Direct shoot regeneration and microtuberization in wild *Cyclamen persicum* Mill. using seedling tissue

Nabila S. Karam *, Mohannad Al-Majathoub

Faculty of Agriculture, Jordan University of Science and Technology, PO Box 3030, Irbid 22110, Jordan

Accepted 8 February 2000

**Abstract**

The influence of cutting method of leaves on direct regeneration in wild cyclamen was studied. Wounded blades, blades with midrib, blades without midrib, central lamina, and petiolated lamina of in vitro leaves were cultured on solid MS media containing 0.1 mg l\(^{-1}\) NAA and 0.22 mg l\(^{-1}\) TDZ. There was a significant effect of leaf cutting method on shoot regeneration; shoots regenerated on all explants except those consisting of blades without midrib. The greatest regeneration percentage (88%) was obtained on leaves with midrib. The influence of type of explant and concentrations of BA and sucrose on microtuberization was also studied. In vitro seedling tuber, petiole, cotyledon, and root explants were cultured on solid MS media supplemented with 0.1 mg l\(^{-1}\) NAA and different concentrations of BA. No tuberization occurred on tuber or petiole explants. Root explants cultured on media containing 1 mg l\(^{-1}\) BA exhibited the greatest microtuberization response. Microtubers were able to sprout within six weeks following initiation of cultures. In vitro microshoots and seedling root explants were cultured in liquid and solid MS media, respectively, containing different concentrations of sucrose. Microshoots failed to produce tubers, whereas root explants showed the capacity for tuber formation. Sucrose concentration had a significant effect on tuberization; 3% sucrose induced 100% tuberization, whereas 9 or 12% sucrose suppressed tuberization. Average weight and size of tubers peaked at 6 and 3% sucrose, respectively. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** Cyclamen; Tuber formation; Juvenile explants; Organogenesis; In vitro
1. Introduction

Wild cyclamen is an endangered species in Jordan. Therefore, it is of utmost importance to preserve the germplasm of this species while maintaining its genotype. In vitro culture systems are considered as helpful tools for clonal propagation of endangered species, production of pathogen-free plants, and long-term storage of germplasm.

Direct regeneration, without an intervening callus stage, is preferred since extensive callus formation and long-term callus culture can lead to somaclonal variation. Using in vitro shoot cultures as a source of explants increases regeneration response compared to explants from field or greenhouse-grown plants (Preece, 1997). The part excised from the explant, size of explant, and wounding of explant surface have been shown to influence shoot regeneration in several plant species (Sharma et al., 1991; Welander and Maheswaran, 1992; Jong et al., 1993; Gosukonda et al., 1995; Bhojwani and Razdan, 1996; Kumar et al., 1998).

Production of resting organs such as tubers facilitates planting or transportation as compared to production of whole plants. The cultivar, plant growth stage, explant type, shaking of culture media (Kobayashi, 1992), and concentrations of growth regulators and sucrose (Chow et al., 1992) in the media may influence regeneration of such resting organs.

In vitro propagation of wild cyclamen has been achieved in our laboratory through organogenesis (Al-Majathoub, 1999). To our knowledge, there is no documented literature on direct shoot regeneration or microtuberization of wild cyclamen. Therefore, this study was initiated to assess the effects of (1) method of cutting leaf explants on direct shoot regeneration and (2) type of explant and concentrations of BA and sucrose on microtuberization of wild cyclamen.

2. Materials and methods

2.1. Plant material

Seeds were extracted from mature fruits of wild cyclamen from Jordan, washed with water for 15 min, dipped in 70% ethanol for 1 min, surface sterilized in 20% sodium hypochlorite solution for 20 min, and finally rinsed in sterile distilled water. Seeds were germinated on half-strength basal MS (Murashige and Skoog, 1962) medium solidified with 0.8% Difco Bacto agar, with pH of 5.8, and containing 20 g l\(^{-1}\) sucrose. Cultures were maintained in dark at 15°C until germination started, then they were transferred to a growth room and maintained at 22±1°C and 16 h light (PPFD=50–60 μmol m\(^{-2}\) s\(^{-1}\)).

In regeneration experiments, the basic culture medium used was a half-strength basal MS medium solidified (unless otherwise mentioned) with 0.8% agar and with pH of 5.8.
2.2. Direct shoot regeneration from leaves

Tubers were excised aseptically from 7-week old seedlings, sectioned into four quarters, and cultured on half-strength basal MS medium containing 0.1 mg l\(^{-1}\) NAA and 2.0 mg l\(^{-1}\) BA (Al-Majathoub and Karam, 1999). Cultures were maintained in dark at 22±1°C. Eight weeks later, microshoots formed on one of the tuber explants were subcultured at 8-week intervals to fresh media of the same composition to establish uniform microshoot cultures.

Fully expanded leaves were excised from microshoots, placed in sterile water to prevent desiccation, and cut in different ways; e.g., scraped using a fine scalpel (wounded blade); cut longitudinally into two parts, one including midrib (blade with midrib) and the other part not including it (blade without midrib); edges of blades removed from all sides keeping a central, 4 mm×4 mm lamina (central lamina); isolating the basal part of lamina with 3 mm of petiole attached (petiolated lamina). Explants were cultured on half-strength basal MS medium supplemented with 0.1 mg l\(^{-1}\) NAA and 0.22 mg l\(^{-1}\) TDZ (Murthy et al., 1998; Al-Majathoub and Karam, 1999). Cultures were maintained in dark at 22±1°C. Eight weeks later, data were collected on shoot formation. There were four replicates (petri dishes) per treatment arranged in a completely randomized design (CRD). For each replicate, four explants were cultured on the medium surface.

2.3. Microtuberization

2.3.1. Effect of explant type and BA concentration

Tuber, petiole, cotyledon, and root explants were excised aseptically from 7-week old seedlings. Petioles and roots were sectioned into 5 mm segments, cotyledons into 3 mm×3 mm sections, and tubers into four quarters. Explants were cultured on half-strength basal MS medium containing 3% sucrose, 0.1 mg l\(^{-1}\) NAA, and different concentrations (0, 1.0, 2.0, or 3.0 mg l\(^{-1}\)) of BA. Cultures were maintained in dark at 22±1°C. Eight weeks later, cultures were evaluated for microtuberization. The experimental design was split-plot with explant type as the main-plot factor and BA concentration as the subplot factor. Each explant type×BA concentration treatment consisted of eight replicates (flasks) that were completely randomized. For each replicate, four explants were placed on the medium surface.

2.3.2. Effect of sucrose concentration

Microshoots formed on a tuber explant on half-strength basal MS medium containing 0.1 mg l\(^{-1}\) NAA and 1.0 mg l\(^{-1}\) BA (Al-Majathoub and Karam, 1999) were subcultured at 8-week intervals to fresh media of the same composition to establish a microshoots stock culture. Uniform microshoots were then cultured in liquid media of the same composition containing different concentrations (30, 60, 90, or 120 g l\(^{-1}\)) of sucrose. Cultures were maintained in dark at 22±1°C with
continuous shaking at 80 rpm. A similar experiment was conducted using root explants from an in vitro seedling and solid medium. Eight weeks later, cultures were evaluated for microtuberization. For each experiment, there were four replicates (flasks) per treatment arranged in a CRD. Each replicate consisted of four explants.

2.4. Statistical analysis

Data were subjected to analysis of variance (ANOVA) and regression analysis by the general linear models procedure using SAS (Statistical Analysis System, 1995, SAS Institute, Cary, NC). Mean separation was performed using the least significant difference method. Percent data were arcsine transformed before performing ANOVA.

3. Results

3.1. Direct shoot regeneration from leaves

Adventitious shoots regenerated directly from the leaf tissue (Fig. 1). There was a significant effect of the method of cutting the leaf on shoot regeneration; shoots regenerated on all explants except those consisting of blades without midrib (Table 1). Best regeneration response was obtained when the leaves were cut with the midrib attached or when they were wounded, where 88 or 81%, respectively, of the leaves formed shoots. Number of regenerant shoots per explant was also affected (Table 1); central lamina explants produced the least number of regenerants when compared to explants exhibiting regeneration.

Table 1

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot regeneration (%)</th>
<th>Number of regenerants per explant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blade with midrib</td>
<td>88a</td>
<td>2.3ab</td>
</tr>
<tr>
<td>Wounded blade</td>
<td>81ab</td>
<td>2.9a</td>
</tr>
<tr>
<td>Central lamina</td>
<td>44bc</td>
<td>0.8bc</td>
</tr>
<tr>
<td>Petiolated lamina</td>
<td>34cd</td>
<td>2.1ab</td>
</tr>
<tr>
<td>Blade without midrib</td>
<td>0d</td>
<td>0c</td>
</tr>
<tr>
<td>Significance</td>
<td>**</td>
<td>*</td>
</tr>
</tbody>
</table>

* Means within columns having different letters are significantly different according to LSD at $P \leq 0.05$.

* Significant at $P \leq 0.05$.

** Significant at $P \leq 0.01$. 

Fig. 1. Shoot regeneration from in vitro leaves of wild cyclamen on solid half-strength basal MS medium supplemented with 0.1 mg l$^{-1}$ NAA and 0.22 mg l$^{-1}$ TDZ.

Fig. 2. Sprouted microtubers produced from a root explant of wild cyclamen on solid half-strength basal MS medium containing 3% sucrose, 0.1 mg l$^{-1}$ NAA and 1.0 mg l$^{-1}$ BA.
3.2. Microtuberization

3.2.1. Effect of explant type and BA concentration

Microtubers started to form 4–5 weeks following initiation of cultures. The root explants had the characteristic appearance of a ‘dog’s bone’ with spherical brown structures formed at the cut ends of the root explant. Tubers were able to sprout within 6 weeks following initiation of cultures, producing a leaf each (Fig. 2).

Efficiency of microtuberization was dependent on explant type and BA concentration (Table 2). No tuberization occurred on tuber or petiole explants. Although both root and cotyledon explants produced tubers, the percentage (42%) of root explants producing tubers was much higher than that (4%) of cotyledon explants. The same trend was observed for number of tubers per explant (Table 2). Significant interaction effects between explant type and BA concentration were detected on frequency of tuber formation and number of tubers (Fig. 3). Although it was exhibited by cotyledon explants, tuberization was not significantly affected by BA concentration. On the other hand, there was a significant effect \( (P \leq 0.01) \) of BA concentration on frequency of tuberization and number of tubers produced by root explants. Both cotyledon and root explants failed to produce tubers when cultured on media without BA. However, frequency of root explants producing

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Explants forming tubers (%)</th>
<th>Number of tubers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Explant type (E)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tuber</td>
<td>0b</td>
<td>0b</td>
</tr>
<tr>
<td>Root</td>
<td>42a</td>
<td>2.5a</td>
</tr>
<tr>
<td>Cotyledon</td>
<td>4b</td>
<td>0.1b</td>
</tr>
<tr>
<td>Petiole</td>
<td>0b</td>
<td>0b</td>
</tr>
<tr>
<td><strong>BA concentration (C)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1.0</td>
<td>20</td>
<td>1.1</td>
</tr>
<tr>
<td>2.0</td>
<td>15</td>
<td>1.0</td>
</tr>
<tr>
<td>3.0</td>
<td>11</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Significance</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>C</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>E×C</td>
<td>***</td>
<td>***</td>
</tr>
</tbody>
</table>

\( ^a \) Means within columns having different letters are significantly different according to LSD at \( P \leq 0.05 \).

*** Significant at \( P \leq 0.001 \).
tubers and number of tubers increased significantly to 71% and 4.6, respectively with addition of 1 mg l\(^{-1}\) BA. Both parameters decreased with increasing BA concentration. Although the highest BA concentration (3 mg l\(^{-1}\)) tested in this study had an inhibitory effect on tuberization from cotyledon explants, 39% of root explants still produced tubers at this concentration. As a function of BA concentration, data were best fitted using the regression equations: percent tuberization (transformed data) = 0.10 + 2.46x − 0.70x\(^2\) (\(r^2 = 0.5\)); number of tubers per explant = 0.16 + 5.33x − 1.58x\(^2\) (\(r^2 = 0.22\)), where x is the concentration of BA (mg l\(^{-1}\)).
### 3.2.2. Effect of sucrose concentration

Microshoots cultured in liquid media failed to produce microtubers, whereas root explants cultured on solid media showed the capacity for tuberization. Sucrose concentration had a significant negative effect \((P \leq 0.001)\) on both percent tuberization and number of tubers per explant. At 30 g l\(^{-1}\) sucrose, 100% of the root explants produced tubers with an average of six tubers per explant. As concentration of sucrose was increased to 60 g l\(^{-1}\), percent tuberization and number of tubers decreased to 37.5% and 1.63, respectively. At 90 or 120 g l\(^{-1}\) sucrose, tuberization was completely suppressed. A significant effect \((P \leq 0.05)\) of sucrose concentration was also detected on both average weight and size of tubers. Average weight of tubers peaked (0.4 g) at 60 g l\(^{-1}\) sucrose, and then decreased at higher concentrations of sucrose. On the other hand, average size of tubers was maximum (0.94 cm\(^3\)) at 30 g l\(^{-1}\) sucrose, then declined with increasing sucrose concentration. As a function of sucrose concentration, data were best fitted using the regression equations: percent tuberization (transformed data) = 5.76 − 1.00x + 0.04x\(^2\) \((r^2 = 0.95)\); number of tubers per explant = 11.42 − 2.27x + 0.11x\(^2\) \((r^2 = 0.61)\); tuber weight = −1.097 + 0.789x − 0.122x\(^2\) + 0.005x\(^3\) \((r^2 = 0.34)\); tuber size = 1.285 − 0.117x \((r^2 = 0.33)\), where \(x\) is the concentration of sucrose (g l\(^{-1}\)).

### 4. Discussion

The presence of midrib in the explant was crucial for adventitious shoot formation. This was clearly evident from the 88% regeneration obtained from blade explants including the midrib compared to the completely inhibited regeneration on blade explants which did not include the midrib. This may be explained by the presence of vascular tissue in the former explants (Dohm et al., 1989; Kumar et al., 1998). The promotive effect of the midrib was also clearly indicated by the 88% regeneration of blade explants including the entire midrib compared to the 44% regeneration of the explants excised from the central region of the leaf which included part of the midrib.

The promotive effect of wounding on direct shoot regeneration has been reported by Gosukonda et al. (1995) and Jong et al. (1993) and may be explained by the increased surface area of tissue exposed to the medium. Low percentage of shoot regeneration from central or petiolated lamina explants may be attributed to reduced size of lamina. Sharma et al. (1991) detected a direct correlation between amount of laminar tissue removed and degree of decline in shoot bud differentiation in mustard greens cultures. These authors also showed that removal of both lobes of the cotyledons resulted in reduction in shoot formation, attributing this to the involvement of some regulatory substance(s) emanating from the lamina in bud differentiation. The presence of petiole also enhanced
regeneration. This was indicated by the 34% regeneration obtained from petiolated lamina explants which did not include a large part of the midrib but included part of the petiole compared to the absence of regeneration on explants without either the midrib or petiole. The promotive effect of petiole on adventitious bud regeneration has been demonstrated in foxglove tree cultures whereby regeneration was completely inhibited in the absence of petiole (Kumar et al., 1998). These authors suggested that besides the lamina, the petiole played a role in shoot initiation. In this respect, Bhojwani and Razdan (1996) reported that the ideal explant to achieve regeneration from cotyledon cultures of mustard greens was the lamina with a short base petiole.

It is possible that the cells most competent to differentiate shoots are located at the petiole base, but to express their totipotency they are dependent on some factor(s) contributed by the lamina. In this respect, Welander and Maheshwaran (1992) reported that laminar segment explants of apple closest to the petiole were more regenerative than those that were farther away from the petiole. Based on these results, it was suggested that an increased density of vascular tissue and thus levels of phytohormones and metabolites near the petiolar region of the explants might be responsible for the increase in shoot regeneration.

Efficiency of tuberization in wild cyclamen was dependent on explant type and BA concentration in the medium. This variation may be due to the degree of cell sensitivity towards growth regulators, which depends on origin of explants and endogenous levels of growth regulators. Root explants were able to produce brown, spherical structures at the cut ends of the explants, which resemble microtubers. Wainwright and Harwood (1985) observed the presence of such spherical structures on root explants of cyclamen ‘Rosamunde’ which they identified as sprouting tubers. Furthermore, unipolar tubers with either a shoot bud or roots and bipolar tubers with both a shoot bud and roots were obtained from leaf blade, petiole, and ovary explants of cyclamen ‘2 NEO’ cultured on media supplemented with NAA and BA (Wicart et al., 1984).

In the current study, roots failed to form microtubers when cultured on BA-free medium. The influence of the balance between auxin and cytokinin in the culture medium should not be precluded. Both auxin and cytokinin were shown to work synergistically to influence bulblet formation in hyacinth (Hyacinthus orientalis L.) (Kim et al., 1981). The stimulatory effect of BA on tuberization in wild cyclamen was indicated by formation of tubers as BA was added to the culture medium containing NAA. Results of the current study are in agreement with those of Kim et al. (1981) who demonstrated that flower bud tissue of hyacinth cultured on a medium deficient of BA and NAA or a medium with a higher level of NAA (1 or 3 mg l\(^{-1}\)) than BA (0.1 or 0.3 mg l\(^{-1}\)) failed to differentiate bulblets. The authors also reported that a reduction in the concentration of either BA or NAA in the culture medium resulted in a significant reduction in bulblet formation. With Dutch iris (Iris hollandica), Hussey (1976) reported slightly
enhanced formation of bulbils as a result of addition of kinetin to a medium containing NAA. Furthermore, Steinitz et al. (1991) failed to obtain corms of gladiolus hybrid (*Gladiolus grandiflorus* Hort.$\times$*Gladiolus tristis* L.) when shoots excised from stock plants kept on a BA-deficient proliferation medium were cultured on a medium deficient of BA. However, when shoots excised from stock plants kept on a BA-containing proliferation medium were cultured on a medium deficient of BA, the authors observed corm formation, which they attributed to the residual carry-over effect of BA present in the proliferation medium.

In fact, there are conflicting reports on the role of cytokinin in storage organ formation in vitro. Steinitz and Yahel (1982) reported complete inhibition of bulblet regeneration in *Narcissus tazetta* when NAA and BA were incorporated into the culture medium. However, the concentrations of NAA (1 mg l$^{-1}$) and BA (10 mg l$^{-1}$) used in that study were much higher than those (0.1 mg l$^{-1}$ NAA and 1–3 mg l$^{-1}$ BA) tested in the current study. Furthermore, complete inhibition of gladiolus corm formation occurred when BA was incorporated into the culture medium (Steinitz and Lilien-Kipnis, 1989). However, the authors concluded that the adverse effect of BA on corm formation deviated from experience gained with in vitro-induced tuberization as being achieved by application of cytokinin in the culture medium. In the current study, tuber regeneration was not successful on microshoots cultured in liquid MS medium containing NAA and BA. This may be due to specific inherent regeneration potentialities among explants (Geier, 1978).

Although it was exhibited on root explants of wild cyclamen on solid media, tuberization was significantly affected by sucrose concentration. Tuberization frequency decreased with increasing sucrose concentration to 6% and was completely inhibited at 9 and 12%. Lipavska and Vreugdenhil (1996) reported decreased dry matter accumulation in potato shoots with increasing sucrose concentration. This was explained by the inhibitory effect of sucrose at high concentrations that resulted in a more negative water potential of the medium, which inhibited growth. However, bulbil formation of *Narcissus* was stimulated by increasing sucrose concentration to 9% (Chow et al., 1992). In a study with gladiolus, Steinitz et al. (1991) demonstrated rapid corm regeneration in liquid cultures containing adequate amount of cytokinin, a gibberellin-biosynthesis inhibitor, and high concentrations of sugar. In our study, weight of tubers produced with 6% sucrose was higher than that with 3% sucrose. This was an expected result due to the less number of tubers produced in a medium containing a higher (6%) amount of sugar.

5. Conclusion

To achieve satisfactory direct shoot regeneration response in wild cyclamen, leaves should be excised from microshoots and cut in such a way that the midrib
is included in the explant. Microtuberization of wild cyclamen may be successfully achieved by excising roots from in vitro seedlings and culturing them on a solid half-strength MS medium containing 3% sucrose, 0.1 mg l⁻¹ NAA, and 1.0 mg l⁻¹ BA. Further research is required for developing a protocol for in vitro propagation of wild cyclamen using directly formed shoot regenerants as a source of material.

Acknowledgements

We acknowledge the Deanship for Scientific Research at Jordan University of Science and Technology (JUST) for funding this project. Cooperation and suggestions of Dr. Rida Shibli (JUST) in carrying out this work are highly appreciated. Sincere appreciation goes to Dr. Hani Ghosheh (JUST) for his guidance in statistical analysis.

References


