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Production podophyllotoxin by root culture of *Podophyllum hexandrum* Royle

Wei Li*, Meng-fei Li, De-long Yang, Rui Xu, Ying-ru Zhang

College of Life Science and Technology, Gansu Agricultural University, Lanzhou, 730070, China.

* Corresponding author. Tel: +86(0)931-7631742; Fax: +86(0)931-7631875; E-mail: liwei@gsau.edu.cn

Abstract

A way of production podophyllotoxin by root culture of *Podophyllum hexandrum* Royle (*P. hexandrum*) was established. In the basis of aseptic plantlets induced from mature embryo of *P. hexandrum* seed, the rhizome of the plantlets were excised to 2.0 cm and then cultured on the Murashige and Skooge medium (MS) supplemented with different concentrations of growth regulators (IBA: 3-Indolebutyric acid, GA_3: Gibberellic acid, Hy: Hydroquinone) and activated charcoal (AC). As the cultures development, the Hy could promote the plantlets growth compared to IBA and GA_3, and the length of rhizome reached by 11.59 cm under the concentration of Hy 2.0 mg/L after 40 days; Supplementation of the medium with AC could significantly hasten the plantlet growth and development compared to other treatments with the length and dry weight of rhizome 13.60 cm and 0.33 g under the concentrations of Hy 2.0 mg/L and AC 0.5 g/L after 40 days; MS + sucrose 30.0 g/L + agar 5.0 g/L + Hy 5.0 mg/L + AC 0.5 mg/L (pH=5.8) was optimum for both biomass and accumulation of podophyllotoxin in root culture of *P. hexandrum*, the content of podophyllotoxin in the root and cultured medium reached 151.50 μg/g and 121.22 μg/g with the accumulation index of podophyllotoxin $e^{0.4487}$ and $e^{0.3329}$ respectively after 50 days. This indicated that production podophyllotoxin by root culture of *P. hexandrum* was feasible and had important application prospect.

Keywords: *Podophyllum hexandrum* Royle; Growth regulators; Activated carbon; Root culture; Podophyllotoxin.

1. Introduction

Podophyllum hexandrum Royle common named Himalayan Mayapple is mainly located at 1500-4200m in the Himalayas such as Afghanistan, Pakistan, Kashmir, North India, Nepal; North America and Western China. In China, it is distributed in the western area Tibet, Yunnan, Qinghai, Gansu province, etc. [1,2]. It is a perennial plant in the barberry family (*Berberidnecae*) and habitats in scrub forests and alpine meadows associating with other plants, usually in humus rich soil. The morphological characteristics of *P. hexandrum* are that grand nodose rhizome, many adventitious roots, length longer than 50cm; stems 30~90cm in height; 2(3) umbrella-like, palmately lobed leaves grow at the top of each plant; Cordiform leaf, 3~5divided, 2~(3) semi-redivided. flowering in May, fruiting in July or August, a white flower in fork of leaves, 6 petals, pink, 5~6cm; ovate red fruit, 4~9cm length, 2~5cm diameter, amount of seeds 80~180, its 1000-seed weight about 20.0g.

Podophyllotoxin is a natural plant secondary metabolites mainly existed in the root of *P. hexandrum* and as well as its congeners and derivatives has pronounced biological activity mainly as anticancer, antineoplastic and anti-HIV drugs, etc [3,4,5]. Due to some disadvantaged biological characteristics that (1) the seed dormancy period is very long. Our studies showed that seed coat and endosperm restriction, physiological after ripping and germination inhibitors were all reasons which caused it impossible to break using traditional methods, only the germination percentage 81.11% with the seed solution in 500mg/L GA_3 for 36 h following with cold stratification for 3 months [6,7]; and (2) the reproductive long cycle that needs at least for 4 to 5 years to overcome the juvenile and during this period the root system and bud begin to become well-developed, but little change on the ground part. What's more, the plants with a single leaf can not carry out sexual reproduction but only with a forked petiole with two leaves and a solitary flower, so the generative propagation of *P. hexandrum* has difficulty taking the traditional measures and techniques [8]; On the other hand, the chemical synthesis of podophyllotoxin is very complicated and rather difficult so that its production can't be applied to under the conditions of modern technology [9,10]; So podophyllotoxin will ultimately depend upon the supply of raw materials. However, plants grown from rhizome cutting of *P. hexandrum* were estimated to take at least 4~5 years to produce rhizome in fair sizes and plants raised from seedling would take even longer [8]. In particular the market demand of podophyllotoxin is increasing and excessive man-made excavation that results in the natural resource facing endangered [4,8].

Since both synthetic podophyllotoxin and traditional cultivation methods are not available in the foreseeable future, the successful production of

podophyllotoxin has paid great attention on using biotechnological approaches such as organs cultures, cell suspension cultures, transgenic hairy roots and biotransformation, etc. Arumugam and Bhojwani [11] proved that using *in vitro* techniques through somatic embryogenesis cultures was a good alternative. Sadowska et al [6] succeeded in obtaining the plantlets and calli by the cultures of embrya and divided embrya. Kadkade [12,13] reported that the production of podophyllotoxin from *P. hexandrum* callus was feasible with the content of podophyllotoxin 0.65%, but it is difficult to establish callus cultural lines and embryogenesis callus. Van Uden et al [14,15,16] reported initiation of cell cultures from roots of *P. hexandrum* and production of podophyllotoxin in undifferentiated callus and cell suspension cultures, the podophyllotoxin content of callus suspension reaching 24.3 μg/ml after 15 days, in addition, feeding the premise material of podophyllotoxin coniferyl alcohol as β-cyclodextrin complex lead to enhanced podophyllotoxin accumulation with a maximum of 0.012%, but the callus was easily browning and difficult to subculture. Transformed hairy roots genetically produced by infection of *P. hexandrum* plantlets with *Agrobacterium rhizogenes* has been proved to be a promising tool for secondary metabolite podophyllotoxin production, especially for the medicinal plants that medicinal value depends on the roots. In addition, both biosynthetic stability and growth rate make it impossible to be used as a continuous source for podophyllotoxin production [17,18]. Giri and Lakshmi Narasu [4] confirmed that production of podophyllotoxin by using hairy roots was the best way as podophyllotoxin is mainly extracted from the root of *P. hexandrum*. Oostdam et al [19] reported that the production of 5-methoxy derivatives of podophyllotoxin from hairy roots cultures of *Linum flavum* could be 5~10 fold higher than untransformed cell suspension cultures. If the characteristics of *P. hexandrum* that its rhizome is grand nodose and its bulb can be cycled every three weeks in the authors' laboratory are considered, the way to produce podophyllotoxin by root culture with the rhizome of *P. hexandrum* excised and then sub cultured on proper medium may be better to produce podophyllotoxin. The present investigation highlights different growth regulators and AC which contribute significantly for growth and development of *P. hexandrum* and accumulation of podophyllotoxin.

2. Materials and Methods

Plant materials, chemicals and instruments

P. hexandrum seeds were collected from the forest (2100m) located in Huichuan, Weiyuan area, Gansu province, China. 3-Indolebutyric acid, Gibberellic acid, Hydroquinone, ethanol and ethyl acetate were all analytical reagent and standard of podophyllotoxin was purchased from Sigma Company (purity ≥ 98.0%). The content of podophyllotoxin was detected by Ultraviolet spectroscopy (UV) (TU-1810).

Preparation of podophyllotoxin standard curve

Weighted the samples of podophyllotoxin 25.00 mg, dissolved and then volume to 250ml with ethanol, made the solution 100 μg/ml. Took the solution 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, 45.0 ml and volume to 100ml with ethanol, respectively.

Initiation of Plantlets

Seed embryo were separated and germinated on MS medium supplemented with sucrose 30.0g/L, agar 5.0 g/L and GA_3 0.5 mg/L (pH=5.8) at 22±1℃ and 16/8 h light (1000LX)/dark regime for the initiation of plantlets.

Initiation of root culture

After the separated embryo culturing about 5 weeks, the two leaves of initiation plantlets started aging and at the same time the bulb derived from rhizome began germinating new leafstalk and changing into three leaves. Then the rhizome of the plantlets were excised to 2.0 cm and inoculated on "MS + sucrose 30.0 g/L + agar 5.0 g/L (pH=5.8)" supplemented with different concentrations of growth regulators (IBA, GA_3, Hy) and AC at 22±1℃ and 16/8 h light (1000LX)/dark regime for subculture

Effect of growth regulators and AC on root growth of *P. hexandrum*

Different concentrations of growth regulators [IBA, GA_3, Hy (0.1~10.0 mg/L)] and AC (0~10.0 mg/L) were used to study their effect on the growth and podophyllotoxin production of *P. hexandrum* root. Measuring the length of root and at regular periods (10^{th}, 20^{th}, 30^{th}, 40^{th} days), Dried at 80 ℃ for 12 h and weighted the dry biomass.

Detection and analysis of podophyllotoxin

Weighted dry biomass of root and leaf (petiole) 2.0 g, respectively, grinded to powder and then put them into Soxhlet to extract the podophyllotoxin with solvent ethyl acetate at 80℃ for 12 h; Weighted cultured medium 50.0 g, heated into liquid and then extracted with ethyl acetate by separatory funnel; the extractions of root, leaf and cultured medium were decompressed and concentrated at 80 ℃, and then the concentrated solutions were dissolved with ethanol to 25 ml. The absorbance (A) was read at 292 nm. Data analysis used EXCEL and SPSS11.5.

3. Results

3.1 Standard curve of podophyllotoxin

Took the ethanol as control, detected the absorbance (A) of different concentrations of standard podophyllotoxin at 292nm. Took

concentrations of podophyllotoxin as x-coordinate and absorbance (A) as y-coordinate, the regression equation of standard podophyllotoxin was Y = 0.0457X - 0.1298 (R^2 = 0.9972), as it is C=21.88A + 2.84. Figure 1 showed that the standard concentration of podophyllotoxin was in good linear relationship from 5μg/ml to 45μg/ml.

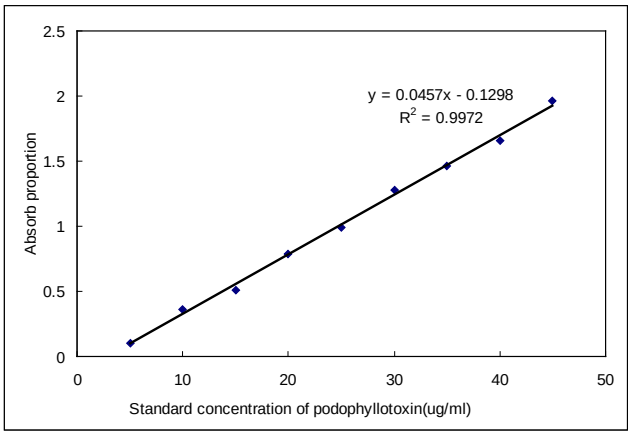

Figure 1. The regression equation of podophyllotoxin.

3.2 Effects of different growth regulators on root growth of *P. hexandrum*

Effects of growth regulators IBA, GA_3 and Hy on root growth were observed. Figure 2 showed that all of the regulators could stimulate the root growth with the development of P. hexandrum plantlets. Growth rate was raised with the concentrations of IBA, GA_3 and Hy increasing on 10th and 20th days, but in opposition on 30th and 40th days, which indicated that growth regulators in high concentrations had to fast the root development and promote the absorption of nutrients and metabolic in order to adapt to the environment and the restoration of pre-excised injury on 10th and 20th days, and then growth rate in high concentrations was slow and the low concentrations began to show positively effect on 30th and 40th days. The rhizome length reached 6.85 cm, 8.12 cm, 11.59 cm that showed better than others treatments at the concentrations of IBA 0.5 mg/L, GA_3 1.0 mg/L, Hy 5.0 mg/L, respectively. And Hy hastened significantly the root growth and development compared to IBA and GA_3 with the rhizome length 8.97 cm, 10.18 cm and 11.59 cm at the concentrations of 0.5 mg/L, 1.0 mg/L and 5.0 mg/L, respectively.

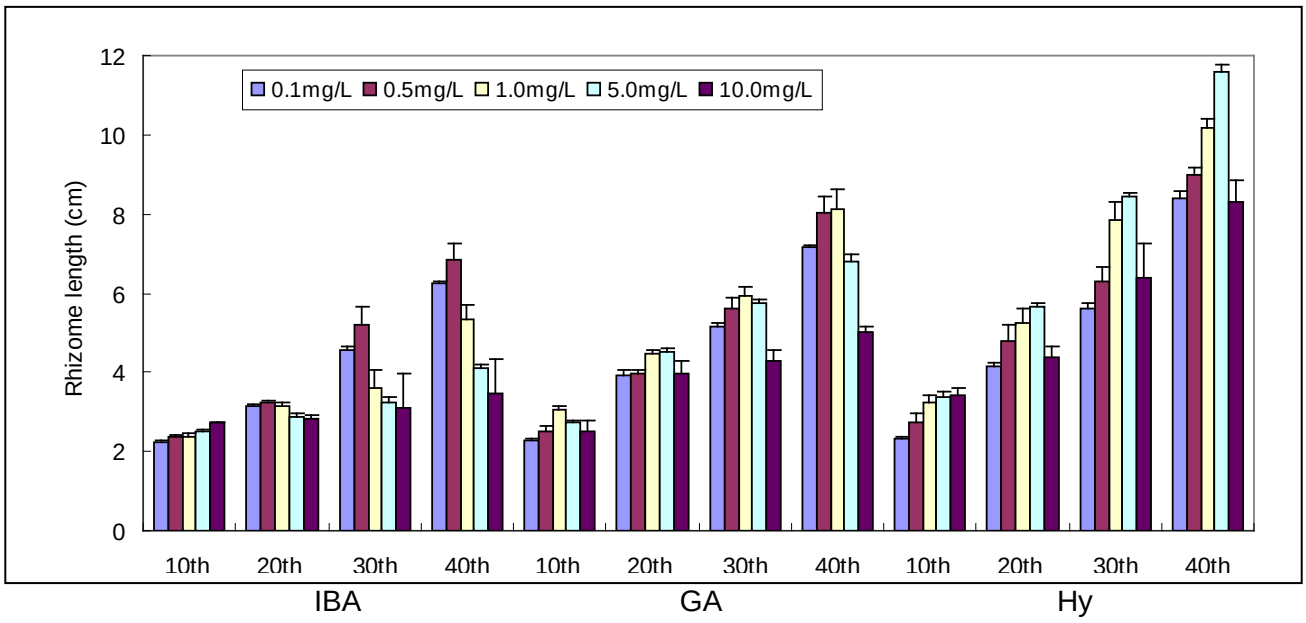

Figure 2. Effect of different concentrations of IBA, GA_3 and Hy on root growth of *P. hexandrum* at different phases. Each Value represents an average of 30 replicates. Bars represent standard error.

3.3 Effect of AC on root growth of *P. hexandrum*

Figure 3 showed that AC could provide a proper environment for the root and promote the growth and development of *P. hexandrum*. At the initiation period the growth rate was raised with the AC concentration increasing (0.5 g/L ~ 5.0 g/L) compared to control, among of which 5.0 mg/L showed better and 10.0 g/L was lower than all of the AC treatments and just higher than the control. On the 20th days AC 10.0 g/L began to show the inhibition compared to the control and the lower concentrations (0.5 g/L ~ 1.0 g/L) took more advantage of the root growth rate than that of 5.0 mg/L. On the 30th and 40th days the rhizome length and growth rate showed the best than other treatments with the rhizome length 13.6 cm and the growth rate 26.69%, which showed that high concentrations of AC could promote the growth at the initiation period and the low concentrations were

advantage for root growth with the development of plantlets.

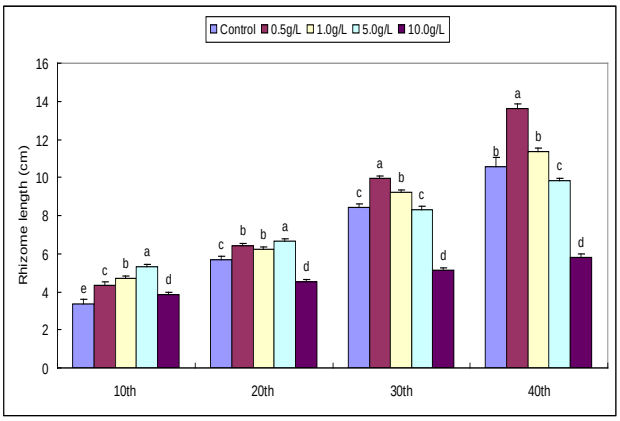

Figure 3. Effect of AC on rhizome length of *P. hexandrum* at different phases. Each Value represents an average of 30 replicates. Bars represent standard error. Means followed by the same letters are not significantly different at 5% using LSD.

Figure 4 showed that the accumulation of dry rhizome weight was increased by adding proper concentration of AC. Generally, the changing of dry rhizome weight was similar to that of root length with AC 0.5 g/L showing the best among the recorded phases. Above of that indicated that the low AC concentration could provide a dark and proper environment that was suitable for almost of plants but inhibit nutrients absorption, accumulation and elongation of rhizome as a result of AC being a strong adsorbent and particles.

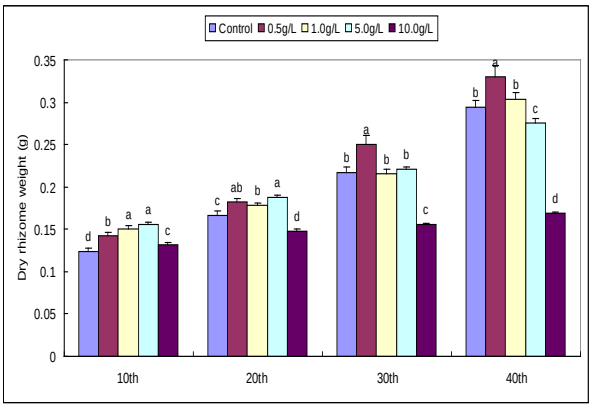

Figure 4. Effect of AC on dry rhizome weight of *P. hexandrum* at different phases. Each Value represents an average of 30 replicates. Bars represent standard error. Means followed by the same letters are not significantly different at 5% using LSD.

3.4 Product formation of podophyllotoxin

Figure 5 showed the analysis of different product formations of podophyllotoxin during the growth of *P. hexandrum* plantlets. The figure showed that the contents of podophyllotoxin in the root and cultured medium were significantly increased with the growth and development of P. hexandrum from the initiation period of 10th days to 50th days, but the content in leafstalk (leaf) hadn't significantly increased. The accumulation index of podophyllotoxin in root was $e^{0.4487}$ and the relationship between the content of podophyllotoxin (y) and the culture times (x) was $y_{(Root)} = 16.464\ e^{0.4487x}$, and the accumulation index of podophyllotoxin in cultured medium was $e^{0.3329}$ and the relationship between the content of podophyllotoxin (y) and the culture times (x) was $y_{(Cultured\ medium)} = 22.714\ e^{0.3329x}$. The content of podophyllotoxin in root reached 25.11, 41.45, 63.43, 101.21 and 151.50 μg/g on the 10th, 20th, 30th, 40th and 50th days, respectively. The content of podophyllotoxin in cultured medium reached 34.24, 41.84, 55.09, 93.20 and 121.22 μg/g on the 10th, 20th, 30th, 40th and 50th days, respectively. The content of podophyllotoxin in leafstalk (leaf) only reached 26.39 μg/g on 50th days. Above of which indicated that the root of *P. hexandrum* could not only accumulate the secondary metabolites of podophyllotoxin in its own tissue and cells but also be released into the external environment.

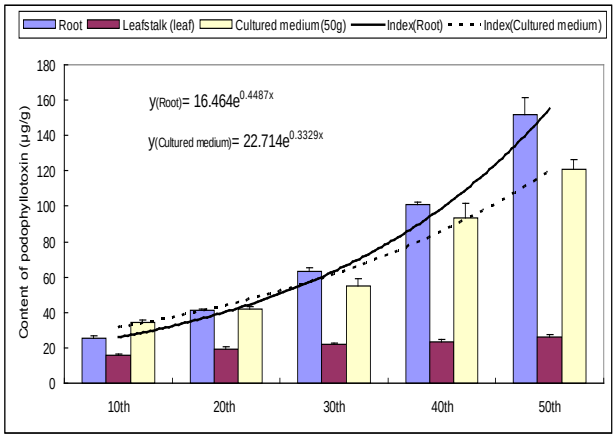

Figure 5. The content of podophyllotoxin in different materials at different growth phases. Note: The content of podophyllotoxin in root or leafstalk (leaf) was 1.0 g and that of in cultured medium was 50.0 g. Each Value represents an average of 30 replicates. Bars represent standard error.

4 Discussions and Conclusions

In this paper we report the establishment of the way of production podophyllotoxin by root culture of *P. hexandrum* through the addition of exogenous hormones IBA, GA_3 and Hy and AC to observe their influences on the growth rate and accumulation of podophyllotoxin. Yang M J et al [20] reported that IBA had a central role in regenerating *Acacia crassicarpa* through organogenesis, the elongated adventitious shoots were rooted at a rate of 96.5% on half-strength MS medium with 0.5 mg/L 3-indolebutyric acid (IBA) in 1 month. Berta E L et al [21] reported that the rooting induction with 14.7 mM

IBA had a significant effect on root regeneration; the highest rooting percentage (46.6%) was obtained with 7 days of induction. GA_3 is well known to hasten vegetative growth and development in almost plants [22]; Maksymowych R et al [23] confirmed the role of GA_3 in enhancement of growth and development of *Xanthium*. Kharkwal et al [24] also reported that an attempt was made to conserve *P. hexandrum* and understand the histological and biochemical changes that were affected by GA_3 in order to bring about this hastening of vegetative growth under ex situ conditions and found that GA_3 could promote mass propagation significantly. Rekha K et al [25] studied the effect of GA_3 inducing changes in slow growing endangered Himalayan plant *Podophyllum hexandrum* and hastening of vegetative growth *P. hexandrum* and found that *P. hexandrum* exhibited delayed emergence of functional leaves or hypocotyl dormancy. However, on GA_3 treatment the functional leaves were found to emerge at a favorable temperature of 25℃ in a higher percentage of seedlings and in a shorter time. Our preliminary studies [26] on the dormancy mechanism *P. hexandrum* seed firstly found the seed extraction hydroquinone (Hy) possessing bioactivities and could stimulus the wheat seedling growth and development and hasten the nutrient substances absorption and accumulation that had the similarity biology characteristics to plant regulators. More importantly, adding proper concentration of Hy in the MS medium *in vitro* culture of seed embryo could hasten and shorten the cycle of the growth and development of *P. hexandrum*. Although the exact role or mechanism for AC in association with agar and growth regulators in the culture medium caused a significant increasing and raised the biomass and development is not yet known, it has been proposed that AC may remove toxic substances generated by plant secondary metabolites such phenols, flavonoids, etc [27]. AC is capable of trapping gases and thus may inactivate ethylene, or other gases, released from the cultured tissues [28]. Thus addition of AC along with growth regulators in the culture medium may absorb phenols, flavonoids etc and thus provide a better growth environment.

In this experiment all the treatment of IBA, GA_3 and Hy could promote the root growth and prolong the root; Hy showed the most significant than GA_3, IBA, subsequently, which further proved that Hy had similarity to auxin on plant growth and development [26]. It also might be shown that Hy is a prerequisite for the promotion of material substance or signalling material, which can promote the synthesis of the premise material or a supporting role, which at last further influences the nutrient absorption and accumulation of the root so as to promote root the growth and development. Addition of AC in association with growth regulators (Hy) in the culture medium resulted in a significant increasing and raised the biomass of root and at the same time prevented the plantlets browning, which showed the same results as the review (Pan M J, et al. Toering A, et al) [27,29]. High level AC inhibited the growth and development of *P. hexandrum* which might be the reason of the competition between the adsorption of AC and that of root; but High level AC (≤5.0 g/L) is directly proportional to the relationship between podophyllotoxin accumulation and cultured medium. After culture 50th days the accumulation index of podophyllotoxin in root and cultured medium reached $e^{0.4487}$ and $e^{0.3329}$ respectively. So the way to product podophyllotoxin mainly derived from the root secondary metabolites would devote significantly to the development of the root culture of *P. hexandrum*, further insight into the precursor feeding materials might be an important way to improve the content of podophyllotoxin and establish an effective root large-scale cultivation to overcome the difficulty obtaining fast growing, sub culture stability, anti-browning cell line and callus for suspension [30,31].

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