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Induction studies of methyl jasmonate and salicylic acid on taxane production in suspension cultures of *Taxus chinensis* var. *mairei*

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Abstract

The induction by methyl jasmonate (MJ) and salicylic acid (SA) of taxol and relevant taxane biosynthesis in suspension cultures of *Taxus chinensis* var. *mairei* was studied both theoretically and experimentally. The theoretical model shows that the apparent number of elicitor molecules binding with hypothetical receptor molecules for MJ in inducing taxol biosynthesis is about 75% lower than that for SA. The apparent binding constant between the elicitor and hypothetical receptor molecules for MJ is 10 orders of magnitude higher than that for SA. MJ increased taxol production more significantly than did SA as observed in our experiments. The induction model is able to predict induction efficiency of an elicitor. SA might apparently increase taxol production by blocking the biosynthesis pathway from baccatin III to cephalomannine, based on the observation that SA promotes cephalomannine production.

Keywords: *Taxus chinensis* var. *mairei*; Suspension culture; Taxane; Methyl jasmonate; Salicylic acid; Induction model

1. Introduction

Taxol, a diterpene found in various tissues of *Taxus* species, and relevant taxanes have received considerable attention as promising anticancer agents owing to their unique mode of action on the microtubular cell system [1]. A major limitation to the therapeutic use of taxol is its limited resource in nature. The production of taxol from plant cell cultures has been regarded as a potential solution to the 'supply crisis' of taxol [2].

Currently, some successful applications of the production of taxol by plant cell culture have been reported in literature [3,4]. The results show that for a better production process design it is of importance to elicit the secondary metabolism by adding various kinds of abiotic and biotic elicitors [5,6]. In general, an elicitor is considered as a signal molecule that acts on plant cells. Elicitor molecules are recognized by specific receptors on the plasma membrane of plant cells [7]. The subsequent binding events trigger a signal-transduction cascade leading to the activation and expression of genes related with the biosynthesis of secondary metabolites [8]. For a better understanding of the induction mechanism, it is necessary to investigate the combination of elicitor molecules with their receptors. It has been reported that jasmonate and salicylate are endogenous signal molecules, which not only elicit plant resistance to pathogens and herbivores but also exogenously induce secondary metabolism pathways [9–12]. Although many articles report that methyl jasmonate (MJ) strongly enhances taxane production through inducing the activation of some key enzymes [13–15], the induction mechanism of MJ on secondary metabolism, especially the binding of MJ with a hypothetical receptor has not been studied. Also, salicylic acid (SA) strongly induces secondary metabolism in plants and plant cell cultures, such as *Hypericum perforatum* L. and *Rubia cordifolia* callus cultures [11,12]. Except for the action of SA on taxol biosynthesis, as reported in our previous paper [16], little information is available about the induction mechanism of SA on taxane production.

Modeling has been proved to be a useful method for analyzing the induction mechanism of elicitors on secondary metabolite biosynthesis [17]. In the present work, the binding of MJ or SA molecules with hypothetical receptors and the elicitation of taxane biosyntheses by exogenous MJ and SA were studied by analyzing induction model parameters and content of taxanes to shed light on the induction mechanism of the two elicitors on taxane biosyntheses.

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2. Materials and methods

2.1. Chemicals

MJ and SA were purchased from Aldrich and Sigma, respectively. All other chemicals were of analytical grade and obtained commercially.

2.2. Cell line and culture conditions

Taxus chinensis var. *mairei* T from the Botany Institute of Chinese Academy of Sciences was grown in modified solid B5 medium (pH 5.8) supplemented with inositol (0.1 mg/l), Vitamin B_1 (10 mg/l), Vitamin B_6 (1 mg/l), nicotinic acid (1 mg/l), sucrose (25 g/l), 6-benzyladenine (0.5 mg/l) and casein acid (1 g/l). Fresh cells (4 g) from the solid medium were collected, inoculated into 50 ml fresh modified liquid B5 medium in 250 ml Erlenmeyer flasks every 15 days and sub-cultured at 25 °C in the dark with continuous shaking at 110 rpm. In order to obtain uniform cells before the induction experiments, six to eight flasks each containing 50 ml cultures of the 5th generation were combined to provide the inoculum in a 500 ml Erlenmeyer flask containing 150 ml fresh liquid medium. After being incubated for 15 days, cells were transferred into 250 ml fresh medium in a 1000 ml Erlenmeyer flask. The cells were collected by vacuum filtration after being cultured for 15 days. Cell samples (4 g) were transferred successively to 50 ml fresh liquid medium in 250-ml flasks and divided into three groups for triplicate experiments. All flasks were capped with filter closures (Beijing Zhentai Horticulture Co., China) consisting of double layers of plastics with four vents covered by ventilated polypropylene filtering membrane. Air and other gases such as ethylene, oxygen and carbon dioxide can freely pass through the closures but bacteria are completely kept out.

2.3. Elicitation procedures

MJ and SA were dissolved in small amounts of methanol and distilled water, respectively. At day 7 or 14 of the culture cycle, filter-sterilized MJ and SA were added into the culture medium to achieve the required concentrations. Samples of the treated and control groups were taken at predetermined time intervals for several analyses. All data were the average of triplicate experiments and the errors were within ±10%.

2.4. Taxane extraction and HPLC analysis

For extraction of intracellular taxanes (10-deacetybaccatin III (10-DAB), baccatin III, taxol and cephalomannine), dried cells (200 mg) were powdered and dissolved in 20 ml methylene chloride/methanol (1:1, v/v) and ultrasonicated for 20 min. The mixture was then extracted three times with 100 ml methylene chloride. For recovery of taxanes from culture medium, 10 ml sample was filtered and the filtrate was extracted three times with 100 ml methylene chloride. The methylene chloride phases in the above two cases were respectively collected and evaporated at room temperature, the remaining taxanes were re-suspended in 1 ml methanol and filtered through a 0.2 μm polymeric filter prior to HPLC analysis. The column (25 cm × 4.6 mm i.d.) was packed with Kromasil C_{18} (5 μm) and eluted with a mixture of methanol and water (65:35, v/v) at 1.0 ml/min. Detection was with a Waters 996 photodiode array detector at 227 nm. Retention time and UV spectral comparison with authentic standards of taxol and other taxanes were used to identify taxol and other taxanes [18]. Taxol and other taxanes were quantified by comparison with external standard curves generated from 30 to 300 μg/ml taxanes [19].

3. Induction model

The binding of the modulators such as ethylene with receptors on the plasma membrane might sensitize hypothetical MJ receptors on the membrane [20]. As MJ solely induces secondary metabolism in plant cells [21,22], MJ molecules may directly associate with hypothetical receptors on the membrane. The signal transduction cascade may then be activated to induce the production of secondary metabolites.

To elucidate the induction mechanisms of MJ and SA on taxol production, the binding of MJ and SA molecules with their respective receptors is analyzed by an induction model based on the induction model of Mirjalili and Linden [17], the inhibition model of Phisalaphong and Linden [23] and the *lac* operon model of Yagil and Yagil [24]. The model is constructed on the basis of the following assumptions: (1) there are the receptors for MJ and SA in plasma membrane; (2) the binding of elicitor molecules to receptors is a reversible process; (3) as a taxol molecule contains several hydrophobic groups, it can bind to proteins by forming hydrophobic bonds [25,26]. It is thus highly possible that taxol molecules bind to the hypothetical receptor to some extent. However, considering the much lower taxol concentration compared to that of elicitors in the present work, the number of receptor molecules binding with taxol molecules was too small and thus neglected for simplicity of calculation.

The two basic equilibrium equations in the model for inducible systems are [24]:

$$nE + R \overset{K_1}{\Leftrightarrow} RE_n \quad (1)$$

$$P + R \overset{K_2}{\Leftrightarrow} PR \quad (2)$$

where n is the apparent number of elicitor molecules binding with a receptor molecule, E is an elicitor molecule, R is an unbound receptor molecule on the plasma membrane. RE_n is a receptor molecule that binds n elicitor molecules, P is a

product molecule (such as taxol molecule), $[P_t]$ is the total concentration of the product in suspension cultures:

$$[P_t] = [P] + [PR] \quad (3)$$

$[R_t]$ is the total concentration of receptor. According to the assumptions, $[R_t]$ is described as

$$[R_t] = [R] + [RE_n] \quad (4)$$

Let $\eta = [P]/[P_t]$, in combination with the expressions of equilibrium constants $K_1 = [R][E]^n/[RE_n]$ and $K_2 = [R][P]/[PR]$, thus

$$\frac{\eta}{1-\eta} - \frac{K_2}{[R_t]} = \frac{K_2}{K_1[R_t]}[E]^n \quad (5)$$

When the concentration of elicitor [E] equals zero, Eq. (5) simplifies to $\eta/(1-\eta) = K_2/[R_t]$. Under these conditions, $\eta \ll 1$, so $K_2/[R_t]$, which becomes the basal level of η, is denoted as η_b. Thus, Eq. (5) can be described as

$$\log\left(\frac{\eta}{1-\eta} - \eta_b\right) = n\log[E] - \log K_1 + \log\eta_b \quad (6)$$

Assuming that K is the apparent binding constant between elicitor and receptor molecules, so $K = 1/K_1$. Then, Eq. (6) is rewritten as

$$\log\left(\frac{\eta}{1-\eta} - \eta_b\right) = n\log[E] + \log K + \log\eta_b \quad (7)$$

If the elicitor promotes the secondary metabolism response, Eq. (7) should be used. If the elicitor acts as an inhibitor in secondary metabolism, Eq. (8) should be applied [24]:

$$\log\left(\frac{\eta}{1-\eta} - \eta_b\right) = -n\log[E] - \log K + \log\eta_b \quad (8)$$

The left hand terms of Eqs. (7) and (8) can be plotted against the logarithm of elicitor concentration to give straight lines. From the slope and intercept of the straight lines n and K can be deduced.

4. Results and discussion

4.1. Taxol production induced by MJ and SA at different concentrations

The content of taxol in time course samples after addition of MJ or SA of different concentrations was detected (Fig. 1), which shows that MJ at concentrations ranging from 25 to 275 μM induced taxol production and the taxol content accumulated to a maximum of 0.143 μM when the MJ concentration was 100 μM. Ketchum et al. [19] observed an optimal MJ concentration of 200 μM for taxol production in *Taxus canadensis* cells. Yukimune et al. [2] and Laskaris et al. [13] reported that 100 μM MJ strongly increased the accumulation of taxanes in suspension cultures of *Taxus media* and *Taxus baccata*. Mirjalili and Linden [17] demonstrated interaction between MJ and ethylene. Thus, the optimal MJ concentration for inducing taxol production is dependent on the cell line and growth conditions.

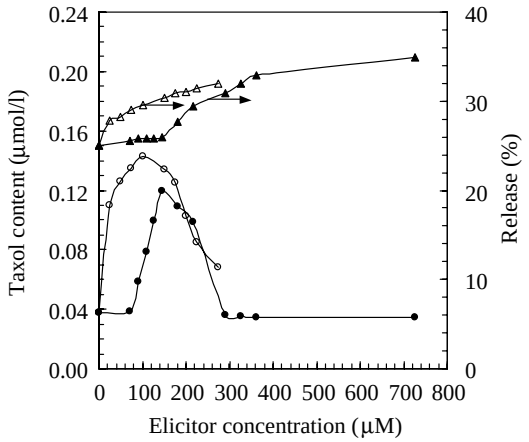

Fig. 1. Taxol contents (in culture medium) and release ratios of the taxol content in the medium to total taxol content (in cells plus medium) after addition of elicitors ((○, △) MJ or (●, ▲) SA) at different concentrations. Elicitors were added at day 7 of cell growth and taxol content was determined at day 14. The arrows indicate the coordinates of the data.

From Fig. 1, it is also seen that SA at concentrations ranging from 72.5 to 290 μM improved taxol production compared to that of the controls. At an SA concentration of 145 μM, the taxol content accumulated to a maximum of 0.119 μM. When SA concentration exceeded 290 μM, taxol production did not increase or even kept at the same level with that of the controls, indicating that the damage of SA to *Taxus* exceeded the tolerance of the cells. The maximal taxol production in the case of MJ is higher than that in the case of SA. This might ascribe to the different induction mechanisms between MJ and SA.

Fig. 1 also shows that the extent of taxol excretion was enhanced with increasing MJ or SA dose. The increased extracellular taxol concentration may be attributed in part to cell lysis caused by the addition of the elicitors, similar with those reported for other elicitors [27].

4.2. Analysis of receptor model parameters

The values of η_b can be experimentally obtained in the absence of elicitor [24] according to $\eta = [P]/[P_t] = [\text{taxol}]/[\text{taxol}]_{max}$. Then, the plots of $\log(\eta/(1-\eta) - \eta_b)$ against log[E] in the case of MJ and SA can be obtained and are shown in Figs. 2 and 3, respectively, and the values of n and K are listed in Table 1. Fig. 2 shows that at MJ concentrations lower than 100 μM, the slope of $\log(\eta/(1-\eta) - \eta_b)$ versus log[E] is positive. This may indicate that the combination of MJ molecules with its receptor plays a decisive role in signal transduction cascade for inducing taxol production. At MJ concentrations ranging from 100 to 225 μM, the slope becomes negative. Generally, the binding of elicitor molecules with their receptors may form an equilibrium state to activate signal transduction cascade at the optimal

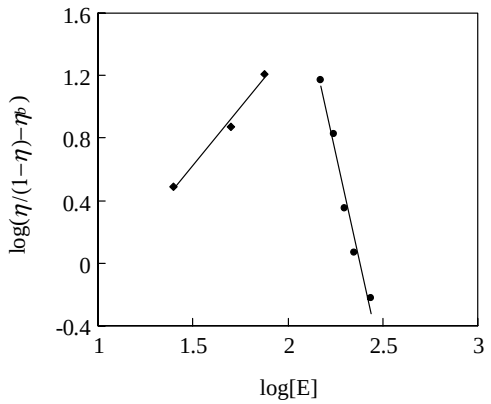

Fig. 2. Plot of $\log(\eta/(1-\eta)-\eta_b)$ vs. log[E] after addition of MJ. MJ concentrations: (◆) 25–100 μM; (●) 100–225 μM.

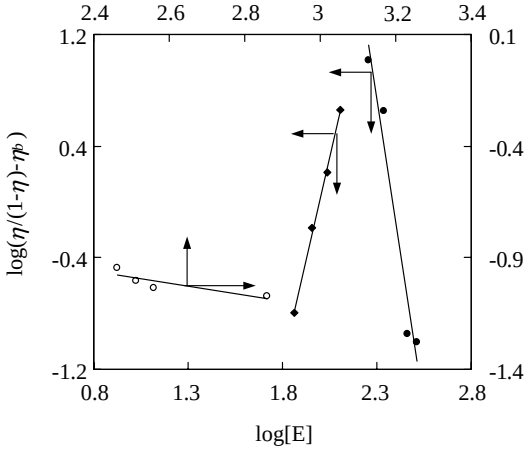

Fig. 3. Plot of $\log(\eta/(1-\eta)-\eta_b)$ vs. log[E] after addition of SA. SA concentrations: (◆) 72.5–145 μM; (●) 145–290 μM; (○) 290–725 μM. The arrows indicate the coordinates of the data.

elicitor concentration. When the elicitor concentration is less than the optimal value, the number of elicitor molecules is not large enough to form this equilibrium state, resulting in lower production of secondary metabolites than that at the optimal concentration. When elicitor concentration is greater than the optimal value, the equilibrium state is broken by a number of elicitor molecules. In this case, a large number of MJ molecules will competitively bind with the limited number of receptor molecules, interfering with signal transduction cascade and thus inhibiting secondary metabolism. The two opposite effects result in the negative slope at 100–225 μM MJ. Table 1 shows that the n value at 25–100 μM MJ is lower than that at 100–225 μM MJ, while the K value in the former case is much larger. The lower n value might be ascribed to the smaller number of MJ molecules for MJ 25–100 μM than that for 100–225 μM. The larger K value further confirms the occurrence of competitive binding of MJ molecules with receptors. The unusual units $(\mu M)^{-n}$ of K at 100–225 μM MJ might be caused by the larger n values (Table 1 and Eq. (8)).

Table 1
The n and K values in Eqs. (1) and (2) for MJ and SA at different concentrations

Elicitor	[E] (μM)	n	K ($(\mu M)^{-n}$)[a]
MJ	25–100	1.49	9.22×10^{-2}
	100–225	5.53	1.18×10^{-14}
SA	72.5–145	5.90	5.48×10^{-12}
	145–290	8.85	2.46×10^{-22}
	290–725	0.24	0.797

[a] The unit of K is deduced from Eqs. (7) and (8).

Fig. 3 shows that at SA concentrations ranging from 72.5 to 145 μM, the slope of $\log(\eta/(1-\eta)-\eta_b)$ versus log[E] is positive. This might indicate that the combination of SA molecules with receptor activates signal transduction cascade inducing taxol production, which is consistent with the results of Fig. 1. At 145–290 μM SA, the slope becomes negative, similar to that in the case of 100–225 μM MJ. This might be attributed to the interference of the large number of SA molecules with the signal transduction cascade for induction of secondary metabolism. The value of n at SA concentrations ranging from 72.5 to 145 μM is lower than that at SA concentrations ranging from 145 to 290 μM, while the value of K in the former case is much larger (Table 1). The reasons for these results are similar to those in the case of MJ. When SA concentrations exceed 290 μM (upper axis of Fig. 3), $\log(\eta/(1-\eta)-\eta_b)$ remains almost unchanged with increasing log[E]. The low n value (Table 1) indicates that few SA molecules combine with the receptor on the plasma membrane. Considering the low cell viability and high membrane permeability (data not shown), these results might be ascribed to the damage of the cell membrane at higher SA concentrations, leading to a reduction in the number of receptor molecules. The receptor model is useful for understanding the relationship among elicitor concentration, binding of elicitor with its receptor and secondary metabolite production.

Table 1 shows that the apparent number of MJ (25–100 μM) molecules combined with the receptor molecules (n) is about 75% lower than that of SA (72.5–145 μM), but the K value of in the case of MJ is ten orders of magnitude higher than that in the case of SA. This indicates that the combination of MJ with the receptor molecules is stronger than that of SA, leading to a more significant increase of taxol production by MJ than by SA (Fig. 1). This further confirms the validity of the induction model in predicting induction efficiency of elicitors.

When analyzing data using the model of Phisalaphong and Linden [23], we found similar variation trends of the values of n and K at different concentration ranges of MJ or SA, and the values of K are 3–7 orders of magnitude higher than those obtained using the present model. This might show that the model of Phisalaphong and Linden [23] is more appropriate for describing the system with the coexistence of MJ and exogenous ethylene, where the binding of ethylene with its receptors sensitizes MJ receptors result-

Table 2
Taxane contents (in cells plus medium) in the presence of MJ or SA at the early phase of cell growth (days 0–14 of cultivation) [32,33][a]

Taxane	Control		MJ-treated		SA-treated	
	Content (μg/g DW)	Molar ratio[b] (%)	Content (μg/g DW)	Molar ratio[b] (%)	Content (μg/g DW)	Molar ratio[b] (%)
10-DAB	47	29.3	167	46.6	234	33.2
Baccatin III	52	30.2	65	16.9	30	3.9
Taxol	51	20.4	203	36.1	171	15.5
Cephalomannine	49	20.1	2	0.4	510	47.4

[a] Elicitors were added at day 7 of cell growth. Taxane contents were determined at day 14.
[b] Molar ratio of taxane to total taxanes (10-DAB, taxol, baccatin III and cephalomannine).

ing in the higher value of binding constant *K* as described by Xu et al. [20].

4.3. Induction of MJ and SA on taxane production at optimal concentrations

Taxane contents at the optimal concentrations of MJ and SA at early (days 0–14 of cultivation) and late (after day 14 of cultivation) cell growth phases are shown in Tables 2 and 3, respectively. When 100 μM MJ was added to the suspension cultures on the seventh day of cell growth, the content of cephalomannine decreased, the content of baccatin III slightly increased and the contents of both 10-DAB and taxol obviously increased (Table 2). Yukimune et al. [2] reported a similar finding. Several papers claimed that taxol is a dead-end metabolite in *Taxus* culture cells and baccatin III is an intermediate of taxol biosynthesis [2,28]. However, Ketchum et al. [19] indicated that baccatin III may be formed as a degradation product of taxol, but there is no direct evidence to confirm this. So, it might be inappropriate if taking baccatin III as either a degradation product or a precursor of taxol. This might lead to an oversimplification of the relationship between baccatin III and taxol due to the highly possible reversible reactions between them as indicated by other researchers [29]. It is thus presumed that MJ may activate the biosynthesis from baccatin III to taxol and inhibit the biosynthesis from baccatin III to cephalomannine. From Table 2, it is seen that the molar ratio of taxol to total taxanes increased from 20.4 to 36.1% and that of 10-DAB to total taxanes increased from 29.3 to 46.6% after the addition of MJ. These results suggest that MJ may promote the production of taxol or 10-DAB. When 145 μM SA was added at day 7, the contents of taxol, 10-DAB and especially cephalomannine were significantly increased but the content of baccatin III was decreased (Table 2). The molar ratio of cephalomannine to total taxanes increased, indicating that SA may promote the production of cephalomannine. This behavior is different from the action of MJ. In comparison, SA is more favorable for the biosynthesis of cephalomannine than is MJ, but less so for taxol production.

Table 3 shows the contents of taxanes after addition of MJ or SA at the late phase of cell growth (after day 14 of cultivation). MJ strongly increased the contents of taxol, baccatin III and 10-DAB but decreased the content of cephalomannine. Moreover, the taxol production at the late phase of cell growth increased more significantly than that at the early phase of cell growth. For better understanding the mechanism of MJ-induced taxol biosynthesis, it is necessary to elucidate the biosynthesis of isopentenyl pyrophosphate (IPP), a universal and key precursor of isoprenoids. Lichtenthaler et al. [30,31] reported that the mevalonate and non-mevalonate biosynthesis pathways were both involved in the biosynthesis of IPP in axenic cultures of duckweed (*L. gibba*) and green tissue cultures of carrot (*D. carota*). We also got a similar finding for taxol biosynthesis in the late growth phase of *Taxus* cells [32,33] with biosynthesis pathways of IPP as described in Fig. 4 [29,32,33]. MJ may have increased the carbon fluxes of the both pathways for taxol biosynthesis at the late phase of cell growth. It is obvious from Table 3 that MJ increased the molar ratio of taxol to total taxanes from 45 to 76.5%, similar to the results at the early phase of cell growth. Therefore, the promotion of MJ to taxol production should apply through the whole growth cycle of cells.

Table 3
Taxane contents (in cells plus medium) in the presence of MJ or SA at the late phase of cell growth (after day 14 of cultivation) [32,33][a]

Taxane	Control		MJ-treated		SA-treated	
	Content (μg/g DW)	Molar ratio[b] (%)	Content (μg/g DW)	Molar ratio[b] (%)	Content (μg/g DW)	Molar ratio[b] (%)
10-DAB	203	34.8	456	17.4	263	13.2
Baccatin III	7	1.1	69	2.4	3	0.1
Taxol	412	45	3153	76.5	1432	45.7
Cephalomannine	170	19.1	146	3.7	1253	41

[a] Elicitors were added at day 14 of cell growth. Taxane contents were determined at day 21.
[b] Molar ratio of taxane to total taxanes (10-DAB, taxol, baccatin III and cephalomannine).

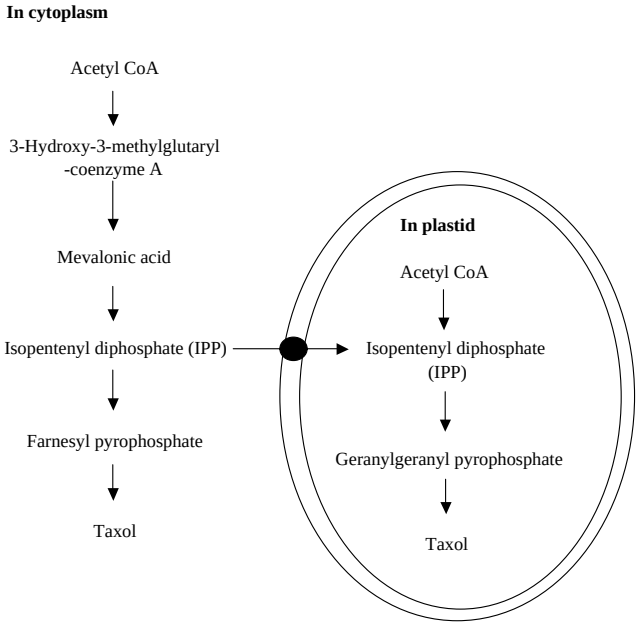

Fig. 4. The likely biosynthesis pathways of IPP for taxol biosynthesis at the late cell growth phase (after day 14 of cultivation) [29,32,33]. The shaded circle indicates the translocators on the plastid membrane for IPP translocating from cytoplasm to plastids. The arrows indicate one or more reaction steps.

It is seen from Table 3 that SA increased cephalomannine content and its molar ratio to total taxanes but decreased the baccatin III content at the late phase of cell growth, similar to the results at the early phase (Table 2). This may indicate that SA promoted cephalomannine production. In the presence of SA, taxol production was about three times higher than that of the control groups, also similar to that at the early phase (Table 2) but different from that induced by MJ. It is thus concluded that MJ is a more effective elicitor in terms of inducing taxol production than SA. This conclusion is also in consistence with the results deduced from the induction model.

5. Conclusions

The values of K, the apparent binding constant between the elicitor and receptor molecules, show that the binding force of MJ molecules with hypothetical receptors is stronger than that of SA for taxol biosynthesis in *Taxus* suspension cultures, which is consistent with the results of taxol production. This result reinforces the finding that MJ is an effective elicitor for improving taxol production as described by Ketchum et al. [22] and confirms the validity of the proposed induction model in predicting the induction efficiency of elicitors. The strongly enhanced cephalomannine production by SA may be relevant to the insignificant improvement in taxol production by SA. Therefore, in the case of SA, the taxol production might be improved by using an appropriate inhibitor to block the biosynthesis pathway from baccatin III to cephalomannine as a result of the increased carbon flux from baccatin III to taxol.

Although it has been recognized that MJ is an excellent elicitor for increasing taxol production [13,19,22], the mechanism of MJ-induced taxol biosynthesis is still not well understood. The results obtained in this work shed a light on understanding the induction mechanism of MJ on taxol biosynthesis. In addition, the results of SA induction on taxane production may be useful for exploring new strategies to improve taxol production. However, it should be noted that the induction model proposed in this work is based on the presumption of the existence of receptors of MJ or SA on the plasma membrane. Although it is highly possible that the MJ and SA receptors exist on the plasma membrane in plant cells as claimed by Gundlach et al. [34] and Birch et al. [35], there lacks a direct evidence of characterizations of such receptors in literature. Moreover, the method for evaluation of η_b used here might result in unrealistic model parameters. Thus, a more sophisticated model is worth considering for a better understanding of the induction mechanism of elicitors on taxane biosyntheses.

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