The establishment of effective regeneration system and *in vitro* culture of *Campanula*

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Abstract
Several species of *Campanula* genus have been cultivated as ornamental plants, and there is an increasing demand for more attractive and novel characteristics. Optimal regeneration protocol is prerequisite for subsequent successful genetic manipulation and generation of new features. In current study the optimal media composition and explant source for shoot regeneration of three *Campanula* cultivars, *C. portenschlagiana* cvs. Blue Ocean (BO) and Royal; *C. carpatica* cv. Improved Blue Uniform (IBU) were determined. The focus of this study was based on avoiding over-supplementation of plant growth regulators (PGRs) especially cytokinins (TDZ). The explant which were examined for shoot regeneration were petioles harvested from two different explant sources; 1) Petioles harvested of three months old tissue-cultured plants, 2) Petioles harvested of etiolated nodal cuts. Furthermore various media compositions have been tested to find out the best composition for shoot regeneration of three cultivars and different NAA (0.5, 1.0 and 2.0 mg L^{-1}) and TDZ (1.0, 2.0, 3.0 and 5.0 mg L^{-1}) concentrations were investigated. According to the results, the most favorable explant source for harvesting petioles were nodal cuts of mature greenhouse plants. The best shoot regeneration obtained from the harvested petioles of nodal cuts in the three cultivars were, 85% IBU, 65% BO and 45.8% Royal.
Keywords**:** shoot regeneration, explant source, TDZ concentration

1. Introduction

Effective regeneration protocol is the prerequisite for subsequent successful genetic manipulation to answer the increasing request for more attractive and new characteristics in ornamental plants. Although approximately 400-600 species exist in the *Campanula* genus (Kuss et al., 2007), only a few species have been the focus of regeneration research (Brandt, 1992; Sriskandarajah et al., 2001; Frello et al., 2002; Joung et al., 2002; Sivanesan et al., 2007; Sivanesan et al., 2011). One of the most critical factors affecting shoot regeneration for *in vitro* culture establishment is the explant source. Although juvenile tissue are generally favored as explant source, mature explant source are required for certain plants (Bonga and von Aderkas, 1992). In some plants due to sterility (Lewis and Lynch, 1989) or relatively high contamination and low germination of seeds in *in vitro* conditions (Gargul, 2006) use of mature tissue as explant source is inevitable. Because of the recalcitrant habit of mature plants, it is necessary to determine which strategy is optimal for overcoming this obstacle. Tissue reinvigoration and the mitigation of recalcitrance in some plants can be forced by the application of PGRs[1] in the culture medium (Murthy et al., 1998) or by the use of a hormone-free medium for some species (Ewald, 1998). Employing adult plants as the source of nodal cuts was previously explored in the *Campanula* species (Sriskandarajah et al., 2008, Paunescu, 2010). According to the current study's knowledge, although the preparation of adult plants as a source of nodal cuts and explants in the *Campanula* species has been performed by the addition of PGRs to the media, a hormone-free medium has not yet been employed for rejuvenation. TDZ[2] is a hormone that effectively promotes shoot regeneration but some disadvantages have been observed when it is used at high concentrations (Lin and

[1] Plant Growth Regulators
[2] Thidiazuron

Chang, 1998). Joung et al. (2002) applied BA[3] to induce shoot formation from *C. glomerata* leaf blades while omitting TDZ in the regeneration media; that study based on its findings, recommended the use of TDZ to increase the regeneration rate and induce shoot formation within a shorter time.

In the *Campanula* species, an increase in the concentration of TDZ up to 10 mg l^{-1} induced shoot regeneration, whereas higher amounts of TDZ significantly reduced shoot regeneration and promoted increased callus formation. In another work, when greenhouse-grown plants were used as a source of explants, all of the explants failed to regenerate shoots (Sriskandarajah et al., 2008). Frello et al. (2002) found a reduced regenerate rate in *C. carpatica* when cotyledons were utilized via the callus phase. Therefore, the manipulation of published *in vitro* culture protocols for the purpose of decreasing hormone application in media is crucial, specifically when using a mature explant source is crucial. In the present work, different explant sources and a variety of concentrations and combinations of PGRs are investigated with the goal of improving shoot regeneration in three *Campanula* species by using a minimal concentration of PGRs particularly TDZ.

2. Materials and methods

2.1. Plant materials

The adult plants of two *Campanula* species, *C. portenschlagiana* cultivars BO[4] and Royal and *C. carpatica* cv.[5] IBU[6], were grown in an experimental greenhouse whose temperature was maintained at 18-22˚C under 337 µmol m^{-2} s^{-1} light. Short-day conditions (8 h light) were simulated to keep the plants in a vegetative stage for the collection of nodal cuts.

2.2. Media optimization for nodal cut shoot growth

Fresh shoots were harvested from all three *Campanula* cultivars that were grown under the present study's greenhouse conditions (see 2.1) and disinfected as described by Sriskandarajah et al. (2008). Each explant was prepared with an 8-10 mm inter-node and one or two nodes. All media were based on MS[7] macro and microelements (Murashige and Skoog, 1962) and prepared as defined by Ghayoor et al. (2015). Two PGR-containing media, 0.5 mg l^{-1} BA and 10 mg l^{-1} TDZ, both supplemented with 0.25 mg l^{-1} NAA[8] (Duchefa, Haarlem, the Netherlands), and one hormone-free medium was compared. In each treatment condition, 6 replicates of 4 explants each were tested. Nodal cuts were grown in the dark at 24˚C. The data were collected after 2 weeks.

2.3. Preliminary study on medium composition for shoot regeneration

To establish the *in vitro* micropropagation of *Campanula*, utilizing mature plants as explant source we have followed the protocol optimized by Sriskandarajah et al. (2008) and applied the described optimum shoot regeneration medium. According to the mentioned study 10 mg l^{-1} TDZ in combination with 0.25 mg l^{-1} NAA was assessed in cv. Royal. 20 petri dishes, each consisting of 4 explants, were investigated.

2.4. Explant source and media composition for shoot regeneration

2.4.1. Explant sources and cytokinin (TDZ) levels

Two different petiole sources were compared in terms of the shoot regeneration capacity in all investigated cultivars. The first type of explant was comprised of petioles harvested directly from plants cultured *in vitro* under 16 hours of 32-48 µmol m^{-2} s^{-1} light and 24 ± 1˚C. The plants cultured *in vitro* were maintained for approximately 3 months in tissue culture conditions. The second type of explant was comprised of petioles that were prepared from two-week-old shoots grown *in vitro* (darkness, 24 ± 1˚C) and derived from the nodal cuts of greenhouse-grown plants. The MS medium was supplemented with 1.0, 2.0 or 3.0 mg l^{-1}

[3] Benzyl Adenine
[4] Blue Ocean
[5] Cultivar
[6] Improved Blue Uniform
[7] Murashige and Skoog
[8] Naphthaleneacetic acid

TDZ. All of the media contained 0.1 mg l^{-1} NAA. Five replicates, each consisting of 4 explants, were investigated. The petioles were maintained in the dark at 24 ± 1˚C to regenerate shoot, and the data were collected after 7 weeks.

2.4.2. Explant sources and auxin (NAA) concentrations

To determine the optimal explant sources for shoot regeneration, two types of petiole sources (described in the previous experiment) were assessed in cv. Royal. A combination of two cytokinins, 5.0 mg l^{-1} TDZ and 5.0 mg l^{-1} BA, supplemented with 0.5, 1.0 or 2.0 mg l^{-1} NAA was used to promote shoot regeneration. In each treatment, 6 replicates of 4 explants each were examined.

2.5. Statistical analysis

The data were subjected to ANOVA by the use of the MSTATC software (Michigan State University, version 1.42). The mean values were separated according to the least significant difference test (LSD) at an alpha level of 5% ($P \leq 0.05$).

3. Results

3.1. Optimization of media for growing nodal explants

The experiment was designed to clarify the effects of growth regulators on the growth of axillary shoots derived from nodal cuts. As fig. 1 shows, in cv. BO supplementation of the medium with 10 mg l^{-1} of TDZ or 0.5 mg l^{-1} BA did not significantly increased the growth of axillary shoots from nodal cuts compared to the hormone-free medium. The lack of significant differences among the tested media indicated that a hormone-free medium is just as effective as the media containing PGRs. In cv. Royal (Fig. 1), no significant differences were observed between the number of shoots from the nodal cuts that were cultivated and regenerated in a hormone-free medium and the nodal cuts cultured in a medium with 10 mg l^{-1} TDZ. In cv. IBU (Fig. 1), the growth of nodal cuts from media containing TDZ or BA was 100%, whereas in the hormone-free medium there was only a 72.1% shoot growth.

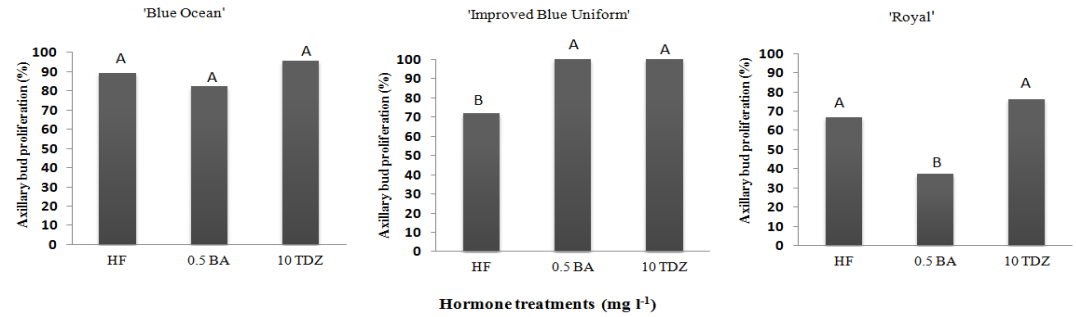

Fig 1 The effect of different media supplemented with growth regulators (mg l^{-1}) and hormone-free (HF) medium on the growth of nodal cuts axillary shoots in three *Campanula* cultivars. The columns marked with same letter within the same chart are not significantly different at $\alpha = 0.05$.

3.2. Preliminary study on shoot regeneration

The purpose of this experiment was to assess the medium composition recommended by Sriskandarajah et al. (2008). 40% of the nodal explants regenerated shoots, 7 weeks after culture but 78.12% of shoots were deformed (Fig. 2e).

Fig 2 Regenerated shoots 6, 7 and 8 weeks after culture (**a**, **b** and **c**), complete developed healthy plants regenerated in 1 mg l^{-1} TDZ (**d**), abnormal regenerated shoots in 10 mg l^{-1} TDZ (**e**).

3.3. Effects of explant source and regeneration media on shoot regeneration

3.3.1. Explant sources and TDZ levels on the shoot regeneration (%)

In the present experiment, two explant sources and three TDZ concentrations were compared. The experiment was designed with a focus of regenerating healthy shoots. In all three tested cultivars, the nodal cuts from greenhouse-grown plants represented a more favorable source for petioles compared to petioles from plants that were cultivated under *in vitro* conditions (Fig. 3). In cvs. BO and Royal, no shoot regeneration was observed from the petioles of tissue culture plants that were grown in a medium containing 1 mg l^{-1} TDZ. On the other hand, cv. IBU 35% of the petioles regenerated shoots in such medium. In cv. Royal, no shoot regeneration occurred in any of the three TDZ concentrations from petioles harvested from plants grown *in vitro* (Fig. 3). Application of higher level of TDZ up to 3.0 mg l^{-1} TDZ enhanced maximum shoot regeneration in all three cultivars from both explant sources.

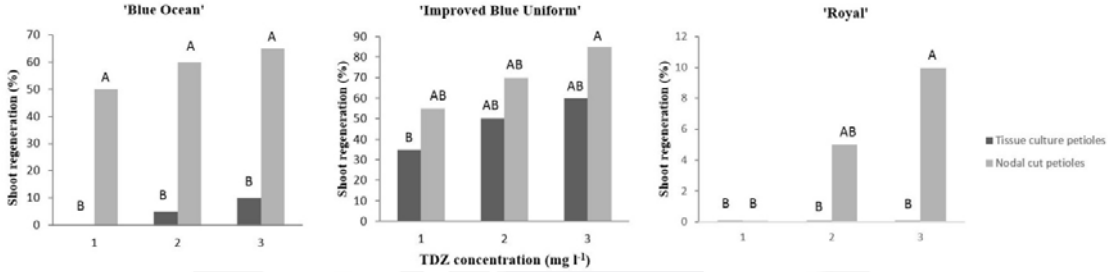

Fig 3 A comparison of the shoot regeneration of petioles harvested from two different explant sources in media containing different PGR combinations. Columns marked with same letter within the same chart are not different significantly at α = 0.05.

All regenerated plants in three cultivars were healthy (Fig 2a, b and c) and no deformed or abnormal plants were detected. The regenerated plantlets were transferred to greenhouse for acclimatization after rooting (Fig. 2d).

3.3.2. Explant sources and NAA concentrations on the shoot regeneration (%)

The results identified an optimal explant source and NAA concentration in a medium to achieve an optimal regeneration in cv. Royal. The use of petioles harvested from the nodal cuts of greenhouse-grown plants significantly improved the shoot regeneration. In contrast, petioles harvested from tissue-culture plants resulted in poorer shoot regeneration in all three media. Increasing the NAA concentration of medium augmented the shoot regeneration efficiently, and using 2.0 mg l^{-1} NAA in combination with 5.0 mg l^{-1} TDZ and 5.0 mg l^{-1} BA boosted the regeneration of shoots from petioles to 45.8% (Fig. 4). The current study's cv. Royal shoot regeneration results confirmed the advantage of using petioles harvested from nodal cuts over petioles from tissue-cultured plants.

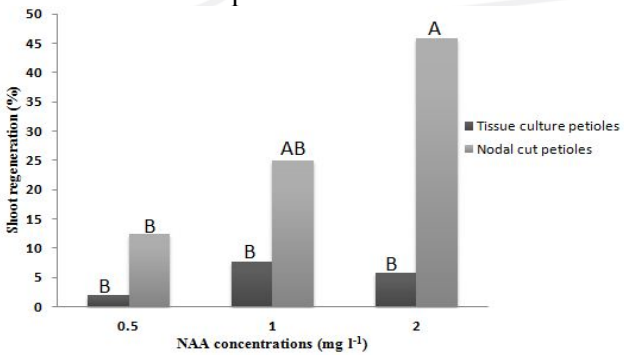

Fig 4 Comparison of effects of two different petiole sources and three NAA concentrations on shoot regeneration in cv. Royal. All media were supplemented with 5.0 mg l^{-1} TDZ and 5.0 mg l^{-1} BA. The columns marked with same letter are not significantly different at α = 0.05.

4. Discussions

To establish nodal cuts, variety of cytokinin and auxin combinations have previously been examined (Alderete et al., 2006, Sriskandarajah et al., 2008, Musallam et al., 2011). The current study's results demonstrated that in cvs. Royal and BO, the use of PGRs did not enhance nodal cut growth in comparison with the hormone-free medium (Fig. 1). By

application of hormone-free medium for reinvigoration the mature nodal explants, the side effects of utilizing media supplemented with high PGRs concentration such as regeneration of abnormal shoots (Fig. 2) were avoided. In previous studies by Sriskandarajah et al. (2008) and Meng et al. (2004) over-supplementation of PGRs was reported as main reason for failure of shoot regeneration. Generally, after the rejuvenation of mature tissues, invigorated explant sources have been favored for harvesting explants compared to the explants from *in vitro* tissue-cultured plants. Our results confirmed the preference of application of invigorated nodal explants compared to three-month-old tissue-cultured plants as explants source (Fig. 3 and Fig. 4). This preference attributed to several underlying factors such as advantages of employing etiolated tissues as explants sources (Li et al, 2009) or loss of cells totipotency due to long-term culture (Bhojwani and Razdan, 1996). Joung et al. (2002) highlighted the contamination problems encountered with *C. glomerata* using material derived from greenhouse-grown plants. Since in the present work, to utilize mature tissues the invigoration phase prior the shoot regeneration was performed, infected shoots were eliminated during the establishment of nodal cuts and healthy grown shoots were chosen for the harvesting of petioles. These advantages can explain the favorability of using nodal cuts.

Sriskandarajah et al. (2001) previously demonstrated that BA induced twice as much callus formation as TDZ in *C. carpatica* and that TDZ promoted shoot regeneration 6 times more efficiently than BA. Furthermore TDZ can enhance shot regeneration in relatively shorter time compared to BA (Joung et al. 2002). Therefore application of TDZ to promote shoots in *Campanula* in high efficiency is necessary.

In several plants, TDZ elicited a stronger effect on regeneration compared to other cytokinins, such as BA (Visser et al. 1992; Aasim et al. 2009). In cv. Royal combination of TDZ and BA promotes the best shoot frequency. Increscent the level of TDZ up to 3 mg l^{-1} increased the frequency of shoot regeneration but application of 10 mg l^{-1} TDZ for shoot induction in cv. Royal decreased the shoot regeneration frequency and caused formation of 78.12 % abnormal shoots. Same results were obtained by Parveen and Shahzad (2010), when supplemented TDZ of shoot regeneration medium were increased (>2.5 μM); the shoot regeneration frequency was decreased. Effect of NAA on induction of shoots was confirmed in several plants; in *Vanda coerulea*, shoot regeneration frequency was higher in media supplemented with a combination of TDZ or BA with NAA when compared to TDZ or BA alone (Jitsopakul et al. 2013). Increasing the NAA concentration decreased the shoot regeneration frequency in *Campanula* cv. Royal, same results were observed in shoot regeneration of

5. Conclusion

The current work described, the efficient method of applying mature *Campanula* plants as explant source with focus on reducing the PGRs content of medium and decreasing the exposure time of explants to the hormones. We are recommending the elimination of the PGRs in nodal explant's culture medium based on the obtained results; that showed no significant advantages of applying PGRs in comparison with hormone-free medium. Also our findings showed that application of lower TDZ concentration for shoot regeneration compared to previous study (Sriskandarajah et al., 2008) results in better shoot regeneration efficiency along with lower costs of commercial operation and obtaining healthy plants.

6. References

Aasim M., Khawar K. M., Ozcan S. (2009). Comparison of shoot regeneration on different concentrations of thidiazuron from shoot tip explant of cowpea on gelrite and agar containing medium. Not Bot Horti Agrobot Cluj-Na, 37: 89-93.

Alderete, L.M., Mori, M., Kato, A., Escandon, A.S., 2006. Establishment of an *in vitro* micropropagation protocol for *Mecardonia tenella*. Electron. J. Biotechno. 9, 263-266.

Bhojwani, S.S., Razdan, M.K., 1996. Plant tissue culture: theory and practice, a revised edition. Elsevier, pp. 125-166.

Bonga, J.M., von Aderkas, P., 1992. Rejuvenation of tissues from mature conifers and its implications for propagation *in vitro*. in: *In vitro* culture of tress, Clonal Forestry I. Ahuja M-R., Libby W. (Eds). Springer, Berlin Heidelberg, pp.182-199.

Brandt, K., 1992. Micropropagation of *Campanula isophylla* moretti. Plant Cell Tiss. Org. 29, 31-36.

Ewald, D., 1998. Advances in tissue culture of adult larch. In Vitro Cell. Dev. _Pl. 34, 325-330.
Frello, S., Stummann, B.M., Serek, M., 2002. Shoot regeneration of *Campanula carpatica* Jacq. (Campanulaceae) via callus phase. Sci. Hortic.-Amsterdam 93, 85-90.
Gargul, J., 2006. Germination. regeneration and transformation studies in *Campanula poscharskyana* and *Campanula portenschlagiana*. Dissertation, University of Agriculture in Krakow, Poland.
Ghayoor Karimiani, Z., Serek, M. Mibus, H., 2015. Transformation of *Campanula carpatica* to alter flower colour and ethylene sensitivity. J. Hortic. Sci. Biotech. 90, 518-524.
Jitsopakul N, Thammasiri K, Ishikawa K (2013) Efficient adventitious shoot regeneration from shoot tip culture of *Vanda coerulea*, a Thai orchid. Sci. Asia. 39, 449-455.
Joung, Y.H., Liao, M.S., Roh, M.S., Kamo, K., Song, J.S., 2002. *In vitro* propagation of *Campanula glomerata*, 'Acaulis' from leaf blade explants. Sci. Hortic.-Amsterdam. 92, 137-146.
Kuss, P., Aegisdottir, H.H., Stocklin, J., 2007. The biological flora of central Europe: *Campanula thyrsoides* L. Perspect. Plant Ecol. 9, 37-51.
Lewis, P., Lynch, M., 1989. Campanulas. Christopher Helm, London.
Li, D.L., Tan, B., Duan, Y.X., Guo, W.W., 2009. Regeneration of transgenic citrus plants from the trimmed shoot/root region of etiolated seedlings. Biol. Plantarum. 53, 578-582.
Lin, C.S., Chang, W.C., 1998. Micropropagation of *Bambusa edulis* through nodal explants of field-grown culms and flowering of regenerated plantlets. Plant Cell Rep. 17, 617-620.
Meng, R., Chen, T.H.H., Finn, C.E., Li, Y., 2004. Improving *in vitro* plant regeneration from leaf and petiole explants of `Marion' blackberry. HortScience. 39, 316-320.
Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plantarum. 15, 473-497.
Murthy, B.N.S., Murch, S.J., Saxena, P.K., 1998. Thidiazuron: A potent regulator of *in vitro* plant morphogenesis. In Vitro Cell. Dev. _Pl. 34, 267-275.
Musallam, I., Shibli, M., 2011. Micropropagation of Caper (*Capparis spinosa* L.) from wild plants. Funct. Plant S. Biotechno. 5, 17-21.
Paunescu, A., 2010. *In vitro* propagation of *Campanula polymorpha* Witas. An endemic plant of carpathian mountains. Analele Universitatii din oradea-Fascicula Biologie. 17, 235-238.
Parveen S., Shahzad A. (2010). TDZ-induced high frequency shoot regeneration in *Cassia sophera* Linn. via cotyledonary node explants. Physiol Mol Biol Plants, 16: 201-206.
Sivanesan, I., Lim, M., Jeong, B., 2011. Somatic embryogenesis and plant regeneration from leaf and petiole explants of *Campanula punctata* Lam. var. rubriflora Makino. Plant Cell Tiss. Org. 107, 365-369.
Sivanesan, I., Lee, Y.M., Song, J.Y., Jeong, B.R., 2007. Adventitious shoot regeneration from leaf and petiole explants of *Campanula punctata* lam. var. rubriflora makino. Propag. Ornam. plants. 7, 210-215.
Sriskandarajah, S., Frello, S., Serek, M., 2001. Induction of adventitious shoots *in vitro* in *Campanula carpatica*. Plant Cell Tiss. Org. 67, 295-298.
Sriskandarajah, S., Mibus, H., Serek, M., 2008. Regeneration and transformation in adult plants of *Campanula* species. Plant Cell Rep. 27, 1713-1720.
Visser C., Qureshi J. A., Gill R., Saxena P. K. (1992). Morphoregulatory role of thidiazuron: substitution of auxin and cytokinin requirement for the induction of somatic embryogenesis in geranium hypocotyl cultures. Plant Physiol, 99: 1704-1707.