ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (2): 328-332, 2008
ISSN 1682-296X

Direct Shoot Formation and Microtuberization from Aseptic Seedlings of *Cyclamen mirabile* Hildebr.

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Abstract: In this study, an efficient propagation method was developed for *Cyclamen mirabile* Hildebr., an endangered endemic species having ornamental importance in Turkey. Sterilized seeds of *C. mirabile* were cultured on full and half-strength (½) Murashige and Skoog (MS) basal media for germination and seedling growth. Tissues excised from 8 week-old seedlings were used as explant source for shoot regeneration and microtuberization. Shoots were obtained from only tuber slices on Bourgin-Nitsch (BN) medium supplemented with auxin and cytokinin. The largest of number of shoots (12.2 shoots/explant) was obtained on ½ MS medium containing 1 mg L^{-1} NAA and 0.5 mg L^{-1} kinetin. Microtubers were formed in the medium promoting shoot proliferation. The highest microtuber formation was obtained on the medium containing 2 mg L^{-1} BA plus 0.1 mg L^{-1} NAA.

Key words: *Cyclamen mirabile,* endemic plant, microshoots, microtubers

INTRODUCTION

Turkey is one of the largest global wild-bulb exporters because of its very rich and diversed plant life including many endemic (bulb) plants. In 1980's, nearly 70 millions of bulbs were exported from Turkey to European bulb market. At the same time, Turkey exported 5 million *Cyclamen* bulbs to the Netherlands, UK and USA *Cyclamen mirabile* Hildebr. is an endemic species. According to Red Data Book of Turkish Plants (Ekim *et al*., 2000), it is in the endangered category. Today, export of endemic *Cyclamen* species (including *C. mirabile*) is banned but enforcing this regulation is extremely difficult because of illegal harvest activities. *Cyclamen mirabile* and the other rare species with beautiful flowers and leaves will always be attractive for bulb markets. Therefore efficient propagation methods must be developed to keep away natural populations of the rare and attractive species from illegal or legal harvest pressure. Ornamental use is not only the reason for making *Cyclamen mirabile* important: Çaliş *et al*. (1997) reported antifungal activities of cyclaminorin, deglucocyclamin and cyclamin saponins obtained from tubers of the species. These discoveries would promote additional extensive harvesting and trading activities on the species.

Plant tissue culture techniques can give efficient propagation systems with minimal disturbance on natural populations and bio-diversity. Additionally, they can provide the know-how of an efficient large scale system for production of plant tissues free from contaminants such as insects, fungi and bacteria. Furthermore, extraction of medicinally important compounds from plant faces some limitations by a series of factors such as diseases, climatical and seasonal conditions. Tissue culture techniques will enable an effective production of active compounds without these problems.

Furukawa *et al*. (2002) obtained calli using aseptic seedlings on the modified MS medium. To our knowledge, there is no literature on direct shoot regeneration and microtuberization of *C. mirabile*. Direct shoot regeneration, without an intervening callus stage, is preferred since extensive callus formation and long-term callus culture can lead to somaclonal variation (Preece, 1997). Rapid production of microtubers could be useful for the production of pathogen-free tubers. The generation of microtubers from *in vitro* plants makes it possible to solve long-term storage, sorting transport, storage and distribution problems and promoting an easier and direct use by the farmers (Jordan *et al*., 2002).

The aim of this study was to determine the effects of explant type, plant growth regulators and their

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concentrations on direct shoot regeneration and microtuberization from aseptic seedlings of *C. mirabile*.

MATERIALS AND METHODS

Mature fruits of *Cyclamen mirabile* were collected from west slopes of Madran Babadağ mountain of province Aydın-Çine (Turkey), between the elevation of 250-500 m. Mature fruits collected from periodically conducted field researches between the years 2003-2005. Seeds were recovered from mature fruits of *Cyclamen mirabile*, washed thoroughly in running tap water for 60 min, dipped in 70% ethanol for 10 min, surface-sterilized in 4.5% sodium hypochlorite solution for 25 min and finally rinsed in sterile distilled water (3-4 times) for 10 min.

Sterilized seeds were sown full and half-strength (½) MS basal media solidified with 0.8% agar, pH 5.8 and containing 3% sucrose. 60 seeds were used in all experiments (repeated 3 times). Radicle emergence was evaluated as an indicator of seed germination. Germinated seeds were counted after 10 weeks of incubation. Germination was expressed in percentage. Cultures were maintained in the dark at 15°C until germination started, then they were transferred to a growth chamber and maintained at 24±1°C under a 16 h photoperiod.

Lamina, petiole, root, intact tuber and tuber sections were excised aseptically from 8-week old seedlings and used for *in vitro* shoot regeneration and microtuberization.

In shoot regeneration and microtuberization experiments, laminas were cut into 5×5 mm sections, petiole and roots into 10 mm-long segments. Explants were cultured on different salt formulations: (Murashige and Skoog, 1962), ½ MS, (Bourgin and Nitsch, 1967; (Linsmaier and Skoog, 1965; Loewenberg, 1969) media, solidified with 0.8% agar, containing 3% sucrose at pH 5.8. All mineral formulations were supplemented with different types and concentrations of plant growth regulators: NAA (0, 0.1, 0.5, 1.0, 2.0, 3.0 mg L^{-1}), NAA (0, 0.1, 0.5 mg L^{-1}) + BA (0.5, 1, 2, 3, 4, 5 mg L^{-1}), KIN (0, 0.1, 0.5 mg L^{-1}) + IAA (0.1, 0.5, 1, 2.5 mg L^{-1}), KIN (0, 0.1, 0.5 mg L^{-1}) + NAA (0, 0.1, 0.5, 1, 2.5 mg L^{-1}), NAA (0, 0.1, 0.5 mg L^{-1}) + TDZ (0.002, 0.02, 0.2, 2.2), 2,4-D (0.1, 0.5, 1, 2.5, 5 mg L^{-1}) + KIN (0, 0.1, 0.5 mg L^{-1}).

Tubers were used as intact or after being sliced into four segments. Tuber segments were transferred to MS, ½ MS, BN, LS and Loewenberg media containing NAA (0, 0.1, 0.5 mg L^{-1}) + BA (0.5, 1, 2, 3, 4, 5 mg L^{-1}), KIN (0, 0.1, 0.5 mg L^{-1}) + IAA (0.1, 0.5, 1, 2.5 mg L^{-1}), KIN (0, 0.1, 0.5 mg L^{-1}) + NAA (0, 0.1, 0.5, 1, 2.5 mg L^{-1}). Four explants were used in all experiments (repeated 4 times). Cultures were maintained in the dark at 24±1°C and or 16 h photoperiod at 24±1°C. Intact tubers cultured on MS or ½ MS media containing NAA (0, 0.1 mg L^{-1}) and BA (0, 1.0, 2.0, 3.0 mg L^{-1}). Five explants were used in each experiment. Cultures were maintained at 24±1°C and in 16 h photoperiod for 8-weeks.

The number of shoots and microtubers per explant (mean and SD) were calculated and differences between means were tested for significance using ANOVA and Duncan's Multiple Range Test at the level of $p \leq 0.05$.

RESULTS AND DISCUSSION

Sterilized seeds were cultured on full and half-strength (½) MS basal media. On MS medium no germination occurred in the first three weeks. A considerable increase in germination was observed between 6th and 7th weeks. Maximum seed germination cultured on MS medium was about 42% at 10th week (Table 1). Seeds cultured on ½ MS medium began to germinate at third week and a marked increase in germination occurred between 5th and 6th weeks. Maximum germination on ½ MS medium was 53% at 8th week. These results showed that ½ MS was more suitable than MS for germination of *C. mirabile* seeds. Mineral requirement during germination depends upon plant species and is probably related to the amount of reserves stored in seeds (Padilla and Encina, 2003). Apparently, *C. mirabile* seeds do not need excessive amount of mineral salts to germinate. Furthermore, seedlings growing on ½ MS were more healthy than on MS (Fig. 1). Our results suggest that the growth of *C. mirabile* seedlings depends on the nutrient store in seeds during ~2 months after germination.

Eight-week old sterile seedlings were used as explant sources for shoot regeneration and microtuberization. Petiolar explants excised from sterile seedlings formed calli on ½ MS medium supplemented with 2 mg L^{-1} NAA at 22±1°C in the dark. The percentage of explants that developed calli were less than 25%. The calli were

Table 1: Germination percentage of *C. mirabile* seeds on MS and ½ MS media

	MS	½ MS
Weeks	Mean germination (%)	Mean germination (%)
1	-	-
2	-	-
3	-	3.3
4	6.6	6.6
5	11.6	13.3
6	18.3	43.3
7	35.0	50.0
8	40.0	53.3
9	40.0	53.3
10	41.6	53.3

Table 2: Effects of BA and NAA on direct shoot regeneration and microtuberization of *Cyclamen mirabile*

Growth regulator (mg L^{-1})			
BA	NAA	No. of shoots per intact tuber explant[a]	No. of microtubers per intact tuber explant[a]
1	0.0	9.60±2.30b[c]	0.6±0.54c
2	0.0	0.60±0.89c	5.20±1.30a
3	0.0	0.40±0.54c	2.40±1.14b
0	0.1	0.00±0.00c	0.00±0.00c
1	0.1	12.20±2.49a	0.80±0.83c
2	0.1	1.40±1.14c	6.00±1.41a
3	0.1	0.80±0.83c	3.00±0.70b
Control[b]		0.00±0.00	0.00±0.00

[a]Values are mean±standard deviation of 4 separate experiments.
[b]Control = ½ MS medium free of growth regulators.
[c]Means in each column followed by different letter(s) are different according to Duncan's Multiple Range Test (p = 0.05)

subcultured at 4-week intervals but they could not show any development. Calli turned brown and died after 1 week. Lamina and root explants which were cultured on all the media became brownish and died after one or two days of incubation. Darkening of explants was explained as a result of phenolic oxidation occurred during cutting. It was reported by many researchers that harmful effects of oxidized phenolic compounds is one of the most common problems which affect the success of *in vitro* cultures (Teixeira *et al*., 1994; Khatri *et al*., 1997).

Shoots were obtained from tuber sections, which were sliced into four segments and cultured on BN media containing 0.1 mg L^{-1} IAA plus 0.5 mg L^{-1} KIN and 0.5 mg L^{-1} NAA plus 0.5 mg L^{-1} KIN at 24±1°C in the dark. The shoot formation rate was 12.5%. Regarding to salt contents, BN and MS media are similar excepting a lesser ammonium concentration in BN than MS medium. Additionally BN medium includes biotin and folic acid. These additional substances probably inhibited phenolic oxidation and stimulated shoot formation from tuber explants. Dias (1999) reported that biotin and folic acid have an absorbing nature of phenolic compounds. Furthermore, promotion of shoot initiation in the dark might be caused by slowing down enzymatic activity responsible for tissue oxidation (Drew, 1986). In addition, the development of shoots may relate to outgrowth from pre-existent meristems of tuber.

To promote shoot regeneration, intact tubers (apical shoots were removed) of sterile seedlings were cultured on different media without cutting to avoid above mentioned risks of the cutting. There was no shoot regeneration on MS and ½ MS media supplemented with NAA alone or without plant growth regulators. Shoot formation was induced in all the media supplemented with BA (Table 2). BA was previously reported as an effective cytokinin for shoot formation of various *Cyclamen* explants (Loewenberg, 1969; Ando and Murasaki, 1983; Hawkes and Wainwright, 1987; Dillen *et al*., 1996). Increasing BA concentration decreased number of shoots per intact tuber but there was no statistical difference between the media containing 2 and 3 mg L^{-1} BA. The maximum shoot formation was attained on ½ MS medium supplemented with 1 mg L^{-1} BA plus 0.1 mg L^{-1} NAA (Fig. 2).

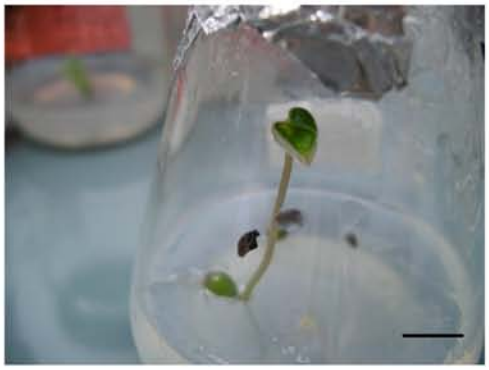

Fig. 1: An 8-week old seedling from the seed germinated on ½ MS medium (Bar: 1 cm)

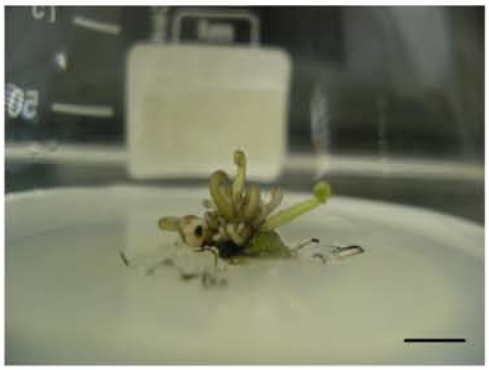

Fig. 2: Shoots regeneration from intact tuber on ½ MS medium containing 1 mg L^{-1} BA plus 0.1 mg L^{-1} NAA (Bar : 1 cm)

In this study, a significant difference in regenerative potential was observed depending on explants and growth regulators as reported previously on other cyclamen cultivars (Geier, 1977; Takamura and Miyajima, 1997; Wainwright and Harwood, 1985). This variation may be explained by degree of cell sensitivity towards growth regulators due to explant origin, endogenous growth regulator levels and auxin and cytokinin oxidases activity (Tran and Trinh, 1990). Shoot organogenesis was observed only from tuber explants indicating specific inherent potentialities among explants (Geier, 1978).

Microtubers were formed from intact tubers in all the media tested for shoot proliferation. Efficiency of tuberization in *C. mirabile* was dependent on BA

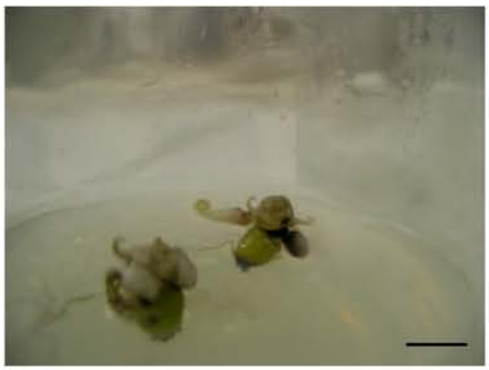

Fig. 3: Microtubers regeneration from intact tuber on ½ MS medium containing. 2 mg L^{-1} BA plus 0.1 mg L^{-1} NAA (Bar: 1 cm)

concentration in the medium. In the case of BA used alone, the highest rate of microtuber formation was obtained at 2 mg L^{-1} BA (5.2 microtuber/tuber). Addition of NAA to the media promoted microtuber formation and the highest microtuber formation was obtained at 2 mg L^{-1} BA and 0.1 mg L^{-1} NAA (6 microtubers/tuber) (Fig. 3). No microtuber formation was observed in the control media without growth regulators. For inducing tuberization *in vitro*, much attention has been payed for the use of cytokinins such as BA, 2-ip, KIN and zeatin (Rosell *et al*., 1987; Lentini and Earle, 1991; Levy *et al*., 1993; Pelacho and Mingo-Castel, 1991; Koda and Okazawa, 1983). Except in the case of in *Dioscorea* spp. microtuberization is stimulated by auxin and inhibited by cytokinin (Jean and Cappadocia, 1992). In the present study NAA was observed to stimulate tuberization in *C. mirabile* induced by BA. Auxins are involved in the cell enlargement and the interaction of cytokinins and auxin guarantees tuber growth (Melis and Van Staden, 1984). The effect of auxins on *in vitro* tuberization depends upon their concentrations. Relatively low concentrations (≤ 5 μM) induce tuberization while higher concentrations are less effective and even inhibitory (Wang and Hu, 1985). In the present study, a low concentration of NAA was used and microtuber formation was stimulated. Results of the current study are in agreement with those of Karam and Al-Majathoub (2000) who demonstrated that root explant of *C. persicum* cultured on ½ MS basal medium supplemented with 0.1 mg L^{-1} NAA and 1.0 mg L^{-1} BA. It is known that tuberization is a plastic and complex event strongly depends on environmental and genotypic factors. Exogenous growth regulators play an important role in this process (Kefi *et al*., 2000).

Microtubers formed a lot of microshoots and these shoots were used a stock for further experiments. In our experiments, roots emerging from microtubers produced brown microtuber-like structures. Wainwright and Harwood (1985) observed the presence of such globular structures on root explants of *C. persicum* cv. Rosemunde and called as sprouting tubers.

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