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In vitro propagation and genetic characterization as effective tools for conservation of *Silene leucophylla*, grown in St. Katherine Protected Area, Sinai, Egypt

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Abstract *Silene leucophylla* is a very rare endemic perennial species growing in the stony habitats at Wadi Tarfa-Solaf microhabitat of St. Katherine Protected areas (SKP), Sinai, Egypt. The present study reports the use of *in vitro* propagation and genetic characterization as an effective strategy for conservation of this rare species. The developed system relied on multiple shoot organogenesis from explants excised from few number of aseptically growing seedlings. A maximum shoot production (150 shoot per single explants) was achieved on Murashige and Skoog’s medium (MS) supplemented with 4.5 benzyl adenine (BA), 0.5 g/l casein and 0.5 mg/l silver nitrate. The obtained results also indicated that a regeneration medium contained 4 mg/l BA; 0.4 mg/l NAA; 0.2 mg/l GA3; 200 mg/l adenine sulfate; 0.5 mg/l silver nitrate; 0.5 g/l casein and 100 mg/l myoinositol favored rooting of the proliferated shoots. In order to devise an appropriate tissue culture media type and regime for true-to-type plantlets, genetic analysis of tissue culture-derived plantlets (vitroplants) was studied using RAPD–PCR analysis and morphological descriptions of vitroplants (tissue culture-derived plantlets) produced on different regeneration media. The results of the molecular
1. Introduction

Silene leucophylla (Family: Caryophyllaceae Bioso), is endangered due to grazing by sheep and Goats as well as over-collection for scientific research. Extensive human impact has resulted in a high incidence of rarity throughout the highly endemic species. According to the restoration report [5,7], the wild populations of S. leucophylla growing in SKP are scattered over seven locations. The population size ranges between 1 and 12 individuals while its total count is only 50 individuals. Other technical report of medicinal plants conservation project [5], the maximum germination percentage of S. leucophylla, although seems to have no specific problems with germination, nevertheless has as low percentage as only 30%. It was noticed that 50% of the gminated seedlings were unable to survive. The inappropriate population size, low seed fitting and the low seedling’s survival rate of this species are among factors which lead to the disappearance of this species from the wild habitats of SKP and to be included among the threatened species list of SKP. The micro-climatic conditions studies indicated that soil supporting S. leucophylla in its natural habitats is alkaline, with low salinity and soluble cations. The prevailing conditions in SKP indicate January as the coldest month and July as the warmest month [6].

Previous efforts to conserve this species using traditional methods indicate very clearly that traditional propagation using seed germination is a problematic and inappropriate approach. Generally, the approach of conservation using seed germination approach is not recommended for many reasons including dormancy, strongly heterozygous nature of some seeds, failure of some plants to set seeds, the fact that one plant is produced by each seed, unavailability of adequate seed materials and low germination rate [16]. On contrary, in vitro culture techniques proved to be effective tools in propagation of rare and recalcitrant species. The successful use of in vitro culture techniques for mass propagation and conservation of rare species dates back to the early 80s. This approach is well reviewed and discussed by Fay and Gratton [10] and Engelmann [8]. Over the years, successful systems for in vitro propagation and cryopreservation of rare species, medicinal plants and wild herbs worldwide were developed [12]. Moreover, botanic gardens and private firms now have tissue culture laboratories for the micropropagation of rare species that are difficult to propagate using traditional methods and produce bioactive substances in vitro [17,19]. Nevertheless, conservation and restoration of rare species in Egypt using in vitro culture techniques is still limited and in the infancy stage. Therefore, the present work aimed at development of in vitro propagation system for S. leucophylla, very rare species endemic in St. Catherine Protected Area, Sinai, Egypt.

2. Materials and methods

2.1. Tissue culture

Few available seeds were collected from five individual plants growing within the same population at Wadi Tarfa-Solaf microhabitat. Seeds were cleaned and subjected to sterilization using different concentrations of commercial Clorox (10%, 20% and 30% Clorox) for 5, 10 and 20 min, to determine the most suitable sterilization conditions. The sterilization was carried out using ethanol 70% for 1 min, Clorox 30% for 10 min and washed thoroughly with sterile distilled water. The seeds were blotted on sterilized filter paper and germinated on basal MS medium [14], supplemented with 30 g/l sucrose and 8 g/l agar. Shoot tip explants of containment-free seedlings were excised and cultured on five different regeneration media. All media contained MS basal salts, 30 g/l sucrose and solidified with 8 g/l agar. The five tested media namely R1 (3 mg/l NAA; 50 mg/l biotin; 5 mg/l thiamine HCl; 170 mg/l NaH2PO4 and 10 g/l sorbitol), R2 (4 mg/l BA; 0.4 mg/l NAA; 0.2 mg/l GA3; 200 mg/l adenine sulfate; 0.5 mg/l silver nitrate; 0.5 g/l casein and 100 mg/l myoinositol), R3 (4 mg/l BA; 1 mg/l Kin; 0.5 g/l casein and 0.5 mg/l silver nitrate), R4 (3 mg/l BA; 50 mg/l adenine sulfate; 70 mg/l thiamine HCl and 170 mg/l KH2PO4 and R5 (4.5 mg/l BA; 0.5 g/l casein and 0.5 mg/l silver nitrate). The regenerated plants were sub-cultured every 3 weeks. During the sub-culturing process, each cluster was separated into single plantlet and transferred to a new fresh medium.

2.2. Morphological description

Morphological evaluation was carried out for five regenerated clones (vitroplants) developed on the different regeneration media, i.e. different phenotypes (clones) originated from shoot explants on five different regeneration media.

2.3. DNA isolation

DNA was isolated from different vitroplants and control seedling (donor plant) using the CTAB (1.4 M NaCl, 0.2% β-mercaptoethanol, 100 mM Tris–Cl and 20 mM EDTA) method of Doyle and Doyle [3]. Briefly, 0.5 g fresh sample was ground to powder in liquid nitrogen with a prechilled pestle and mortar, suspended in 1 ml preheated CTAB buffer and incubated at 65 °C for 1 h with occasional shaking, then centrifuged for 10 min at 1000 rpm. The supernatant were transferred to a new tube containing 0.5 ml of (chloroform- isomyl) 24:1, then centrifuged for 15 min at 14,000 rpm and the aqueous layer was transferred to a new sterilized tube. The ice cold isopropanol was added to precipitate the nucleic acid (RNA, DNA), incubated at –20°C overnight and centrifuged at 14,000 rpm for 20 min. The supernatant was
discarded and the pellet was washed carefully twice with cold 70% ethanol, dried at room temperature and re-suspend in 100 μl of sterile de-ionized distilled water.

2.4. Agarose electrophoresis

DNA concentration was determined by electrophoresis of 6 μl of DNA, containing 1 μl of 6x loading buffer (0.25 g bromo-phenol blue and 100 ml of 30% glycerol), along serial known concentrations of lambda DNA at 100 V for approximately 30 min using TAE buffer (242 g Tris–base, 57.1 ml glacial acet- tic acid and 100 ml EDTA (0.5 M pH 8.0). Agarose gel was stained using 0.2 μg/ml ethidium and visualized under UV light (Gel Doc, Biometra, Germany).

2.5. RAPD–PCR analysis

RAPD analysis was carried out as described by Welsh et al. [20] with minor modifications. Briefly, PCR amplification was performed in 25 μl reaction mix containing 20-40 ng genomic DNA, 0.5 unit Taq polymerase (Sigma), 0.2 mM each of dATP, dCTP, dGTP, dTTP, 5 Pmol random primer and appropriate amplification buffer. The reaction was assembled on ice, overlaid with a drop of mineral oil. Amplification was performed for 45 cycles using Biometera Uno thermal cycler, as follows: One cycle at 95 °C for 3 min and then 44 cy- cles at 92 °C for 2 min, 37 °C for 1 min and 72 °C for 2 min (for denaturation, annealing and extension, respectively). Reaction was finally incubated at 72 °C for 10 min and further incubated on 4 °C. Five primers were preselected based on their ability to amplify S. leucophylla genome and produced reproducible amplification patterns. The amplification products were analyzed by electrophoresis in 2% agarose in TAE buffer, stained with 0.2 μg/ml ethidium bromide and photographed under UV light. The buffer was added to the agarose, then heated in a microwave for melting, then cooled to 60 °C and the ethidium bromide was added. Sample was prepared by using 10 μl PCR-product and 2 μl 10× loading buffer. A 100 bp DNA ladder (Axygen) was used.

3. Results

3.1. Establishment of in vitro culture and shoot organogenesis

The best sterilization conditions were attained using 30% Clo- rox for 10 min, which gave the highest percentage of contamination-free seedling as well as the highest germination rate. The germination rate was 40% in the first week, 80% in the second week and 100% in the third week. The obtained data indicated that all tested media produced cluster of shoots...
however the difference among them was in the number of shoots per single explants, shoot density and root formation. It was observed that the best shoot production was recorded on medium R5 (4.5 mg/l BA, 0.5 g/l casein hydrolysate and 0.5 mg/l silver nitrate). Each explant produces up to 153.0 shoots after five subcultures, followed by medium R2 (4 mg/l BA, 0.4 mg/l NAA and 0.2 mg/l GA3) which gave 142.7 shoots per single explant (Fig. 2). The obtained data also indicated that R2 medium favored rooting of the proliferated shoots (Fig. 3). In the first month, the number of proliferated shoots per explant was approximately 4–7 shoots per single explant. Statistical analysis shows that the increments in the number of proliferated shoots through the different subcultures is highly significant, as indicated by increase between all pairs of subcultures in media R2, R3, R4 and R5 (Table 1). A significant increase in 1st, 2nd and 4th pairs in medium R1 was also noticed. This result indicates high response for all tested media in shoot production. The effect of the tested media on root formation was different than its effect on shoot production (Table 2). Medium R2 was the best medium for this purpose followed by medium R4 (111.7 and 21.00 roots/cluster, respectively).

3.2. Morphological description of vitroplants

Different phenotypes of tissue culture-derived plantlets (vitroplants) were collected and morphologically described. The recorded morphological features indicated that the investigated

![Figure 2](image1.png) Mean number of proliferated shoots per single explant on the five different media.

![Figure 3](image2.png) Mean number of roots per cluster on the five tested regeneration media.
vitroplants had similar morphology except minor differences in size, plant length and appearance. There is no feasible difference among leaf morphology of in vivo (control mother plant) and in vitro (vitroplants). Detailed recorded morphological features of vitroplants were photodocumented and summarized in Table 3.

3.3. Genetic analysis

In this experiment, four vitroplants, described in Table 3 and designated as elongated growth (R2 and R3) and cushion growth (R4 and R5) were subjected to genetic analysis at the DNA using RAPD–PCR. DNA of control germinated seedling was used as reference DNA. Bands with the same mobility were treated as identical fragments (monomorphic). Weak bands with negligible intensity and smear bands were both excluded from final analysis. Fig. 4 demonstrates the RAPD profiles obtained with four different primers (OPA1, OPB2, OPB4 and OPG2). Seven reproducible polymorphic bands were detected using primers OPB2, OPB4 and OPG2. Analysis of the developed RAPD-based fingerprints indicated that all of 100% of clones produced on R4 medium were true-to-type. On contrary, all clones produced on either R2 or R5 media showed genetic variations at the DNA level. In this context, no major genetic variations at the DNA level were detected in clones produced on R3 media. It could be concluded that distinct genetic variations between the donor mother plant (reference DNA) and vitroplant produced on R2 and R5 medium were recorded, as indicated by the presence or absence of polymorphic bands in RAPD profiles, generated by PCR amplifications using the primers OPB2, OPB2 and OPG2 (Fig. 4). Therefore, the best medium for in vitro propagation and mass production of true-to-type plants from S. leucophylla is R4 medium.

4. Discussion

In vitro propagation of wild threatened plant species is proving to be successful approach [15]. Studies on this species have been so limited due its seed rarity caused by some reproductive
difficulties leading to scare its seed setting [1]. Liu et al. [13] emphasized the need of using biotechnological methods, which can greatly facilitate conservation and sustainable utilization of genetic diversity. Plant tissue culture has, therefore, largely been adopted for mass production of selected elite varieties and to conserve endangered and threatened species. The results of present study assure the possible mass propagation and consequently conservation and restoration of *S. leucophylla*. The results of tissue culture indicated that R5 medium (4.5 mg/l BA, 0.5 g/l casein and 0.5 mg/l silver nitrate) is the best medium for the production of maximum number of shoots per single explant, followed by R2 medium (4 mg/l BA, 0.4 mg/l NAA and 0.2 mg/l GA3). The superiority of R5 medium might be due to silver nitrate and casein. The positive impact of silver nitrate and casein on shoot organogenesis *in vitro* is reported by Escalettes and Dosba [9] and El-Bahr et al. [4], respectively. On the other hand, the recorded highest percentage of rooting of proliferated shoots on R2 medium might be due to the presence of NAA and GA3. Due to somaclonal variations associated with *in vitro* culture techniques, the genetic stability of tissue culture-derived plants should be monitored at the DNA level. RPAD–PCR technique is believed to be reliable simple technique used by many research groups to examine genetic variability [11]. Results of the present study indicated that abnormal phenotypes might and might not associated with genetic variations at the DNA level. This can be explained on the basis that clones of many species have a high level of morphological plasticity in

<table>
<thead>
<tr>
<th>Pairs</th>
<th>R1 SD</th>
<th>R1 Sig. (2-tailed)</th>
<th>R2 SD</th>
<th>R2 Sig. (2-tailed)</th>
<th>R3 SD</th>
<th>R3 Sig. (2-tailed)</th>
<th>R4 SD</th>
<th>R4 Sig. (2-tailed)</th>
<th>R5 SD</th>
<th>R5 Sig. (2-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subculture 1–Subculture 2</td>
<td>5.1962</td>
<td>0.3140*</td>
<td>0.5774</td>
<td>0.0034**</td>
<td>0.5774</td>
<td>0.0051**</td>
<td>0.5774</td>
<td>0.0051**</td>
<td>0.5774</td>
<td>0.0025**</td>
</tr>
<tr>
<td>Subculture 2–Subculture 3</td>
<td>6.9282</td>
<td>0.0460*</td>
<td>0.5774</td>
<td>0.0034**</td>
<td>1.0000</td>
<td>0.0041**</td>
<td>1.1547</td>
<td>0.0051**</td>
<td>1.1547</td>
<td>0.0025**</td>
</tr>
<tr>
<td>Subculture 3–Subculture 4</td>
<td>4.0000</td>
<td>0.0742</td>
<td>5.1962</td>
<td>0.0073**</td>
<td>2.0000</td>
<td>0.0041**</td>
<td>2.0000</td>
<td>0.0015**</td>
<td>2.3094</td>
<td>0.0025**</td>
</tr>
<tr>
<td>Subculture 4–Subculture 5</td>
<td>3.7859</td>
<td>0.0047**</td>
<td>5.0000</td>
<td>0.0012**</td>
<td>2.6458</td>
<td>0.0024**</td>
<td>3.2146</td>
<td>0.0016**</td>
<td>6.0277</td>
<td>0.0012**</td>
</tr>
</tbody>
</table>

Table 3  Morphological characterization of different *Silene leucophylla* vitroplants developed on the five tested regeneration media.

<table>
<thead>
<tr>
<th>Description</th>
<th>R1</th>
<th>*R2</th>
<th>*R3</th>
<th>*R4</th>
<th>*R5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant appearance</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elongated</td>
<td>Few (−2)</td>
<td>Few (−2)</td>
<td>Few (−2)</td>
<td>numerous</td>
<td>Numerous</td>
</tr>
<tr>
<td>Cushion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Internodes length</td>
<td>Long (7 mm)</td>
<td>Short (3 mm)</td>
<td>Short (2 mm)</td>
<td>Very short (1 mm)</td>
<td>Very short (&lt;1 mm)</td>
</tr>
<tr>
<td>Stem</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root</td>
<td>adventitious Fibrous root</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>leaf</td>
<td></td>
<td></td>
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<tr>
<td>Stipule</td>
<td>Estipulate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base</td>
<td>Sheathing (connate at base)</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Petiole</td>
<td>Petiolate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Base of blade</td>
<td>Symmetrical</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Leaf blade</td>
<td>Simple</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Leaf margin</td>
<td>Enure</td>
<td></td>
<td></td>
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<tr>
<td>Apex</td>
<td>Acute to acuminate</td>
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<tr>
<td>Venation</td>
<td>Reticulate pinate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf insertion</td>
<td>Radical</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>arrangement</td>
<td>Alternate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shape of leaf</td>
<td>Spathulate</td>
<td></td>
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</tr>
</tbody>
</table>

* Vitroplants subjected to genetic analysis (RAPD–PCR).
response to environmental conditions [2]. The results of the present work also confirmed that optimization of \textit{in vitro} culture techniques must be carried out not only using tissue culture criteria, such as no. of shoots per explants and highest regenerative capacity, but also genetic stability at the DNA level of tissue culture-derived plants. In the present study, the best medium for maximum shoot production is R5 medium but the best medium based on the results of genetic analysis is R4 medium. Similar conclusion was previously reported by Saker et al. [18].

In conclusion, the present study demonstrates that mass propagation via multiple shoot organogenesis coupled by simple, routine and fast genetic analysis using RAPD–PCR are effective integrated and applicable systems for conservation of \textit{S. leucophylla}. This system can directly assist in various conservation efforts, including restoration of this threatened plant.

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**References**


