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Effect of Explant Source and Growth Regulators on *in vitro* Callus Growth of *Taxus baccata* L. Washingtonii

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Summary

The effects of explant source, medium composition and growth regulators were examined in order to optimize the induction and selection of fast growing callus lines of European yew (*Taxus baccata* L. Washingtonii). Callus cultures were induced from isolated mature zygotic embryos or from segments of juvenile branches. Following two months of growth on induction medium (MS + 3.0 mg/L NAA, 0.5 mg/L kinetin, 100 mg/L arginine, 2.5 % sucrose and 0.8 % agar), callus proliferation was induced in 86.4 % of embryo explants and 100 % of branch-cutting explants. The growth potential of established callus lines was found to vary in response to genetic potential and culture medium composition. The growth rate of stem-derived callus obtained on induction medium was superior to that obtained using all other tested media modifications (duplication time 9.6 days). However, the growth of embryo-derived callus lines was enhanced by increasing the iron content from 27.8 to 55.6 mg/L $FeSO_4 \cdot 7H_2O$ in the maintaining MS medium (duplication time for line E2 was 8.5 days). In two out of three embryo-derived lines, tissue growth was further improved by transferring onto modified B5 medium (duplication time for lines E2 end E5 was 4 and 5.7 days, respectively). HPLC analysis confirmed the presence of the anticancer agent cephalomannine in calli grown on B5 medium and a taxane-like substance in calli grown on MS medium.

Key words: callus induction, European yew, MS medium, taxane, zygotic embryo

Introduction

European yew (*Taxus baccata* L.) belongs to the genus *Taxus*, well known because it contains paclitaxel, one of the most promising natural anticancer agents (*1*). However, this species is endangered and prone to extinction in Europe, due to the small size and senescent status of most populations (*2,3*). Today, taxanes are accessible by synthetic and semisynthetic procedures and they are isolated from fast growing *Taxus* species in the field (http://www.21cep.com/sft/pxsft.htm). However, such sources are time- and labour-consuming. On the other hand, reduced pools of natural adult trees available for the extraction, and low levels of paclitaxel and related taxanes in *Taxus* tissue, underline the need for an alternative source of taxanes, such as plant cell and tissue culture. The direct manipulation of plant cell and tissue culture systems has many advantages over the

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conventional isolation of secondary metabolites from the intact plant (*4*). In fact, the manipulation of tissue culture conditions has already resulted in enhanced biomass production and increased biosynthesis of taxanes in several other yew species (*5–8*).

The selection of callus lines with optimal growth and efficient taxane production is a long-term process, mostly because of the recalcitrant behavior of *Taxus* spp. under *in vitro* conditions. Factors influencing tissue growth are explant source and medium composition. Young stem cuttings of adult trees were commonly used as primary explant sources for callus induction (*8,9*). However, using this kind of primary explant, the selection of fast-growing tissue lines requires a large number of available adult trees. With the aim of improving the selection of fast growing callus lines, we evaluated growth variability among callus lines induced from zygotic embryos obtained from a single tree. We presumed that the heterogeneity of zygotic embryos would provide a sufficient number of different cell lines, even when only one adult tree is available.

With respect to callus induction, MS medium is not among the most effective media (*10*) and it frequently suppresses callus growth. However, *T. cuspidata* calli grown in MS medium displayed significantly higher paclitaxel yields than those grown in other tested media including Gamborg's B5 (*11*). This result encouraged us to attempt to optimize the induction and selection of *T. baccata* callus lines on modified MS medium for fast growing cultures. Taxane content was preliminary analyzed using HPLC.

Materials and Methods

Plant material

Mature seeds and young branches (season's growth until June) were collected from an adult *T. baccata* L. Washingtonii tree. In order to isolate mature zygotic embryos, seeds were washed with tap water for 15 min and surface sterilized in 1 % Izosan (a commercial, chlorine releasing product, Pliva-Zagreb) for 15 min, rinsed three times with sterile water and soaked in 1 % H_2O_2 for 4 days at 4 °C. The seeds were then washed with 6 % H_2O_2 for 10 min and then with sterile distilled water for 15 min prior to embryo isolation. Whole embryos were put in culture medium. Branches were surface sterilized in 1 % $HgCl_2$ for 10 min, washed in 0.5 % $CaCl_2$ for 5 min, rinsed three times with sterile water and dried on sterile filter paper. One-cm long segments of young branches without needles were placed horizontally on the medium. Cultures were grown in the dark, at 24 °C.

Culture medium

The induction medium was 1×MS (*12*) supplemented with 3 mg/L NAA, 0.5 mg/L kinetin, 100 mg/L arginine and 2.0 % sucrose. The pH value was adjusted to 5.8 prior to autoclaving. Sigma purified agar was added at a concentration of 0.8 %. The medium complements we varied during experiments were: (a) sucrose concentration (2, 2.5 or 3 %), (b) type of auxin and its concentration (3 mg/L NAA, 2 or 3 mg/L 2,4-D), (c) presence or absence of 1 % insoluble polyvinylpyrrolidone (PVP), and (d) concentration of iron (27.8 or 55.6 mg/L $FeSO_4 \cdot 7H_2O$). The effect of basal salt mixtures on callus growth was compared using maintaining MS medium and modified B5 medium (*9*). Calli were subcultured monthly, in test tubes (ϕ 30 mm) containing solidified medium. Each tube was inoculated with 0.5 g of callus. Fresh weight increase (fwi) was calculated as the difference between the final and the initial fresh weight, and was monitored for more than three years. After transferring to a modified medium composition, we waited for at least three culture cycles before tissue fresh weight was measured. Callus growth rate was defined as the time of duplication of the fresh weight (dt). The results were statistically analyzed by multifunctional Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) at the 95 % level of confidence. Data are presented as means ± standard error (SE). SE was calculated from the mean squared error from the ANOVA.

Taxanes extraction and HPLC analysis

Tissue samples were collected after 30 days of subculture and frozen at –80 °C. Approximately 2 g of tissues were extracted with 5 mL of 80 % MeOH, centrifuged (9000 rpm, 10 min) and dried at 25 °C. The residue was dissolved in 5 mL 15 % MeOH and pre-purified using a Sep-Pak Vac 3cc (500 mg) C18 column (Waters). Taxanes eluted with 75 % MeOH, were analyzed by HPLC following a procedure described previously (*13*). Taxanes were detected at 227 nm, using a PDA detector (Waters). Taxanes in the samples were identified on the basis of characteristic absorption spectra (210–300 nm), retention times and co-chromatography with standards (10-deacetylbaccatine III – Sigma, baccatin III – Sigma, paclitaxel – Sigma, cephalomannine – Yuman Hande Tech. Development) and were quantified by means of external standards.

Results

The best callus induction in mature zygotic embryos (100 %) was obtained using MS medium supplemented with 3.0 mg/L 2,4-D, 0.5 mg/L kinetin and 2.0 % sucrose (Fig. 1). Increase in sucrose concentration of 3.0 mg/L in the defined MS medium reduced callus induction by 20 % (Fig. 1). Less efficient but none-the-less good callus induction (85 %) was obtained using media supplemented with 2 mg/L 2,4-D or 3 mg/L NAA. Callus proliferation on the whole embryo surface was coupled with red coloration of embryos and release of phenolic exudates in all media tested. Embryos that did not respond remained whitish in color. The induced calli were friable and dark yellow in color.

When young branches of *T. baccata* were used as primary explants, callus induction was obtained in 100 % of the explants cultured in MS medium supplemented with 3 mg/L NAA, 0.5 mg/L kinetin and 2.5 % sucrose. Callus tissue had the same morphology as calli induced from embryos, although the color of these highly proliferating stem-derived callus (SDC) was pale yellow to white. Since the applied medium proved to be effective,

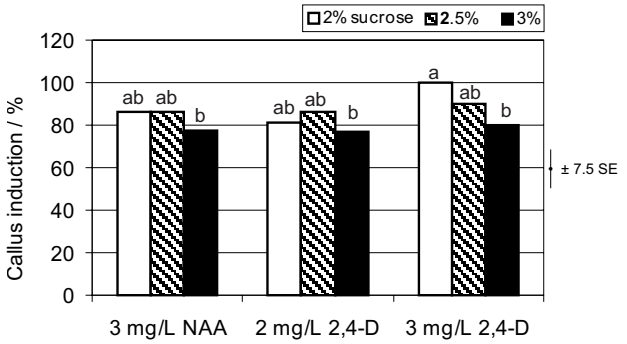

Fig. 1. The effect of auxin and sucrose content on callus induction (%) in mature zygotic embryos of *T. baccata* cultured in the induction medium specified in Materials and Methods. The data shown are the means of three replicate experiments (30 embryos per experiment). Means labeled with the identical letters are not significantly different at the 95 % level of confidence. The standard error (SE) bar is estimated from the mean squared error in the analysis of variance.

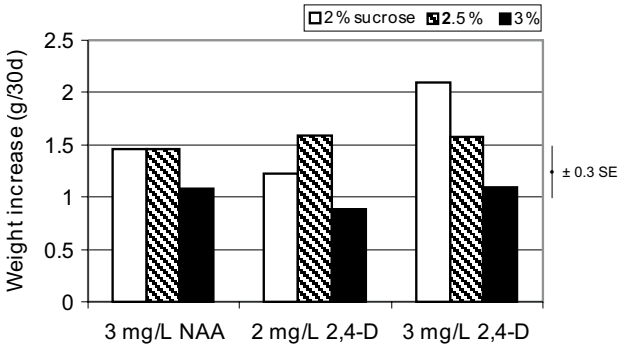

Fig. 2. Fresh weight increase in stem-derived callus lines of *T. baccata* in relation to different auxin and sucrose levels in the induction MS medium. The data presented are the means of 6 measurements. Means labeled with identical letters are not significantly different at the 95 % level of confidence. The standard error (SE) bar is estimated from the mean squared error in the analysis of variance.

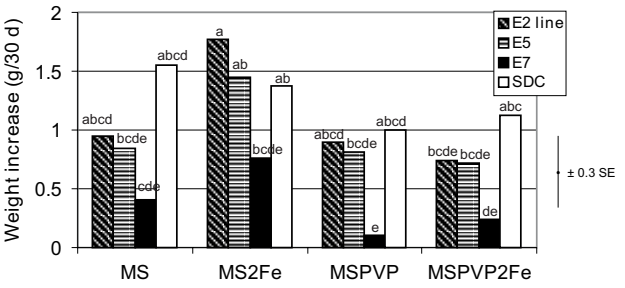

Fig. 3. The effect of 1 % PVP and iron levels on fresh weight increase in stable callus lines of *T. baccata* (embryo-derived cell lines E2, E5, E7 or stem-derived callus, SDC). The maintaining medium (MS) was supplemented with 55.6 mg/L $FeSO_4 \cdot 7H_2O$ (MS2Fe), 1 % PVP (MSPVP) or both (MSPVP2Fe). The data shown are the means of 6 measurements. Means labeled with identical letters are not significantly different at the 95 % level of confidence. The standard error (SE) bar is estimated from the mean squared error in the analysis of variance.

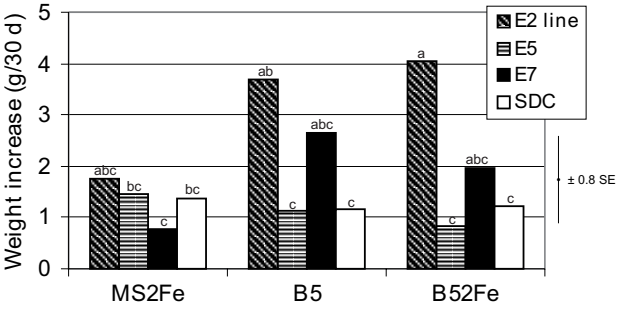

Fig. 4. Effect of the basal medium on fresh weight increase in stable callus lines of *T. baccata*. Embryo-derived callus lines (E2, E5 or E7) and the stem-derived callus line (SDC) were grown in maintaining MS medium supplemented with 55.6 mg/L $FeSO_4 \cdot 7H_2O$ (MS2Fe), Gamborg B5 medium with 27.8 or 55.6 mg/L $FeSO_4 \cdot 7H_2O$ (B5 or B52Fe, respectively). The data shown are the means of four measurements. Means labeled with identical letters are not significantly different at the 95% level of confidence. The standard error (SE) bar is estimated from the mean squared error in the analysis of variance.

we did not test any other medium formulae for callus induction in stem explants. However, to obtain optimal tissue growth, stem derived calli were further cultured on differently modified MS medium (Fig. 2). The fastest growth of stem derived calli was obtained on MS medium supplemented with 3 mg/L 2,4-D and 2 % sucrose (fwi = 2 g, dt = 7.4 days). Sucrose concentration of 3 mg/L reduced callus growth in all media tested (Fig. 2).

From more than a hundred embryo-derived callus (EDC) lines, three fast growing cell lines were selected (E2, E5 and E7). These stable callus lines had been cultured on MS medium supplemented with 3 mg/L 2,4-D, 0.5 mg/L kinetin and 2.5 % sucrose (maintaining medium) and their growth potential was followed for three years (Figs. 3 and 4).

The effect of iron concentration and the addition of 1 % PVP on callus growth was assayed (Fig. 3). The addition of 55.6 mg/L $FeSO_4 \cdot 7H_2O$ to the maintaining MS medium (formula MS2Fe) instead of 27.8 mg/L (control), enhanced the growth of the embryo-derived callus lines (Fig. 3). The best growing potential was exhibited by line E2 (fwi = 1.8 g, dt = 8.5 days). On the contrary, a higher concentration of iron reduced tissue growth of stem-derived callus from fwi = 1.5 g (control) to fwi = 1.4 g. The addition of 1 % insoluble PVP to MS medium (formula MSPVP) did not affect growth of the EDC lines but significantly reduced proliferation of the line SDC (Fig. 3). In the presence of PVP, both types of callus released »red/brown-colored« exudates into the medium, but not when calli grew on MS medium without the addition of PVP. The addition of 55.6 mg/L $FeSO_4 \cdot 7H_2O$ in the MSPVP medium (formula MSPVP2Fe) did not reduce the negative effect of PVP on the growth potential of *T. baccata* callus tissue (Fig. 3).

The effect of basal salt mixtures on callus growth was estimated after transferring a portion of the calli from maintaining MS medium onto modified B5 medium (*9*). Two out of three EDC lines (E2 and E7) dis-

played reduced browning and faster tissue growth after transferring to modified B5 medium (Fig. 4). The fresh weight increase of line E2 was 3.7 g (dt = 4.0 days) and that of line E7 was 2.7 g (dt = 5.7 days). However, we found B5 medium to be less effective for stem-derived callus growth than modified MS medium. Increased concentration of $FeSO_4 \cdot 7H_2O$ (55.6 mg/L instead of 27.8 mg/L, the control) in B5 medium did not improve the proliferation of any callus lines except for line E2 (fwi = 4.1 g, dt = 3.8 days).

Taxane content was analyzed using HPLC. Preliminary measurements were done on embryo-derived and stem-derived callus cultures grown on three different media: MS, MSPVP and B5. Among the tested taxanes, only cephalomannine was detected and only in cultures grown on B5 medium (0.49±0.05 μg/g fresh weight). The presence of a taxane-like substance with the retention time of cephalomannine was detected in the majority of calli grown on MS (0.37±0.25 μg of paclitaxel equivalents /g fresh weight), and MSPVP medium (0.46±0.26 μg of paclitaxel equivalents /g fresh weight). The spectrum of the unknown substance was similar to paclitaxel and was recognized as a taxane-like spectrum by the computer program (Millennium^{32}, Waters).

Discussion

Using modified MS medium we obtained a significantly higher percentage of callus induction from mature zygotic embryos of *T. baccata* (77.5–100 %), in comparison with the 15 %-induction obtained in *Taxus cuspidata* by using B5 medium (*9*). In addition, the frequency of stem-derived callus induction on MS medium in our experiments was 100 %. The reported frequency of callus induction in stem segments of *T. baccata* is more than 75 % (*7*) or 85 % in *T. cuspidata* (*9*). The best callus induction in both kinds of primary explants was achieved using MS medium supplemented with 3 mg/L 2,4-D, 0.5 mg/L kinetin and 2.0 % sucrose. Sucrose at 3 % reduced induction and growth of *T. baccata* callus, possibly due to osmotic shock or repression of biosynthetic pathways (*2,14*).

During the study, we selected four stable, fast growing callus lines of *T. baccata* with doubling times from 7 to 10 days. As previously published (*15*), an average *Taxus* cell growth rate is from 13 to 20 days. The high efficiency of callus induction and rapid growth of the callus lines recovered from embryos argue for more frequent utilization of zygotic embryos in callus induction. Also, the use of zygotic embryos provides an additional source of cell lines with diverse growth potential and taxane content, even when only a single mother tree is available. The cell lines initiated from *T. canadensis* zygotic embryos were successfully selected for taxane production (*14*).

Compared to the maintaining MS medium we used, modified B5 medium (*9*) improved growth of two EDC lines (E2 and E7). However, in our experiments, the growth potential of stem-derived callus and the E5 cell line did not change in response to the replacement of MS with B5 medium. As reported previously, there was no difference between the growth potential of stem derived calli grown on B5 or MS medium (*6,7*). According to our results, it appears that basal salts mixtures have a stronger effect on the proliferation of embryo-derived calli than on calli recovered from stem segments.

Secretion of some pigments or phenolic compounds into the medium has been shown to have a deleterious effect on the growth and viability of *Taxus* cells *in vitro*. Addition of 1.5 % insoluble PVP, a phenolic-complexing agent, to B5 medium partially relieved this problem with no impact on the growth of stem-derived *T. cuspidata* callus (*9*). In our experiments, the addition of 1.5 % of insoluble PVP to MS medium inhibited the growth of stem-derived *T. baccata* callus but had no growth effect on the two out of three embryo-derived callus lines. On the other hand, increased iron content (55.6 mg/L instead of the customary 27.8 mg/L $FeSO_4 \cdot 7H_2O$) improved the growth of embryo-derived, but not that of stem-derived, callus lines of *T. baccata*.

Among the tested taxanes, only a small amount of cephalomannine was detected and only in the cultures grown on B5 medium. In the majority of calli grown on MS and MSPVP medium the presence of a taxane-like substance with the retention time of cephalomannine and a spectrum similar to paclitaxel was found but further analysis would be necessary for unequivocal identification. Fast tissue growth is not commonly associated with secondary metabolite production. The desired production of paclitaxel may be achieved after prolonged tissue culturing, when the tissue reaches the stationary growth phase (*16*), or by altering the growth conditions (*11,17*).

Conclusion

The MS-based media used in the study proved to be highly effective in terms of *T. baccata* callus induction and enhanced callus growth. Our results show that zygotic embryos make an equally good source of callus recovery explants as the commonly used stem segments. Moreover, the use of zygotic embryos increases variability among cell lines in the process of selection. We show that callus cultures recovered from different types of primary explants exhibit different sensitivity to cell culture conditions. An increment in iron concentration in MS medium, or in B5 medium, increases the growth of embryo-derived callus lines and, at the same time, decreases the growth of the stem-derived callus line. In addition, the presence of PVP in MS medium inhibits the growth of stem-derived callus but not of the embryo-derived callus lines. Selected callus cultures of *T. baccata* retained fast growth throughout three years, as well as the capability to produce cephalomannine (on B5 medium) and a taxane-like substance (on maintaining MS medium). Stable, rapid growing callus cultures of *Taxus baccata* selected in our experiments promise the possibility of improved taxane production in *T. baccata* tissue culture in future studies.

References

1. G. M. Cragg, S. A. Shepartz, M. Suffness, M. Grever, *J. Nat. Prod. 56* (1993) 1657–1668.
2. D. García, R. Zamora, J. A. Hodar, J. M. Gomez, J. Castro, *J. Biol. Conserv. 95* (2000) 31–38.

3. J. C. Svenning, E. Magard, *Biol. Conserv. 88* (1999) 173–182.
4. J.-H. Kim, J.-H. Yun, Y.-S. Hwang, S. Y. Byun, D.-I. Kim, *Biotechnol. Lett. 17* (1995)101–106.
5. A. G. Fett-Neto, S. J. Melanson, S. A. Nicholson, J. J. Pennington, F. DiCosmo, *Bioeng. Biotechnol. 44* (1994) 967–971.
6. R. E. B. Ketchum, D. M. Gibson, G. Gallo, *Plant Cell Tissue Organ Cult. 42* (1995) 185–193.
7. E. R. M. Wickremesinhe, R. N. Arteca, *Plant Cell Tissue Organ Cult. 35* (1993) 181–193.
8. A. Zhir, K. Maciejewska, M. Jaziri, J. Homés, M. Vanhaelen, *Med. Fac. Landbouw Univ.* Ghent, (1995) 2111–2214.
9. A. G. Fett-Neto, F. DiCosmo, W. F. Reynolds, K. Sakata, *Bio/Technology, 10* (1992) 1572–1575.
10. J. J. Zhong, *Plant Tissue Cult. Biotechnol. 1* (1995) 75–80.
11. A. G. Fett-Neto, S. J. Melanson, K. Sakata, F. DiCosmo, *Bio/Technology, 11* (1993) 731–734.
12. T. Murashige, F. Skoog, *Physiol. Plant. 15* (1962) 473–497.
13. S. Baebler, M. Camloh, M. Kovac, M. Ravnikar, J. Zel, *Planta Med. 68* (2002) 1–2.
14. H. Q. Wang, J. T. Yu, J. J. Zhong, *Process. Biochem. 35* (2000) 479–483.
15. M. Jazir, A. Zhiri, Y. W. Gou, J. P. Dupont, *Plant Cell Tissue Organ Cult. 46* (1996) 59–75.
16. T. J. Hirasuna, L. J. Pestchanker, V. Srinivasan, M. L. Shuler, *Plant Cell Tissue Organ Cult. 44* (1996) 95–102.
17. R. E. B. Ketchum, D. M. Gison, R. B. Croteau, M. L. Shuler, *Biotechnol. Bioeng. 62 (*1999) 97–105.

Utjecaj početnog eksplantata i regulatora rasta na kulturu kalusnoga tkiva europske tise (*Taxus baccata* L. Washingtonii)

Sažetak

Ispitan je utjecaj eksplantata i sastav hranidbene podloge na rast kalusnih linija europske tise. Kalusne kulture potaknute su na eksplantatima čitavih zrelih zigotnih embrija i odsječaka jednogodišnjih grančica odrasle biljke. Nakon dva mjeseca uzgoja na indukcijskoj podlozi (MS s dodatkom 3,0 mg/L NAA, 0,5 mg/L kinetina, 100 mg/L arginina, 25 g/L saharoze i 8 g/L agara) proliferacija kalusa postignuta je na 86,4 % embrijskih eksplantata i na 100 % eksplantata grančice. Rast ustaljenih kalusnih linija ovisio je o genetskom potencijalu i sastavu hranidbene podloge. Najbolji prirast svježe mase kalusa dobivenog iz tkiva grančice ostvaren je na indukcijskoj podlozi (vrijeme udvostručenja mase kalusa bilo je 9,6 dana). Kalus induciran iz embrija rastao je brže na podlozi MS s povećanim udjelom željeza (55,6 mg/L umjesto 27,8 mg/L $FeSO_4 \cdot 7H_20$) pa je masa tkiva linije E2 udvostručena za 8,5 dana. Prirast mase tkiva u dvije od tri embrijske kalusne linije poboljšan je supkultiviranjem na modificiranu podlogu B5 (tkivna masa linija E2 i E5 udvostručena je nakon 4, odnosno 5,7 dana). Analizom HPLC u kalusnim kulturama uzgajanim na podlozi B5 dokazana je prisutnost antikancerogene tvari cefalomanina, a u kalusnom tkivu raslom na podlozi MS prisutnost neodređene tvari slične taksanu.