Enhancement of Paclitaxel Production by Combination of in situ Extraction with an Organic Solvent and an Elicitation in a Suspension Callus Culture

Shinjiro YAMAMOTO*, Yuichi GOTO, Tomohiko KAWANO, Shuhei HAYASHI and Shintaro FURUSAKI
Department of Applied Life Science, Sojo University, 4-22-1 Ikeda, Kumamoto 860-0082, Japan

For the effective production of paclitaxel, the behavior of a suspension culture of *Taxus baccata* callus was investigated in an organic solvent-medium two-phase culture system. Lauryl alcohol (LA) was used as the solvent to extract paclitaxel in situ from the medium in the culture, where the volume fraction of LA was 10%. Paclitaxel production in the culture with in situ extraction with LA was improved from that without LA maintaining the callus growth. An elicitor, methyl jasmonate (MJ), was added to the two-phase culture system, resulting in the enhancement of both the production amount of paclitaxel and the callus growth rate with an increase in the MJ concentration. Paclitaxel production rate in the culture with LA including 22 mM MJ was greater than that without LA and MJ by a factor of more than two.

1. Introduction

Paclitaxel (Taxol®) is a natural product which is used as an excellent antitumor drug for ovarian and breast cancers [1, 2]. Moreover, paclitaxel has been reported to exhibit inhibitory effects against a variety of tumor cells [3] and to have a potential to treat Alzheimer's disease [4], suggesting that the demand for paclitaxel is increasing. Currently, paclitaxel is manufactured by a semi-synthetic method, which synthesizes paclitaxel by chemical modification of a precursor such as 10-deacetylbaccatin III extracted from the needles of *Taxus* yew trees [2]. The paclitaxel is expensive because the production amount is limited by the plant source. As an alternative paclitaxel production process, total synthesis of paclitaxel [5, 6] has been studied, however, it requires 40 steps and the yield was low. Therefore, total synthesis is not feasible for large scale production [7]. Recently, sustainable production processes attract a great deal of attention. Plant cell (callus) culture is one of the promising methods for this purpose because it is harmless to trees and the environment since it is not necessary to collect a large amount of yew leaves. Callus can be easily induced from a part of plant tissue and simply cultivated using conventional cell culture equipment. A large scale culture of the plant cell makes it possible to produce sufficient quantities of paclitaxel and to meet the increasing commercial demand.
In the callus culture, the paclitaxel produced by cells reduces the callus growth, and paclitaxel productivity is also reduced due to feedback inhibition. To avoid this inhibition, two-phase culture systems using solid absorbents or organic solvents added to the medium have been proposed [8-12]. Polymeric solid resins such as nonionic Amberlite can recover in situ plant metabolites effectively from the culture medium and subsequently lead to productivity enhancement [13, 14]. Introduction of liquid organic solvents such as hexadecane [8] and a derivative of glycerol ester (tricaprylin) [9] were effective for the selective in situ extraction of paclitaxel from the culture medium. Hydrocarbons of higher logP values as well as higher alcohols increased the paclitaxel productivity in the two-phase system [15].

Elicitors, which are compounds of biotic or abiotic origin, induce selective production of secondary metabolites of plant cells [16-21]. Methyl jasmonate (MJ) is known to enhance paclitaxel synthesis in the callus culture of *Taxus* species [18]. Recently, improved paclitaxel production by a combination of in situ extraction with an organic solvent (oleic acid/terpineol=1:1, v/v) and elicitation by MJ in cell suspension cultures of *T. chinensis* has been reported [22]. However, the callus growth was reduced in the culture. Generally, callus growth ceased or decreased in the culture when paclitaxel production was induced by adding MJ. For effective paclitaxel production, the culture conditions which enhance paclitaxel production while maintaining callus growth should be explored. We have been investigating an effective organic solvent for this purpose and found suitable hydrocarbons of higher logP values as well as higher alcohols [15]. In this research we performed the callus culture in an organic solvent-medium two phase system including MJ. The combination effects of the in situ extraction with the organic solvent and elicitation by MJ on both the callus growth of *Taxus baccata* and its paclitaxel production were examined. Lauryl alcohol, which enhanced the callus growth and paclitaxel productivity in our previous research [15], was used as an organic solvent.

2. Experimental

2.1. Callus and Medium

The callus, which was kindly supplied by Dr. M. Seki, The University of Tokyo, Japan, was used in this research. The callus induced from *Taxus baccata* was maintained in a solid medium containing an agar and in a modified Gamborg’s B5 [23] medium as described previously [15]. The callus was subcultured on the solid medium at 25 °C in the dark.

2.2. Chemicals

Lauryl alcohol (LA; Wako Pure Chem. Co., Osaka, Japan) was used as the organic solvent because it enhanced the callus growth and paclitaxel production according to a previous study [15]. Methyl jasmonate (MJ; Wako Pure Chem. Co., Osaka, Japan) was applied as an elicitor. LA and MJ were filter-sterilized by using a 0.22 µm hydrophobic membrane filter (Millipore, Japan) prior to use in the culture.

2.3 Callus culture

Culture was performed in the LA-medium two-phase system. Modified Gamborg’s B5 medium without agar as described above was used for the suspension callus culture. Culture was started by inoculation of cells, which had been subcultured in a rotary shaker (NR-150, Tai tec, Saitama, Japan) for 10-14 d, in a 100 mL Erlenmeyer flask containing 20 mL of the medium at a
rotational speed of 110 rpm and a cell concentration of 20-25 g-fresh cell weight per L (g-FCW/L). Firstly, 2 mL LA (i.e. 10 vol%) and 0.1 mM MJ were added at the initiation of the callus culture to investigate the effect of the combination of in situ extraction with LA and simultaneous elicitation by MJ. The reason for the use of 0.1 mM MJ in the experiment was that this MJ concentration was reported to be optimal for paclitaxel production in Taxus media callus culture (Yokimune et al. [21]). Conventionally, MJ was added to the culture medium with ethanol because of its low solubility in water. To compare this with the present culture conditions where MJ and LA were added to the medium, culturing by adding MJ only with methanol was also performed. Methanol, 5 µL per mL of culture medium, was added in this case with MJ to the medium after cell inoculation because the solubility of MJ in the aqueous medium is low. Methanol was ineffective in promoting callus growth and paclitaxel production (data not shown). After a 7 d culture period, the fresh cell weights of the callus and paclitaxel concentrations in both the medium and LA were measured.

Next, the effect of the MJ concentration on callus growth and paclitaxel production in the culture with in situ extraction using LA was examined. Since MJ is a relatively hydrophobic compound, it can be solubilized in LA. A callus culture in the absence of LA and MJ was conducted here as the control culture. After the 14 d culture period, the fresh cell weight of callus and the paclitaxel concentrations both in the medium and in LA were measured.

To express the effectiveness of the combination of in situ extraction with LA and elicitation by MJ, the callus growth rate and paclitaxel production rate were used. To simplify these rates, overall callus growth rate \( V_{FCW} \) and overall paclitaxel production rate \( V_P \), which were estimated from the increased amounts of callus growth and paclitaxel during a defined culture period, were defined by Eqs. (1) and (2), respectively.

\[
V_{FCW} \text{[g-FCW/L/d]} = \frac{\Delta FCW}{\Delta t} \quad (1)
\]
\[
V_P \text{[mg-paclitaxel/L/d]} = \frac{\Delta P}{\Delta t} \quad (2)
\]

where \( \Delta FCW \), \( \Delta P \) and \( \Delta t \) are the increased amount of the fresh cell weight, the increased amount of paclitaxel and the defined culture periods (7 or 14 d), respectively.

Since most of the paclitaxel synthesized by the callus was secreted into the medium (data not shown), the paclitaxel concentration in the callus was ignored. In the LA-medium two phase system the total amount of paclitaxel was estimated from the paclitaxel concentrations in the medium and LA.

2.4. Analysis

Extraction and analysis of paclitaxel in both the medium (aqueous phase) and LA (organic phase) were conducted according to the procedure by using a reverse-phase high performance liquid chromatography (HPLC) system with a cyanopropyl-silica-gel column (TSKgel CN-80TS, 4.6x250 mm, Tosoh, Japan) as reported previously [15]. The MJ partitioned in LA did not interfere in the HPLC analysis of paclitaxel extracted from LA. The callus was separated from the culture broth of the sample by filtration and then washed with water. The fresh cell weight (FCW) was determined by weighing the callus.
3. Results and Discussion

3.1. Effect of \textit{in situ} extraction with LA and elicitation on paclitaxel production and callus growth

Firstly, an experiment on the effect of \textit{in situ} extraction by LA and elicitation by MJ on paclitaxel production was carried out. Fig. 1 shows the experimental results during a 7 d culture period after initiation of the callus culture and simultaneous elicitation by 0.1 mM MJ in the LA-medium two phase system. The overall paclitaxel production rate in the culture with \textit{in situ} extraction with LA was improved compared to that of the control. Moreover, the overall paclitaxel production rate in the culture with the combination of \textit{in situ} extraction and elicitation was higher than that with \textit{in situ} extraction only and that by elicitation only, indicating a favorable combination effect of \textit{in situ} extraction and elicitation on paclitaxel production. This effect may be because paclitaxel production was enhanced by MJ and the produced paclitaxel was simultaneously extracted with LA. The overall callus growth rate in the culture with the combination of \textit{in situ} extraction and elicitation was

![Fig. 1](image1.png)  \textbf{Fig. 1}  \textit{Effect of \textit{in situ} extraction with LA and elicitation by MJ on the overall paclitaxel production rate.}  0.1 mM MJ = the culture including 0.1 mM MJ and methanol; LA only = the culture with \textit{in situ} extraction with LA only; LA+0.1 mM MJ = the culture with \textit{in situ} extraction with LA and elicitation by 0.1 mM MJ; control = the culture with no treatment

![Fig. 2](image2.png)  \textbf{Fig. 2}  \textit{Effect of \textit{in situ} extraction with LA and elicitation by MJ on the overall callus growth rate.}  0.1 mM MJ = the culture including 0.1 mM MJ and methanol; LA only = the culture with \textit{in situ} extraction with LA only; LA+0.1 mM MJ = the culture with \textit{in situ} extraction with LA and elicitation by 0.1 mM MJ; control = the culture with no treatment
the same as that of the control (Fig. 2). On the other hand, the callus growth rate in the culture containing MJ was higher than that for other culture conditions. Generally, the callus growth ceased or decreased by adding MJ because of the increased paclitaxel concentration in the medium, however, our present data showed the opposite result. The callus of *T. baccata* used in the present research secretes most of the paclitaxel synthesized during the culture, which is different from other *Taxus* species which accumulate paclitaxel inside the cell. Though paclitaxel inhibits the callus growth, the precursors of the paclitaxel such as 10-deacetylbaccatin III and baccatin III accumulated in the cells may also have an inhibitory effect against callus growth. If this is true, our experimental result can be explained as follows. The intracellular precursors were quickly converted to paclitaxel by MJ and subsequently the produced paclitaxel was secreted into the medium and then extracted with LA. Though this phenomenon was not clearly understood, decreased intracellular concentration of the precursors through the metabolism induced by MJ may lead to the decreased inhibition and to increase a callus growth rate regardless of the enhanced paclitaxel production. The callus growth rate in the culture with *in situ* extraction and elicitation was lower than that with elicitation only despite of the same MJ concentration of 0.1 mM. Since MJ is preferentially partitioned into LA rather than the medium due to its hydrophobicity, the MJ concentration in the culture medium containing both LA and MJ was lower than that containing MJ only, which might result in relatively higher concentration of the intracellular precursors and consequently in limitation of the callus growth.

### 3.2 Effect of MJ concentration on paclitaxel production and callus growth in the culture with *in situ* extraction

Next, the effect of MJ concentration on paclitaxel production and callus growth was examined. Fig. 3 shows the experimental results for a 14 d culture period after initiation of the callus culture and simultaneous elicitation by various concentrations of MJ in the LA-medium two phase system. An increase in the MJ concentration ranging from 0.1 to 22 mM resulted in an increase in paclitaxel production rate. The overall paclitaxel production rate in the culture with LA containing 22 mM MJ was more than two fold greater than that without LA and MJ. A similar trend of increasing callus growth rate was observed on increasing the MJ concentration (Fig. 4). As described above, the intracellular inhibitory precursors of paclitaxel were converted to paclitaxel, secreted into the medium and accumulated in the LA phase, resulting in the improvement in the callus growth rate. Zhang and Xu reported that *in situ* extraction with oleic acid/terpineol and elicitation by MJ present in the callus culture improved paclitaxel production. However, callus growth decreased [22]. Our proposed culture with *in situ* extraction with LA and elicitation by MJ was effective in promoting both callus growth and paclitaxel production. This method could be a promising way to realize large scale culture for the paclitaxel manufacturing process.

The time of addition of MJ to the medium, the order of addition of LA and MJ and the amount of LA added to the medium are important factors for improvement in both paclitaxel production and callus growth. We are now investigating these factors. The partition of MJ between LA and the medium, which is related to paclitaxel synthesis and callus growth, should be measured.
Fig. 3  Effect of in situ extraction with LA and elicitation by MJ on the overall paclitaxel production rate. LA only = the culture with in situ extraction with LA only; LA+0.1-22mM MJ = the culture with in situ extraction with LA and elicitation by 0.1-22 mM MJ; control = the culture with no treatment.

Fig. 4  Effect of in situ extraction with LA and elicitation by MJ on the overall callus growth rate. LA only = the culture with in situ extraction with LA only; LA+0.1-22mM MJ = the culture with in situ extraction with LA and elicitation by 0.1-22 mM MJ; control = the culture with no treatment.
4. Conclusion

The effect of a combination of in situ extraction by LA and elicitation by MJ in the callus culture of T. baccata on paclitaxel production and callus growth was investigated. Combination effects of in situ extraction and elicitation on paclitaxel production and callus growth were observed. The paclitaxel production rate by the culture with in situ extraction and elicitation was more than two fold higher than that for the control culture without extraction and elicitation. The greater the amount of MJ in the culture with in situ extraction, the larger was the amount of paclitaxel production and callus growth.

Acknowledgment

This work was partially supported by a Grant-in-Aid for Scientific Research (B) (No. 14350440) from the Japan Society for the Promotion of Science (JSPS). We would like to thank Dr. Minoru Seki for his kind supply of Taxus baccata callus.

References


