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EXPERIMENTAL PAPER

Effect of plant growth regulators and explant types on induction and growth of callus of *Primula veris* L.

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Summary

Primula veris L. (*Primulaceae*) is a well-known medicinal herb. The callus induction response of three explant types: roots, cotyledons, and hypocotyls of four-week-old cowslip seedlings were evaluated. The highest statistically different callus induction rate was 93.6% and was obtained from root explants on MS medium supplemented with 0.1 mg/l BA and 5.0 mg/l PIC. Calli also appeared on 83.3% of cotyledon explants on MS medium supplemented with 1.0 mg/l BA and 3.5 mg/l 2,4-D and on 81.0% of root explants on MS medium supplemented with 0.1 mg/l KIN and 2.0 mg/l 2,4-D. These values were not statistically different. The average time required for callus initiation was 4 to 6 weeks, however, it depended on the explants type. The most suitable condition for callus proliferation and growth was MS medium with 0.5 mg/l TDZ and 0.1 mg/l NAA, and with 1.0 mg/l BA and

2.0 mg/l or 3.5 mg/l 2,4-D. No light conditions proved to be more favourable for cowslip calli induction and growth.

Key words: *cowslip, plant growth regulators, callus induction, proliferation*

INTRODUCTION

Primula veris L. (*Primulaceae*) is a source of valuable pharmaceutical raw materials that are used for the production of expectorant and diuretic drugs because of its high content of triterpene saponins and phenolic glycosides in its rhizomes and roots. The presence of several methoxyflavones in its flowers and leaves was also reported [1-5]. Presence of similarly rare flavones from the glandular leaf exudates of *P. elatior* was reported as well [6]. Some of them have cytostatic, antimutagenic, and anticarcinogenic activities [7-10]. Extracts of lipophilic flavones derived from the leaves of *P. veris* were found to influence the viability and migration of HeLa cells that are overexpressing constitutively active PKCe [11-12]. Its main component, zapotin, was found to be an antiproliferative compound, which may provide promising results in the treatment of cancer with overexpressed levels of PKCe [13].

Primula veris is a rare species in many countries and is protected by law. Additionally, collection of cowslip raw materials from natural resources does not guarantee high quality, as concentrations of the biologically active compounds, similarly to many other medicinal plants, are very often not stable [14-15]. Plant tissue cultures are an alternative and effective way to obtain high quality raw materials. Hitherto, *Primula vulgaris,* [16-17], *P. elatior,* [16], *P. obconica,* and *P. malacoides* [18] have been successfully propagated from calli. *In vitro* regeneration of *P. veris* from a seedling soot apex was also described [19] .

The aim of our study was (1) to investigate the effect of plant growth regulators and explants type on calli induction and (2) to develop an efficient protocol for the production of high frequency calli of *P. veris*.

MATERIALS AND METHODS

The origin of cowslip seeds, as well as the protocol of sterilization and germination of the seeds from which the seedlings were cultured *in vitro*, have been described [19]. Cotyledon, hypocotyl and root explants of four-week-old seedlings have been used. The sterile explants were placed on 20 ml of MS medium [20] or B5 medium [21] in 6 cm diameter 100-ml Erlenmeyer flasks. Six plant growth regulators (PGRs), benzyladenine (BA), kinetin (KIN), thidiazuron (TDZ), 2,4-dichlorophenoxyacetic acid (2,4-D), picloram (PIC), and 1-naphthaleneacetic acid (NAA) in different concentrations were tested (tab. 1). The callus induction response was evaluated for 60 explants of each type and was examined in three replications.

Table 1.

Callus induction rate (%) in dependence of explant type and the applied medium

Medium number	Medium/PGR [mg/l]	Seedling explants		
		Cotyledons	Hypocotyls	Roots
1	MS-1/KIN 0.1 2,4-D 2.0	15.4 ij*	23.5 hi	81.0 b
2	MS-2/BA 0.1 PIC 5.0	18.5 ij	71.4 bc	93.6 a
3	MS-3/BA 0.1 2,4-D 10.0	8.5 j	49.0 ef	71.4 bcd
4	MS-4/BA 1.0 2,4-D 2.0	47.2 efg	45.0 efg	67.5 cd
5	MS-5/BA 1.0 2,4-D 3.0	58.3 cde	45.0 efg	33.3 gh
6	MS-6/BA 1.0 2,4-D 3.5	83.3 b	57.1 def	50.0 ef
7	B5/TDZ 2.0 2,4-D 4.0	12.5 ij	14.3 ij	42.9 fg

* – means followed by the same letters are not significantly different at $p=0.05$

All media were supplemented with 3.0% sucrose and were solidified with 0.8% Difco Bacto Agar. Medium pH was adjusted to 5.6 before autoclaving at 121°C for 20 min. Cultures were maintained in the growth chamber in darkness or under a 16-h photoperiod (cool-white fluorescent light lamps, 60 μM m^{-2} s^{-1}) at 21±2°C.

Callus formation frequency was calculated after 6 weeks of culture inoculation. The obtained data were submitted to two-way ANOVA. Prior to statistical analysis the obtained percentage data were arcsine transformed (Z=asin (X)), where X was the percentage of callusing explants in each sample, and submitted to Lilliefors (p>0,05) and Levene (p>0,05) tests to assess the ANOVA assumptions of normality and homoscedasticity. Mean separation was conducted by post-hoc Tukey's test ($p=0.05$).

RESULTS

The average time required for the callus induction from the examined explant types was 4 to 6 weeks of culture in darkness. The callus induction rate differed depending on the explant types, hormone combinations, and their interactions (tab. 1, 2). ANOVA showed that the combination of 0.1 mg/l BA and 5.0 mg/l PIC (MS-2) prompted the highest percentage of callus induction (93.6%) from roots and that response was significantly different from those on other several media. The high rate of callus induction from the root explants was recorded on KIN 0.1 mg/l and 2,4-D 2.0 mg/l in MS-1 medium (81.0%). The callus could also be induced from

hypocotyls and cotyledons in media containing different concentrations of BA and PIC or 2,4-D (MS-2 and MS-6), the frequency was from 71.4% to 83.3% and these responses were significantly different from almost all other media. Significantly lower callogenesis potential expression (12.5–14.3%) was noticed for cotyledon and hypocotyl explants cultured on 2.0 mg/l TDZ and 4.0 mg/l 2,4-D in Gamborg B5 medium (MS-7) (tab. 1).

Table 2.

Effect of medium, plant growth regulators composition and explant types on the average callus induction rate

Source	SS	DF	MS	F	*p*-value
Medium	1.3070	6	0.2178	91.0995	$p<0.001$
Explant	1.1242	2	0.5621	235.0675	$p<0.001$
Interaction	2.4004	12	0.2000	83.6556	$p<0.001$
Error	0.1004	42	0.0024		
Total	4.93197	62			

Callus morphology varied depending on each explant origin. The earliest callus tissue, which looked almost white and transparent, appeared after 4 weeks on root explants that were cultured on 0.1 mg/l KIN and 2.0 mg/l 2,4-D in MS-1 medium (fig. 1a). At the same time, callus was formed on root explants on MS-2 medium supplemented with 0.1 mg/l BA and 5.0 mg/l PIC and it was yellowish and of loose structure (fig. 1b). Callus induction from hypocotyls was observed on MS-2 medium after 6 weeks of culture, and conditions with light turned to be more effective in that combination (fig. 1c).

The obtained primary calli were subcultured every 4 weeks, however, calli did not always grow on the same media as those used for its induction. Abundant callus tissue induced on root explants on MS-1and MS-2 media turned brown and failed to thrive after 2 weeks from the first passage on the same types of media. The permanent callus cultures were obtained from roots on 1.0 mg/l BA and 2.0 mg/l 2,4-D (MS-4) (Fig. 1d), from hypocotyls on 1.0 mg/l BA and 3.0 mg/l 2,4-D (MS-5) (fig. 1e), and from cotyledons on 1.0 mg/l BA and 3.5 mg/l 2,4-D (MS-6) after several passages on the same media (fig. 1f). A highly efficient proliferation and growth of calli was observed when calli induced on root explants were transferred onto MS medium supplemented with 0.5 mg/l TDZ and 0.1 mg/l NAA and subcultured every 4 to 6 weeks (fig. 1g). The conditions without light proved to be more suitable for initiation of calli and for its proliferation and growth. Callus tissue regenerated from roots on MS medium supplemented with 0.5 mg/l TDZ and 0.1 mg/l NAA when transferred to light conditions after three passages on the same type of medium, differentiated well into numerous shoots (fig. 1 h). Obtained shoots passaged onto a medium with no plant growth regulators grew and rooted successfully.

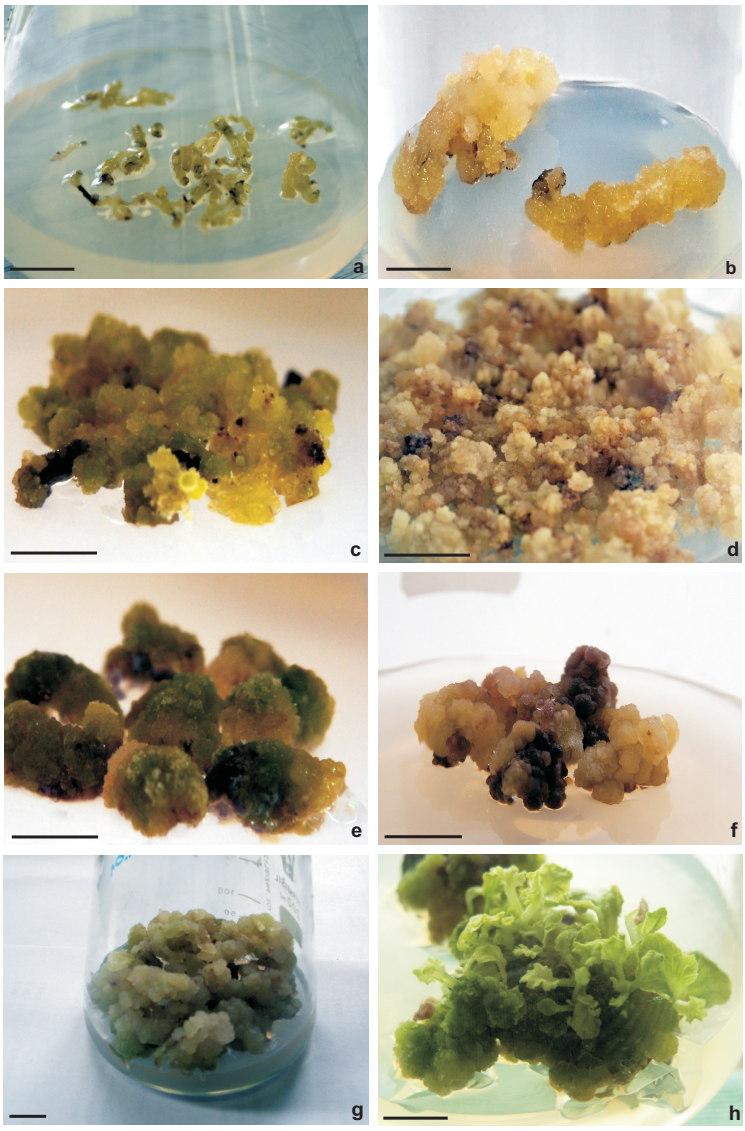

Figure 1.

Callus induction, proliferation and shoot regeneration in *Primula veris*:
a: white-transparent callus induced from roots on MS-1 medium with 0.1 mg/l KIN and 2.0 mg/l 2,4-D;
b: callus formation on roots on MS-2 medium with 0.1 mg/l BA and 5.0 mg/l PIC;
c: yellow-green callus obtained in light from hypocotyls on MS-2 medium with 0.1 mg/l BA and 5.0 mg/l PIC;
d: stabilized callus culture regenerated from roots on MS-4 with 1.0 mg/l BA and 2.0 mg/l 2,4-D;
e: dark-green compact stabilized callus regenerated in light from hypocotyls on MS-5 medium with 1.0 mg/l BA and 3.0 mg/l 2,4-D;
f: yellow-brown calli obtained from cotyledons on MS-6 with 1.0 mg/l BA and 3.5 mg/l 2,4-D;
g: white-cream abundant stabilized callus regenerated from roots on MS medium with 0.5 mg/l TDZ and 0.1 mg/l NAA; h: shoot regeneration on callus transferred into the regeneration medium with 0.5 mg/l TDZ and 0.1 mg/l NAA.
Scale bar = 1.0 cm.

DISCUSSION

There are only a few reports about successfully establishing callus cultures for *Primula* plants [16-18]. According to Mizuhiro et al. [18], the friable callus was induced from petioles of *P. obconica* and from leaf fragments of *P. malacoides* on MS medium supplemented with 0.1 mg/l BA and 5.0 mg/l PIC, as well as on MS medium supplemented with 0.1 mg/l BA and 10.0 mg/l 2,4-D. Our results confirmed the same combinations of plant hormones to be highly effective for callus initiation from *P. veris* root and hypocotyl explants. In addition, our results proved that 0.1 mg/l KIN or 1.0 mg/l BA combined with 2.0 mg/l and 3.5 mg/l 2,4-D, respectively were the best cytokinins for callus induction on root or cotyledon explants and that the time needed for calli induction was 3 to 4 weeks of culture. In his studies on *P. veris*, Okršlar et al. [22] observed effective callus induction from root explants on Anderson medium with KIN (4.5 μM) and 2,4-D (45.0 μM), and on MS medium with BA (4.5 μM) and NAA (45.0 μM), but after a much longer time of 2 to 6 months. In turn, Schween and Schwenkel [16] reported callus induction on hypocotyl and leaf explants from seedlings of *P. vulgaris* and *P. elatior* on MS medium with various concentrations of TDZ and 2,4-D, after only 1 to 3 weeks. They also found that 2.0 mg/l TDZ or 2.0 mg/l KIN combined with 2.0 mg/l and 4.0 mg/l 2,4-D or 8.0 mg/l PIC, respectively, were the best cytokinins for callus induction on hypocotyl and leaf explants. In our study, 0.1 mg/l KIN combined with 2.0 mg/l 2,4-D was also very effective in callus initiation from cowslip root explants but we did not confirm the high TDZ effectiveness in callus induction from tested *P. veris* seedling explants. However, unlike low efficiency of 2.0 mg/l TDZ combined with 2.0 mg/l–3.5 mg/l 2,4-D in callus induction (unpublished data), it was found in our work that 0.5 mg/l TDZ combined with 0.1 mg/l NAA or 1.0 mg/l BA combined with 2.0 mg/l or 3.5 mg/l 2,4-D were the most effective cytokinins for proliferation, growth, and successful establishment of cowslip permanent callus cultures.

CONCLUSIONS

In conclusion, an efficient protocol for inducing and obtaining permanent callus cultures of *Primula veris* was developed. Three types of seedling explants were examined and their roots proved to be the most suitable for callus induction and its growth. BA combined with PIC was the most effective plant hormone combination inducing calli, while effectiveness of BA combined with 2,4-D in callus induction depended on hormone concentrations and explant types. TDZ combined with NAA proved to be the most effective for proliferation and establishing permanent culture of cowslip callus.

REFERENCES

1. Huck CW, Huber CG, Lagoja IM, Ongania KH, Scherz H, Bonn GK, et al. Isolation and structural elucidation of 3',4',5'-trimethoxyflavone from the flowers of *Primula veris*. Planta Med 1999; 65: 491.
2. Huck CW, Huber CG, Ongania KH, Bonn GK. Isolation and characterization of methoxylated flavones in the flowers of *Primula veris* by liquid chromatography and mass spectrometry. J Chromatogr A 2000; 870: 453-462.
3. Stecher G, Huck CW, Stöggl WM, Bonn GK. Phytoanalysis: a challenge in phytomics. Trends Anal Chem 2003; 22: 1-14.
4. Budzianowski J, Morozowska M, Wesołowska M. Lipophilic flavones of *Primula veris* L. from field cultivation and in vitro cultures. Phytochemistry 2005; 66:1033-1039.
5. Budzianowski J, Morozowska M, Wesołowska M. Comparative study of lipophilic flavones in *Primula veris* L. from natural localities, field cultivation and in vitro culture. Abstracts of the 5th International Symposium on Chromatography of Natural Products (ISCNP). The Application of Chromatographic Methods in Phytochemical and Biomedical Analysis 2006; 19-22 June 2006, P-15: 66. Lublin, Poland.
6. Budzianowski J, Wollenweber E. Rare flavones from the glandular leaf exudate of the oxlip, *Primula elatior* L. Nat Prod Commun 2007; 2(3):267-270.
7. Edenharder R, Tang X. Inhibition of the mutagenicity of 2-nitrofluorene, 3-nitrofluoranthrene, and 1-nitropyrene by flavonoids, coumarins, quinines and other phenolic compounds. Food Chem Toxicol 1997; 35: 357-372.
8. Lee JE, Safe S. 3',4'-Dimethoxyflavone as an aryl hydrocarbon receptor antagonist in human breast cancer cells. Toxicol Sci 2000; 58: 235-242.
9. Mata-Greenwood E, Ito A, Westenburg H, Cui B, Mehta RG, Kinghorn AD et al. Discovery of novel inducers of cellular differentiation using HL-60 promyelocytic cells. Anticancer Res 2001; 21: 1763-1770.
10. Tokalov SV, Kind B, Wollenweber E, Gutzeit HO. Biological effects of epicuticular flavonoids from *Primula denticulata* on human leukaemia cells. J Agr Food Chem 2004; 52: 239-245.
11. Paszel A, Okarma M, Stryczyńska M, Budzianowski J, Rybczyńska M. The influence of lipophilic flavones of *Primula veris* on proliferation of HL60 cells overexpressing MPR. Acta Biochim Pol 2007; 54, Suppl. 4:156. Abstr. 42nd Meeting of the Polish Biochem. Soc., Szczecin, Poland, 18-21 Sept. 2007.
12. Totoń E, Hoffman J, Budzianowski J, Rybczyńska M. Influence of lipophilic flavones of *Primula veris* on proliferation, invasion and viability of HeLa cells overexpressing PKCεA/E. Acta Biochim Pol 2007; 54, Suppl. 3, Abstracts of the fifth International Conference: Inhibitors of Protein Kinases and Workshop Session: Novel Molecular Design and Simulation Methods, 23–27 June 2007, 47 (P17). Warsaw, Poland.
13. Szymanowska N, Totoń E, Hoffman J, Budzianowski J, Rybczyńska M. Antiproliferative effect of zapotin on HeLa cells overexpressing constitutively active PKCe. Acta Biochim Pol 2008; 55, Suppl. 3: 129. Abstr. 43nd Meeting of the Polish Biochem. Soc. And the 10th Conf. Pol. Cell Biol. Soc., Olsztyn, Poland, 07-11 Sept. 2007.
14. Kozłowski J. Kompleksowa interpretacja zmian zawartości związków biologicznie czynnych w roślinach i surowcach zielarskich. Poznań: Instytut Przemysłu Zielarskiego 1985: 1-55.
15. Morozowska M. Vegetative development, flowering, fruiting and saponin content in cultivated cowslip (*Primula veris* L.) plants. Herba Pol 2004; 50 (2): 28-35.
16. Schween G, Schwenkel HG. In vitro regeneration in *Primula* ssp. via organogenesis. Plant Cell Rep 2002; 20: 1006-1010.
17. Schween G, Schwenkel HG. Effect of genotype on callus induction, shoot regeneration, and phenotypic stability of regenerated plants in the greenhouse of *Primula* ssp. Plant Cell Tissue Org Culture 2003; 72: 53-61.
18. Mizuhiro M, Kenichi Y, Ito K, Kadowaki S, Ohashi H, Mii M. Plant regeneration from cell suspension-derived protoplasts of *Primula malacoides* and *Primula obconica*. Plant Sci 2001; 160: 1221-1228.
19. Morozowska M, Wesołowska M. In vitro clonal propagation of *Primula veris* L. and preliminary phytochemical analysis. Acta Biol Cacov Bot 2004; 46: 169-176.
20. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tissue culture. Physiol Plant 1962; 15: 473-497.

21. Gamborg OL, Miller RA, Ojima K. Nutrient requirement of suspension cultures of soybean root cells. Exp Cell Res 1968; 50: 151-158.
22. Okršlar V, Plaper J, Kovač M, Erjavec A, Obermajer T, Rebec A, et al. Saponins in tissue culture of *Primula veris* L. In Vitro Cell Dev–Pl 2004; 34: 644-651.

WPŁYW REGULATORÓW WZROSTU I RODZAJU EKSPLANTATÓW NA INDUKCJĘ KALUSA I WZROST PIERWIOSNKA LEKARSKIEGO (*PRIMULA VERIS* L.)

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S t r e s z c z e n i e

Primula veris L. (*Primulaceae*) to znana roślina lecznicza. Opracowano metodę otrzymywania *in vitro* tkanki kalusowej z trzech rodzajów eksplantatów: korzeni, liścieni i hypokotyli z czterotygodniowych siewek pierwiosnka lekarskiego. Najwyższy, istotny statystycznie i wynoszący 93,6% indukcji tkanki kalusowej uzyskano z eksplantatów korzeniowych na podłożu Murashige i Skooga (MS) zawierającego 0,1 mg/l BA i 5,0 mg/l PIC. Indukcję kalusa obserwowano także na 83,3% eksplantatów liścieniowych na pożywce MS uzupełnionej 1,0 mg/l BA i 3,5 mg/l 2,4-D oraz na 81,0% eksplantatów korzeniowych na pożywce MS zawierającej 0,1 mg/l KIN i 2,0 mg/l 2,4-D. Średni czas potrzebny do inicjacji kalusa wyniósł

4 do 6 tygodni i zależał od rodzaju eksplantatu. Podłoża MS uzupełnione 0,5 mg/l TDZ i 0,1 mg/l NAA oraz 1,0 mg/l BA i 2,0 mg/l lub 3,5 mg/l 2,4-D okazały się najodpowiedniejszymi dla dalszego rozwoju i wzrostu zregenerowanej tkanki kalusowej. Warunki bez dostępu światła były korzystniejsze dla indukcji i wzrostu tkanki kalusowej pierwiosnka lekarskiego.

Słowa kluczowe: *pierwiosnek lekarski, roślinne substancje wzrostowe, indukcja kalusa, proliferacja*