations prescribed for a variety of musculoskeletal conditions (e.g., arthritis, rheumatism), and as a general tonic to increase energy, improve overall health and longevity (Chatterjee and Pakrashi, 1995). Many pharmacological studies have been conducted to investigate the properties of Ashwagandha in an attempt to authenticate its use as a multi-purpose medicinal agent. Western research supports its poly-pharmaceutical use, confirming antioxidant, anti-inflammatory, and anti-stress properties in the whole plant extract and several separate constituents (Mishra et al., 2000).

A large scale and unrestricted exploitation of the natural resources to meet its ever-increasing demand by the pharmaceutical industry, coupled with limited cultivation and insufficient attempts for its replenishment, have culminated in the marked depletion of the species. Since, the requirement of W. somnifera biomass rapidly increased over the last few years, concrete measures are needed to conserve this valuable species. Recently, alginate encapsulation technology for the production of synthetic seeds in conjunction with micropropagation has become a viable approach for in vitro conservation (Ahmad et al., 2012).

Synthetic seed technology is an exciting and rapidly growing research area in plant cell and tissue culture (Datta et al., 1999). Production of synthetic seed endowed with high germination rate under in vitro conditions bears immense potential as an alternative...
of true seeds. Technology offers many useful advantages on a commercial scale for the variety of crop plants, especially crops for which true seeds are not used or readily available for multiplication or true seeds are expensive, hybrid plants and plants which are prone to infections. The technology would be useful for multiplying genetically engineered plantlets (transgenic plants, somatic and cytoplasmic hybrids, sterile and unstable genotypes).

Plantlets develop within culture vessels on a medium containing ample sugar and nutrient to allow for heterotrophic growth under low levels of light and high relative humidity. These conditions result in the formation of plantlets with abnormal morphology, anatomy and physiology (Kozai and Smith, 1995). In the course of hardening, the tissue culture plants gradually overcome these inadequacies and adapt to ex vitro conditions. Plant survival, growth and productivity are reported to be intimately coupled with the aerial environment through processes such as energy exchange, loss of water vapor in transpiration and uptake of CO2 in photosynthesis (Stoutjesdijk and Barkman, 1992). Therefore, role of oxidative stress and protective enzymatic systems in relation to progression of acclimatization process is an essential step to be studied. These criteria would be able to supply more objective information than agronomic parameters or visual assessment when evaluating for component traits of complex characters.

Genetic stability and maintenance of germplasm is one of the most important pre-requisite in the in vitro propagation of plant species. The occurrence of cryptic genetic defects arising via somaclonal variation in the regenerants can seriously limit the broader utility of the micropropagation system (Salvi et al., 2001). It is therefore imperative to establish genetic uniformity of synthetic seed derived plantlets to suggest the quality of the plantlets for its commercial utility. Several strategies have been developed to assess the genetic purity of tissue cultured raised plants such as morphological descriptions, physiological supervisions, cytological studies, isoenzymes etc., (Gupta et al., 1999). Polymerase chain reaction (PCR) based techniques such as random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) are immensely useful in establishing the genetic stability of in vitro regenerated plantlets in many plant species (Ahmad and Anis, 2011; Faisal et al., 2012). RAPD and ISSR markers are very simple, fast, cost-effective, highly discriminative and reliable. They require only a small quantity of DNA sample and they do not need any prior sequence information to design the primer. Since, uniformity of the tissue culture raised progeny is the major concern to maintain the quality of germplasm, we have adopted RAPD/ISSR technique (Williams et al., 1990) for the evaluation of genetic integrity among regenerated plantlets.

So far, there is only one report available on the development of synthetic seed system in W. somnifera using apical buds (Singh et al., 2006b). However, no studies available on the possible role of oxidative stress, protective enzymatic system with their corresponding isoenzymes as well as the analysis of genetic fidelity of sysexed derived plantlets of W. somnifera have been reported.

Therefore, the present study has been conducted to optimize the parameters for the production, conservation and their conversion potential under in vitro conditions after cold storage using synthetic seed technology to ensure steady supply of quality plants. Moreover, the physiological, enzymatic activity and the genetic stability of the synthetic seed derived plantlets were also assessed during acclimatization.

2. Materials and methods

2.1. Encapsulation material

Nodal segments with axillary buds approximately 1 cm dissected aseptically from in vitro established (8 weeks old) cultures of W. somnifera were used as explants for encapsulation (Fatima and Anis, 2012).

2.2. Encapsulation matrix

Different concentrations 2, 3, 4 and 5% (w/v) of sodium alginate were prepared using liquid MS medium. For complexation 25, 50, 75 100 and 200 mM Calcium chloride solutions were prepared. Both, the gel matrix and complexing agent were sterilized by autoclaving at 121 °C (15 lbs) for 15 min after adjusting the pH to 5.8.

2.3. Encapsulation, planting media and culture conditions

Encapsulation was accomplished by mixing the nodal segments from in vitro regenerated shoots into the sodium alginate solution and dropping them into the calcium chloride solution. The droplets containing the explants were held for at least 30 min to achieve polymerization of the sodium alginate. The alginate beads were then collected, rinsed with sterile liquid MS medium and transferred to sterile filter paper in petri-dishes for 5 min under the laminar airflow hood to eliminate the excess of water and thereafter planted into petri-dishes containing sowing medium (M1–MS) composed of MS nutrient medium with the various concentrations and combinations of BA (0.5, 1.0, 2.5, 5.0 and 10.0 μM) and NAA (0.5 μM), respectively (Fatima and Anis, 2012).

2.4. Low temperature storage

Synthetic seeds (encapsulated nodal segments) were transferred in petri-dishes containing agar medium M3 (BA 2.5 μM + NAA 0.5 μM) and stored in a laboratory refrigerator at 4 °C. Five different low temperature exposure times (2, 4, 6 and 8 weeks) were evaluated for regeneration. After each storage period, encapsulated nodal segments were placed on MS medium with or without growth regulators for conversion into plantlets. The percentage of encapsulated nodal segments forming shoot and root were recorded after 4 weeks of culture to regeneration medium (M3).

2.5. Hardening and acclimatization

Plantlets with shoot and roots were removed from the culture medium, washed gently with tap water and transferred to thermocol cups containing sterile soilrite, moistened with 1/2 MS lacking organic supplements placed under low light intensity of 25 μmol m−2 s−1. Potted plantlets were covered with a transparent polythene membrane to maintain high humidity and irrigated every 3 days with half strength MS salt solution lacking vitamins and PGRs for 2 weeks. The membranes were removed after 2 weeks in order to acclimatize plants to field conditions. After 4 weeks, acclimatized plants were transferred to pots containing normal soil, maintained in a greenhouse and finally transferred to field under full sun.

2.6. Chlorophyll and carotenoids estimation

The chlorophyll a and b and carotenoids from leaf tissue were estimated by using the method of McKinney (1941) and Maclachlan and Zalik (1963) respectively. About 100 mg fresh tissues from interveinal areas of leaves were taken after 0 (persistent leaves), 7, 14, 21 and 28 (fully expanded leaves) days of acclimatization. These leaves were grind in 5 ml acetone (80%) with the help of mortar and pestle. The suspension was filtered with Whatman filter paper number-1, if necessary the supernatant was regrinded, washed and filtered, the total filtrate was taken in graduated test
tubes and final volume was made up to 10 ml with 80% acetone. The optical density (O.D.) of chlorophyll solution was read at 645 and 663 nm wave lengths and for carotenoids, the O.D. was read at 480 and 510 nm wavelengths with the help of a spectrophotometer (UV-Pharma Spec 1700, Shimadzu, Japan).

2.7 Leaf gas exchange measurements

The net photosynthetic rate ($P_N$) of in vitro regenerated plants was measured during different stages (0, 7, 14, 21 and 28 days) of acclimatization using portable Infra Red Gas Analyzer (IRGA, LICOR 6400, Lincoln, USA) on the basis of net exchange of CO$_2$ between leaf and atmosphere by enclosing the leaf in the leaf chamber, and monitoring the rate at which the CO$_2$ concentration changed over a short time intervals (10–20 s). The net photosynthetic rate was expressed as $\mu$mol CO$_2$ m$^{-2}$ s$^{-1}$.

2.8 Antioxidant enzymes extraction and assay

To determine antioxidant enzyme levels, 0.5 g fresh leaf tissue was homogenized in 2.0 ml extraction buffer containing 1% polyvinylpyrrolidone (PVP), 1% Triton x-100, and 0.11 g ethylene-diamine tetraacetic acid (EDTA) using pre-chilled mortar and pestle. The homogenate was filtered through four layers of cheese cloth and centrifuged at 15,000 rpm for 20 min. The supernatant was used for enzyme assays. Extraction was carried out in dark at 4°C.

2.9 Superoxide dismutase (SOD) estimation

Superoxide dismutase (SOD) activity was determined by method of Dhindsa et al. (1981) with slight modifications. SOD activity in the supernatant was assayed by its ability to inhibit the photochemical reduction. The reaction mixture consisting of 0.5 M potassium phosphate buffer, 200 mM methionine, 1 M sodium carbonate, 2.5 mM nitroblue tetrazolium (NBT) solution, 3 mM EDTA, 0.1 ml enzyme extract, 60 $\mu$M riboflavin, and 1.0 ml DDW were incubated in a test tube under a 15 W fluorescent lamp (Phillips, India) for 10 min at 25–28°C. Reaction mixture containing all the above substances along with enzyme, placed in dark served as blank A whereas blank B contained all the above substances except enzyme and placed in light along with the sample. Absorbance of samples along with blank B was read at 560 nm against the blank A. A 50% reduction in color was considered as one unit of enzyme activity. SOD activity of extract was expressed as enzyme units (EU) $\mu$mol H$_2$O$_2$ min$^{-1}$ protein $^{-1}$.

2.10 Estimation of catalase (CAT) activity

Catalase (H$_2$O$_2$:H$_2$O$_2$ oxidoreductase; EC 1.11.1.6) activity in the leaves of regenerated plantlets was determined by the method of Aebi (1984) with slight modifications. Reaction mixture containing 0.5 M potassium phosphate buffer, 3 mM EDTA, 0.1 ml enzyme extract, and 3 mM H$_2$O$_2$. The reaction was allowed to run for 5 min. Catalase activity was determined by monitoring the disappearance of H$_2$O$_2$ and measuring the decrease in absorbance in 25°C. CAT activity was calculated by using extinction coefficient ($\varepsilon$) 0.036 $\mu$mol$^{-1}$ cm$^{-1}$ and expressed in enzyme units (EU) $\mu$mol H$_2$O$_2$ per min at 25°C.

2.11 Estimation of peroxidase

Peroxidase content (EC 1.11.1.7) was determined by Bergmeyer et al. (1974). About 0.2 mg fresh leaves sample were collected and homogenized in a mortar and pestle with 5 ml chilled phosphate buffer (50 mM pH 7.8). The homogenate centrifuged 10, 000 rpm for 20 min at 4°C. The supernatant was stored at 4°C and used for the peroxidase assay. The assay mixture contains 0.1 M phosphate buffer (pH 7.8), 4 mM pyrogallol, 3 mM H$_2$O$_2$ and crude enzyme extract. Transfer the reaction mixture into a suitable cuvette and measure the absorbance at 420 nm using Spectrophotometer (UV-Pharma Spec 1700, Shimadzu, Japan). The enzyme was expressed as nmol (H$_2$O$_2$ destroyed) mg$^{-1}$ (protein) s$^{-1}$. The peroxidase content was determined as follows:

\[
\text{Unit/g material} = \left( \frac{A_{420} - A_{600}}{V \times \varepsilon \times W} \right)
\]

wherein, $A_{420}$ is the Dilution factor (21); 1000 is the conversion factor from nm to g; $V$ is the final volume of reaction mixture; $\varepsilon$ is the molar extinction coefficient of purpuragallin at 420 nm; $V_i$ is the volume of enzyme used; $C_i$ is the enzyme concentration in mg/ml.

It measures the oxidation of pyrogallol to purpuragallin by peroxidase when catalyzed by peroxidase at 420 nm.

\[
\text{H}_2\text{O}_2 + \text{pyrogallol} \rightarrow \text{H}_2\text{O} + \text{purpuragallin}
\]

2.12 Estimation of carbonic anhydrase (CA) activity

The enzyme CA catalyses the reversible hydration of carbon dioxide (CO$_2$) to give the bicarbonate ion. It was assayed using the method of Dwivedi and Randhawa (1974).

\[
\text{H}_2\text{O} + \text{CO}_2 \rightarrow \text{H}^+ + \text{HCO}_3^-
\]

Plant leaves were sampled from each sample randomly. Leaves were cut into small segments (1 cm$^2$) at a temperature below 25°C. After mixing them, 200 mg leaf pieces were weighed and cut further into small pieces (2–3 mm length) in 10 ml 0.2 M cysteine in a petri dish at 0–4°C. After being cut, the solution adhering at their surface was removed with the help of a blotting paper followed by transfer immediately to a test tube, having 4 ml phosphate buffer of pH 6.8. To this 3.4 ml 0.2 M sodium bicarbonate (NaHCO$_3$) in 0.02 M sodium hydroxide (NaOH) solution and 0.2 ml 0.002% brothymol blue indicator was added. After shaking, the tube was kept at 0–4°C. For 20 min.

2.13 Estimation of lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) content was determined using a modified protocol of Cakmak and Horst (1991). The lipid peroxidation product in leaf samples was expressed as MDA (malondialdehyde) content. Approximately 0.5 g leaf tissue was homogenized with 5 ml 0.1% trichloroacetic acid (TCA), and centrifuged at 15,000 rpm for 5 min. Then, 1 ml aliquot of the supernatant was mixed with 4 ml 0.5% (w/v) thiobarbituric acid (TBA), prepared in 20% (w/v) TCA, and incubated in boiling water for 30 min. Thereafter, it was immediately cooled on ice to stop the reaction, and centrifuged at 12,000 rpm for 30 min. The supernatant was placed in a UV-VIS spectrophotometer (UV-1700 PharmaSpec) to determine the absorbance at 532 nm and corrected for non-specific turbidity by subtracting its absorbance at 600 nm. The concentration of lipid peroxides together with oxidatively modified proteins of plants were thus quantified in terms of MDA level using an extinction coefficient of 155 mM$^{-1}$ cm$^{-1}$ and expressed as nmol g$^{-1}$ fresh weight. TBARS content was determined as follows.

\[
\text{TBARS content (nmol g}^{-1} \text{ fresh weight)} = \frac{(A_{532} - A_{600})V \times 1000}{\varepsilon \times W}
\]
wherein, $e$ is the specific extinction coefficient ($155 \text{ mM}^{-1} \text{ cm}^{-1}$); $V$ is the volume of the extraction medium; $W$ is the fresh weight of leaf; $A_{600}$ is an absorbance at 600 nm; $A_{532}$ is an absorbance at 532 nm.


The genetic stability of the recovered plants was studied by RAPD and ISSR techniques. Ten micropropagated plants from field transferred progeny were randomly selected alongwith mother plant and evaluated for genetic homogeneity. Genomic DNA was extracted from young leaves of *W. somnifera*, following the cetyl-methylammonium bromide (CTAB method) described by Doyle and Doyle (1990). The extracted DNA was tested for purity ($A_{260}/A_{280}$ ratio) on a UV–vis spectrophotometer (UV-1700 Pharma Spec, Shimadzu, Japan, Kyoto).

A set of thirteen ISSR (UBC, Vancouver, BC, Canada) and 40 RAPD (Kit OPB and OPC) primers were used for initial screening. PCR reactions for RAPD/ISSR markers amplification were performed on a thermocycler (Biometra, T Gradient Thermoblock, Germany). The PCR amplification mixture (20 µl) contained 10X buffer (2 µl), 25 mM MgCl$_2$ (1.2 µl), 10 mM dNTPs (0.4 µl), 2 µM primers, 3 Unit Taq polymerase (0.2 µl) and 40 ng Template DNA. PCR amplification program consisted of 45 cycles including a 94 °C denaturation step of 5 min, a 35 °C annealing step of 1 min and a 72 °C elongation of 1 min. A final extension was followed at 72 °C for 10 min. DNA amplification products were fractioned by electrophoresis in 0.8% (w/v) agarose gels with 4 µl ethidium bromide in TAE buffer (pH 8.0) run at 50 V for 2 h and visualized on a UV transilluminator (Bio Rad, Hercules, CA, USA). In order to assess the consistency of band profiles DNA isolation and PCR reactions were carried out three times. Only well defined and reproducible bands were scored. Bands with the same migration were considered to be homologous fragments, regardless of intensity.

2.15. Statistical analysis

All the experiments were repeated thrice with 20 nodal segments for each treatment. The data obtained were analyzed using statistical software, SPSS Version 16 (SPSS Inc. Chicago, USA) and means were compared using Duncan’s multiple range test (DMRT) at 0.5% level of significance. All the results were expressed in mean ± standard error.

Fig. 1. Plant regeneration from encapsulated nodal segments of *W. somnifera*. (A) Artificial seeds of *W. somnifera* obtained by the encapsulation of nodal segments in 3% sodium alginate and 100 mM calcium chloride. (B) Culture showing shoot emergence from synthetic seeds after 2 weeks of culture. (C) Germinated synthetic seed with shoot on MS + BA (2.5 µM) + NAA (0.5 µM) after 4 weeks of culture. (D) Acclimatized plantlets derived from encapsulated nodal segments.
Table 1
Effect of different concentrations of sodium alginate with optimum concentration of calcium chloride (100 mM) on the formation and conversion of synthetic seeds of *W. somnifera* after 4 weeks of culture on MS medium. Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different (*P* > 0.05) using Duncan’s multiple range test.

<table>
<thead>
<tr>
<th>Sodium alginate (w/v)</th>
<th>Conversion response</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.00 ± 0.00d</td>
<td>Fragile beads difficult to handle</td>
</tr>
<tr>
<td>2</td>
<td>0.00 ± 0.00d</td>
<td>-do-</td>
</tr>
<tr>
<td>3</td>
<td>92.4 ± 0.64*</td>
<td>Beads with soft texture</td>
</tr>
<tr>
<td>4</td>
<td>76.6 ± 0.90b</td>
<td>Hard beads</td>
</tr>
<tr>
<td>5</td>
<td>58.1 ± 0.49*</td>
<td>Hard beads</td>
</tr>
</tbody>
</table>

3. Results and discussion

The encapsulated beads differed morphologically with respect to texture, shape and transparency with different combination and concentrations of sodium alginate (2–5%) and CaCl$_2$. 2H$_2$O$_2$ (25–200 mM). The assessment of the effects of various concentrations of sodium alginate and calcium chloride was prerequisite in order to standardize the preparation of characteristic beads. An encapsulated mass of 3% sodium alginate with 100 mM CaCl$_2$. 2H$_2$O was found most suitable for the formation of clear, uniform beads within an exchange duration of 30 min (Fig. 1A; Tables 1 and 2). Higher concentration of sodium alginate (4% or 5%) and calcium chloride (200 mM) were found to be unsuitable because the resulting beads formed were too hard, isodiametric and also inhibits the conversion rate while, the lower concentration of sodium alginate (1 or 2%) and CaCl$_2$ (25 or 50 mM) not only prolonged the ion exchange (polymerization) duration but also resulted in the formation of fragile beads that were difficult to handle (Tables 1 and 2). Sodium alginate preparations at lower concentration were unsuitable, probably because of a reduction in their gelling ability after exposure to high temperature during autoclaving (Larkin et al., 1988). A successful propagation system routed through encapsulation is based on significant evaluation of factors affecting gel matrix and also on sodium alginate and CaCl$_2$, 2H$_2$O, which plays an important role in complexion and capsule quality (Singh et al., 2006a). The present results are in corroboration with previous findings (Siddique and Anis, 2009).

Nodal segments encapsulated in 3% (w/v) sodium alginate and 100 mM CaCl$_2$. 2H$_2$O exhibited shoot regeneration after 2–3 weeks (Fig. 1B), cultured on five different media (M1–M5), described above. The frequency of shoot regeneration varied with medium composition. The highest frequency (86.2%) of conversion of encapsulated buds was achieved on MS medium (M3) supplemented with BA (2.5 μM) and NAA (0.5 μM) after 4 weeks of culture (Figs. 1C and 2). Conversion into plantlets was achieved after 6 weeks of culture on the same medium. Shoots developed were phenotypically normal with distinct nodes and internodes. There was no regeneration occurred on hormone free medium (M1) whereas, higher concentration of hormone (M5) in a medium showed the emergence of weak shoots with stunt growth. This is in accordance with the previous findings (Kavyashree et al., 2006).

Storage duration (2, 4, 6 and 8 weeks) was also found to influence the regeneration frequency of encapsulated axillary buds at 4°C. A highly desirable feature of encapsulated nodal segment is their ability to retain viability in terms of sprouting and conversion potential even after a considerable period of storage required for germplasm exchange. The effect of different storage duration on encapsulated nodal segment at 4°C is summarized in Fig. 3. An alginate matrix also served as an artificial endosperm, thereby providing nutrients to the encapsulated explants for regrowth. Antonietta et al. (1999) reported that the synthetic endosperm should contain nutrients and a carbon source for germination and conversion.

Table 2
Effect of different concentrations of calcium chloride with optimum level of sodium alginate (3%) on the formation and conversion of synthetic seeds of *W. somnifera* after 4 weeks of culture on MS medium. Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different (*P* > 0.05) using Duncan’s multiple range test.

<table>
<thead>
<tr>
<th>Calcium chloride (mM)</th>
<th>Conversion response</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>0.00 ± 0.00d</td>
<td>Fragile beads difficult to handle</td>
</tr>
<tr>
<td>50</td>
<td>0.00 ± 0.00d</td>
<td>-do-</td>
</tr>
<tr>
<td>75</td>
<td>84.4 ± 1.44*</td>
<td>Very soft but handled easily</td>
</tr>
<tr>
<td>100</td>
<td>97.0 ± 0.51*</td>
<td>Soft beads and easy to handle</td>
</tr>
<tr>
<td>200</td>
<td>69.4 ± 0.20*</td>
<td>Hard beads</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of different medium (M1–M5) on *in vitro* regeneration form sodium alginate encapsulated nodal cuttings of *W. somnifera* after 4 weeks of culture. Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different (*P* > 0.05) using Duncan’s multiple range test.

Fig. 3. Effect of cold storage (4°C) on *in vitro* regeneration from alginate encapsulated nodal cuttings of *W. somnifera* on M3 medium. Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different (*P* > 0.05) using Duncan’s multiple range test.
A decline in percentage conversion frequency (62.8%) of synthetic seed was observed as the storage period increased beyond 4 weeks. This decline in the conversion response could be attributed to the inhibition of tissue respiration by the alginate matrix (Redenbaugh et al., 1984) or a loss of moisture due to the partial desiccation during storage (Danso and Ford-Lloyd, 2003). Encapsulated nodal segments were viable up to (50.5%), even after 8 weeks of cold-dark storage (Fig. 3). These observations corroborate with earlier findings by Faisal and Ains (2007). The present study could be considered as an improved encapsulation technique for W. somnifera using nodal explants. In the present investigation, the conversion frequency of synseed was quite higher (86.2%) after 4 weeks of cold storage in comparison to other reports on W. somnifera (Singh et al., 2006).

The synthetic seeds demonstrated high adventitious rooting capacity after sowing. The regenerated micro-shoots rooted when excised and subjected to 1/2 MS medium containing NAA (0.5 μM). Rooted plantlets with 4–5 fully developed leaves, retrieved from encapsulated nodal segments were transferred to thermocups containing soilrite. The plantlets were covered with transparent polybags and acclimatized by adopting the standard procedure. After 4 months, they were transferred to pots containing normal garden soil and maintained in greenhouse with 90% survival rate (Fig. 1D). Micropropagation is restricted by often high percentage of plants lost or damaged during ex vitro transplantation (Pospisilova et al., 1999). The plantlets are susceptible to various stresses. A switch to autotrophy and changes in stomata functioning and cuticle composition has been reported during acclimatization (Huynenbroeck et al., 1998). The imposition of environmental stresses increased the rate of production of reactive oxygen species (ROS). ROS are inevitable byproducts of aerobic metabolism which cause lipid peroxidation and consequently membrane injuries, protein degradation, enzyme inactivation, damage to DNA etc. To counter the hazardous effects of reactive oxygen species, plant cells develop a complex antioxidant defense and enzymatic scavenging system composed of antioxidant enzymes and metabolites such as superoxide dismutase (SOD), catalase (CAT) and peroxidases (POXs) etc.

During transfer of tissue cultured raised plantlets from in vitro to ex vitro, the change in pigment concentration (Chlorophyll and carotenoid contents) was estimated and it was observed that with an increase in number of days of acclimatization, the pigment contents increased significantly. The Chl a content was low (0.23 ± 0.01) mg g⁻¹ on 0 days of acclimatization whereas, it was increased up to (0.42 ± 0.02) mg g⁻¹ after 21 days and was maximum (0.45 ± 0.01) mg g⁻¹ at 28 days of transfer (Fig. 4). Decreased in chlorophyll level during the first week of transplantation was accompanied by poorly developed chloroplast and disorganized grana. Enhancement in pigment contents may be attributed to the induction of chlorophyll synthesis enzyme required for chlorophyll biosynthesis. Similar results have been reported by Pospisilova et al. (1999) and Jeon et al. (2005).

Carotenoid plays an important role in protection of chlorophyll pigments under stress conditions (Kenneth et al., 2000) which might be generated during acclimatization. Carotenoid contents increased gradually during the period of transplantation. The maximum carotenoid level (0.10 ± 0.01) mg g⁻¹ was observed after 28 days of acclimatization (Fig. 4). Increase in carotenoid levels is not unexpected as carotenoids are reported to be involved in protecting the photosynthetic machinery from photo-oxidative damage (Jeon et al., 2005).

During transplantation of plantlets, the change in net photosynthetic rate (PN) was observed after 7, 14, 21 and 28 days of acclimatization. PN as measured decreased in the first week after transplantation and increased thereafter. The highest net photosynthetic rate (4.10 ± 0.14) μmol CO₂ m⁻² s⁻¹ was obtained after 28 days of transplantation to ex vitro environment (Fig. 5). The decline in photosynthetic rate during the first week after the transfer from in vitro to ex vitro condition indicates that climatic conditions create stress in micropropagated plants. Similar results were observed earlier in Rosa hybrida (Genoud-Gourichon et al., 1999).

Carbonic anhydrase activity was assessed during acclimatization process and was found to increase during hardening process and reached (1.90 ± 0.10) mM CO₂ g⁻¹ fresh mass⁻¹ after three weeks of transfer (Fig. 6). The enzymes play a determinant role in transport and exportation of sugar with the plant (Aragon et al., 2005).

Acclimatized plantlets of W. somnifera showed a time dependent increase in superoxide dismutase, catalase and peroxidase activity. SOD, POX and CAT are the key enzymes involved in the detoxification of the deleterious oxygen species. Changes in SOD activity was observed during the first 14 days after transplantation and reached maximum (9.20 ± 0.10) mg⁻¹ protein min⁻¹ at 28 days of acclimatization (Fig. 7). SOD is believed to play a crucial role in the antioxidant systems as it catalyses the dismutation of O₂ into H₂O₂ and O₂ (Bowler and Van Montagu Inze, 1992). In response
to this, the cellular machinery generates free radical scavengers (SOD) that effectively prevent membrane oxidation and damage to biological molecules. In addition, peroxidase (POXs) and catalase (CAT) activity increased during the whole period of acclimatization and reached to its maximum value (4.40 ± 0.10; 4.20 ± 0.10) mg⁻¹ protein min⁻¹ respectively, at 28 days of transfer as compared to control plantlets (Fig. 8). The increase in antioxidants activities (SOD, POX and CAT) was noticed upto 28 days of acclimatization and it got stabilized thereafter. The augmentation in catalase activity could be explained by peroxisomal proliferation, where this enzyme is localized (Willekens et al., 1995). Increased catalase levels also suggest its role in the photo-respiratory detoxification of hydrogen peroxide through the mitochondrial electron transport system (Scandalios, 1990). POXs catalyses various reactions where H₂O₂ is used as one of their substrates including cell wall lignification (Lee et al., 2007).

The ROS-induced peroxidation of lipid membrane is a reflection of stress-induced damage at the cellular level (Jain et al., 2001). An enhanced level of lipid peroxidation, as indicated by MDA content, was observed in Withania leaves after transplantation to ex vitro environment, clearly indicating an oxidative stress. MDA is a common product of lipid peroxidation and a sensitive diagnostic index of oxidative injury (Janero, 1990). During transplantation, the MDA contents in leaves was found to be low (2.53 ± 0.13) μmole mg⁻¹ s⁻¹ after 7 days but the MDA level significantly increased to (3.86 ± 0.06) μmole g⁻¹ FW after 28 days of acclimatization (Fig. 9). The increase in TBARs (Thiobarbituric acid reactive substances) content observed in this study may be an indicator of the regeneration of ROS. Similar findings are in accordance with Faisal and Anis (2009). Increase in lipid peroxidation was reported in many plants under various environment stresses (Prasad, 1996).

RAPD and ISSR finger printing of ten randomly selected, in vitro raised plants and mother plant was carried out. Well resolved, clear and distinct banding patterns were manually scored from the gel profiles and included for final analysis. Bands with same mobility were treated as identical fragments and weak bands were excluded from the final analysis. RAPD and ISSR were chosen because of their simplicity and cost-effectiveness. They amplify different regions of the genome providing broad analysis of genetic stability or variation in plants. Out of 20 RAPD primers screened from each Kit B

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**Fig. 6.** Changes in carbonic anhydrase activity (mM CO₂ (g⁻¹) fresh mass s⁻¹) during acclimatization of W. somnifera plantlets. Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different (P=0.05) using Duncan’s multiple range test.

**Fig. 7.** Changes in SOD (Unit mg⁻¹ protein min⁻¹) activity during acclimatization of W. somnifera plantlets. Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different (P=0.05) using Duncan’s multiple range test.

**Fig. 8.** Changes in CAT (μmol min⁻¹ mg⁻¹ protein) and POX (μmole mg⁻¹ (protein) s⁻¹) activity during acclimatization of W. somnifera plantlets. Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different (P=0.05) using Duncan’s multiple range test.

**Fig. 9.** Changes in MDA (μmol g⁻¹ FW) concentration during acclimatization of W. somnifera plantlets. Values represent means ± standard error of 20 replicates per treatment in three repeated experiments. Means followed by the same letter are not significantly different (P=0.05) using Duncan’s multiple range test.
and Kit C, 16 and 18 primers produced clear, reproducible bands and well resolved banding pattern, respectively (Table 3). On the basis of banding pattern and resolution, primers OPB04 and OPC08 were used for further analysis. Primer OPB04 produced 9 monomorphic bands (Fig. 10A), while primer OPC08 produced 11 monomorphic bands (Fig. 10B; Table 3).

For ISSR analysis, 13 ISSR primers were screened for the regenerated plants. All the 13 primers gave clear, unambiguous and reproducible bands and were used for ISSR-PCR. Primer UBC-866 amplified maximum 13 monomorphic bands (Fig. 11A), while primer UBC891 produced 9 monomorphic bands (Fig. 11B; Table 4). No polymorphism was detected during the ISSR analysis of tissue culture raised plantlets. All the RAPD and ISSR tested primers produced monomorphic pattern across all the plants and the mother plant, confirming the genetic uniformity of the micropropagated plantlets. Our results corroborate with the earlier reports on genetic

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Fig. 10. A profile of polymerase chain reaction (PCR) amplification products from lane 1–10 micropropagated plants of W. somnifera using randomly amplified polymorphic DNA (RAPD) primer OPB04. A profile of polymerase chain reaction (PCR) amplification products from lane 1–10 micropropagated plants using randomly amplified polymorphic DNA (RAPD) primer OPC08.

Fig. 11. A profile of polymerase chain reaction (PCR) amplification products from lane 1–10 micropropagated plants using inter sequence repeat (ISSR) primer UBC 866. A profile of polymerase chain reaction (PCR) amplification products from lane 1–10 micropropagated plants using inter sequence repeat (ISSR) primer UBC891. M, Marker (ADNA/EcoRI + HindIII indicated in bp); P, Donor plant; Lane 1–10, Micropropagated plants.
stability of synthetic seed derived plantlets of Cineraria maritime (Srivastava et al., 2009) and Rauwolfia serpentina (Faisal et al., 2012).

4. Conclusion

The present protocol highlights the development of high frequency shoot recovery in W. sommifera from encapsulated nodal segments after 4 weeks of storage. The technique offers a simple way of handling cells and tissues, protecting them against strong external gradients and proves an efficient delivery system. Synthetic seeds are also expected to offer an appropriate recipient system for alien gene transfer in micro-projectile based gene delivery system. Since, antioxidant metabolism has been shown to be important in determining the ability of plants to survive in oxidative stresses, therefore, an up regulation of these enzymes would help to reduce the buildup of ROS. This factor could be a key to design adequate methods to improve acclimatization process. The ability to generate transgenic plants provides a powerful tool to increase the level of stress tolerance by increasing the expression of the native genes of the antioxidant enzymes, the natural defense system in plants. The basic information on different biochemical changes during acclimatization process of in vitro raised plantlets is also essential for further molecular breeding. The present protocol explores the possibility of preserving the genetic stability of the selections and promotes true to type genotype for exchange of germplasm between laboratories.

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