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Original Contribution

EFFECTS OF PLANT GROWTH REGULATORS ON THE CALLOGENESIS AND TAXOL PRODUCTION IN CELL SUSPENSION OF *TAXUS BACCATA* L.

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ABSTRACT

Taxol is an anticancer drug for treatment of a variety of human cancers. The effects of explant source and growth regulators were examined in order to optimize the induction and selection of fast growing callus lines of *Taxus baccata*. Explants were prepared from stems, needles and base of needle of yew tree. We used basal B5 medium with addition of different combination of Auxins and Cytokinins include NAA, 2,4-D, Kin, BAP and GA3. Growth index was used to evaluate the callus growth after 2 month of culture. Callus derived from stem segments displayed the best growth in defined media. Results showed that among all different combination of hormones, the best growth response was obtained from a treatment with 4 mg/l 2,4-D, 0.5 mg/l kin that was 9.286 g callus. We also examined the effect of different combination of hormones in cell suspension culture and 3 suitable medium were selected. These medium include growth medium with 2,4-D (1 mg/l), Kin (1 mg/l) and GA3 (1 mg/l), Taxol production medium with 2,4-D (1 mg/l) and Kin (1 mg/l) and Baccatin III production medium with 2,4-D (2 mg/l) and Kin (0.5 mg/l). Results showed that with the plant hormones amount of Taxol and Baccatin III were increased 3.1 and 5 fold respectively.

Key words: *Taxus baccata*, explants, Auxine, Cytokinin, callus, Baccatin III, Paclitaxel.

INTRODUCTION

Taxol (Paclitaxel) a secondary compound with complex diterpenoic structure is a potent antimitotic drug employed for the treatment of different cancers. Taxol has a unique way of preventing the growth of cancer cells through inhibiting microtubule dissociation, due to its binding affinity to tubulin. It was originally extracted from the bark of *Taxus brevifolia*, a slow growing yew native to the North-Western of Pacific area (1). Paclitaxel has been found in the stem bark, roots and needles of several other yew species. While all *Taxus* species contain Paclitaxel, but amount of Paclitaxel and other related compounds are vary greatly among *Taxus* species (1).

Due to the low yield of Paclitaxel in nature and environmentally destructive prospect of large-scale harvesting of yew trees, alternative sources of Paclitaxel have been sought. Its complex chemical structure means that the total chemical synthesis is not considered economically feasible (2). However, semi-synthesis of Paclitaxel from more abundant Taxanes, for example, via the conversion of Baccatin III was isolated from the needles of yew trees, have provided an immediate and renewable source of the drug (3). Alternatively, the yield of Paclitaxel and its synthetically useful progenitors in *Taxus* cell culture systems, is described in several reports (4, 5), offers another possibility, but the yields need to be improved.

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Secondary metabolite production in plant cell cultures is a process which is usually dependent on a growth phase. For example, in case of Paclitaxel yields in *Taxus* cell cultures, where the production of this secondary product mainly takes place when the linear growth phase has finished and the culture is in its stationary growth phase (6, 7). In such cases, a two-stage culture system would seem to be adequate in order to stimulate secondary metabolite production. First, plant cells are cultured in medium that has been optimized for their growth, and second, after the medium has been removed, the cell biomass then continues its growth in a production medium that mainly stimulates the biosynthesis of secondary metabolites. At the same time, this system has the advantage of permitting the addition of biosynthetic precursors and elicitors when the secondary metabolite production is at its highest, that is, during the second stage of the culture (8). So having a suitable medium is very important for cells growth and Taxol production (8).

Selection of callus lines with optimal growth and efficient Taxanes production is a long-term process for *Taxus* spp. under *in vitro* conditions, factors influencing tissue growth, are explant source and medium composition.

In this work, at first we studied the combinations of different plant hormones and explants sources in callus production. Then in cell suspension culture of *Taxus baccata* hormones were tested on Taxol and Baccatin III production. Results were evaluated for determination of growth with dry weight and for Taxol and Baccatin III amount with HPLC.

MATERIAL AND METHOD

Plant Material and Callus Culture

Explants of *Taxus baccata* tree from the botanic garden of the University of Tehran (Karaj, Iran) were used as the plant material for callus induction. The explants included young stem and needles and base of needles that connect the needles to stem.

For callus culture The explants were washed with distilled water, immersed for 60 seconds in 70% ethanol, and surface sterilized by immersion in sodium hypochloride (3% with a few drops of Tween 20) (MERK, Germany) for 20 minutes and then explants were rinsed three times with sterile distilled deionized water. Briefly, the sections of young stem, needle and base of needle (length 0.8-1.0) of *Taxus baccata* were prepared. For callus induction stems were halved longitudinally and placed with a cut surface in contact with the medium. Needles and base of needles were cut and placed with outer surface in contact with the medium. During this induction process, the cultures were kept at 25 °C in dark condition.

To obtain a homogenous callus, several subcultures of calli were done every 20 to 25 days.

Culture Medium

The basal medium was Gamborg's B5 medium describe previously (9, 10, 11) supplemented with sucrose (25 g/l), plant agar (6.5 g/l), double time of B5 vitamins, Myoinositol (100 mg/l) and PVP (500 mg/l).(all from DUCHEFA, Netherland)

Plant Hormones were tested in 4 individual experiments following a factorial experiment based on completely randomized design. The First one contained combination of NAA (1, 2 mg/l), BAP (0, 0.5, 1 mg/l) and GA3 (0, 0.5, 1 mg/l) the second included combination of NAA (1, 2 mg/l), Kin (0, 0.5, 1 mg/l) and GA3 (0, 0.5, 1 mg/l) , the third contained combination of 2,4-D (2, 4 mg/l), Kin (0, 0.5, 1 mg/l) and GA3 (0, 0.5, 1 mg/l) and the forth included combination of 2,4-D (2, 4 mg/l), BAP (0, 0.5, 1 mg/l) and GA3 (0, 0.5, 1 mg/l), for analyzing the effect of plant growth regulators on callus initiation and growth. The pH of the callus induction medium was adjusted to 5.8 prior to autoclaving. (all from DUCHEFA, Netherland)

Cell Suspension Culture

The cell suspension was maintained in basal Gamborg's B5 medium like callus culture. Different routine maintenance of culture was performed in 250 ml flasks by transferring 2.5 g fresh weight callus into 50 ml liquid medium. Flasks were placed in rotary shaker at 110 rpm in dark at 25 °C (12). In order to study the effect of different concentrations of hormones two experiments were performed following a factorial experiment based on completely randomized design. First one included combination of 2, 4-D (1-4 mg/l), Kin (0, 0.5, 1 mg /l), GA3(0, 0.5, 1 mg/l) and second one included combination of NAA (1-4 mg/l), 2,4-D (0, 0.2, 0.5, 1 mg/l), Kin(0, 0.2 mg/l).

Taxol and Baccatin III Extraction and Determination

Methods were based on those described previously (13). Cell and medium in the culture flasks were separated by filtration. Extracellular Taxol was extracted with dichloromethane and organic phase

was then separated from the aqueous medium by a separation funnel. It was done two times. The dichloromethane was then evaporated to dryness with rotary (Heidolph, Germany) and the solid residue was dissolved in methanol.

The Taxol and Baccatin III content in the extract solution was analyzed by HPLC (SYKAM, Germany) with UV detection at 227 nm. The HPLC column was Kromasil C18 of 250 × 4.6 mm dimension.(GRACE, U.S.A) The mobile phase was methanol: water in a 70:30 volume ratio with 0.01 % TFA(MERK, Germany) at a flow rate of 1 ml/min (14). The Taxol extract solutions were all filtered through a 0.45 µm membrane before being injected into the system. The quantification of Taxol and Baccatin III was based on external standards. (Taxol standard: COLBIOCHEM, USA)
(Baccatin III: SIGMA, Germany)

Callus Growth Measurement

In order to minimize differences in growth that may have been the result of variation in inoculums size, a growth index was used to evaluate the callus growth after 2 month of culture. The growth index was defined as following:

$G.I = (Weight_{final} - Weight_{initial}) / Weight_{initial}$

Growth Measurement In Suspension Culture

At first cells and medium were separated. Cells were kept at 50° C for 3 hours to be dried completely.

Statistical analyzes

The SAS 8.1 and Excel software 2007 were utilized for statistical analysis. The Student-test or one-way ANOVA analysis followed by the Duncan multiple comparison tests were used for statistical evaluations. A P value of less than 0.05 was assumed for significant differences. Every treatment was done with 3 replications.

RESULTS

Explant Source and Callus Development

Our results showed that approximately in all callus culture treatments calli started to develop after about 2 weeks of the culture. In needle culture Initiation of calli occurred from the region where the needles were stripped off. Longitudinal stem sections showed intense cellular proliferation, possibly due to the larger area provided for uptake of medium components and the calli developed from the division of cambium and outer parenchyma tissues of the stem. In the case of needle explants, smaller calli were produced. This might be related to the relatively small amount of meristems and parenchyma in needles (15). Callus growth of the Sections prepared from the base of needles ranged between the stems and needles. It may be because of small amount of meristem and parenchyma than stem and more than needles. Comprising the callus index among all treatments in 4 experiments between three kinds of explants showed that the highest callus growth rate was for stem explants and after that base of needles and needle was better **(Fig 1)**.

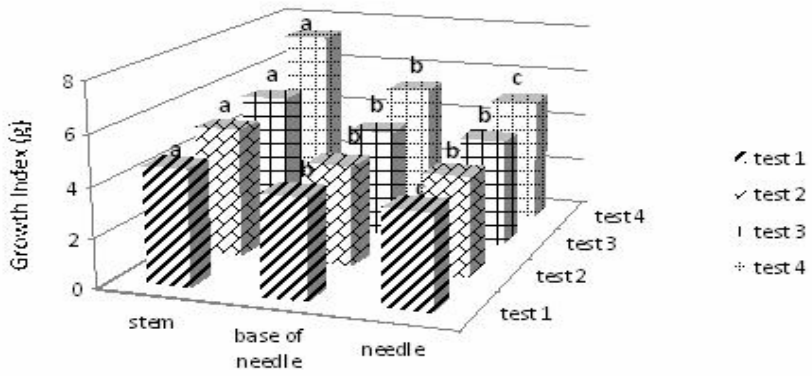

Figure 1: Comprising the callus index for 4 experiments between three kinds of explants. First test contained combinations of NAA (1, 2 mg/l), BAP (0, 0.5, 1 mg/l) and GA3 (0, 0.5, 1 mg/l) the second included combinations of NAA (1, 2 mg/l), Kin (0, 0.5, 1 mg/l) and GA3 (0, 0.5, 1 mg/l) , the third contained combinations of 2,4-D (2, 4 mg/l), Kin (0, 0.5, 1 mg/l) and GA3 (0, 0.5, 1 mg/l) and the forth included combinations of 2,4-D (2, 4 mg/l), BAP (0, 0.5, 1 mg/l) and GA3 (0, 0.5, 1 mg/l).

One of the problems in callus culture was browning the explants especially during the first 2 weeks of culture. Browning of the explants, initiated callus and culture medium was regularly observed. This process is a result of the production and oxidation of phenolic compounds by the explants. Frequent sub culturing might reduced this problem but not completely. This browning led to die of cells so affected on callus index. For reducing this problem PVP as an antioxidant were used.

On the other hand we compared the amount of phenolic compounds in explants that collected in spring with ones collected in autumn and winter. Results showed that spring explants produced less phenolic compounds than others. That has been noted before by others in *T.cuspidata* (11).

The slow growth rate of callus initiation and growth was observed for all three kinds of explants in the present study. This slow growth might be due to the presence of certain growth inhibitors. Needles callus was growing more slowly than stem Callus as was noted in earlier investigation (16). Calluses with subculture intervals of 25-30 days were being maintained for more than one year.

Plant Growth Regulators Ratio and *T.baccata* Callus Growth

Different ratios and concentrations of plant hormones affected the growth of different kind of explants. Results showed that between four series of experiments the last one include 2,4-D (2, 4 mg/l), kin (0, 0.5, 1 mg/l) and GA3 (0, 0.5, 1 mg/l) had the best result **(Fig 1)**.

After the second month among all the plant regulator combinations the best growth response was obtained by stem explants cultured in medium with 4 mg/l 2,4-D , 0/5 mg/l kin. The callus index for this medium was 9.286 g after 60 days of growing. The highest callus index were 5.296 g and 5.069 g for base of needles and needles respectively (data was not shown).

Plant Growth Regulators Ratio and *T.baccata* Cell Growth

After assaying 68 different suspension culture media in both experiments three suitable medium for growth, Taxol and Baccatin III production were selected. Among all of these 68 media one with 2,4-D (1 mg/l), Kin (1 mg/l) and GA3 (1 mg/l) showed the best result in growth. For Cells cultured in this medium **(Fig 2)** the linear growth phase lasted for 14 days and after that the stationary phase was began. A biomass production of 35.333 g of dry weight / L was measured on day 14 that is the end of linear phase and start of stationary phase. A biomass production of 34.166 g of dry weight / L was measured on day 21. As can be deduced, this presented a growth rate of 0.702 g day^{-1} at the end of linear growth phase and a growth rate of 0.412 g / day at the end of the culture. These growth rates are quiet good in comparison with reports of 17-day doubling time in cell culture of *T.cuspidata* (17) and 14-day doubling time in cell cultures of *T. Media* (18) and 7.8 day doubling time in *T.media* (8). On the other hand when the cell suspension grew in the production medium for Taxol a biomass yield of 34.165 g of dry weight / L was measured on day 14 and after that stationary phase was started and 34.06 g of dry weight / L was measured in day 24 which represented a growth rate of 0.618 day^{-1} **(Fig 3)**. Concentration of hormones in this medium were 2,4-D (1 mg/l) and Kin (1mg/l). In suitable medium for Baccatin III production as shown in diagram 3 the maximum growth was in day 7 and biomass production was 31.066 g of dry weight at the end of linear phase and after that growth rate was decreased and stationary phase was started. The growth rate for this medium at the end of linear phase was 0.795/ day and at the end of the culture was 0.257/ day. Hormones amount in this medium were 2, 4-D (2 mg/l) and Kin (0.5 mg/l).

We assayed all 68 medium with HPLC for Taxol and Baccatin III. Results showed that among all media 2 of them were better than others, one for Baccatin III and one for Taxol.

It can be seen that the most productive state for both media was on day 21, and from day 14 amount of Taxol and Baccatin III were increased, that is the start of growth stationary phase. The maximum yields of Taxol was 811.222 µg/l in medium with 1 mg/l 2,4-D and 1 mg/l Kin. It was 260.97µg/l in control that is B5 medium without hormones **(Fig 4)**.

Maximum Baccatin III was 3.116 mg/l in medium with 2 mg/l 2,4-D and 0.5 mg/l Kin. In control 0.627µg/l was obtained **(Fig 5)**. It was reported before that in *T.media* cell culture, medium with 2 mg/l 2,4-D and 0.1 mg/l BAP was optimum for both Taxol and Baccatin III production with maximum amount of 2.09 and 2.56 mg/l respectively (8).

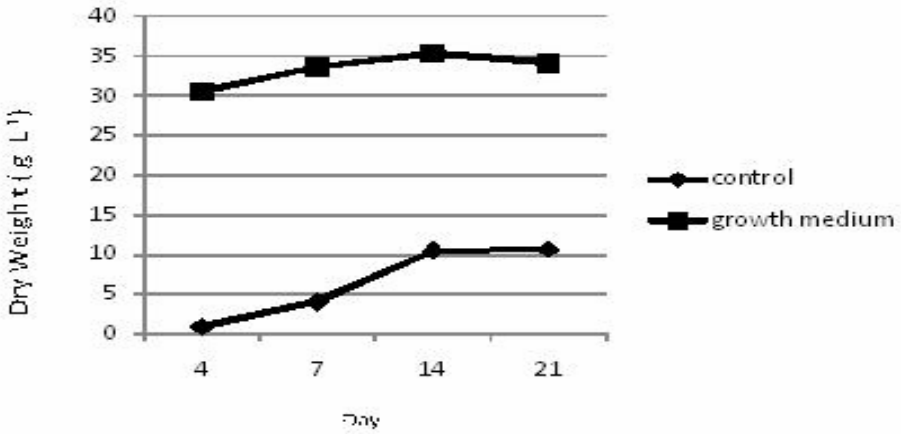

Figure 2: Cells dry weight (g/l) in selected medium for cell growth (2,4-D (4 mg/l) + Kin (0.5 mg/l)).

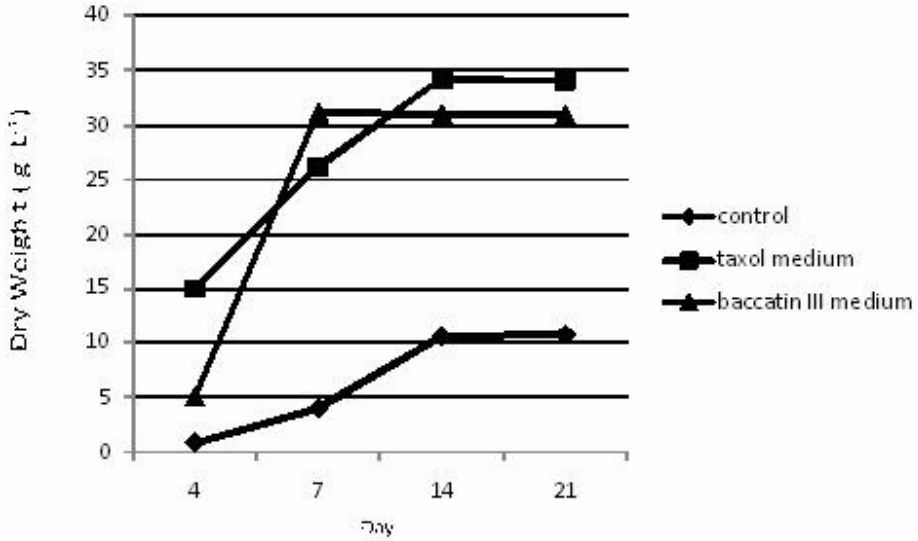

Figure 3: Cells dry weight (g/l) in selected mediums for Taxol (2,4-D (1mg/l) + Kin (1mg/l)) and Baccatin III (2,4-D (2 mg/l + Kin (0.5 mg/l)) production.

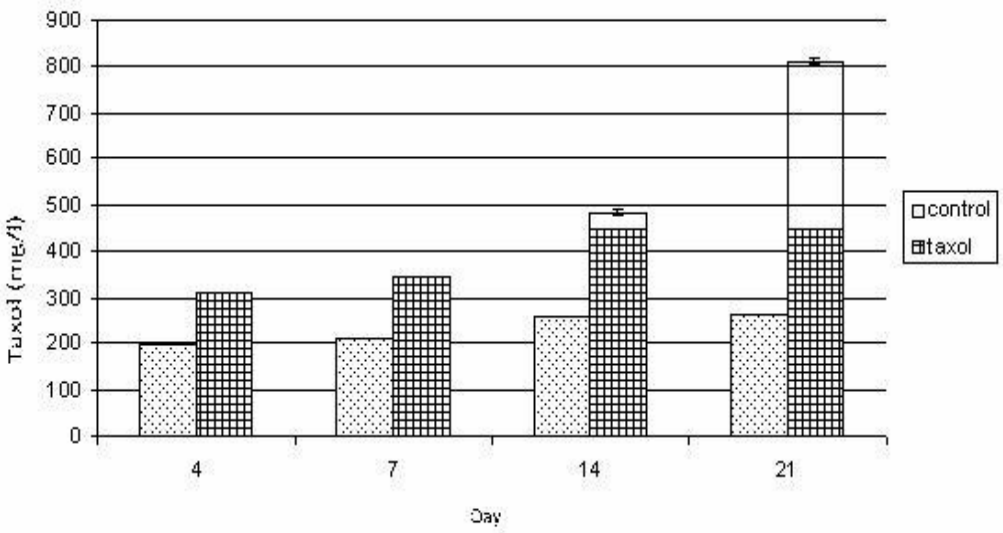

Figure 4: Extracellular Paclitaxel in Taxol production medium (1mg/l 2, 4-D and 1mg/l Kin) in comparison with control. Data represent average values from 3 replicates ± SD. Control is B5 medium without hormones.

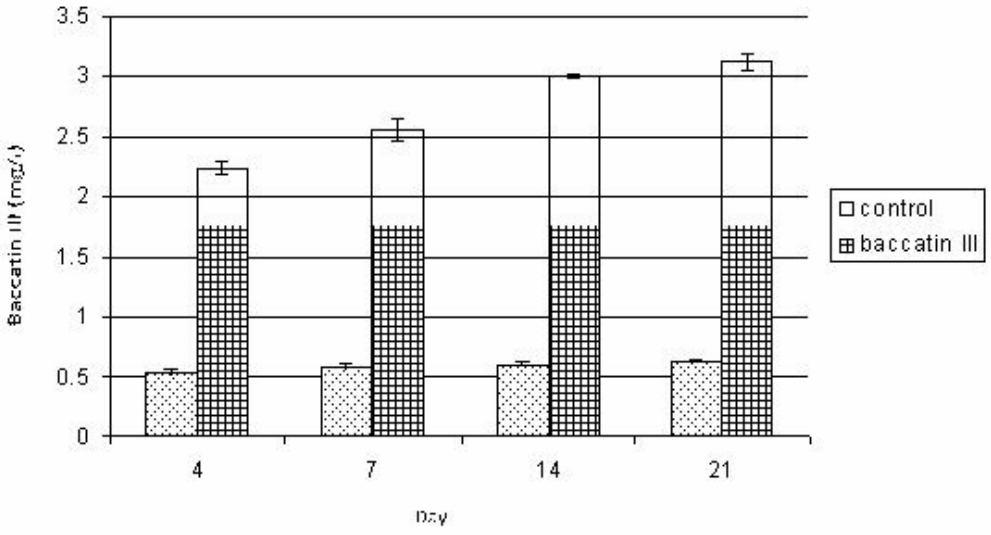

Figure 5: Extracellular Baccatin III in Baccatin III production medium (2 mg/l 2,4-D and 0.5 mg/l Kin) in comparison with control. Data represent average values from 3 replicates ± SD. Control is B5 medium without hormones.

We can see that in all three selected mediums 2,4-D and Kin were existed but just in growth medium GA3 was existed. It shows that GA3 is not necessary for Taxol and Baccatin III production. Fetto-neto et al., (16) reported that GA3 stimulate callus growth without affecting Taxol production.

DISCUSSION

The present study demonstrated the possibility of obtaining and maintaining callus of *Taxus baccata* for periods about one year. Callus culture results showed that the sections of young stem exhibited a better callusing response than the other tested part of the plant as has earlier been reported for *T.baccata* (15) so stems culture is more useful for producing fast growing and high quality callus.

In callus culture browning was one of our problems that solved with PVP and sub culturing. It seems that PVP was effective in partially adsorbing phenolic compounds produced by the explants (15, 16).

As we can see in all derived suitable mediums combination of Auxin and Cytokinin were effective. Significant Auxine and Cytokinin interactions affecting the growth of callus in several plant species are well documented (19). We concluded that among Auxines 2,4-D was more effective than NAA in callus initiation and development. The good effect of 2,4-D on callus growth of *T.baccata* may be related to role of this plant hormone in blocking morphogenesis and favors differentiation and callus developments. Between Kin and BAP, first one was more effective. This was in agreement with Cusido et al., (1999) and Jha et al., (1997) results for *T.baccata* (8, 11). Our results showed that GA3 in 0.5 mg/l is effective for callus growth but more than this is not useful.

In cell suspension culture we saw reduction in Taxol yield when cells were in logarithmic growth phase. It may be related to the usual apparent loss of ability to accumulate secondary products as plant tissue undergoes active cell division. Possible factors involved in this phenomenon include repression of regulatory genes in non-specialized cells, diversion of substrates from secondary metabolism, non-operational end-product transport mechanisms, and lack of proper storage sites or unregulated catabolism of synthesized products (20).

Our data indicated that Taxol and Baccatin III yield in cell culture can be increased 3.1 and 5 fold respectively by manipulating hormones concentration of culture medium. There are several approaches to be considered to increase these yields by cell culture system, such as elicitor treatment, immobilized culture and permeabilization of cultured cells. By comprising between obtained Taxol and Baccatin III we concluded that Baccatin III concentration in *Taxus baccata* is more considerable than Taxol. Since semi synthesis is commercial method for Taxol production and Baccatin III is used on it, producing the Baccatin III by this method from *Taxus baccata* can be useful.

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