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Influence of Different Sugar Regimes on the Growth of Callus Culture of *Taxus baccata* L. and the Production of Taxanes

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ABSTRACT Influence of fructose addition to the cultivation medium on the production of taxanes and the growth of callus culture of *Taxus baccata* was studied. The cultures showed an ability to adjust to the substitution of some of the sucrose in the media by fructose and the fresh biomass accumulation was higher on the media containing different concentrations of fructose during the second cultivation period.

Key words: Exogenous carbohydrates, plant tissue culture, taxanes, taxol, *Taxus*

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Introduction

Yew trees have been a subject of increased interest during last twenty years as they seem to be the only natural source [including their endophytic fungi (1)] of toxic diterpenoids called taxanes of which Taxol® exhibits a significant activity against several types of cancer and has been approved by FDA for treatment of ovarian and metastatic breast cancer in 1993. Taxol for medicinal purposes is either isolated from the bark of *Taxus brevifolia* or produced by semisynthesis from 10-deacetylbaccatin III isolated from a renewable source III needles - and chemically prepared side chain. Even though the supply is certainly not sufficient. Therefore there has been an extensive search for alternative ways to produce taxol and related compounds. Taxol and other taxanes producing cell cultures iniciated from different *Taxus* species are considered a promising alternative for obtaining these compounds from plant material.

In 1989 Christen and co-workers first published that cell cultures of *Taxus spp.* produce taxol (2) and since then the number of publications on taxane production in cell or callus cultures has been continuously increasing. Some of them deal with influence of different carbohydrates in a range of concentrations in media on growth of the cultures and taxane production as well. The cultures are initiated and subcultivated usually on media containing sucrose. It has been suggested that addition of fructose to the medium can either improve growth of *Taxus spp.* cell cultures (3) or stimulate taxane production (4). The main goal of this work was to determine the influence of partial and total substitution of sucrose in the medium by fructose on the production of taxanes and the growth of callus culture of *Taxus baccata.*

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Materials and Methods

Plant material and cultivation conditions

Embryos asseptically excised out of seeds of *Taxus baccata* were used as primary explants for this callus culture. The culture was subcultivated every 6 - 8 weeks on fresh nutrient E6 medium according to Eriksson (5) containing 1% sucrose, 450 mg/l glutamine, 3 mg/l 2,4-dichlorofenoxyacetic acid, 0.5 mg/l kinetine and 0.5mg/l N^6-benzylaminopurine solified with 2 g/l

Phytagel (Sigma) on which the best results concerning taxane production while sustaining satisfactory growth were achieved in our laboratory (6). The culture was maintained on this medium for more than two years before these experiments. Considering the purpose of this work (determination of growth curves) the cultivation of the callus on membrane rafts (Sigma Chem. Co.) placed inside Magenta GA7-3 vessels with liquid media was chosen for the experiment. The culture was kept at 24°C in the dark.

In the experiments three sugar regimes were tested on our callus culture - 0,75% sucrose (S)+0,25% fructose (F), 0,5%S+0,5%F and 1%F. 1%S was used as a control. Each variant -A, B and C - represented 12 vessels. When the cultures were well out of the exponencial phase, 6 membrane rafts of each variant were transferred onto a fresh medium containing only fructose and 6 were transferred onto a fresh medium of the same compositition on which the callus was cultivated until the transfer. Figure 1 shows an arrangement of the experiment.

Growth measurements

The initial inoculum of 2 g of the callus was transferred on a pre-weighed membrane raft which was placed into the vessel containing 60 ml of the medium. The membrane rafts were weighed twice a week and growth curves were determined as an increase of growth value ($GV = \Delta W/W_{inicial}$) during the cultivation period.

Determination of sugars in the media

Samples of 1 ml of the media were asseptically pipetted out of the vessels which were not used for determination of growth characteristics. The samples were filtered through a 0.45 μm filter and stored in Eppendorf microtubes freezed at -18°C until an HPLC analysis. De-frozen samples were agitated and 15 μl of the solute were used for the analysis. Sucrose, glucose and fructose were measured using a HPLC system (Spectra Physics) equipped with a refractive index detector (Shodex *RI-71*). The column was Hi Plex Ca^{2+}, 300 × 7, 7 mm (Polymer Laboratories) and its temperature was 80°C. The guard columns were Hema-Bio 1000 SB+Q (Watrex). The mobile phase was deionized Mili-Q water (Milipore) and the flow rate was 0.5 $ml.min^{-1}$.

Extraction and HPLC analysis of taxanes

Harvested cells were homogenized and extracted in methanol for three days. The extracts were dried *in vacuo*. The samples were redissolved in dichlormethane-methanol mixture (1:1) and analysed by HPLC.

All the media were passed through a SEP-PAK C_{18} cartridge (Waters, U.S.A.). The cartridge was washed with methanol (5 ml) and water (10 ml) before passing the media and then again with water (5 ml). Taxanes were eluded from the cartridge by methanol (5 ml). The extract was evaporated in vacuo, redissolved in dichlormethane-methanol mixture (1:1) and analysed by HPLC.

Analysis was performed on a reverse-phase column (Labio Praha ODS Biosfer, 7 μm, 250 × 4 mm). The linear gradient elution profile started with methanol-wateracetonitrile (A: 20:67:13) and ended with methanolwater-acetonitrile (B: 20:27:53) within 50 min and included a 5 min wash in both solvents to re-establish the initial condition. The flow rate was 1 $ml.min^{-1}$ and all chromatograms were plotted at 227 nm, an absorption maximum of taxol, using a

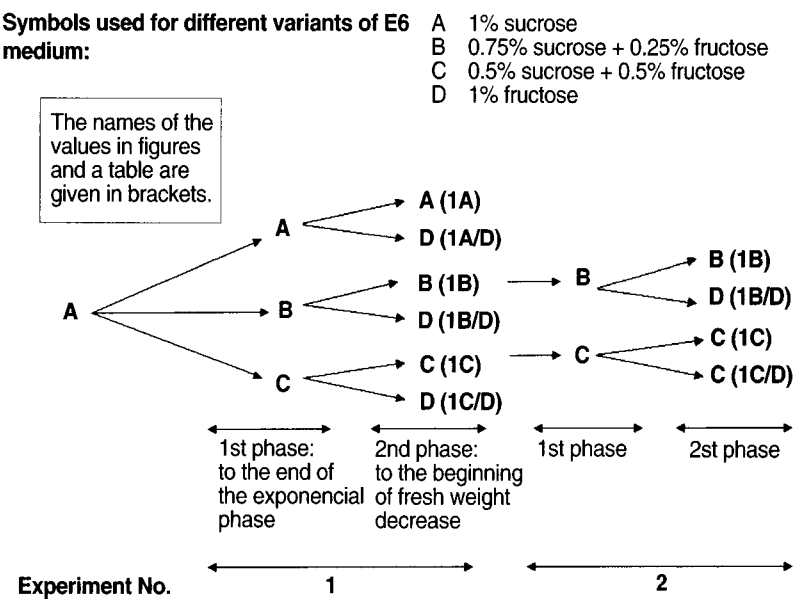

Figure 1. Structure of the experiments.

high performance liquid chromatography system equipped with a photodiode array UV detector (Waters, U.S.A.). Identification of the taxanes in samples was carried out by retention time and UV spectra.

Results and Discussion

Growth of the cultures

The growth curves on GV basis were determined for both phases of the two experiments.There were no dramatic differences between the cultures on media with different ratio of sucrose and fructose concerning the growth dynamics. There has only been a slight shift in the time when the cultures on different medium variants were well out of the exponencial phase in the 1st phase of the experiment No. 1, but this difference was not at all apparent in the experiment No. 2. The differences in osmotic pressure of a medium (due to an exchange of some of sucrose by a monosaccharide) at the beginning of cultivation that could also possibly influence the growth rate on individual variants of media were soon (by the 10th day of cultivation - until then the cultures grew very slowly anyway) wiped out by an extracellular hydrolysis of sucrose.

In the experiment No. 2 the final fresh biomass accumulation was higher on the same variants of media (Figure 2) which could have been caused by the cultures' adjustment to the presence of fructose in the media. But this difference was not, however, proved for dry biomass accumulation (Figure 3).

Carbohydrate uptake kinetics

Changes of sucrose, glucose and fructose levels in the media were detetermined during the first phase of the experiments for three variants of the cultivation medium - A (Figure 4A), B (Figure 4B) and C (Figure 4C). Sucrose was depleted from the media by the 18th day of cultivation in case of being the only available sugar and by the 11th day in case of a regime of 0.5% sucrose + 0.5% fructose. At the same time increased concentrations of glucose and fructose in the media of which glucose was preferentially taken up by the cells. Fast sucrose hydrolysis and preferential uptake of glucose, but only if sucrose alone was present in the medium, were reported for other *Taxus spp.* cell cultures (7, 8, 9) Even though the cells do not take up sucrose as a disaccharide, the importance of sucrose as a carbon and energy source for the callus culture may be demonstrated by the fact that the cultures produce an extracellular

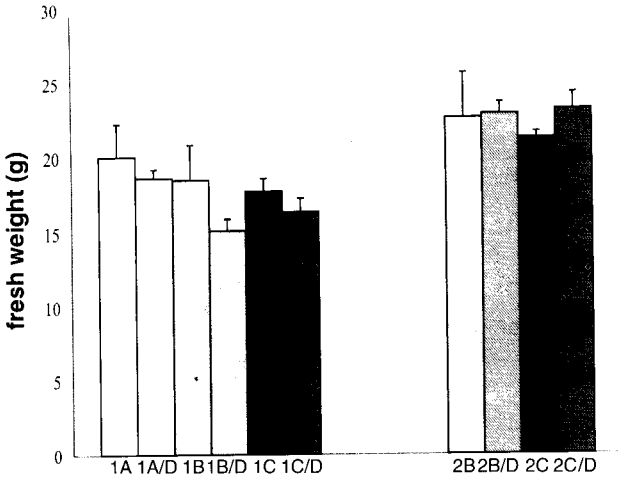

Figure 2. Biomass accumulation at the end of both experiments (1 nad 2) - on fresh weight basis. The first group of columns represent the first experiment and the second one the second experiment. Letters express different carbohydrate regimes -**A**-1% sucrose (*S*), **B**-0.75% sucrose+0.25% fructose(F), **C**-0.5%S+0.5%F, **D**-1%F. The bars represent standard deviations of the mean.

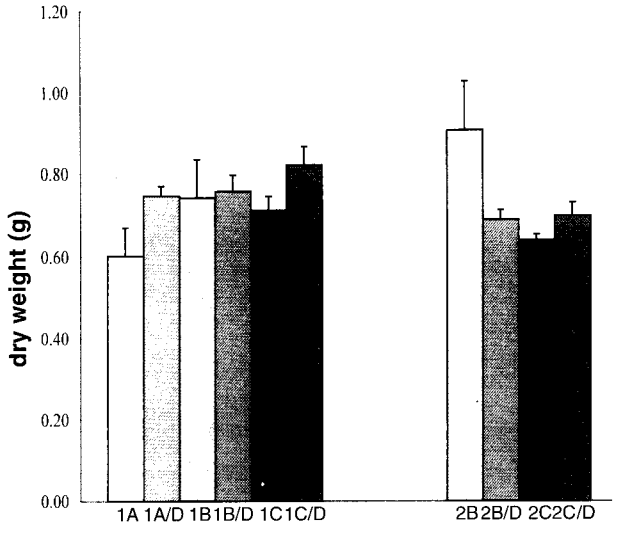

Figure 3. Dry matter accumulation at the end of both experiments (1 nad 2) - on fresh weight basis. The first group of columns represent the first experiment and the second one the second experiment. letters express different carbohydrate regimes - **A** - 1% sucrose (S), **B** -0.75% sucrose + 0.25% fructose (F), **C** - 0.5%S+0.5%F, **D**-1%F. The bars represent standard deviations of the mean.

invertase immediatelly after being subcultured on the fresh media and sucrose is hydrolyzed very fast in spite of a presence of a monosaccharide in the media.

Taxane production

The total taxane production at the end of both experiments are shown in Table 1. The reference No. 6 contains the list of taxanes isolated from the callus culture. The greater fresh biomass production in the experiment No. 2 was connected with a decrease of taxane production in comparison with the same variants in the experiment No. 1. In this experiment the highest total amount of taxanes was determined on the medium with 0.5%S + 0,5%F. Such a high value was due to the presence of 243 μg/g dry weight of one substance in the extracted medium - baccatin III. However, this taxane was not present in greater amounts than units of μg/g in any other sample. As can be seen in Table 1 the ratio between amounts of intracellularly accumulated taxanes and taxanes detected in the media varies considerably. The results from previous studies and the opinions of their authors differ, too. Only traces of taxanes detected in the media were reported (10, 6). It was implied then that these low amounts of taxanes are released into the media due to the cell lysis. On the contrary, there are other studies reporting higher amounts of taxanes in the media. For example, 66% of total taxol amount were detected in the medium in the stationary phase for the suspension culture of *Taxus cuspidata* (7). And Hirasuna and his co-workers report for one cell line of *Taxus baccata* even higher fraction of total taxol amount detected in the medium - more than 90% (4). Authors of both of these studies suggested the taxanes were excreted to the media by living cells. Such unambiguous results were not obtained in case of our callus culture. We presume that due to the low solubility of taxanes in water the storage site in the cell for them may be the cell wall (it is in the intact plant, anyway) and they can eventually diffuse into the medium on the basis of some complex balances. Even the quality of the cell wall of rapidly dividing cells in the tissue culture can contribute to this phenomenon.

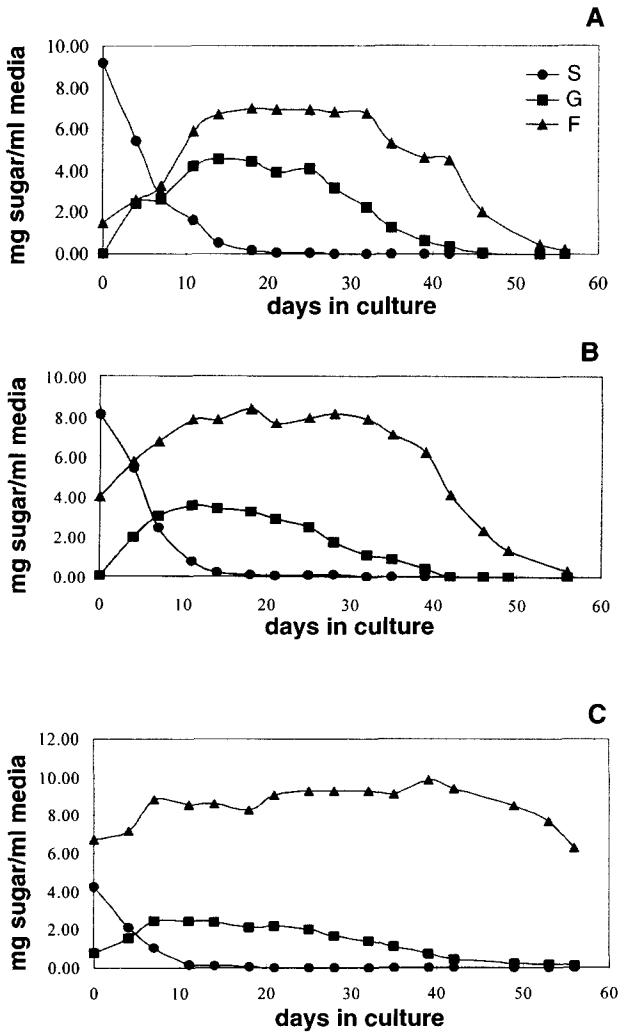

Figure 4. Changes of sucrose (S), glucose (G) and fructose (F) levels in the culture medium during the 1st phase of the experiment No. 1. **A**-medium supplemented with 1%S, **B**-0.75%S+0.25%F, **C**-0.5%S+0.5%F.

It is apparent from both experiments that presence of fructose as an only sugar or in combination with sucrose does not significantly increases production of taxanes in this culture and inferments that fructose might function as a stressor leading to a higher secondary metabolite accumulation in case of being the only available carbohydrate cannot be confirmed.

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