Different modes of plant regeneration and factors affecting in vitro bulblet production in Ornithogalum virens.

Pradeep Kumar Naik and Sanghamitra Nayak*

Department of Bioinformatics, Jaypee University of Information Technology, Waknaghat, Solan – 173215, Himachal Pradesh, India

* Corresponding author, email: sanghamitran@yahoo.com

ABSTRACT: Efficient procedures are outlined for plant regeneration through direct shoot bud formation and indirect organogenesis through callus formation in Ornithogalum virens Lindl using bulb scale as explant. Murashige and Skoog’s (MS) medium containing 1 mg/L (5.4mM) NAA and 2 mg/L (4.4mM) BA was most effective in direct induction of shoot buds from explant. Callus cultures were raised from the bulb scale segment on MS medium supplemented with 2 mg/L (9.0mM) 2,4-D. Shoot regeneration from callus was optimum on the medium containing 2 mg/L (10.7mM) NAA and 0.5 mg/L (2.2mM) BA. Shoots developed roots on MS basal medium devoid of growth regulators. Regenerated plants grew profusely in MS liquid medium and were successfully transferred to pots. Bulblets were induced at the base of the regenerants upon transfer to MS basal medium supplemented with enhanced concentrations of sucrose (45 to 90 g/L). Direct induction of bulblets also occurred on the bulb scale grown on the MS media supplemented with 1 mg/L (5.4mM) NAA, 2 mg/L (8.9mM) BA and 60 g/L sucrose. Size of bulblets could be increased by decreasing the salt strength of MS basal medium to half. The effect of in vitro induced bulblet size on the ex vitro survival rate was also reported. Bulblets produced in vitro could be transplanted directly to potted soil. Chromosome analysis of direct explant-derived plants, callus-derived regenerates, and plants sprouted from in vitro-induced bulblets revealed only diploid cells with normal karyotypes comprising 2n = 6 chromosomes.

List of abbreviations: NAA, a-naphthalene acetic acid; BA, 6 benzyladenine; 2,4-D, 2,4 dichlorophenoxyacetic acid.

KEYWORDS: Ornithogalum virens, tissue culture, bulblet induction.

INTRODUCTION

Ornithogalum is a genus of bulbous plants native to the Mediterranean and South Africa. Ornithogalum species, commonly known as “Star of Bethelhem” have been cultivated commercially for a long time for their high ornamental value as cut-flowers and are becoming increasingly popular. Ornithogalum virens Lindl is economically important for its ornamental value. Besides, its low chromosome number (2n = 6) and simple karyotype makes this species an ideal material for in vitro studies involving chromosome behaviour during organ differentiation. It grows in India in temperate regions of the Himalayas at an altitude of 2000 m. Conventionally, the species is propagated vegetatively using mother bulbs, but the rate of propagation is very slow, producing 4-6 plants in a year. The horticultural interest in Ornithogalum virens necessitates the development of in vitro method to speed up the propagation rate. Tissue culture technique is powerful tool which can be employed as an alternative to the conventional method of vegetative propagation with the objective of enhancing the rate of multiplication of desired genotypes1,2. The tissue culture technique has been successfully applied for rapid propagation of some Ornithogalum species3,4. The present paper deals with procedures for different modes of regeneration of plantlets directly from bulb scale explant and from callus, as well as in vitro production of bulblets. In vitro bulblet formation has been reported in O. umbellatum3 and in other bulbous plant types such as tulips5, Narcissus6, Lachenalia7, Lilium8, Hyacinthus9. Bulblets have several advantages. They are harder thereby increasing the survival rate after transplanting to soil. They are easy to handle and can be sown as seeds. They can be transported dry and no extra time or facilities are needed for hardening off. In this paper we have studied the factors affecting the formation of bulblets.
in vitro, as well as transplantation success, which is one of the most important criteria in determining the success of cost-effective commercial micropropagation of Ornithogalum virens.

**MATERIALS AND METHODS**

**In vitro Regeneration**

The bulbs of O. virens Lindl were taken as explants and outer scales were peeled off and discarded. The lower halves of bulbs containing the basal disc were cut into 4 sectors, washed thoroughly in 5% Teepal detergent for 15 min, surface sterilized with 0.1% mercuric chloride for 30 min, and rinsed 5 times in sterile distilled water. Each sector was further dissected to 2-3 explants, each explant with 1-2 scales joined at the base by a small part of the disc. The explants were aseptically inoculated onto Murashige and Skoog's basal medium supplemented with different concentrations and combinations of NAA and BA, as mentioned in Table 1 and 2, for induction of shoots directly from explant and from callus. Regenerated shoots were rooted on MS medium devoid of any growth regulators and were maintained by regular subculture in liquid MS medium. In vitro grown plants were thoroughly washed in tap water, dipped in 0.2% "Bavistin" (a fungicide) solution for 10 min and transferred to potted soil in shade for acclimatization.

**Induction of Bulblets**

Scales of in vitro grown bulbs and culture derived regenerates of about 3-4 cm long were inoculated onto MS medium supplemented with sucrose (30, 60 and 90 g/L) for in vitro formation of bulblets. For direct regeneration of bulblets, bulb scale explant were grown on media containing 1 mg/L NAA and 2 mg/L BA and different concentrations of sucrose (30, 60 and 90 g/L). The effect of different concentrations of sucrose on the percentage of shoot producing bulbs was studied. Different salt strength of media (full MS, ½ MS and ¼ MS) were used to determine the effect of salt strength of media on in vitro bulb formation. Bulblets thus produced could directly be transplanted to the pots containing mixtures of soil and sand (1:1).

**Culture Conditions**

For solidification of media 0.8% bacto-agar was used. Media were autoclaved at 121°C and 1.05 kg/cm² for 20 min and the pH was adjusted to 5.7. Cultures were inoculated at 25 ± 1°C and grown under white fluorescent light with 55 μmol m⁻² s⁻¹ light intensity. Each experimental set consisted of 15 replicates. Means were calculated by taking an average of 3 successive experiments.

**Cytological Analysis**

For analysis of chromosome number of regenerated plants, root tips were pretreated with a 2:1 mixture of saturated paradichlorobenzene solution: 0.002 M 8-hydroxyquinoline for 3 h, fixed overnight in 1:3 acetic acid: ethanol, and stained using the aceto orcein technique. A detailed karyotypic study was made on the basis of the length of chromosome and position of the centromere as determined by F%, which denotes the ratio in percentage of the short arm to total length of each chromosome. Chromosome morphology was determined following the classification of Battaglia. Chromosome counts were made on 8-10 countable metaphase plates per root tip. Fifty regenerated plants from each group i.e. direct explant derived plant, direct bulblet sprouted plants and callus regenerated plants, were analysed.

**Statistical Analysis of Data**

Data were subjected to analysis of variance and statistical significance of different treatment means was determined.

**RESULTS AND DISCUSSION**

**In vitro Regeneration**

Shoot buds were induced directly (Fig 1a) from the bulb scale when explanted on MS medium supplemented with NAA (0.5 - 1 mg/L) and BA (0.5 – 3 mg/L) (Table 1). Application of NAA alone had little effect on shoot bud induction, but the combination of NAA (1 mg/L) and BA (2 mg/L) was most effective in inducing an average of 10.4 ± 0.1 shoot buds in about 73% of explants within 30 days of culture (Table 1). This result showing requirement of NAA and BA in direct shoot bud formation is in agreement with that of Hussey. The mean number of shoots formed per explant in this medium was significantly higher (p = 0.001) than those formed in media with other hormonal combinations (Table 1). On the other hand, the combinations of another auxin (IAA, 0.5 – 1 mg/L) and cytokinin (kinetin, 0.5 – 3 mg/L) were not effective in direct regeneration of shoot buds from the explant (data not shown). In O. virens, direct regeneration of shoot buds were conditioned to the attachment of the basal disc portion with the bulb scale, as reported earlier in O. thyrsoides and O. umbellatum.

Callus was induced from the bulb scale of O. virens at the basal region in proximity with the disc when cultured on MS basal medium supplemented with 1-4 mg/L 2,4-D. MS basal medium supplemented with 2 mg/L 2,4-D was most effective showing callus induction from 75% of explant in 2-3 weeks. Requirement of 2, 4-D for callus induction in O. virens is in contrast to the other reports in Ornithogalum species,
where callus initiation was favored by the presence of NAA in medium\(^3\). Faster proliferation of callus (Fig 1b) occurred by repeated sub-culturing on the MS basal medium with 2 mg/L 2, 4-D.

Shoot buds (Fig 1c) were induced from 2 months old callus grown on MS media when 2,4-D was replaced by a combination of NAA (1, 2 or 3 mg/L) and BA (0.1, 0.5 or 1 mg/L) (Table 2). Callus grown on the media containing 2 mg/L NAA and 0.5 mg/L BA showed the best response, inducing 7.4 ± 0.2 shoots per culture (Table 2) from about 82% of the calli within 3-4 weeks. The requirement of auxin and cytokinin in combination for shoot bud formation from calli of *O. virens* is like that of *O. umbelatum*\(^4\), but unlike *O. thyrsoides*\(^3\), where shoot induction occurred on the basal medium devoid of any growth regulators.

Shoot buds obtained both through direct and indirect organogenesis developed into shoots which attained the length of 3-4 cm within 15 days. Elongated shoots about 4 cm long were excised from the base and placed in MS basal medium for rooting. Roots were formed in 3-4 days. Completely regenerated plants were maintained in MS liquid medium, where they multiplied (Fig 1d) rapidly through formation of lateral shoots, resulting in production of 8-10 plants from a...
in vitro on MS basal medium supplemented with enhanced levels of sucrose (45-90 g/L) (Fig 6). Of all the concentrations of sucrose used, MS medium supplemented with 60 g/L sucrose was found to be optimum for formation of bulbs in vitro. The percentage of in vitro grown shoots (explants) forming bulbs was approximately 70%. Tiny bulbs were visible at the base of shoots after 30 days of explantation on MS medium. No bulbs could be induced in vitro on MS medium containing a normal amount of sucrose (30 g/L). Sucrose is considered to be stored in the form of starch in the storage tissue i.e bulb scales of most bulbous plants. Sucrose has been found to promote formation of various storage organs (bulbs, corms, tubers and rhizomes) in most cases studied so far.

**Table 2.** Effect of different combinations of NAA and BA on multiple shoot formation from callus.

<table>
<thead>
<tr>
<th>MS+Growth regulator (mgL⁻¹)</th>
<th>Percent of regenerating callus shoot per callus (mean ± SEM)*</th>
<th>Number of regenerating callus per callus (mean ± SEM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAA</td>
<td>BA</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.0</td>
<td>0.1</td>
<td>30.5 ± 0.5</td>
</tr>
<tr>
<td>2.0</td>
<td>0.1</td>
<td>61.3 ± 0.5</td>
</tr>
<tr>
<td>3.0</td>
<td>0.1</td>
<td>40.2 ± 0.3</td>
</tr>
<tr>
<td>1.0</td>
<td>0.5</td>
<td>66.2 ± 0.5</td>
</tr>
<tr>
<td>2.0</td>
<td>0.5</td>
<td>82.2 ± 0.6</td>
</tr>
<tr>
<td>3.0</td>
<td>0.5</td>
<td>47.2 ± 0.5</td>
</tr>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>70.5 ± 0.2</td>
</tr>
<tr>
<td>2.0</td>
<td>1.0</td>
<td>80.5 ± 0.2</td>
</tr>
<tr>
<td>3.0</td>
<td>1.0</td>
<td>50.1 ± 0.4</td>
</tr>
<tr>
<td>F value</td>
<td>490.90</td>
<td>255.70</td>
</tr>
<tr>
<td>P value</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

The differences in the mean values among the treatment groups are statistically significant (p < 0.001). *Data represents 15 replicates for treatment in three repeated experiments.

Fig 2. Bulb formation at the base of in vitro grown plants of *O. virens* after 60 days of culture.

Fig 3. Induction of bulblets on bulb scale of *O. virens* grown on MS + 1 mgL⁻¹ + 2 mgL⁻¹ BA + 60 gL⁻¹ sucrose.

The differences in the mean values among the treatment groups are statistically significant (p < 0.001). *Data represents 15 replicates for treatment in three repeated experiments.*

**Induction of Bulblets**

Regeneration of plants of *O. virens* was accompanied by bulb formation (Fig 2) at the base of shoots grown in vitro on MS basal medium supplemented with enhanced levels of sucrose (45-90 g/L) (Fig 6). Of all the concentrations of sucrose used, MS medium supplemented with 60 g/L sucrose was found to be optimum for formation of bulbs in vitro. The percentage of in vitro grown shoots (explants) forming bulbs was approximately 70%. Tiny bulbs were visible at the base of shoots after 30 days of explantation on MS medium. No bulbs could be induced in vitro on MS medium containing a normal amount of sucrose (30 g/L). Sucrose is considered to be stored in the form of starch in the storage tissue i.e bulb scales of most bulbous plants. Sucrose has been found to promote formation of various storage organs (bulbs, corms, tubers and rhizomes) in most cases studied so far.

**Direct Bulblet Formation on Bulb Scale**

Bulblets were produced directly on the bulb scale (Fig 3) when the later was grown on MS medium supplemented with NAA (0.5, 1, 2 mg/L), BA (0.5, 1, 2 mg/L) and elevated levels of sucrose (45-90 g/L). MS basal medium supplemented with NAA (1 mg/L), BA (2 mg/L) and sucrose 60 g/L was found to be most effective in inducing 12-15 bulblets of different sizes (2-10mm) within 4-5 weeks. Bulblet production directly on bulb scale explants has also been reported in other species, including *O. umbellatum*.

**In vitro Bulblet Size**

Bulblets formed in vitro at the base of regenerated plants or directly on bulb scale were of varying sizes,
i.e. small (2-3 mm), medium (4-6 mm) and large (7-10 mm). Sizes of bulblets could be increased by decreasing the salt strength of MS medium (Table 3). It was observed that the percentage of medium size bulblet formation on medium with 45% sucrose increased from 3-64%, and that with 60% sucrose, it increased from 5-56% when the salt strength of the medium was reduced to half (Table 3). An effect of mineral salts on in vitro bulblet formation has also been reported in Lilium speciosum.

**Transplantation of Bulblets into Soil**

All in vitro plantlets with bulbs induced in culture showed a survival rate of about 80% when transferred to the potted soil. But, survival rates of bulblets produced directly on explants varied for different size bulblets. Small bulblets (2-3 mm diam) showed a survival rate of 40-50%, whereas, the larger bulblet (4-10 mm diam) had a 70-80% survival rate. The effect of in vitro induced bulblet size on ex vitro survival rate has also been reported in Lachenalia. Transplanting of in vitro plantlets is more labor intensive then planting bulblets directly on soil. However, in order to successfully implement the use of in vitro generated bulblets rather then in vitro rooted plantlets of *O. virens* for commercial production, all stages of in vitro bulblet production must be optimized.

**Cytological Analysis**

Regenerants obtained directly from bulb scale from callus and those sprouted from in vitro induced bulblets were subjected to cytological analysis. A total of 50 plants from each group was analysed. It was found that all plants were diploid (2n = 6) (Fig 4) with normal karyotypes (Fig 5a–c). Detail karyotypic analysis revealed no gross structural changes in any chromosomes of regenerated plants of *O. virens* when compared with the karyotype of the source plant. This suggests the relative stability of all types of regenerants

**Table 3.** Strength of medium on size of bulb formation in vitro.

<table>
<thead>
<tr>
<th>Strength of medium</th>
<th>Sucrose</th>
<th>Percentage of bulbs formed in each size class.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Small (2-3 mm)</td>
</tr>
<tr>
<td>Full MS</td>
<td>30</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>92</td>
</tr>
<tr>
<td>Half MS</td>
<td>30</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>41</td>
</tr>
<tr>
<td>1/4th MS</td>
<td>30</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>77</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>70</td>
</tr>
</tbody>
</table>

**Fig 4.** Diploid cell showing six chromosomes in root tip of a regenerated plant.

**Fig 5.** Normal karyotypes in root tips of regenerated plants.  
(a) Direct explant derived plant  
(b) Callus derived plant  
(c) Plant developed from in vitro induced bulblets.

**Fig 6.** Effect of sucrose concentration in the medium on in vitro bulb formation in *O. virens*.
obtained through different modes of regeneration. Among other Liliaceous ornamentals, genetic stability of explant and callus derived regenerants has also been reported in *Ruscus hypophyllus*\(^\text{18}\), *O. thyrsoides*\(^\text{19}\) and *O. umbellatum*\(^\text{4}\).

Thus, the process of regeneration of plantlets from bulb scale explant of *O. virens* through different modes of regeneration ensures rapid multiplication of true-to-type traits for commercial use.

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**REFERENCES**