PLANT TISSUE CULTURE

Highly efficient microtuber formation, direct shoot regeneration, and root induction in *Cyclamen persicum* Mill. from *in vitro* seedling-derived tuber and leaf segments

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Abstract

The ornamental crop *Cyclamen persicum* is one of the important perennial pot plants in the world, which is commercially propagated by seeds. The present study developed a highly effective method for microtuber formation and direct shoot regeneration using *in vitro* leaves and tubers from three different cultivars: Pure White, Neon Pink, and Dark Violet. Explants were transferred to ½ Murashige and Skoog basal medium, which contains different levels of benzyladenine (BA) or thidiazuron (TDZ), for direct shoot regeneration or microtuber formation. Finally, the percentage of explants with direct regeneration and the average number of shoots or microtubers in each explant were determined. The highest number of directly regenerated shoots (27.1) was observed on leaf explants of cultivar Pure White at 0.25 mg L^{-1} TDZ and 0.1 mg L^{-1} α-naphthaleneacetic acid (NAA). The highest number of proliferated microtubers (17.1) was observed from the culture of tuber cuts in the media supplemented with 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA in darkness in cultivars Neon Pink and Pure White. The best rooting regarding the number and the length of the roots resulted from 0.5 or 1.0 mg L^{-1} NAA. Finally, the rooted shoots were transferred to the soil and 58% of them acclimated successfully to the greenhouse conditions.

Keywords Cyclamen · Direct shoot regeneration · Microtuber · Micropropagation · Benzyladenine · Thidiazuron

Introduction

Cultivars of *Cyclamen persicum* Mill. are of commercial importance, while other cyclamen species (such as *C. coum*) may be of interest for medicinal use (Speroni *et al*. 2007; Foubert *et al*. 2008; Quave *et al*. 2008). Potted *C. persicum* has become a common houseplant, especially in Japan and Europe (Schwenkel 2001; Mizunoe *et al*. 2015). Potted cyclamen plants available on the market are usually obtained from growing F1 hybrid seeds. However, growing demand and attractiveness to keep *C. persicum* as an ornamental plant on the one hand and the costs and manual labor of F1 hybrid seed production on the other are of concern to cyclamen planters (reviewed by Jalali *et al*. 2012). Compared to many other flowering houseplants, vegetative propagation of *C. persicum* is more difficult since mass propagation by cutting stems and tubers is almost impossible (Schwenkel and Winkelmann 1998).

Clonal propagation by plant tissue culture might be a way out of the mentioned concerns by producing clones of the mother plants, thus preventing inhomogeneity. For the last 30 yr, researchers have been working on ways to grow *C. persicum* using *in vitro* tissue culture of explants. Accordingly, aseptic seedlings have no contaminants, and they have been considered to have a high organogenic potential in *C. persicum* (Kiviharju *et al*. 1992; Karam and Al-Majathoub 2000b, a; Abu-Qaoud 2004; Da Silva 2016).

Somatic embryogenesis has been investigated as the most common method for *C. persicum* micropropagation (Kreuger *et al*. 1995; Schwenkel and Winkelmann 1998; Winkelmann *et al*. 2000, 2004; Hohe *et al*. 2001; Pueschel *et al*. 2003; Seyring and Hohe 2005; Prange *et al*. 2010; Winkelmann 2010; Jalali *et al*. 2012; Rode *et al*. 2012; Kocak *et al*. 2014; İzgü *et al*. 2016). Nevertheless, using somatic embryos to generate clones of mother plants is problematic. Drawbacks are because these embryos do not have the seed coat and

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endosperm that may allow them to survive in medium- or long-term preservations and poor media (Mwangi *et al.* 2013). Additionally, this technique is usually associated with callus production that, in many cases, results in somaclonal variation (Ruffoni *et al.* (1998); Winkelmann and Serek 2005; Borchert *et al.* 2007).

There are very few reports on attempts to induce direct organogenesis from cyclamen tissues (Abu-Qaoud 2004; Seyring *et al.* 2009; Tütüncü and Mendi 2022). *In vitro* culture of fertilized ovules isolated from cyclamen ovaries have been performed to investigate the effect of irradiated pollen on regeneration; however, the experiment was not successful due to a lack of *in situ* parthenogenesis in these ovaries (Tütüncü and Mendi 2022). In another study, shoot induction has been examined from six cyclamen species using explants derived from greenhouse-grown plants including young leaves, petioles, flower buds, and peduncles (Seyring *et al.* 2009). Moreover, induction of shoots from *in vitro* tubers has been performed on *C. mirabile* Hildebr., investigating a broad type of basal culture media (Yamaner and Erdag 2008) including Murashige and Skoog (MS) and ½ MS (Murashige and Skoog 1962). Additionally, *in vitro* shoot regeneration and microtuberization of *C. persicum* Mill. were evaluated using seedling tissues of the cultivar 'Concerto' on MS basal medium. Three levels of benzyladenine (BA) and four levels of thidiazuron (TDZ) were used with three different explants. But the highest shoot regeneration response of this cultivar was not more than 59% (Abu-Qaoud 2004).

Direct shoot or microtuber regeneration could eliminate unwanted variation resulting from the callus induction phase and would have the highest commercial potential for *C. persicum* as an ornamental plant. Therefore, the current study focused on the optimization of microtuber propagation and direct shoot regeneration from three commercial cultivars of *C. persicum*. These methods are of great potential importance to investigate the ornamental character enhancement of the cultivars in future studies.

Materials and Methods

Source of Chemicals and Equipment The equipment were provided by the biotechnology laboratory of agricultural faculty at Tarbiat Modares University in Tehran, Iran, while the plant growth regulators, MS medium, and other phytochemicals were sourced from internal distributors of Sigma-Aldrich in Tehran, Iran. The agar was sourced from Ibresco Life Science (Karaj, Iran).

Seed Sterilization and Germination The experiments have been performed on three F1 cultivars of *C. persicum* including Pure White, Neon Pink, and Dark Violet provided by Nahal Gostar Royan Co (Qazvin, Iran). The F1 hybrid seeds

were rinsed for 15 min with sterile water and were immersed for 10 min in alcohol (70%) before being disinfected for 20 min in a sodium hypochlorite solution (3.0% w/v). Following the latter, seeds were rinsed three times (5 min each) with sterile distilled water and sowed on MS medium, then dispensed in test tubes. Media were supplemented with 3.0% (w/v) sucrose, 100.0 mg L^{-1} myoinositol, and 9.5 g.L^{-1} agar as a gelling agent. The media were transferred then to dark conditions at 4°C until seed germination initiated.

Explant Preparation and Regeneration Treatments Aseptic seedlings were kept in glass tubes to reach the four-leaf stage, and explants were extracted from tubers or the youngest fully developed true leaves of *in vitro* seedlings.

Leaf explants were cut and extracted right beside the mid-vein. Five segments (with a size of 5 mm^2 each) were extracted from each leaf blade and put upside-down on plates containing four different treatments of cytokinins (1.0, 2.0, and 3.0 mg L^{-1} BA or 0.25 mg L^{-1} TDZ), for a direct shoot or microtuber regeneration. Only one concentration of TDZ was included in the current study according to the best concentration reported in the literature for shoot induction (Karam and Al-Majathoub 2000b, a; Abu-Qaoud 2004). Two control media (without cytokinin, but with auxin, and without hormone) were also applied. Each treatment had three replicates with five leaf sections. The tubers of aseptic seedlings were also used for microtuber (secondary tubers develop on leaf or tuber segments) formation and direct shoot regeneration treatments. Since every seedling develops a single tuber and therefore limits the number of proper tubers, each tuber treatment had three replicates with only four cuts of a single tuber explant per each Petri dish.

The regeneration treatments with either tuber or leaf explants contain 0.1 mg L^{-1} NAA as the source of auxin (Karam and Al-Majathoub 2000a; Prange *et al.* 2008; Yamaner and Erdag 2008).

All the regeneration trials, both with leaf or tuber explants, have been incubated for an initial 4 wk in darkness, and then half of them were transferred to the controlled growth chamber with a photoperiod of 16 h of light and 8 h of darkness at 22 to 25°C for another 4 wk. The other half was kept in the dark for an additional 4 wk. The percentage of explants with a direct shoot or microtuber regeneration and the average number of shoots or microtubers in each explant were determined after 8 wk.

Experiments established in ½ MS media and experiment factors and their relative abbreviations are as follows: Hormonal concentrations (mg L^{-1}): without hormone (H0), without cytokinin (H1), BA 1 (H2), BA 2 (H3), BA 3 (H4), and TDZ 0.25 (H5). All hormonal treatments were supplemented with 0.1 mg L^{-1} NAA, except for H0. Cultivars: Dark Violet (C1), Neon Pink (C2), and Pure White (C3). Light conditions: an initial 4 wk of darkness followed by 4

wk of photoperiod (16 h of light and 8 h of darkness) (L1) and 8 wk of darkness (L2).

The regeneration experiments were done in Petri dishes. After passing the initial 8 wk, the explants, which went through shoot or microtuber regeneration, were transferred to the test tubes to have more upper space for new developing structures.

Rooting Treatments of Shoots The *in vitro* rooting of regenerated shoots was accomplished on ½ MS medium containing 0.1, 0.5, or 1.0 mg L^{-1} NAA or IAA. Before starting the rooting treatments, regenerated shoots from the leaf explants on 0.25 mg L^{-1} TDZ were transferred to the same medium and kept for another 4 wk to reach a height of approximately 2 cm. The rooting media were dispensed in magenta boxes and were prepared for the culture of shoots. The boxes with cultured shoots were transferred then to a growth chamber with 16 h of light and 8 h of darkness photoperiod at 22 to 25°C after an initial week in darkness. The number of roots and the mean of the highest roots were determined after 8 wk.

***Ex Vitro* Acclimation of Plantlets** The success rate of acclimation was calculated from the shoots originally regenerated from the leaf explants on 0.25 mg L^{-1} TDZ treatment and passed the rooting phase (in 0.5 mg L^{-1} NAA) before the acclimation. For this purpose, the rooted shoots reaching the lid of the glass jar (approximately 10-cm height) were taken out from the rooting media. The residual media was washed out from the roots to avoid fungi contamination. The plants were then transferred to the pre-irrigated small pots containing cocopeat, peat moss, and perlite (1:1:2) mixture. To gradually adapt the *in vitro*–grown plantlets to the greenhouse condition (26 ± 2 °C), each pot was covered by a transparent plastic cup for the first 5 d. From the second day on, the cover gets holes every day to allow the gas exchange and gradual exposure to the new environmental conditions. After the 5th day, the cover cup was totally removed, and plantlets were irrigated again.

Statistical Analysis After 8 wk of regeneration treatments, data have been collected in both types of explants of all three cultivars. The treatments were arranged as a completely randomized design factorial. Factors (and relative abbreviations), which had been applied, were mentioned in the "Materials and Methods" section. The arcsine-transformation was used to the percentage data to stabilize variance. Besides, for the data that were not normally distributed, the log-transformation was performed. Data were analyzed using the analysis of variance (ANOVA), and all statistical analysis was performed using the SAS v 9.4 software. Graphs were also drawn using Excel software.

Results

Effect of Cytokinin Treatments on the Direct Shoot and Microtuber Regeneration from *In Vitro* Leaf Explants The three-way interaction of cytokinin treatments, light conditions, and cultivars on direct shoot regeneration from leaf explants was significant ($p \leq 0.01$) as well as the interaction between cytokinin concentrations and cultivar on direct microtuber regeneration from leaf explants ($p \leq 0.01$). The treatment of leaf explants with 0.25 mg L^{-1} TDZ (and 0.1 mg L^{-1} NAA) in cultivar Pure White resulted in the highest shoot number(s) per leaf explant (27.1) in comparison to the other treatments (Fig. 1). This shoot number resulted from the leaf samples (Fig. 2*b*), which were transferred from the initial 4 wk of darkness to a photoperiod of 16-h light and 8-h darkness and kept for additional 4 wk (Fig. 2*c*).

The maximum microtuber regeneration from leaf explants (9.7) was observed in the treatment of cultivar Pure White on media supplemented with 2.0 mg L^{-1} BA or 0.25 mg L^{-1} TDZ (both containing 0.1 mg L^{-1} NAA). The microtuber regeneration was induced from leaf segments, which were kept for the entire 8 wk in darkness (Fig. 2*d*). The treatment of explants with 1.0 mg L^{-1} BA, 0.25 mg L^{-1} TDZ, or 2.0 mg L^{-1} BA showed the maximum regeneration percentage of 100, 100, and 91.66 in each leaf explant, respectively.

Effect of Cytokinin Treatments on Direct Shoot Regeneration and Microtuber Formation from *In Vitro* Tuber Explants According to the variance analysis, the interaction between the cytokinin concentrations and the cultivars on direct shoot regeneration from tuber cuts was significant ($p \leq 0.05$). The same interaction was significant ($p \leq 0.01$) on direct microtuber formation on initial tuber explants. The mean comparisons revealed that the treatment of cultivar Dark Violet in 1.0 mg L^{-1} BA (and 0.1 mg L^{-1} NAA) had the highest number of shoots (5.9) per tuber cut (Figs. 3 and 4*d*). According to the mean comparison, the treatment of tuber explant in 1.0 mg L^{-1} BA (and 0.1 mg L^{-1} NAA) in cultivar Pure White had the maximum new microtuber formation (17.1) per initial tuber explant in darkness (Figs. 3 and 4*e*).

Effect of Auxin Treatments on Root Formation of Regenerated Shoots The effects of auxin type on the average number and length of the roots ($p \leq 0.01$ and $p \leq 0.05$, respectively) were significant. Moreover, the effect of auxin concentrations on the average length of the roots was significant ($p \leq 0.01$), as well.

The treatment of shoots with 1.0 mg L^{-1} NAA resulted in the maximum number of roots (11.8) per shoot. In contrast, the treatment of shoots with 1.0 mg L^{-1} IAA with 2.09 roots per shoot showed the lowest average number. The NAA also resulted in the longest root length (0.86 cm) per shoot, and
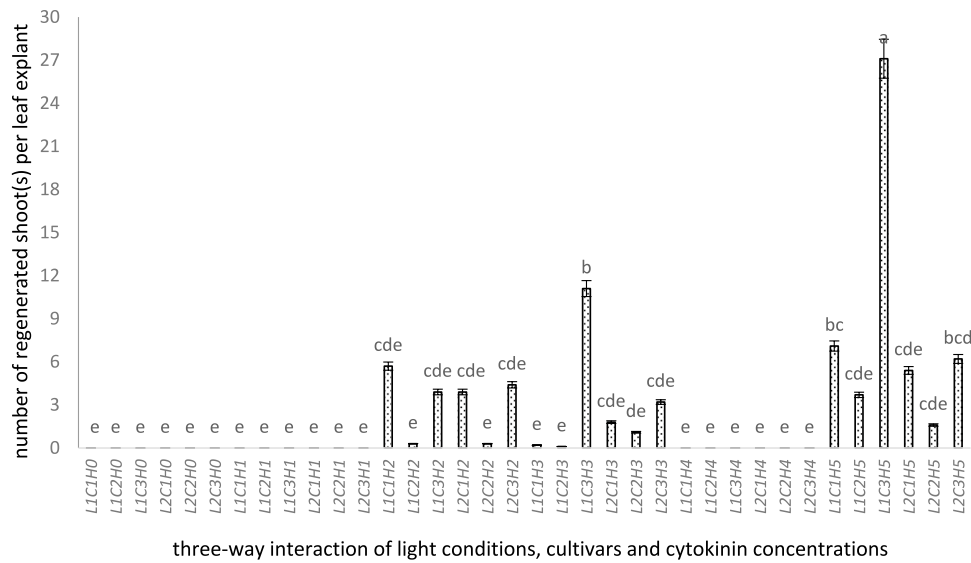
Figure 1. The means of the three-way interaction of light conditions, cultivars, and cytokinin concentrations on the average number of the regenerated shoot(s) per *in vitro* leaf explant of *Cyclamen persicum* Mill. Different *letters* on *top* of each *bar* indicate significant differences between treatments. Experiments established in ½ Murashige and Skoog medium and relative abbreviations are as follows: Hormonal conc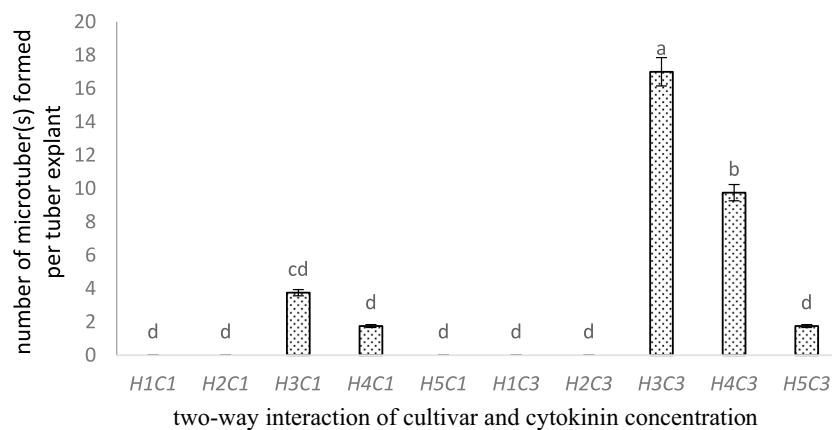entrations (mg L^{-1}): without hormone (H0), without cyto- kinin (H1), benzyladenine 1 (BA 1; H2), BA 2 (H3), BA 3 (H4), and thidiazuron 0.25 (TDZ 0.25; H5). All hormonal treatments are supplemented with 0.1 mg L^{-1} naphthaleneacetic acid (NAA), except for H0. Cultivars: Dark Violet (C1), Neon Pink (C2), and Pure White (C3). Light conditions: an initial 4 wk of darkness followed by 4 wk of a photoperiod of 16 h of light and 8 h of darkness (L1), and 8 wk of darkness (L2).
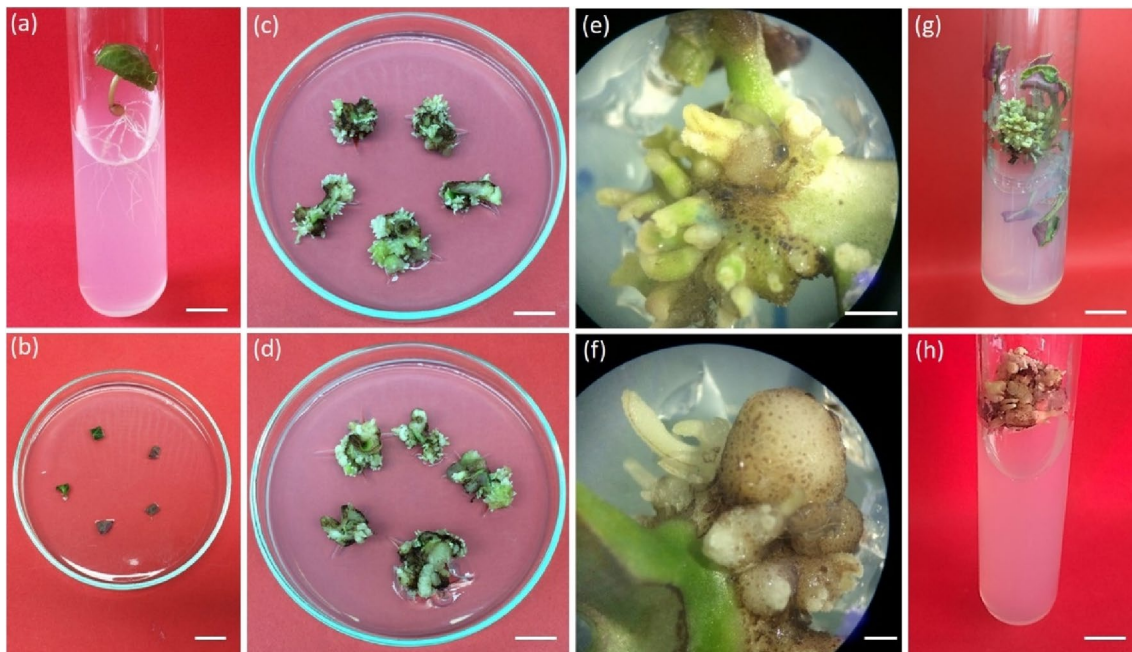
Figure 2. Direct shoot regeneration and microtuber formation from *in vitro* leaf explants of *Cyclamen persicum* Mill. cultivar Pure White. (*a*) 4-wk-old *in vitro* seedling; (*b*) *in vitro* leaf explants on the regeneration medium in Petri dishes; (*c*) regenerated shoots on leaf explants after 8 wk (the first initial 4 wk in darkness and the next 4 wk in a photoperiod of 16 h of light and 8 h of darkness); (*d*) regen- erated microtubers on leaf explants following 8 wk in darkness; (*e*) regenerated shoots under the stereo microscope, and (*f*) regenerated microtubers under the stereo microscope; (*g*) regenerated shoots transferred to the new media in test tubes; (*h*) regenerated microtu- bers transferred to the new media in test tubes. *Scale bars* = 1 cm in *a*, *b*, *e*, *f*, and 1 mm in *c* and *d*.

Figure 3. The means of two-way interaction of cultivar and cytokinin concentration on the average number of microtuber(s) formation per *in vitro* tuber explant of *Cyclamen persicum* Mill. All tuber segments were kept for 8 wk in darkness. Abbreviations shown in the *x-axis* are as follows: without cytokinin (H1), benzyladenine 1 (BA 1; H2), BA 2 (H3), BA 3 (H4), and thidiazuron 0.25 (TDZ 0.25; H5). All hormonal treatments are supplemented with 0.1 mg L^{-1} naphthaleneacetic acid (NAA). Cultivars: Dark Violet (C1) and Pure White (C3). The cultivar Neon Pink did produce enough *in vitro* seedling tubers, as the explant source, at the beginning of the experiment and therefore has been excluded from this experiment.

the IAA showed the shortest (0.45 cm) one. However, the maximum length of the roots (0.92 cm) per shoot was 0.5 mg L^{-1} of each examined auxin.

***Ex Vitro* Acclimation of Plantlets** The rooted shoots reaching approximately 10-cm height could successfully acclimate to the soil and greenhouse conditions (Fig. 5). In the current study, out of 300 rooted young plantlets, 174 plants were adapted to the greenhouse environment. Those were phenotypically similar to the plants grown from the cultivation of F1 hybrid seeds.

Discussion

The use of aseptic plantlets of *C. persicum* has been investigated in many studies as the source of explants for shoot regeneration (Bach *et al.* 1998; Karam and Al-Majathoub 2000a; Abu-Qaoud 2004; Prange *et al.* 2008). Nevertheless, there is barely a report available dealing with direct regeneration from *in vitro* explants (studied mainly in (Abu-Qaoud 2004)). Most of the published reports exhibit indirect regeneration passing a callus induction phase, which may lead to undesired somaclonal variations.

Figure 4. Direct shoot regeneration and microtuber formation from *in vitro* tubers of *Cyclamen persicum* Mill. (*a*) cuttings of *in vitro* tuber on regeneration medium; (*b*) regenerated shoots on tuber cuttings in cultivar Dark Violet; (*c*) formation of microtubers on tuber cuttings in cultivar Pure White; (*d*) regenerated shoots under the stereo microscope; and (*e*) new microtubers under the stereo microscope. *Scale bars* = 1 cm in *a*, *b*, *c*, and 1 mm in *d* and *e*.
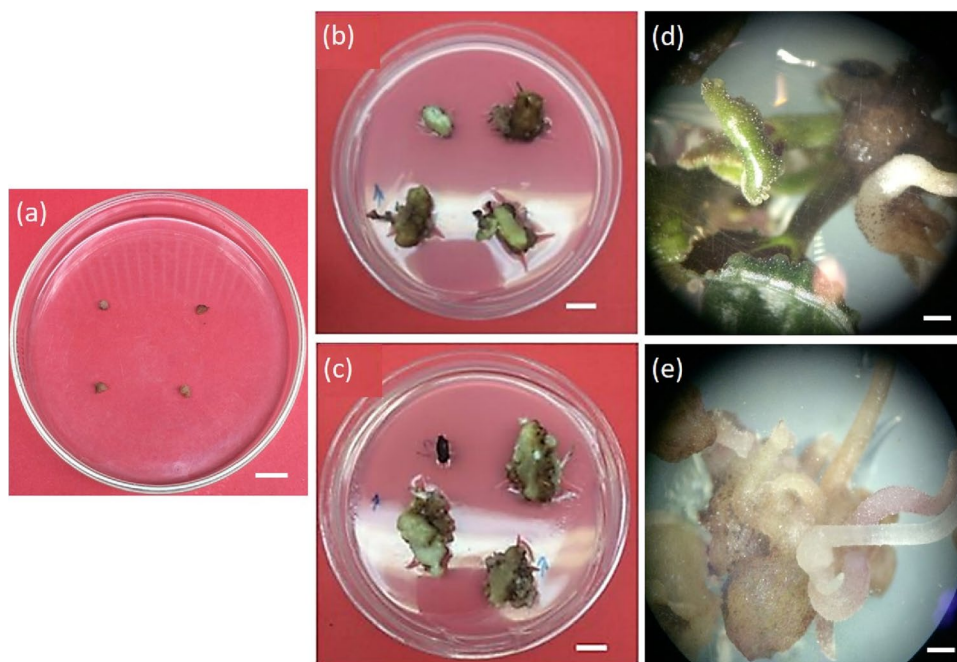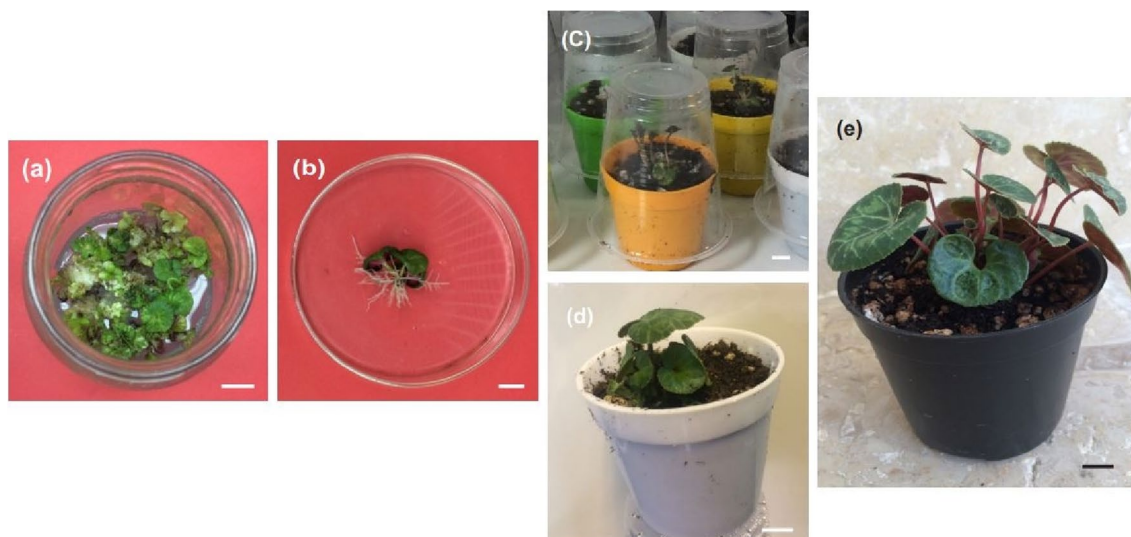
Figure 5. Acclimation of rooted shoots of *Cyclamen persicum* Mill. to the soil and greenhouse conditions. (*a*) Regenerated shoots rooting in a glass jar containing appropriate medium; (*b*) a single rooted shoot ready for transferring to the soil; (*c*) acclimating plantlets; (*d*) and (*e*) acclimated plantlets of two different ages. *Scale bar*=1 cm.

Direct Shoot and Microtuber Regeneration from *In Vitro* Leaf Explants The *in vitro* leaf explants of *C. persicum* were able to differentiate in ½ MS media supplemented with TDZ or BA hormones. The proportion of explants that had directly developed shoot buds often exceeded 95%. However, the most structures regenerated on leaf explants that have been carefully examined were shoot buds showing the lateral leaves initials under the stereo microscope (Fig. 2*e*). These structures were obviously distinct from the somatic embryos that differentiate usually from callus in the presence of 2,4-D and pass the four developmental stages of globular, heart, torpedo, and cotyledon (Wicart *et al*. 1984), although the presence of embryo structures cannot be fully excluded.

TDZ treatments gave a significantly higher number of direct shoot regeneration from leaf segments than BA-supplemented media. The highest regenerated shoot number was obtained with 0.25 mg L^{-1} TDZ (and 0.1 mg L^{-1} NAA), which produced 27.1 shoots per leaf explant in the cultivar Pure White. Four weeks of a photoperiod of 16 h of light and 8 h of darkness followed by 4 wk of darkness resulted in the regeneration of shoots but not microtuber, from leaf explants of all three examined cultivars. In contrast, the leaf segments treated with the same cytokinin incubated for 8 wk in darkness showed microtuber regeneration on which the shoots emerge later on in investigated cultivars. BA has been reported before as an effective cytokinin for shoot regeneration from leaf sections in the *C. persicum* plant but with a lower regeneration measure than TDZ (Abu-Qaoud 2004). TDZ has been used to induce shoot regeneration in leaf sections of several plant species as well (Parveen and Shahzad 2010; Ahmed and Anis 2012). Additionally, it has been

used to induce callus production from isolated protoplast of *C. persicum* (Morgan 1998). The current study confirmed the stimulating effect of TDZ on shoot formation from leaf explants. A similar effect was reported by Abu Qaoud (Abu-Qaoud 2004). Moreover, the current study confirmed a much higher average of regenerated shoots per leaf segment (27.1) in comparison to the mentioned report (10.8).

BA exhibited a significant effect on the microtuberization of leaf explants in cultivars Neon Pink and Pure White (the maximum number of new microtuber formations (9.7) per initial leaf segment). A similar effect was reported in a study before but in a much lower measure than the current study. In the previous work, the average number of tuberous structures produced per leaf explant ranged from 2 to 3 (Abu-Qaoud 2004).

In the current study, no shoot regeneration was observed in the control media without TDZ or BA. The same result has been reported before (Karam and Al-Majathoub 2000b) and, similarly, the explant type, the cytokinin concentration, and their dual interaction had significant effects on the percentage of the shoot regeneration and the number of shoots per explant (Karam and Al-Majathoub 2000b). One of the differences between BA and TDZ is the potential of TDZ in the induction of shoot regeneration even in darkness, while BA can induce shoot regeneration more effectively in light (Mundhara and Rashid 2002). This is in agreement with the result of the current study in which BA was not as efficient as TDZ in shoot regeneration from leaf segments. This might be due to the initial 4-wk darkness period in all regeneration experiments.

Different cultivars have shown different responses to hormonal treatments for direct shoot regeneration and

microtuber formation. The reason could rely on distinctive internal hormonal balance and regulation in each cultivar (Mariotti *et al.* 2021).

Direct Shoot Regeneration and Microtuber Formation from *In Vitro* Tuber Cut Explants

In the current study, the highest number of shoots per *in vitro* tuber explant was observed in 1.0 mg L^{-1} BA (and 0.1 mg L^{-1} NAA) in cultivar Pure White. Other studies have also reported the effectiveness of BA for shoot regenerations from *in vitro* tubers (Fukui *et al.* 1988; Takamura and Miyajima 1997). Increasing BA concentration (to 3.0 mg L^{-1}) resulted in a significant reduction in the number of shoots regenerated from tuber cut explants. Microtubers could be successfully induced in both types of explants (leaf and tuber) of *C. persicum*, although the higher potential of shoot regeneration in the current study was related to the leaf explants compared to the tuber explants. It was shown that 8-wk incubation of initial tuber cut explant in darkness resulted in the formation of new microtubers (Fig. 4*e*) on which the shoot buds appeared later. Although the initial emerging structures may look callus-like, a closer look at the tissue proves the presence of unipolar tuberous structures that have been reported earlier in *C. persicum* (Wicart *et al.* 1984).

Root Formation of Regenerated Shoots in the Presence of NAA

Root formation was observed in shoots that had been transferred to auxin (only)-supplemented media. The ½ MS medium supplemented with 0.5 or 1.0 mg L^{-1} NAA showed the highest average numbers and the lengths of the roots. It has been reported that rooting of *C. persicum in vitro* shoots in the media supplemented with 0.5 or 1.0 mg L^{-1} auxin was successful (Karam and Al-Majathoub 2000b). This also proves the result in the current study from the rooting experiment.

Ex Vitro Acclimation of Plantlets

In the current study, more than 58% of rooted young plantlets were successfully adapted to the greenhouse conditions. Young plantlets were kept for 6 wk in the soil having 3 to 5 newly developed leaves and were not apparently distinct from plants grown from the cultivation of F1 hybrid seeds. It implies that the absence of the callus induction step may well help in retaining true-to-type plants without the need for germinating the F1 hybrid seeds in each propagation season. Moreover, the removal of the individual seed germination phase for each micropropagation round of *C. persicum* will save a lot the time and the cost of production.

Conclusions

In this study, the direct shoot regeneration, microtuber propagation, and microtuber regeneration from *in vitro* explants of *C. persicum* were described in a higher measure than reported before for other cultivars. Shoot and microtuber regeneration was observed in all three cultivars of Pure White, Neon Pink, and Dark Violet from aseptic leaf or tuber explants but in different efficiencies due to differences in cytokinin types and concentrations. This system of direct (shoot or microtuber) regeneration without passing the callus induction phase could be very advantageous for micropropagation and transformation approaches of commercial cultivars in *C. persicum.*

Declarations

Conflict of Interest The authors declare no competing interests.

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