Nutritional and seasonal requirements for callus growth in *Taxus baccata*

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Summary: Callus cultures derived from young stems of two varieties of *Taxus baccata* cv. *aureovariegata* (genotype I) and Taxus baccata L. (genotype II, III) were induced. Gamborg´s B5 medium was supplemented with different concentrations of auxin (2,4-D) in combination with cytokinins (kinetin or topolin) and with a phenolic-binding compound (PVP) to prevent callus darkening and growth inhibition. Stem explants displayed different responses to *in vitro* culture depending on plant genotype and on the season. Genetic variability was observed in the growth rate of calli initiated from all three genotypes of the same *Taxus* species. We found the best growth of callus cultures originated from the genotype III in defined media. After the first subculture the majority of the cream-coloured primary callus turned brown and ceased its growth. However, the long-term culture was initiated.

Introduction

The European yew – *Taxus baccata* L. (Taxaceae) – presently scarce in the wild is widely cultivated as an ornamental tree. A large grove of this species from "Harmanecká tisina" a nature reserve (Slovakia), has been described (*Kresánek* & *Dugas*, 1985). In addition to the red aril, all other parts of yew contain highly poisonous alkaloid toxic for all animals.

The discovery of paclitaxel, a diterpene pseudalkaloid, initially isolated from the bark of *Taxus brevifolia* Nutt. by *Wani* et al. (1971) started new medicinal use of yew. Showing broad antitumour spectra paclitaxel (Taxol®; Bristol-Myers Squibb Co.) and its derivative docetaxel (Taxotere®; Rhone-Poulenc Rorer Co.) as one of the most important anticancer agents has been approved for use in more than 40 countries (*Holmes* et al., 1995, *Seki* et al., 1997).

Both paclitaxel and docetaxel are the only plant secondary metabolites known to prevent mitosis by inducing tubulin polymerisation to form stable, non-functional microtubules (*Schiff* et al., 1979). This is in contrast to the classic phytochemical mitotic blocks such as colchicines and vinca alkaloids that act by destabilizing and depolymerising microtubules.

The enormous demand for supply of paclitaxel to the clinical trials has raised an environmental discussion. The isolation of the drug from its original source – the bark of the slow-growing *Taxus brevifolia* – results in the mass destruction of yews. Although the total chemical synthesis of paclitaxel was announced, it seems to be unfeasible on an industrial scale (*Holton* et al., 1994; *Nicolaou* et al., 1994). Therefore, the biotechnological approach could solve the increased demand for this compound. The production of paclitaxel and related taxanes by *Taxus* tissue cultures has already been patented (*Christen* et al., 1991).

The aim of our work was to characterize the induction and the growth of *T. baccata* callus cultures derived from 3 different genotypes on selected hormone regimes.

Material and methods

Young stem explants from three different genotypes of *Taxus baccata* L. (genotypes II, III) and *Taxus baccata* cv. *aureovariegata* (genotype I) growing on a homogenous site at the Botanical Garden of P.J. Šafárik University, Slovakia, were used. The explants were excised from young stems collected during all seasons of one year.

Stems stripped of needles were washed with tap water to remove dust and solid particles and surface sterilized by immersing in 5% solution of the detergent (SAVO) overnight, 70% ethanol for 5 minutes, 0.2% $HgCl_2$ for 15 minutes and rinsed three times with sterile distilled water.

Stems aseptically dissected into approximately 1 cm long segments were immersed in the medium with one of the transversally sectioned ends and incubated in the dark at 22 °C. Explants were grown in glass tubes, each containing 5 ml of medium.

The explants were cultivated in B_5 medium (*Gamborg* et al., 1968) containing 2% (w/v) sucrose, 0.6% (w/v) agar and 1.5% (w/v) polyvinylpyrrolidone (PVP), a phenolic-binding compound. The media were supplemented with 2,4-

dichlorphenoxyacetic acid (2,4-D) at concentration ranging from 2 to 8 mg/l in combination with 0.1, 0.5 or 1 mg/l kinetin (Kin) or topolin (Top), prepared with double distilled water, adjusted to pH 5.6 with NaOH and HCl and autoclaved at 121 °C for 15 minutes.

After 28 days of culture the percentage of explants showing callogenesis was calculated (+ good, – weak or no growth). Each treatment is based on 70–102 observations.

For the assignment of the growth index (G_I), the exact weight of primary callus discarded from segments was used as inoculums and placed on 5 ml of fresh medium in glass tubes. After 28 days of culture the fresh weight was determined. To minimize differences in growth due to variation in inoculums size, results are presented as a growth index:

$$G_I = (W_{t\ Final} - W_{t\ Initial}) / W_{t\ Initial}$$

For each genotype, we used 9–25 observations for each treatment. Experimental results were analysed by analysis of variance.

At the end of each experiment, we used the best growing calli for the establishment of the long-term culture.

Results and discussion

Induction of callogenesis

In our first experiment the concentration of the auxin/cytokinin ratio changed successively from one treatment to the following. The explants were derived from young stems of *T. baccata* cv. *aureovariegata* (genotype I) collected from March to September. At the end of the growth period, some seasonal changes in the ability of explants to initiate the growth of primary callus were observed *(Table 1)*.

In the spring (March-April) the percentage of explants exhibited good growth of primary callus varied between 71.6–94.1%.

The increment in the induction of callogenesis was typical for experiments carried out in May and June when yews are sprouting young shoots. According to our results this period of year is the least suitable for the initiation of callus cultures from stem explants. Only 1.4, 5.7 and 25.5% exhibited good callus growth in compare with the percentage of explants cultured on the same media in September or April (75, 75 and 90.5%).

The explants derived from the youngest shoots (2–4 weeks old) collected in May and June showed very low or no callogenesis *(Table 2)*.

Table 2 Effect of various concentrations of the cytokinin/auxin combination on the initialisation of callogenesis of explants derived from 2–4 weeks old shoots of the genotype I. The percentage of explants showing callogenesis after the period of 4, 7 and 9-weeks culture on B5 medium in experiments initialised in the period of May – June is shown.

Kin (mg/l)	0,5	1		0,5	1	0,5	1
2,4-D (mg/l)	6	6	8	6	6	6	6
Season	*End of May – beginning of June*					*Middle of June*	
Cultivation period	4 weeks			9 weeks		7 weeks	
+ (%)	0	0	0	0	0	10.1	13
– (%)	100*	100*	100	100*	100*	89.9	87

* The number of observation n = 22

Using all three genotypes (I, II, III) the effect of two different cytokinins (Kin, Top) in combination with 2,4-D was assessed. After 28 days of cultivation callus occurred on more then 54% explants *(Table 3)*. Overall, the induction effect of topolin was stronger for genotypes I and II, while similar concentration of topolin resulted in a lower percentage of explants exhibiting callus formation of the genotype III.

Primary callus derived from young stem explants was pale yellow to dark brown in colour. Pale yellow calli were subcultured.

Table 1 Effect of various concentrations of the cytokinin/auxin ratio on the initialisation of callogenesis of young stem-explants derived from the genotype I. The percentage of explants showing callogenesis after the period of 4-weeks culture on B5 medium in various seasons is described.

Kin (mg/l)	0.1				0.5			1			
2,4-D (mg/l)	2	4	6	8	6		8	6		8	
Season	**March**				*June*	*Sept.*	*April*	*June*	*Sept.*	*April*	*May*
+ (%)	91.1	94.1	87.5	71.6	1.4	75	73.5	5.7	75	90.5	25.5
- (%)	8.9	5.9	12.5	28.4	98.6	25	26.5	94.3	25	9.5	74.5

Table 3 The percentage of explants exhibited callogenesis after 4-weeks treatment with two different cytokinins (Kin, Top) on B5 medium supplemented with 2,4-D 6 mg/l.

Genotype	I				II				III			
Kin mg/l	0.5	1	–	–	0.5	1	–	–	0.5	1	–	–
Top mg/l	–	–	0.5	1	–	–	0.5	1	–	–	0.5	1
Season	*September*		*October*		*September*		*October*		*September*		*December*	
+ (%)	75	75	92.6	94.1	73.3	77.1	70.6	95.3	76.8	78.9	54	62
– (%)	25	25	7.4	5.9	26.7	22.9	29.4	4.7	23.2	21.1	46	38

Growth measurements

After 28 days of culture the growth index was calculated based on the fresh weight growth data. First, the influence of the ratio of auxin (2,4-D) and two different cytokinins (Kin, Top) on the growth of callus cultures derived from the genotype I, was examined. We obtained the best growth response with the ratio of 6: 0.1 (mg/l 2,4-D: mg/l Kin). There was a significant difference in callus growth for this treatment when compared to 2,4-D: Kin ratio of 6: 1 and when topolin was substituted for kinetin *(Figure 1)*.

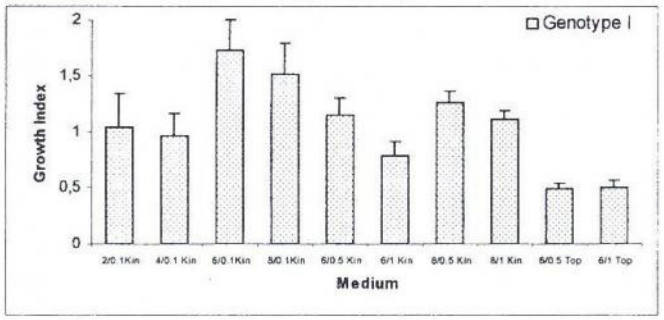

Figure 1 Callus growth of the genotype I on media with different ratio of 2,4-D: Kin (Top). Bars are the standard errors of the means (95% confidence interval).

As shown in *Figure 2a* a genetic variability between genotypes I, II and III in growth response was observed. Although grown under similar conditions donor plants may display significant differences in the biomass production *in vitro* (*Fett-Neto* and *DiCosmo* 1997). For most treatments we found the best growth of callus derived from genotype III with statistically significant differences at the ratio of 6: 0.5 (mg/l 2,4-D: mg/l Top). For the combined results of all three genotypes, media with the half dose of cytokinins (both Kin and Top) supported better callus growth with significant differences among them *(Figure 2b)*. Presently we are using the B_5 media with this hormone regime for routine subculture.

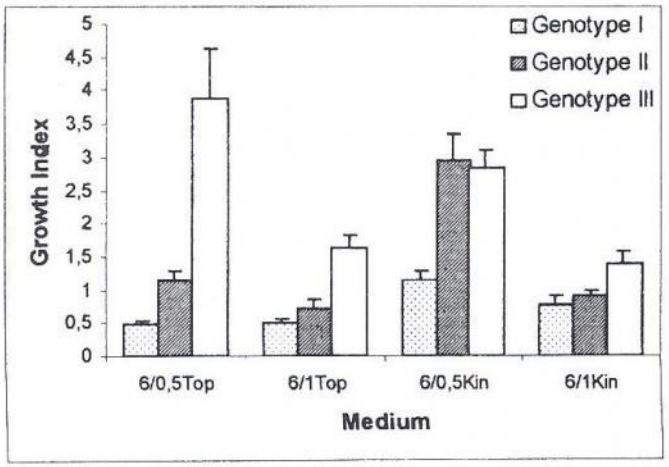

Figure 2a Growth index means of callus derived from individual genotypes. Bars are the standard errors of the means (95% confidence interval).

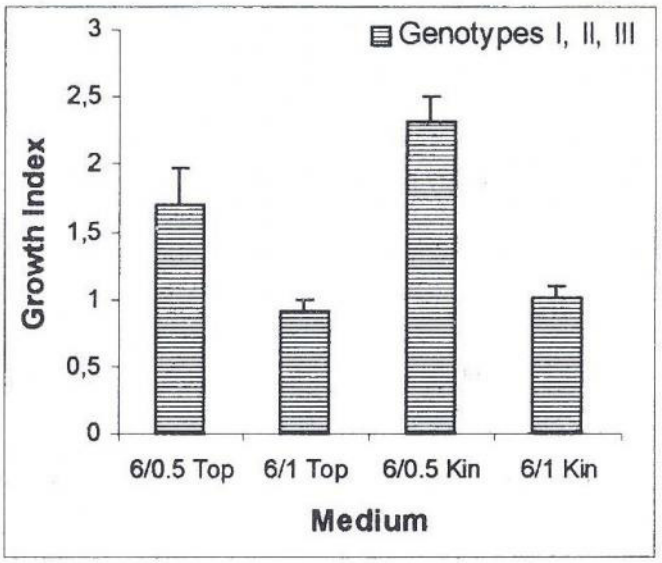

Figure 2b Growth index means from all three genotypes. Bars are the standard errors of the means (95% confidence interval).

The use of stem segments as nurse culture resulted in nearly identical treatment means with significant effect on callus growth *(Figure 3)*.

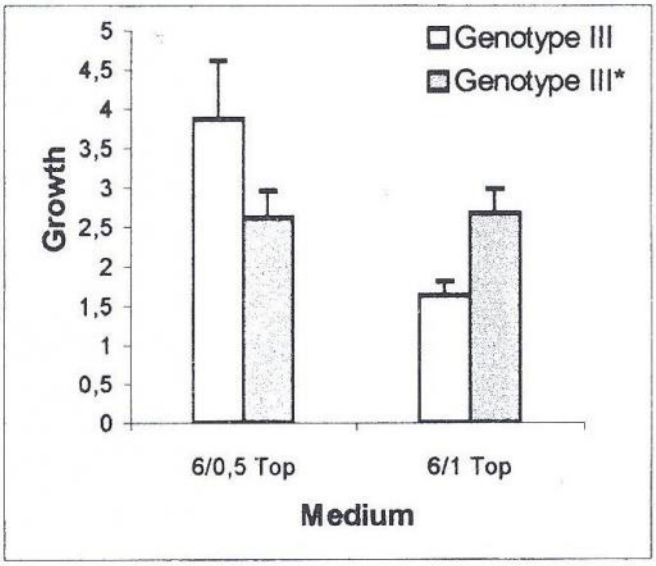

Figure 3 The comparison of the growth of callus (derived from genotype III) without and with the use of nurse culture (*). Bars are the standard errors of the means (95% confidence interval).

Conclusion

The objective of our work was to demonstrate the ability to induce callogenesis from young stems during all seasons of the year and a potent genetic variability in the growth response of callus cultures derived from different genotypes of the same *Taxus* species.

The optimisation of hormone regime has lead to improvement in the growth of *Taxus baccata* calli.

Although many protocols for cultivation of *Taxus* cell cultures have been established, the growth ability seems to be very genotype-dependent.

These features are essential for establishing of fast-growing callus cultures over long periods of time. It should be a good starting point for initiation of cell suspensions, an alternative source of paclitaxel and related taxanes.

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