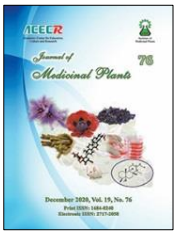
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Research Article

Enhancement of Taxol production by applying amino acid complex along with chitosan in suspension culture of *Taxus baccata* L.

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ABSTRACT

Background: Taxol (Paclitaxel) has been highly recommended to control and treat a wide range of cancers. *Taxus baccata* L. *is* primary and natural origin of *Taxol.* **Objective:** Due to severe restriction and prohibition of cutting *T. baccata*, the alternative way for Taxol production is to apply in vitro culture method which was implemented at the current study. Here, for the first time, the effect of amino acid complex as elicitor along with chitosan in cell suspension culture of *T. baccata* for enhancing Taxol production was studied. **Methods:** Young leaves of *T. baccata* as explants were cultured in different callus induction treatments. At the next step, the calli from the best callus induction treatment were transferred to cell suspension cultures containing chitosan and amino acid complex alone or in combination with each other. Taxol content in treatments were measured by HPLC. **Results:** MS medium supplemented with 2,4-D at 2 mg/L and NAA at 1 mg/L proved to be the best treatment of callus induction (100 %), fresh weight (495 mg) and dry weight (272 mg) of calli. Also, HPLC analysis confirmed the maximum production of Taxol (1.96 mg/g) in MS medium having 2 ml/L amino acids complex with 10 mg/L chitosan. **Conclusion:** Applying amino acid complex as elicitor with chitosan is suggested for enhancing Taxol production in cell suspension culture of *T. baccata*.

1. Introduction

Taxol, a secondary metabolite with a complex diterpenoid structure of the *Taxus baccata* (English yew), has been a very successful anticancer drug since it was initially approved for the treatment of breast and ovarian cancers [1-2]. Considering the low vegetative growth and reproductive rate of this plant, its very low Taxol

Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; NAA, Naphthalene Acetic Acid; HPLC, High Performance Liquid Chromatography; BAP, 6-Benzylaminopurine; SA, Salicylic Acid; MeJa, Methyl Jasmonate

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content and a highly challenging purification process, Taxol production in this way seems not very effective [3-5]. However, the most promising alternative for the production of Taxol is the use of cell cultures derived from different *Taxus* species. Therefore, tissue culture offers a practical alternative to Taxol production. Being capable of performing a precise control of the production conditions, applying production stimuli such as elicitors and the ease of extraction in short time, are the main advantages of this system [6-7]. The use of elicitors of plant defense mechanisms has been one of the most effective strategies for improving the productivity of Taxol and many other useful secondary metabolites in plant cell cultures [8]. In plant cell cultures, an elicitor can be defined as a compound introduced in small concentrations to a living system to promote the biosynthesis of the target metabolite. Traditionally, elicitors have been classified in two types; abiotic or biotic, according to their chemical nature and exogenous or endogenous origin, and notably include yeast extract, methyl jasmonate, salicylic acid, vanadyl sulphate and chitosan [9]. Chitosan is obtained by partial deacetylation of solid chitin under alkaline conditions or by enzymatic hydrolysis with chitin deacetylase [10]. Some studies confirm that applying chitosan as an elicitor enhances the production of secondary metabolites in cell suspension cultures of different plants. For example, adding chitosan improved flavonoid content in *Andrographis paniculata* cell suspension cultures [11]. Different concentrations of chitosan enhanced psoralen biosynthesis in cell cultures of *Psoralea corylifolia* and *Conium maculatum* as well as the dicentrine content in *Stephania venosa* [12-14]. Finally, chitoheptaose, a chitosan derivative, markedly increased paclitaxel production in *Taxus cuspidata* cell cultures [15]. Although amino acids have been used as biostimulant for increasing qualitative and quantitative characteristics in many plants, there are a few reports stating the use of amino acids as elicitor in plant cell suspension cultures. For example, increasing Taxol production in *T. cuspidata* cultures by adding phenylalanine to the medium [16] and Taxol production in *T. canadensis* and *T. cuspidata* by applying phytosulfokine (5 amino acid peptide) [17].

Here, for the first time, the effect of applying amino acid complex as elicitor in combination with chitosan for Taxol production in cell suspension cultures of *T. baccata* was studied.

2. Material and methods

2.1. Plant material and sterilization

Healthy young leaves (1-2 cm segments) collected from *Taxus baccata* (English yew) grown at college of agriculture and natural resources, located in Karaj, were used as explants. Explants were washed thoroughly with running tap water for 30 min. Then, they were surface sterilized by treating modified methods including 70 % ethanol for 1 min and 1.5 % (v/v) sodium hypochlorite for 15 min [18]. Explants were finally rinsed three times with sterile double-distilled water to remove any traces of the surfactants. Then, they were cultured on establishment medium which was solid MS medium [19] and were kept for two weeks at 25 ± 2 ºC under 16-h photoperiods with a light intensity of 3000 lux.

2.2. Callus induction

The Explants were cultured on solid MS medium fortified with different levels of 2,4-dichlorophenoxyacetic acid (2,4-D) (0 and 2 mg/L), naphthalene acetic acid (NAA) (0 and 1 mg/L) and activated charcoal (0 and 2 g/L). The cultures were maintained in the dark at 25 ± 2 °C

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[20]. The percentage of callus induction, callus fresh weights (FW) and callus dry weights (DW) were recorded 4 weeks after culture. The successfully induced callus was separated from the explants and cultured separately until used for the establishment of cell suspensions.

2.3. Cell suspension culture

Cell suspension culture was initiated by applying a modified methods including transferring calli from the best treatment of callus induction media (80-100 mg) to a 50-mL Erlenmeyer flask containing 15 mL of liquid MS medium supplemented with 2 mg/L 2,4-D, 1 mg/L NAA. The suspension cultures were incubated on a rotary shaker at 120 rpm and 25 ± 2 ℃ under 16-h photoperiods with a light intensity of 3000 lux. Prior to autoclaving (121 °C, 20 min), all media were adjusted to pH = 5.6-5.8 [21].

2.4. Elicitation of cell suspension culture

Two elicitors including Amino acid complex (liquid form, INAGROSA Company, Spain) and chitosan (Sigma, 85 % deacetylated) were used to study the elicitation effect on Taxol production by cell suspension cultures of *T. baccata*. Elicitation was applied on day 21 after inoculation, when the cell cultures were in the middle of rapid growth phase. To determine the most effective elicitor concentration for induction of Taxol, both elicitors were added to cultures at final concentrations of 0 and 10 mg/L for chitosan and 0, 1 and 2 ml/L for amino acid complex individually and in combination. Samples were taken 72 h after adding elicitors. The cell biomass were freeze-dried and used for Alkaloid extraction. It is worth saying that the amino acid complex is consisted of 19 necessary free amino acids (3750 mg/L). The list of these free amino acids with their concentrations (w/w %) is mentioned below: glycine (1.8 %), valine (5.1 %), proline (8.4 %), alanine (13.21 %), aspartic acid (4.5 %), arginine (8.4 %), glutamic acid (0.9 %), lysine (5.1 %), leucine (16.51 %), isoleucine (4.5 %), phenylalanine (5.1 %), methionine (4.2 %), serine (3.9 %), threonine (3 %), histidine (3 %), glycocoll (9.6 %), tyrosine (1.5 %), glutamine (0.9 %), cysteine (0.3 %), other (0.08%).

2.5. Alkaloid extraction and HPLC analysis

Taxus baccata extracts were prepared by mixing freeze-dried cell cultures (5 g) with 25 mL acetonitrile solution treated with 60 min sonication. The extracts were then centrifuged at 20,000 rpm at 4 °C for 15 min. The supernatant was then collected and filtered for HPLC analysis.

Taxol was quantified by its optical density peaks at 227 nm, using Paclitaxel (Sigma Aldrich Company, UK) as standard compounds for calibration. HPLC analysis was performed on Knauer HPLC system (1200 series, UV detector K-2501). A volume of 50 µl of samples was injected in 18 reverse-phases Phenomenex column (Gemini NX-C18, 5 mm, 4.6 × 250 mm). The mobile phase for alkaloid elution was 20 % methanol and 80 % deionized water.

2.6. Statistical analysis

The experiments were set up on a completely randomized design with four replicates per treatment and five explants per replicate for callus induction and three replications for each elicitation treatment. Statistical differences were assessed based on analysis of variance (ANOVA) using SPSS (version 18, USA). Differences among means were analyzed using Duncan multiple range test at a probability level of $P < 0.01$. The values are expressed as the mean ± standard error (SE).

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3. Results

Callus induction was observed after two weeks with swelling and then formation of mass on the surface of the explants. The color of calli was dark cream and the texture was friable after four weeks (Fig. 1).

Based on analysis of variance, only hormone combination influenced the frequency of callus induction, fresh weight and dry weight. Activated charcoal did not have significant effect on callus induction characteristics (Table 1).

Mean comparison of the effect of hormone combination on callus induction (%), fresh and dry weights showed that the best treatment belonged to 2 mg/L 2,4-D along with 1 mg/L NAA applied in MS medium (Fig. 2). The range of mean callus induction (%), fresh weight (mg) and dry weight (mg) in different treatments were from 20 to 100 %, 15 to 495 and 11.25 to 227, respectively.

The results obtained from analysis of variance related to the extracted Taxol detecting by HPLC method showed that the effect of interaction between amino acid complex and chitosan was significant at 5 % probability level (Table 2).

The highest accumulated amount of Taxol was 1.96 mg/g which was about three times higher than non-elicited cells (0.64 mg/L). The maximum Taxol was gained in amino acid complex concentration of 2 ml/L with chitosan at 10 mg/L treatment (Fig. 3).

HPLC chromatogram of control treatment (Amino acid complex at 0 ml/L with chitosan at 0 mg/L) and the best treatment (Amino acid complex at 2 ml/L with chitosan at 10 mg/L) have been shown (Fig. 4). The presence of Taxol in recombinant cells was validated by the confirmatory spiking HPLC analysis with the commercial standard of Taxol.



Fig. 1. Cream and friable callus obtained from young leave of *T .baccata*

Table 1. The variance analysis of the effect of 2,4-D, NAA and Activated charcoal on callus induction.

Source of variation	df	Mean of Squares		
		Callus Induction	Fresh Weight	Dry weight
2,4-D	1	7812.500**	286903.125**	122512.500**
NAA	1	17112.500**	350703.125**	159612.500**
Activated charcoal	1	361.250^{ns}	2628.125^{ns}	281.2500^{ns}
2,4-D × NAA	1	13612.500**	279378.125**	115200.000**
2,4-D × Activated charcoal	1	151.2500^{ns}	2050.3125^{ns}	450.000^{ns}
NAA × Activated charcoal	1	612.500^{ns}	1665.3125^{ns}	0.000^{ns}
2,4-D × NAA × Activated charcoal	1	112.500^{ns}	2257.8125^{ns}	1012.500^{ns}
Error	24	82.916	253.6458	149.8333
CV (%)		23.8	42.5	48.3

** Significant at $P \leq 0.01$; ns: not significant

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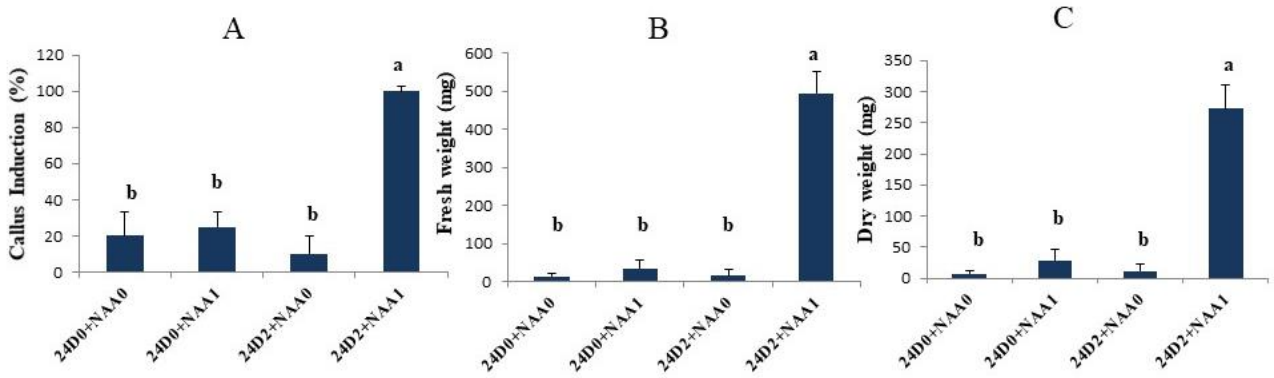


Fig. 2. Effect of different concentration and combination of 2,4-D and NAA on callus induction of *T. baccata*. A: frequency of callus induction, B: fresh weight, C: dry weight. Values followed by different letters in each trait are significantly different at $P \leq 0.05$.

Table 2. The variance analysis of the effect of Amino acid complex and chitosan on production of Taxol

Source of variation	df	Mean of Squares (Taxol Production)
Amino acid complex	2	0.463**
chitosan	1	1.993**
Amino acid complex× chitosan	2	0.024**
Error	12	0.002
CV (%)		2.9

** Significant at $P \leq 0.01$; ns: not significant

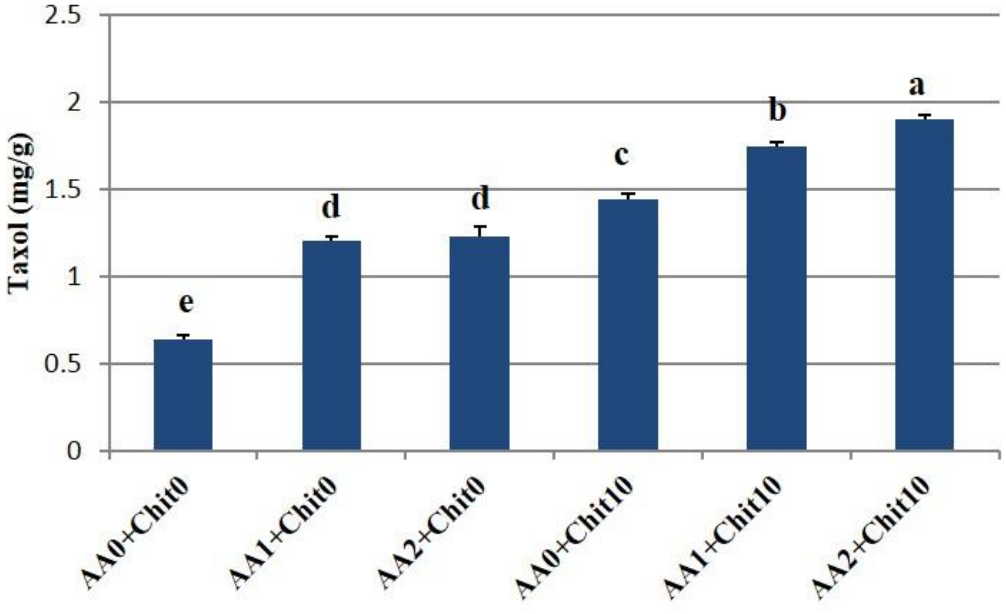


Fig. 3. Effect of different concentration and combination of Amino acid complex and chitosan on production of Taxol 72 h after elicitation. AA stands for amino acid complex and Chit stands for chitosan. Values followed by different letters in each trait are significantly different at $P \leq 0.05$.

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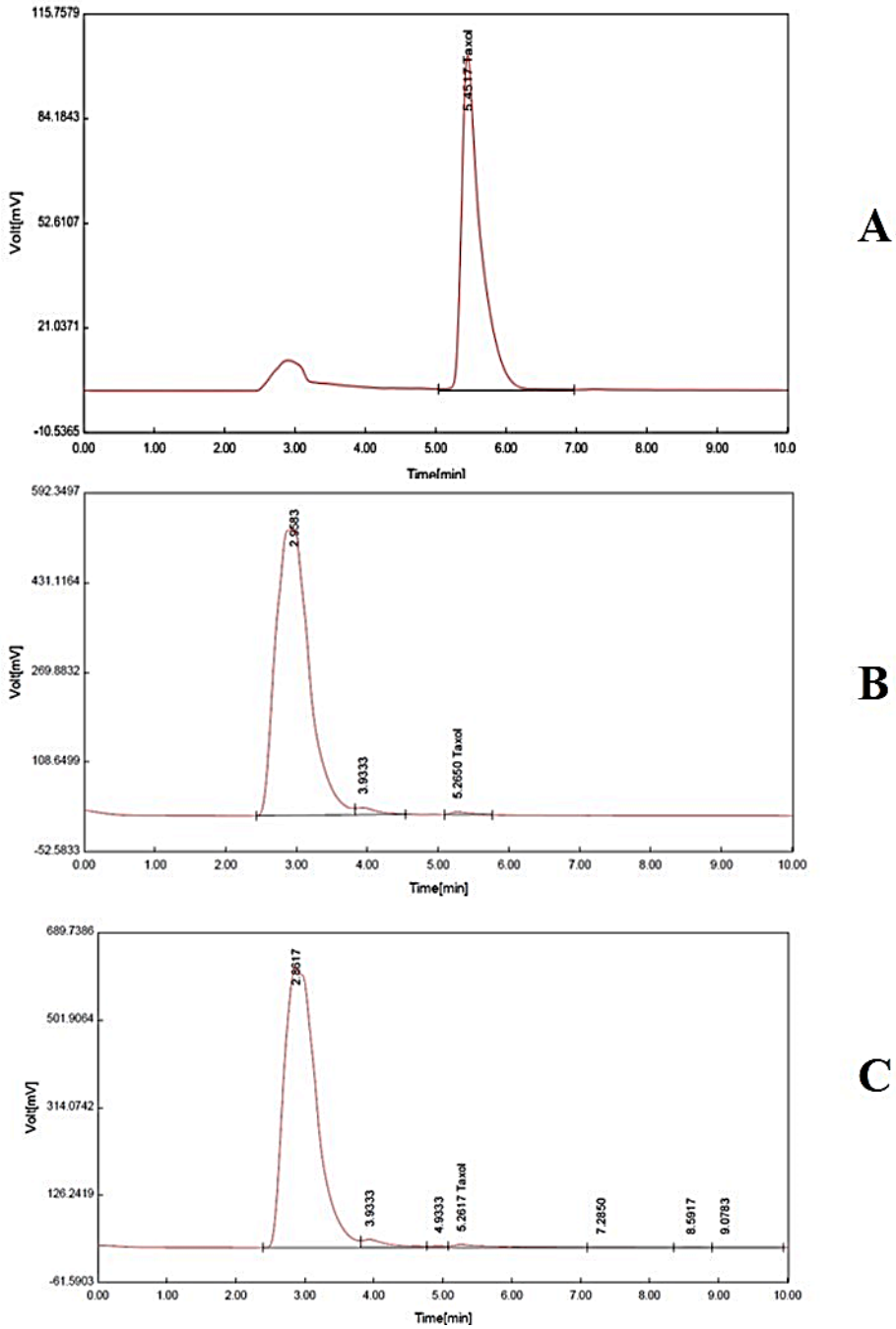


Fig. 4. HPLC chromatograms of *T. baccata* cell suspension cultures (A) Standard Taxol (B) control treatment (C) amino acid complex at 2 ml/L + chitosan at 10 mg/L treatment.

4. Discussion

It has been observed that young tissue is more responsive or prone to callus initiation than mature plant parts or young tissue from adult trees [22]. For the same reason, young leaves were selected as explants. In the present study, MS medium was used as a basal medium. Previous studies have shown that different basal media including MS have been employed for initiation and maintenance of callus cultures.

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Different plant growth regulators have also been used for callus induction [23]. For example, Gamborg's B5 medium (B5) with 2 mg/L of NAA and 0.1 mg/L of BAP was optimum for callus growth of *T. baccata* [24]. Callus induction of *T. chinensis* was achieved on MS medium with 0.1 mg/L 2,4-D, 0.5 mg/L NAA, 0.5 mg/L BAP and supplemented with casein hydrolysate [25]. In this study, MS medium with 2 mg/L 2,4-D along with 1 mg/L NAA and without any other supplement was led to callus induction.

Elicitation is one the most effective techniques currently being used for improving the biotechnological production of secondary metabolites. Elicitors are compounds that stimulate any type of plant defense and promote secondary metabolism [26]. In order to increase secondary metabolites (paclitaxel and related taxanes) in *Taxu*s sp. different elicitors have been applied in considerable research efforts. For example, boosting paclitaxel production by adding SA to the cell culture medium of *T. chinensis* [27]; improving yields of this taxane in a two-stage cell suspension culture of *T. baccata* using two different concentrations of SA [21]; enhancing yields of paclitaxel and other taxane with various fungal extracts, alone or in combination with other elicitors [28-29]; increasing Taxol biosynthesis and excretion to the medium 55 times higher than non-elicited cultures of *T. media* [30]; increasing in Taxol (5.1-fold) and total taxanes (3.5-fold) in the SA-pretreated calli of *T. baccata* cultured on the medium containing 2 % glucose, compared to the control [18] and improving Taxol production (2.5 times more than the control) by applying polyethylene glycol (3 %) in a *T. baccata* L. callus cultures [31]. Chitosan is another elicitor which has been used to improve paclitaxel production in cell suspension cultures of *T. chinensis* [32-33]. Chitosan, the most important derivative of chitin, is obtained by partial deacetylation of solid chitin under alkaline conditions or by enzymatic hydrolysis with chitin deacetylase [34].

To the best of our knowledge, there is no report about applying amino acid complex (all necessary amino acids together) as elicitor in cell suspension culture. With reference to the reports, they are limited and confined to applying single amino acids or low chain peptides [35-36]. For example, increasing Taxol production in *T. cuspidata* cultures by adding phenylalanine to the medium [16]; applying Phytosulfokine-α (PSK-α), a 5-amino acid peptide, in *Taxus* cell suspension cultures which strongly elicited Taxol production with synergistic interaction with MeJa [17]. However, there are considerable reports about applying free amino acids as biostimulants in sustainable agriculture. Biostimulants are products which are often referred to as positive plant growth regulators or metabolic enhancers for plant nutrient availability/uptake/assimilation and use efficiency, abiotic stress tolerance as well as product quality [37-38]. There are several categories of biostimulants, such as products based mainly upon microbial inoculants, humic and fulvic acids, vitamins, seaweed extracts, protein hydrolysates and amino acids [39]. Amino acids as biostimulant have several roles including acting as osmolytes, regulation of ion transport as well as modulation of stomatal opening and promotion of nitrogen assimilation in plants via coordinated regulation of carbon and nitrogen metabolism [40].

In the current study, however, all necessary free amino acids as a complex has been used for the first time as elicitor in cell suspension culture of *T. baccata*. As per results obtained, this amino acid complex had a positive interaction effect

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with chitosan to improve Taxol production. The role and mechanism of free amino acids to enhance Taxol production is suggested to assess scientifically. Though, most of the studies about the enhancing effect of elicitation on secondary compound production in plant cell cultures have been empirical (because of its complexity) without exploring the cellular response at a molecular level [9]. It is also suggested to study amino acid complex alone or in combination with other elicitors to increase different secondary metabolites from various plant species.

5. Conclusion

In conclusion, amino acid complex can be used as a novel elicitor in companion with chitosan in cell suspension cultures of *T. baccata* to enhance Taxol production. It is also suggested to study the effect of this promising elicitor alone or in combination with other elicitors for promoting the production of secondary metabolites from various plant species.

Author contributions

Experiment performing and data gathering: Bahar Amirkavei Najafabadi

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Editing: Nasim Zarinpanjeh, Nassrin Qavami, Mahammad Ali Ebrahimi

Conflict of interest

Authors declare that there is no conflict of interest.

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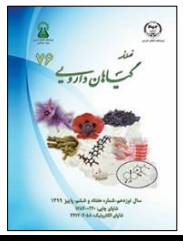
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مقاله تحقیقاتی

ارتقا تولید تاکسول از طریق کاربرد ترکیبی از اسیدهای آمینه و کیتوزان در کشت تعلیق سلولی سرخدار (*Taxus baccata* L.)

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سرخدار

چکیده

مقدمه: تاکسول (Paclitaxal) به منظور کنترل طیف گسترده‌ای از سرطان‌ها توصیه می‌شود. سرخدار (*Taxus baccata* L.) منشأ اولیه و طبیعی تاکسول است. **هدف:** با توجه به محدودیت شدید در برش سرخدار، روش جایگزین برای تولید تاکسول استفاده از روش کشت درون شیشه‌ای است. در اینجا، برای اولین بار اثر ترکیبی از اسیدهای آمینه به عنوان الیسیتور به همراه کیتوزان در کشت تعلیق سلولی سرخدار برای افزایش تولید تاکسول مورد بررسی قرار گرفته است. **روش بررسی:** برگ‌های جوان سرخدار به عنوان ریزنمونه در تیمارهای مختلف القای کالوس کشت شدند. کالوس‌ها به دست آمده از بهترین تیمار کالزایی به کشت‌های تعلیق سلولی که دارای کیتوزان و ترکیت اسیدهای آمینه به تنهایی و یا در ترکیب با یکدیگر بودند، منتقل شدند. میزان تاکسول در تیمارها با استفاده از روش کروماتوگرافی مایع با کارایی بالا اندازه گیری شد. **نتایج:** محیط کشت MS به همراه 2,4-D به میزان دو میلی‌گرم در لیتر و NAA به میزان یک میلی‌گرم در لیتر، بهترین تیمار از نظر درصد کازایی (۱۰۰ درصد)، وزن تر (۴۹۵ میلی‌گرم) و وزن خشک (۲۷۲ میلی‌گرم) بود. همچنین، آنالیز HPLC حداکثر تولید کالوس در محیط کشت MS به همراه ۲ میلی لیتر در لیتر از ترکیب اسیدهای آمینه و ۱۰ میلی‌گرم در لیتر کیتوزان را تأیید کرد. **نتیجه‌گیری:** استفاده از ترکیب اسیدهای آمینه به عنوان الیسیتور همراه با کیتوزان به منظور افزایش تولید تاکسول در کشت تعلیق سلولی پیشنهاد می‌شود.

مخففها: 2,4-D، ۴،۲- دی‌کلروفنوکسی استیک اسید؛ NAA، نفتالن استیک اسید؛ HPLC، کروماتوگرافی مایع با کارایی بالا؛ BAP، ۶- بنزیل آمینو پورین؛ SA، سالیسیلیک اسید؛ MeJa، متیل جاسمونات

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