**IN VITRO PROPAGATION AND CRYOPRESERVATION OF SNOW MOUNTAIN GARLIC ENDEMIC TO HIMALAYAN REGION**

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**ABSTRACT**

Present study reports a protocol for in vitro propagation and cryopreservation of a medicinal herb, Snow Mountain Garlic (*Allium sativum*). A three stage sterilization treatment was found to be the most effective leading to maximum number of uncontaminated explants. Different combination of growth hormones in MS medium was used for shoot proliferation and multiplication. Maximum number of shoots were obtained in MS medium supplemented with 1.0 mg/l of Kinetin (5.0±0.55 shoots per explant). Effective rooting of plantlets was obtained on MS media supplemented with 0.1mg/l NAA. Rooted microshoots were acclimatized successfully in pots with the survival rate of 90 %. In vitro bulblet formation was highest in the MS medium supplemented with 12% sucrose (3.8±0.65) whereas it was only 1.7±0.65 when the medium contained 9% sucrose concentration. For in vitro conservation a 3% sodium alginate and 100mM CaCl$_2$ was found most suitable for encapsulation of explants. The highest survival rate of explants (36%) was obtained when the encapsulated beads were precultured in 0.4 M sucrose for 3 days and then immersed in liquid nitrogen for 60 minutes. This is the first report on developing a protocol for in vitro propagation and cryopreservation of Snow Mountain Garlic which can be further used for genetic transformation studies.

**Key words:** *Allium*, micropropagation, in vitro, cryopreservation, synthetic seeds

**INTRODUCTION**

Garlic, *Allium sativum* L. belonging to the Alliaceae family is an important plant widely used in the preparation of food, as a condiment because of its ability to improve the taste of food. It has been used since ancient times for the medicinal purposes as its regular consumption dilates the blood vessels and enhances physical potency [1]. It has compound Allicin that provide antibiotic, antitumor, antibacterial, antiviral, anti-oxidant, anti-aging properties and reduction of cardiovascular disease risk factors [2,3]. One of its types is Snow Mountain Garlic, (also called as Kashmiri garlic) is an important herb which is found in the snow covered mountains of the Himalayas at an altitude of 6000 feet above sea level. This species
has the ability to survive in extremely low temperature of -10°C and very little oxygen. In ancient times the mountaineers used the Snow Mountain Garlic to raise their energy levels and detoxify body in extreme cold weather. Now it is cultivated for global human consumption. Cultivated garlic being sexually sterile is generally propagated vegetatively. Since a single seed bulb gives one plant so availability of large number of seed bulbs is limitation for its propagation on large scale. Thus, in vitro propagation is an important tool in conservation, and production of high number of genetically alike plants that can be cultivated from only one single plant stock [4,5]. Ex situ conservation techniques like cryopreservation is a safe and cost-effective gene banking strategy for long-term preservation though the availability or development of efficient cryogenic protocol which yields a high percentage of recovery is a basic requirement.

MATERIALS AND METHODS

Plant material
The plant material used in present study was the cloves of Snow Mountain Garlic (Allium sativum) which were brought from Kashmir Valley and were used for all the experiments (Fig 1).

Establishment of aseptic cultures
The outer, dry, papery bulb scales of the cloves were removed. Healthy cloves were surface-sterilized by washing them in 70% ethanol for 20-30 seconds, KMnO₄ (1 ppm for 2 minutes) and then in 0.5% HgCl₂ solution for 3 min, containing two drops of Tween 20 per 100 ml. These were agitated for 10 min in a sterile flask. Then the cloves were washed three times for 2 min in sterile distilled water. The shoot bases were excised from the cloves aseptically inside a laminar flow cabinet, with sterile surgical blades and were then used as explants. These explants were placed on basal MS nutritive media [6] with 3% sucrose and 0.8% agar for their initial establishment and were monitored for the contamination.

Shoot proliferation and multiplication
After the initial aseptic establishment, the shoot cultures were transferred on to the modified MS medium supplemented growth regulators viz. BAP (6-benzylaminopurine) and Kn (Kinetin). Different concentrations of BAP (0.0mg/l, 0.2mg/l, 0.5mg/l and 1.0mg/l) and Kn (0.0mg/l, 0.5mg/l and 1.0mg/l) were used either singly or in combinations for shoot proliferation and multiplication (Table 1). The concentration of GA₃ (2.0mg/l) was kept constant in all the combinations. Data was recorded after 28 days of subculturing. Every possible care was taken to prevent any further contamination.

In vitro bulblet formation
Cluster of multiple shoots of Snow Mountain garlic were divided into single shoots. These single shoots were then cultured on MS medium supplemented with different concentrations of sucrose (3%, 6%, 9% and 12%) for in vitro bulblet formation. The percentage of bulblet formation was recorded after 8 weeks.
**Root induction and Transplantation**

Rootable shoots were excised from lavishly multiplying shoot clusters and were then transferred singly to culture tubes containing MS medium supplemented with different concentrations of NAA (0.1 mg/l, 0.5 mg/l and 1.0 mg/l). The percentage of root induction was recorded after 30 days of culture. After the roots were well developed, the rooted plants were taken out of culture tubes, washed gently to remove agar and then transferred to the pots with a mixture of sand and soil in the ratio of 2:1. The plantlets in the pots were covered with jars to maintain the humidity. After 2 weeks, the jars were removed and the established plants were then transferred to soil in the field conditions and their survival rate was observed.

**Cryopreservation using encapsulation and dehydration**

Excised shoot tips from *in vitro* grown plants were encapsulated in alginate beads which were composed of 3% sodium alginate (w/v) in liquid MS media at pH 5.7. The beads were allowed to polymerize for 20 min in MS medium with 100 mM CaCl$_2$ and 0.4 M sucrose. The beads were then pretreated in liquid MS medium with 0.4 M sucrose for 48 hrs on a rotary shaker (50 rpm) and were blotted dry on sterile filter paper and desiccated for 6 h (approx. 20% moisture content) in a glass petri dish under laminar flow (0.6 m/sec) at ambient temperature of ~25°C and at 35 ± 2% relative humidity (%RH). Dried beads were placed in 1.2 ml cryovials (10 beads per cryovial) and plunged directly into liquid nitrogen (LN$_2$) for 30 min and 60 min. The cryovials were then rewarmed at 45°C in water bath for 1 min and at 25°C in water bath for 2 min. The beads were rehydrated in liquid MS medium for 5 min and then transferred onto recovery medium (MS without growth hormones) in petriplates. These petriplates were kept in diffused light for 5 days and then were transferred to normal light conditions. The petriplates were checked for the greening of explants after 2 weeks of post culture. After 21 days the beads with green explants were transferred to the test tubes containing media with growth hormones (BAP 0.5mg/l & Kinetin 0.5mg/l). Elongated shoots were further sub cultured after 35 days.

**Data collection and analysis**

The average number and length of shoots per explant and the total number of shoots produced were calculated and used for evaluation of the different treatments. The shoots and roots were removed from the cultures to measure the number and length of shoots and roots. The statistical analysis is based on mean values and standard error.

**RESULTS AND DISCUSSION**

**Shoot establishment and multiplication**

The surface sterilization treatment given to explants using single sterilant for different time periods was not applicable, since it could not completely control the contamination. A three stage sterilization process which included the combination of sterilants i.e. 70% alcohol (for 30 sec), KMnO$_4$ (1 ppm for 2 minutes) and HgCl$_2$ (0.5% for 3 minutes) was found to most effective leading to maximum uncontaminated explants. All explants gave shoot buds within 30 days of inoculation in MS medium containing either BAP or Kinetin or both. The MS medium supplemented with BAP and kineitin resulted in maximum number of shoots. This is because the additions of growth regulators like BAP and kineitin affects shoot formation in a dose dependent manner.
The average number of shoots per explant varied with the concentration and combination of growth regulators. The average number of shoots per explant was less when the MS medium was supplemented with BAP and Kinetin both as compared to medium supplemented with either BAP or Kinetin alone (Table 1). The MS medium supplemented with 1.0 mg/l Kinetin and 1.0 mg/l BAP gave least number of average shoots per explant (1.5±0.10). Kinetin has been found to be more effective in microshoot formation as compared to BAP in earlier studies also [7]. The average shoot length (in cm) was maximum in the medium supplemented both with 0.5 mg/l Kinetin and 0.5 mg/l BAP (9.8±0.22) while the MS medium supplemented with 1.0 mg/l Kinetin and 1.0 mg/l BAP gave average shoot length of 2.3±0.42 (Table 1).

### Table 1: Effect of different concentration and combination of growth regulators in shoot multiplication and shoot length

<table>
<thead>
<tr>
<th>MS+Growth regulators (mg/l)</th>
<th>No. of inoculated tubes</th>
<th>No. of proliferated shoots</th>
<th>Average No. of shoots per explant*</th>
<th>Average Length* (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP(0.5)+Kn(0.5)</td>
<td>25</td>
<td>10</td>
<td>1.5±0.15</td>
<td>9.8±0.22</td>
</tr>
<tr>
<td>BAP(0.2)+Kn(0.5)</td>
<td>25</td>
<td>12</td>
<td>3.0±0.40</td>
<td>6.8±0.31</td>
</tr>
<tr>
<td>BAP(0.5)+Kn(1.0)</td>
<td>25</td>
<td>20</td>
<td>2.15±0.20</td>
<td>7.8±0.88</td>
</tr>
<tr>
<td>BAP(1.0)+Kn(1.0)</td>
<td>25</td>
<td>20</td>
<td>1.5±0.10</td>
<td>2.3±0.42</td>
</tr>
<tr>
<td>BAP(1.0)+Kn(0.5)</td>
<td>25</td>
<td>18</td>
<td>2.12±0.19</td>
<td>3.6±0.73</td>
</tr>
<tr>
<td>BAP(1.0)+Kn(0.0)</td>
<td>25</td>
<td>18</td>
<td>4.34±0.17</td>
<td>6.9±0.22</td>
</tr>
<tr>
<td>BAP(0.0)+Kn(1.0)</td>
<td>25</td>
<td>15</td>
<td>5.0±0.55</td>
<td>6.2±0.34</td>
</tr>
</tbody>
</table>

*Each value represents mean±SE of three replicates

### Rooting and Acclimatization

Rootable shoots were separated and cultured singly on the rooting medium. All shoots produced visible roots (Fig 3). The root induction in garlic was observed in each MS medium supplemented with 0.1 mg/l, 0.5 mg/l and 1.0 mg/l NAA. Highest number of roots was observed on MS media containing 0.1 mg/l NAA, after 5-7 days of culturing as compared to MS media containing 1.0 mg/l NAA. In addition, root length was also affected by NAA in the medium. A lower level of NAA (0.1 mg/l) resulted in elongated but few roots. The rooted plants transferred to small plastic cups resulted in 90 % survival rate after three weeks of transplantation. Bekheet [8] also reported the high survival rate after using perlite and peatmoss in the ratio 1:1 instead of sand and soil (2:1). The plantlets got adjusted after 2-3 weeks and were allowed to grow further for 7-8 weeks and subsequently transferred to fields.

Fig 2: Shoot Proliferation after 4 weeks of culture on MS medium containing 0.5 mg/l Kinetin and 0.5 mg/l BAP
In vitro propagation and cryopreservation of snow mountain garlic endemic to Himalayan region

In vitro root regeneration on MS media supplemented with 1.0 mg/l NAA

Fig 3: In vitro root regeneration on MS media supplemented with 1.0 mg/l NAA

In vitro bulblet formation

The development of bulbets from shoots can contribute to the successful acclimatization of in vitro propagules and the bulbs formed are better adapted to storage conditions. MS medium supplemented with 12% sucrose was found optimal for bulbet growth as 80% of explants show bulbet formation as compared to explants supplemented with low concentration of sucrose (Table 2). Also the number of bulbs per explant was more in medium supplemented with 12% sucrose i.e. 3.8±0.65 as compared to MS medium with 9% sucrose concentration (1.7±0.67).

Previous studies on other Allium species have also reported that medium supplemented with higher concentration of sucrose resulted in in vitro bulbet formation [9,10]. Haque et al. [11] reported that with the increase in sucrose concentration upto 9% in the medium, 90% shoots resulted in bulbet formation whereas 12% sucrose concentration resulted in 50% heavier bulbets. In present study, 12% sucrose was found to be optimal for in vitro bulbet formation as there is an increase in the synthesis of starch and total carbohydrates as reported by Langens-Gerrits et al. [12]. Different concentrations of jasmonic acid added to MS medium have also potential for in vitro bulblet formation in garlic [13].

<table>
<thead>
<tr>
<th>MS+Sucrose conc.</th>
<th>Bulbet formation</th>
<th>No. of bulbs/culture (mean±S.E)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS+0%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MS+3%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MS+6%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MS+9%</td>
<td>40%</td>
<td>1.7±0.67</td>
</tr>
<tr>
<td>MS+12%</td>
<td>80%</td>
<td>3.8±0.65</td>
</tr>
</tbody>
</table>

Table 2: Effect of sucrose concentration on in vitro bulbet formation

Encapsulation-dehydration

A 3% sodium alginate and 100mM CaCl₂ was found most suitable for encapsulation and conversion of explant into complete plantlets (Fig 5). Beads formed using lower concentration of sodium alginate (1.5%) and 50mM CaCl₂ were too fragile to handle and beads formed from a high concentration of sodium alginate (5.0%) were too hard for conversion of explants to plantlets.
However, Castillo et al. [14] reported the uniformity in size and shape of beads with 2.5% sodium alginate for the somatic embryos of papaya while Ghosh and Sen [15] achieved a maximum conversion of encapsulated somatic embryos of Asparagus cooperi with 3.5% sodium alginate. The highest survival rate of explants (36%) was obtained when the beads were precultured with 0.4 M sucrose for 3 days and then immersed in liquid nitrogen for 60 minutes whereas when the beads followed by same treatment were plunged in liquid nitrogen for 30 min gave a survival rate of 32% (Fig 6). Similar results were reported by Subaih et al. [16] in date palm (33.3-40.0%) with 0.1 M sucrose after 2 or 4 h of dehydration. However when the concentration of sucrose was increased in MS medium (0.5 M) the survival rate of the explants decreased (Table 3). This is due to high concentrations of osmoticum in the medium that causes a negative water potential, and reduces the optimal turgor pressure needed for cell division and thus inhibits growth [17]. However, Baghdadi [18] reported a 100% survival rate when the Crocus calli was precultured on 0.1 and 0.5 M sucrose irrespective of the preculture duration whereas if the encapsulated beads are kept in a medium with high concentration of sucrose (0.1 to 1.0 M) and dehydration for minimum one day, then it leads to their high survival rate [19].

Hence, this appears to be the first report on developing a protocol for in vitro propagation and cryopreservation of Snow Mountain Garlic. The protocol developed in this study is suitable for the genetic transformation studies of this economically important food value crop and can be used in the conservation of this valuable species.

Table 3: Effect of the concentration of sucrose and time period in liquid nitrogen on the survival rate of explants

<table>
<thead>
<tr>
<th>MS+Sucrose conc.</th>
<th>Duration in liquid N₂ (min)</th>
<th>No. of beads/cryovial</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS+0.1M</td>
<td>30</td>
<td>10</td>
<td>0%</td>
</tr>
<tr>
<td>MS+0.2M</td>
<td>30</td>
<td>10</td>
<td>3%</td>
</tr>
<tr>
<td>MS+0.3M</td>
<td>60</td>
<td>10</td>
<td>20%</td>
</tr>
<tr>
<td>MS+0.4M</td>
<td>30</td>
<td>10</td>
<td>36%</td>
</tr>
<tr>
<td>MS+0.5M</td>
<td>60</td>
<td>10</td>
<td>26%</td>
</tr>
</tbody>
</table>

Fig 5: Explants encapsulated in alginate beads

Fig 6: Explants encapsulated in alginate beads
ACKNOWLEDGEMENT
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REFERENCES
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