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Direct Regeneration in Cyclamen Persicum Mill. Using Seedling Tissues

الاستنبات المباشر لنبات قرن الغزال Cyclamen persicum. Mill باستخدام بادرات داخل الانابيب

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Abstract

In Vitro shoot regeneration and microtuberization of *Cyclamen persicum* Mill. were studied using seedling tissues. Tuber, leaf and petiole sections of aseptic seedlings of cultivar 'Concerto' were established on Murashige and Skoog (MS) basal medium. Three levels of benzyladenine (BA) (4.4, 8.8, 13.3 µM) and four levels of thidiazuron (TDZ) (0.5, 1, 2, 4 µM) were used with the three different explants. All regeneration media were supplied with 5.4 µM naphthalene acetic acid (NAA). No shoot regeneration was observed in media without cytokinins. The greatest percent shoot regeneration (100 %) was obtained from tuber sections cultured on media supplemented with 4.4 µM BA and 4 µM TDZ. No regeneration was obtained with petiole sections. Microtubers were formed with leaf explant. The higher microtuberization response was obtained with leaf explants cultured on media supplemented with 2 and 4 µM TDZ (41.6 and 58.3, respectively). Tuberous structures (microtubers) were able to sprout and leaves continued to grow on these structures. After an acclimatization period, the plantlets were transferred to the greenhouse and continued their growth normally.

Abbreviations: BA-benzyladenine; MS. Murashige and Skoog (1962) medium NAA- naphthalene acetic acid; TDZ – thidiazruon;

ملخص

تمت دراسة تأثير نوعين من السيتوكاينين على استنبات (Regeneration) نبات قرن الغزال *Cyclamen persicum.* Mill صنف ''Concerto'. زرعت بذور الصنف على وسط موراشيكي وسكوك (MS). تم استخدام الدرنات، والاوراق وحوامل الاوراق من البادرات النابتة. كما تم استخدام ثلاثة مستويات من البنزل ادنين (BA) (٤,٤ ، ٨,٨ ، ١٣,٣ مايكرومول) وأربعة مستويات من الثيادايزرون (TDZ) (٠,٥،

٤,٢٨ مايكرومول) وقد تمت اضافة ٥,٤ مايكرومول من نافثالين حامض الخليل (NAA) في جميع البيئات المستخدمة لتجارب الاستنبات. لم يحدث تمايز في البيئات التي لا تحتوي على السيتوكاينين، علما انه تم الحصول على ١٠٠% تمايز سوقي باستخدام البنزل ادنين والثيادايزرون في حالة الدرنات. لم تعط حوامل الاوراق أي استنبات. حصل تكوين لاشكال درنية من أنسجة الأوراق، حيث حصل أعلى مستوى عند ٢، ٤ مايكرومولار TDZ، وبنسبة ٤١,٦ و٥٨,٣% على التوالي. استمرت الاوراق في النمو والتطور، وتم نقل النباتات، بعد الأقلمة، إلى البيت الزجاجي، حيث استمر نموها بصورة طبيعية.

Introduction

Cyclamen persicum Mill. is an important flowering pot plant. It is commercially propagated by seeds. Seed propagation is usually associated with cultivar variation. In addition, hybrid seed production is expensive; therefore, cloning of this plant is preferred. Micropropagation is considered as a helpful tool for clonal propagation.

Micropropagation of *Cyclamen* through organogenesis and somatic embryogenesis has been reported (2,4,10,13-14,21-23). Somatic embryogenesis is still the most used method for *Cyclamen* micropropagation; however, this technique is associated with callus production that, in many cases, resulted in somaclonal variation (4,6,13,18,20). In addition, low regeneration ability has been reported with this technique. Among 11 *Cyclamen* cultivars tested, only 'Annekel' cultivar showed reasonable regeneration ability (22).

Organogenesis from variuos explants and sources of *Cyclamen* plant have been reported. Among explants used were tuber tissue (5,13,22), petiole (1,8,11-12). Various parts from aseptic seedlings (11-13,16,21,24) , flower organs (11-12), root explant (11-12) and leaf explant (2,9-12).

Among all presented reports, the regeneration percentage was still not satisfactory for commercial propagation of known cultivars. Among 18 *Cyclamen* cultivars tested, eight regenerated less than three shoots per explant (9). In another study, with wild *Cyclamen* plants, direct shoot regeneration with a high percent (88%) on leaf sections were obtained using both NAA and TDZ (11-12); therefore, this work was initiated as an effort to improve the regenability of *Cyclamen* plant using thidiazuron and benzeladenine with tuber, leaf and petiole explants.

Materials and Methods

Mature seeds of *Cyclamen persicum.* Mill. Cv 'Concert' from Royal Flower Company were used in this study. The seeds were sterilized by immersion for 10 minutes in 10% chlorox (5.25 Sodium hypochlorite) followed by three rinses in sterile distilled water. The seeds were germinated on (MS) [17], media salt supplemented with 30 g/L sucrose, 100 mg/L myoinositol, and 8 gm/L Difco Bacto agar as a gelling agent. The pH was adjusted to 5.7 prior to autoclaving; the medium was dispensed in 25 x 18mm test tubes. Plant materials for regeneration experiments were taken from three sources: one source was the tuberous stem that forms after four weeks of germination (Fig 1). Each tuber was divided transversely into two portions; only the upper portions were used in the regeneration trials. The tuber sections were planted onto MS basal media supplied with 4.4, 8.8, and 13.3 µM benzyladenine (BA) or (0.5, 1, 2, and 4 µM thidiazuron (TDZ), a control medium (without cytokinin was also used), therefore, a total of eight treatments were used. Each treatment was replicated 10 times. The treatments were arranged in a completely randomized design (CRD).

Figure (1): *In vitro* tuberous stem of Cyclamen plant that developed after four weeks of seed germination.

The cultures were incubated in a growth room at a temperature ranging between 22- 24 C^o and under 50 µmolem $^{-2}$ s^{-1} light intensity for 16 hours duration.

After one month of incubation, the percent of explant producing shoots, and the average number of produced leaves were recorded.

The remaining seedlings were kept until true leaves were developed. After 8 weeks of planting, fully expanded leaves were used as a second explant. The leaf petioles were separated from the leaves, divided into two sections and planted as another explant. Each leaf blade was divided into four quarters and planted onto 9 x 15 mm petridishes containing MS basal media supplies with 5.4 µM naphthalene acetic acid (NAA) and three levels of BA (4.4, 8.8, 13.3 µM) or four levels of TDZ (0.5, 1,2,4µM). A control medium was included in the experiment. The levels of BA and the levels of TDZ with the control were considered as treatments. Each treatment was replicated five times (5 petridishes with four leaf sections). The same treatments were used with the petiole sections; however, with only 4 petridishes per treatment. Treatments were arranged in a completely randomized design (CRD). All cultures were kept under dark conditions for one month at 22-24 C^o. Cultures were then transferred to light condition in the growth room.

After one month, data were collected for percent of explants producing tuberous structure (TS), average number of TS per explant, average number of leaves produced per TS and the callus development on scale basis (0- 5). All data were analyzed using the SAS software program according to one way analysis of variance. Significant results were followed by means separation with the Duncan's Multiple Range test at 5% probability level [19].

Results and Discussion

Both BA and TDZ exhibited a significant effect on shoot regeneration, average leaf number and callus formation (Table 1). No shoots were developed on media without hormones (control). A high percent of shoot regeneration (83-100%) was obtained without significant difference between BA and TDZ. Similar effect of both BA and TDZ was observed with the average number of leaves produced. The higher leaf number was obtained with 2 and 4 µM TDZ (8.2 and 10.8 respectively), which differs significantly from other cytokinin levels. Callus formation was significantly similar in all treatments except with media supplied with 0.5 and 1 µM TDZ.

Direct regeneration occurred from tuber and leaf explants taken from aseptic seedlings. Aseptic seedlings of *Cyclamen* were used as a source of explant in several studies (2,8,11-12,24). Aseptic seedlings have no contaminants and they are characterized by a high potential of organogenesis. Benzyladenine was used by other researchers to enhance regeneration of shoots in *Cyclamen* plants (22), working with organogenesis of 'Anneke' *Cyclamen* cultivar, found that the highest shoot number was obtained from tuber explants with BA at 1 µM. In another study, (5) were able to obtain 100% shoot regeneration with BA at 10^{-4} M in half MS salt with bulb tissues. These findings matched to what has been shown in this study.

Table (1): Effect of benzyladenine and thidiazuron on *in vitro* regeneration from tubers of *Cyclamen*. Cv. Concerto.

Treatment	**Conc.µM**	**Percent of explants forming shoots**	**Average No. of leaves per explant**	**Callus production (1-5) scale**
Control	0.0	0.0 b*	0.0 d	3.0 a
BA	4.4	100 a	6.5 bc	2.8 a
	8.8	83 a	6.6 bc	2.6 a
	13.3	83 a	7.8 b	2.3 a
TDZ	0.5	93 a	5.0 c	1.8 b
	1.0	100 a	6.5 bc	1.8 b
	2.0	97 a	8.2 ab	2.6 a
	4.0	100 a	10.8 a	3.0 a

* Means within coloumns followed by different letters are significantly different according to Duncan's Multiple Range test. P = 5%.

No regeneration was obtained with petiole explants regardless of the cytokinin used. Callus was formed on many explants, but without further morphogenical development. BA and TDZ exhibited significant effects on microtuberization on leaf explants (Fig. 2a). Both tuberous number (TS) and leaf number per TS were influenced (Table 2). Microtuberization was achieved on media supplemented with TDZ and only with 8.8 and 13.3 µM BA (Fig.2-a). The greatest significant percent of tuberous formation was observed on media supplied with 2 and 4µM TDZ (41.6 and 58.3 respectively). The average number of TS produced per explant ranged from 2-3. However, in case of BA level, only one TS was produced per regenerated explant. The average number of leaves produced per tuber (Fig.2-b) showed similar results. TDZ treatments gave significantly a higher number of leaves than BA treatments. The highest significant number (8.6) was obtained with 2 µM TDZ followed by 4 µM TDZ which produced 5.8 leaves per TS.

Figure (2-a): ***In vitro*** tuber structures that regenerated from leaf section of Cyclamen

Figure (2-b): ***In vitro*** Cyclamen plant leaves developed from regenerated tuber structure from leaf section

Table (2): Effect of benzyladenine and thidiazuron on *in vitro* regeneration from leaf sections of *Cyclamen*. Cv. Concerto.

Treatment	Conc.µM	Percent of explants forming TS[1]	Average No. of TS per explant	Average No. of leaves per TS
Control	0.0	0.0 c*	0.0 b	0.0 c
BA	4.4	0.0 c	0.0 b	0.0 c
	8.8	5.5 bc	1.0 b	2.5 c
	13.3	8.5 b	1.0 b	2.0 c
TDZ	0.5	8.3 b	2.0 a	2.0 c
	1.0	16.7 b	2.5 a	2.5 c
	2.0	41.6 a	3.0 a	8.6 a
	4.0	58.3 a	2.5 a	5.8 b

* Means within coloumns followed by different letters are significantly different according to Duncan's Multiple Range test. P = 5%.

The use of both leaf blade and petioles for *in vitro* culture of *Cyclamen* is common. Using such explants will not severely damage the stock plants. In contrast, the use of the tuberous stem would mostly induce death to the stock plant. In this study, petioles did not give any regeneration within the levels of both BA and TDZ used. This finding agrees with [6,11-12], where less regeneration was obtained with petioles. In contrast, etiolated petioles resulted in higher regeneration than non-etiolated [1]. As shown in this study, the use of petioles as an explant proved to be not effecient under the conditions of the experiment. On the other hand, the use of leaf sections proved to be active in regeneration. TDZ and BA were crucial for regeneration. However TDZ was more effecient. This finding strongly agreed with other researchers' results. BA was used as a cytokinin for shoot regeneration from leaf section in *Cyclamen* plant, but with low regeneration [9]. In contrast, [2] reported that leaves were the best explant for direct organogenesis in *Cyclamen* using 2iP as a cytokinin.

In our current study, as high as 58 % leaf explants produced tuberous structure and up to 8 leaves were produced per tuber with the incorporation of TDZ in the media. TDZ was used to induce shoot regeneration in leaf sections of several plant species [3]. TDZ was more effecient in the induction of direct shoot regeneration and microtuberization in wild *Cyclamen persicum* than BA with several explants, shoot formation occurred in several explants cultured on media containing TDZ at both .022 and 0.22 mg/L. No regeneration was observed with media containing BA [11-12]. These results are conform with our findings. TDZ was also used to induce callus production of isolated protoplast of *Cyclamen* persicum [15].

This study confirmed the stimulating effect of TDZ on shoot and tuber formation. Moreover, tuber explants have higher potential of adventitious shoot formation than leaf section. In addition, this work showed the ability of *Cyclamen persicum* plants to be successfully germinated, grown and differentiated on MS media with both TDZ and BA. The regenerated shoots continued to grow and several rooted on media without cytokinin. They were successfully transferred and grown in the greenhouse. Up to 30% of the plants continued their growth and gave normal flowers.

Finally, the regeneration system described in this study enhanced the vegetative propagation of *Cyclamen* plant. However, in order to utilize this protocol for mass production , more cultivars should be investigated with other factors.

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