Establishment of callus induction system, histological evaluation and taxifolin production of Larch

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Abstract

Taxifolin in larch is an important natural active ingredient, which prevents and treats liver diseases and cardiovascular diseases, and has many health benefits and application values such as anti-tumor, anti-virus, and anti-aging. However, the traditional extraction method is not conducive to the protection of the ecological environment, and also limits the industrial production of taxifolin from larch. Therefore, the purpose of this research is to establish a larch callus culture system and obtain taxifolin from callus. The shoots of the stem-tips of Larix olgensis were used as explants to be cultured. The light conditions, optimal medium and hormone ratio for callus induction were screened to evaluate the callus induction characteristics of larch and its influence on the accumulation of taxifolin. The results showed that: callus formation started on 28–51 days, and the best conditions for inducing callus were explored: 12 h/d light culture MS + 2,4-D 2 mg/L + 6-BA 1 mg/ L + KT 0.1 mg/L; the callus induction rate is 50%-80%; the highest condition for producing taxifolin 6-BA 1 mg/L + NAA 0.1 mg/L, reaching 0.414%. In conclusion, this study represents a suitable induction condition for larch callus culture and taxifolin accumulation.

Key Message

In this study, a stable and efficient larch callus culture system was established to lay the foundation for the industrial production of taxifolin, which may also be the best strategy for obtaining taxifolin in the future.

Introduction

Taxifolin (TAX), a dihydroflavonol, is an important natural active ingredient, which has generally been isolated from larch root. In view of its structure containing more phenolic hydroxyl groups, TAX has a variety of biological activities, including anti-inflammatory, antioxidant, anti-proliferation and anti-anaphylaxis effects(Kim et al. 2008; Ahn et al. 2010; Ivanov et al. 2014)

Therefore, TAX has high development potential, such as food additives, health foods and medicines. TAX was first extracted from Douglas fir bark by Japanese scholars using hot water extraction(Fukui et al. 1966). In recent years, TAX has also been detected in some fruits, but the content is extremely low. At present, larch is still the main source of TAX, and it is also an important tree species for protecting the ecological environment. As more and more countries authorize taxifolin to enter the food and health products industry, the demand for TAX is also increasing, while its output is far from meeting the needs of the market (Wang et al. 2011). Therefore, the research on improving the production of taxifolin has important practical significance. In fact, the traditional method of producing dihydroquercetin still uses larch as raw material by hot water extraction or ultrasonic extraction, and these methods not only has low extraction efficiency, but also a large amount of larch raw materials are consumed, which is very unfavorable to the protection of the environment. Therefore, it is of great significance to actively explore the use of forest resources and explore new resource alternatives and production methods.

The genus Larix is almost all tall deciduous trees belonging to the Pinaceae, most of which are forest species and mainly used in construction, decoration, papermaking and other industries (LEWANDOWSKI et al. 1991). In recent years, with the continuous deepening of research, some tree species are used for extracting resin, refining turpentine, extracting natural active substances and other chemical or pharmaceutical raw materials.

However, due to the long growth cycle of larch, the selection of new varieties takes a long time, and the effect is slow, it is difficult to improve the traits of the offspring, and the accumulation period of natural active substances is long, which limits the application range of larch(Bonga and Pond 1991). With the development of science and technology, obtaining medicinal secondary metabolites has become a new direction through tissue culture technology in contemporary plant biotechnology research(Zhao et al. 2001). Therefore, it is of great significance to genetically improve larch species, prepare active ingredients and explore the biosynthetic pathways of secondary metabolites by establishing an efficient and stable tissue culture system for larch species.

Based on the above research background, the purpose of this study is to establish a uniform and stable larch callus culture system through plant tissue culture technology, and to investigate the influence of different hormone combinations on the content of taxifolin in callus. According to our investigation, the research on the induction of callus and the production of taxifolin by larch in vitro has not been reported yet.

Materials And Methods

Explant material preparation

The material of Larix olgensis was provided by Changbaishan Forest Farm in Jilin Province, and the research was carried out in the Natural Products Laboratory of the College of Chinese Medicinal Materials, Jilin Agricultural University. The materials were washed thoroughly in running tap water for 15 minutes, followed by a sterilized scalpel to cut off the shoots (0.5-1 cm) and soak them in 70% (v/v) ethanol for 5 minutes, and then young shoots were washed with sterile distilled water for 2 minutes. Afterwards, these explants were sterilized in a laminar flow cabinet using 6% NaClO□purchased from Qingdao Rishui Biotechnology Co., Ltd.□ solution for 40 minutes. Finally, rinse with sterile distilled water three times for 30 seconds each time.

Induction of LarchCallus

Sterilize the ultra-clean workbench in advance for 40 minutes and ventilate for 10 minutes to enter the ultra-clean workbench. Afterwards, wipe the plate, scalpel, and tweezers with an cotton containing 75% ethanol, and then use an alcohol lamp to sterilize it for later use. The aseptic shoots of Larch were selected as explants for callus culture(Salajová and Salaj 2005), and the culture conditions are as follows: Murashige and Skoog medium(Murashige and Skoog 1962) are used as the medium for explant growth, according to table 1 configuration with different concentrations and the combination of 2,4-

dichlorophenoxyacetic acid (2,4-D), 6-benzylamino adenine (6-BA), naphthaleneacetic acid (NAA) and agonist modulator (KT, purchased from Beijing solebao Technology Co., Ltd), and the pH value is adjusted to 5.8±0.1. The light time was 12 h/d and the callus induction characteristics were recorded such as callus formation rate, fresh and dry callus weight after 50 days of culture in a constant temperature incubator at 25°C. In addition, the light microscope was performed biweekly to check the callus morphology (Nikon Eclipse NI, Dusseldorf, Germany).

Histological observation of callus induction in the process

For histological examination, callus cultures were selected with good growth and different induction levels and fixed with 2.5% glutaraldehyde for 24 hours at room temperature. After fixation, wash 3-4 times with 0.1M PBS buffer (pH 7.2) solution, and the tissue samples were dehydrated according to the graded ethanol series (10%, 30%, 50%, 70%, 90%, 95%) for 15 minutes, and then place in absolute ethanol overnight. After that, the tissue samples were replaced with isoamyl acetate for 30 minutes and dried in a constant temperature dryer for 60 minutes, and then they were observed and photographed by scanning electron microscope (SEM) after spraying gold by ion sputtering.

Effects of different photoperiod on callus induction rate

The uniformly growing callus were selected and divided into three groups, 10 bottles in each group, and inoculated in MS + 6-BA 1.0 mg/L+2,4-D 2.0 mg/L+ KT 0.1 mg/L solid medium. They were cultured in a constant temperature incubator with three lighting conditions: 24 h/d full light culture, 12 h/d light culture, and 24 h/d full dark culture. The light intensity was 50 μmol/(m^2•s) and the culture temperature was 25±1 ℃, and then the callus induction rate was counted after 50 days.

Extraction and analysis of taxifolin from callus

Fresh callus was freeze-dried at -20 ℃ and 1.45 mbar (Christ alpha 1-2 LD plus, Germany) for 24 h. The dried callus was ground into powder with a mortar and sifted through 40 mesh for standby. Each sample was weighed accurately with 0.2 g, and taxifolin was extracted by adding 30 ml methanol and 90 Hz ultrasound for 30 min. After that, the solution was filtered, and the filtrate was evaporated to dryness, dissolved in methanol and diluted to 10 ml Brown volumetric flask. The treated solution was filtered through 0.22 μmmicroporous membrane for HPLC detection. HPLC analysis was carried out on Waters alliance 2695 with Waters UV detector 2489 (Waters Technology Shanghai, China). The injection volume of the sample was 20 μL and the column temperature was kept at 30 ℃. The binary elution solvent consisted of A (methanol) and B (water): 45%: 55%, and gradient elution procedure was used. A cosmosil column 02485-81 C18 (250 mm × 4.6 mm / 5 μm) was used. The flow rate was maintained at 1 ml/min. The UV spectrum of taxifolin was obtained with 290 nm detection wavelength.

Statistical Analysis

All the displayed data are average ±standard deviation. Statistical significance was determined by one-way analysis of variance (ANOVA). Then use SPSS18.0 software to carry out the least significant difference (LSD) multiple comparison test, where $P<0.05$ is considered significant. Statistical graphs were produced via software of GraphPad Prism 6.0.4 software (GraphPad Software, Inc, San Diego, USA).

Results And Discussion

Characteristic analysis of callus

Previous researchs and analysis have shown that the induction period and growth of callus were different, which depended on hormone treatment, concentration and growth regulators(Teszlák et al. 2005). As shown in Table 2, the induction range of callus is 50% - 80%, and that is the culture type with growth regulator (70% -80%) is easier to form callus, and the highest induction rate is 83.4%, which occurs in T2: MS + 2,4-D 2 mg/L + 6-BA 1.0 mg/L + KT 0.1 mg/L. In addition, callus induction occurred at 28.58±2.97 - 51.07±3.49 days after inoculation, and the fastest callus occurred at about 28 days at T5: MS+2,4-D 0.1 mg/L +6-BA 2.0 mg/L, which may be due to the effect of 6-BA on promoting organogenesis and undifferentiated tissue growth(Fu-heng et al. 2014). Besides, the fresh and dry weights callus formed in each treatment represented the growth of callus, and the growth of callus in each treatment was different. The results of this study are shown in Fig. 1, the highest fresh weight obtained was 3.30 ± 0.23 g, while the highest dry weight was 1.25 ± 0.19 g at T10: MS + 2,4-D 2.0 mg / L + NAA 1.0 mg /L, this indicated that auxin (as NAA, 2,4-D) had the greatest impact on the fresh and dry weight of callus, which may be due to the continuous proliferation of tissue cells caused by auxin, and appropriate concentration of auxin promoted its growth(Lu et al. 1983; Stasolla and Yeung 2003).

Furthermore, the characteristics of callus formation were observed by optical microscope from the beginning of callus formation in the study, and observed that the callus formed by larch was small in shape and rich in small vacuoles, which was similar to the previous reports(Attree et al. 1992; Mehring et al. 2020). Simultaneously, it was observed that the surface texture of callus formed in T2, T6 and T11 with relatively high induction rate changed from brittle to hard, and the surface gradually transited from yellow fluent callus to brown callus, and finally to light brown callus (Fig. 2, Tab. 2). The comparative analysis of T2 and T11 showed that with the increase of mitogen in the combination of 6-BA and 2,4-D, the division rate of callus increased and the number of callus increased, and the texture of callus was loose and suitable for differentiation culture in the future. In the combination of NAA and 6-BA, although the induction rate reached 78.5%, the browning was serious in the later stage, and the hard texture was not conducive to the later subculture. Considering comprehensively, Considering comprehensively, T2: MS + 2,4- D 2 mg / L + 6-BA 1.0 mg / L + KT 0.1 mg / L were selected as the best culture for larch shoot callus induction condition.

Histological analysis of the surface structure of Larch callus

The surface structure of callus is constantly changing in different culture cycles(Moumou et al. 1992). As shown in Fig. 3, the same larch callus exhibits roughly three different structures with different growth

cycles. The callus cultured for 10 days had obvious bulges on the surface, cracks on the bulging surface, and loose arrangement (Fig. 3a-c); After 20 days of culture, the surface of the callus began to bulge and began to dent, and the arrangement became dense, moveover this structure indicated that there were different developmental periods on the same piece of callus (Fig. 3d-f)After 30 days of continuous culture, the callus was arranged tightly, and the structure of embryoid body will appear at the crack, and the shape and size of the embryoid structure are not much different, mainly globular (Fig. 3g-i), which was related to the development stage of callus.

Analysis of the influence of light treatment on callus induction rate

Light plays an important role in callus formation, differentiation and synthesis of secondary metabolites(Jao et al. 2005; Shiga et al. 2009).The results showed that the callus induction rate of larix gmelinii callus was 26.3% in dark culture at 24 h/D, and most of the callus were brown with the extension of culture time, which indicated that the callus was hard, and its fresh weight and dry weight increased first and then decreased, and the fresh weight reached the maximum value (2.78 ± 0.67 g) at 40 days of culture (Fig. 4a and b, Fig. 5a), while the dry weight also reached the peak at 40 days (0.68 ± 0.15 g); The callus induction rate under 24 h/d light culture is 47%, but most of the callus were light yellow brown and the surface color of callus was dark, and fresh weight and dry weight increased steadily and the maximum fresh weight was 2.27 ± 0.53 g while the maximum dry weight was 1.67 ± 0.22 g (Fig. 4c and d, Fig. 5b); The callus induction rate of 12 h/d light culture was 64.4%, which was 17.4% higher than that of 24 h/d light culture. Moreover, the callus induced was light yellow, yellow-green and denser. The maximum fresh weight reached 2.94±0.62 g (Fig. 4e and f, Fig. 5c). Therefore, 12 h/d light culture was used as the most suitable light condition for inducing larch callus in this study.

Exogenous hormone different concentrations regulate taxifolin accumulation in larch callus

In order to explore the content change of Larix callus, the change of taxifolin content in larch callus, several hormone combinations with high induction rate were selected and analyzed by HPLC. As shown in Fig. 6, the taxifolin content of 6-BA 1 mg / L + NAA 0.1 mg / l was 0.477% (Fig. 6a); The taxifolin content of 2,4-D 2 mg / L + 6-BA 1 mg /L was 0.113% (Fig. 6b), while the dihydroquercetin content of 2,4-D 4 mg / L + 6-BA 2 mg / L was only 0.037%(Fig. 6c). The analysis of the results showed that low dose of 6-BA and NAA were beneficial to the accumulation of taxifolin in callus, and increasing the concentration of 2,4-D inhibited the accumulation of taxifolin and other secondary metabolites. In addition, the content of taxifolin in the root of 30-year-old Larix olgensis was about 0.7%, while the content of taxifolin in callus produced by plant tissue culture was up to 0.4%, indicating that inducing callus is an efficient and quick method to produce secondary metabolites, and the productivity has been improved(Ahmad et al. 2020). In conclusion, the establishment of a stable and efficient callus culture system of Larix can lay a foundation for industrial production of taxifolin, which may be the best strategy for obtaining taxifolin in the future.

Conclusion And Outlook

In this study, the shoot was used as the explant source of callus induction, and the callus culture system of larch was established by plant tissue culture technology. These evidences showed that MS medium with 2,4-D and 6-BA hormones and kinetin (KT) showed a high callus induction potential, and the induction rate reached 83.4%. In addition, depending on the culture conditions, the texture and surface color of the obtained callus are also different, the texture changes from brittle to hard, and the surface gradually transitions from yellow fluent callus, to brown callus, and finally to light brown callus. Besides, it was also found that the photoperiod also had a great influence on the callus formation, and 12 h/d light culture was the most suitable light condition for inducing callus of larch, and the induction rate is 17.4% higher than other conditions. Importantly, this study investigated for the first time the effects of different hormone combinations and concentrations on the content of taxifolin in callus, and these data also supported the potential of larch callus to produce an excellent alternative source of taxifolin. In conclusion, this study provides a new research method for the preparation of natural products, which also establishs the foundation for the future research on the biosynthesis of taxifolin in larch callus.

Declarations

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Author Contributions Wencong Liu perceived and planned the experiment; Xinglong Liu, Yinan Zheng and Yingchun Zhao transcribed the chief document text and investigated figures; Chen Xueyan and Chuanbo Ding mainly performed statistics and figures drawing; Wu Ming, Ling Dong, Qiteng Ding and Shuang Xu checked and modified the manuscript. All authors review and legalize the content of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Tables

Table 1 Combination ofphytohormones

Code	Baisc medium	Combination ofphytohormones			
		2,4-D mg/L	6-BA mg/L	NAA mg/L	KT mg/L
T1	MS	2.0	0.5	-	-
T2	MS	2.0	1.0	-	0.1
T3	MS	2.0	2.0	-	-
T4	MS	0.5	1.0	-	0.1
T5	MS	0.1	2.0	-	-
T6	MS	-	1.0	0.1	-
T7	MS	-	1.0	0.5	-
T8	MS	-	1.0	1.0	-
T9	MS	2.0	-	0.5	-
T10	MS	2.0	-	1.0	-
T11	MS	2.0	-	-	0.1

Table 2 Callus growth rate, callus texture, callus induction rate characteristics under the same conditions

growth regulators	Average of callus formation (day)	Callus morphology		Response (%)
		Texture callus	Nature of the	
T1	32.70±4.77	Less Friable	Yellow fluencies callus	72.6
T2	34.16±2.20	Friable	Yellow fluencies callus	83.4
T3	49.89±5.32	Compact	Brownish yellow callus	64.1
T4	43.26±1.72	Friable	Light yellow callus	74.6
T5	28.58±2.97	Less Friable	Yellow fluencies callus	67.9
T6	41.66±3.64	Friable	Light yellow callus	78.5
T7	45.37±2.69	Compact	Brownish yellow callus	66.4
T8	51.07±3.49	Compact	Brownish yellow callus	62.1
T9	43.19±3.78	Friable	Light yellow callus	64.4
T10	45.50±3.13	Compact	Light brown callus	58.9
T11	46.69±1.45	Compact	Light brown callus	73.8

Figures

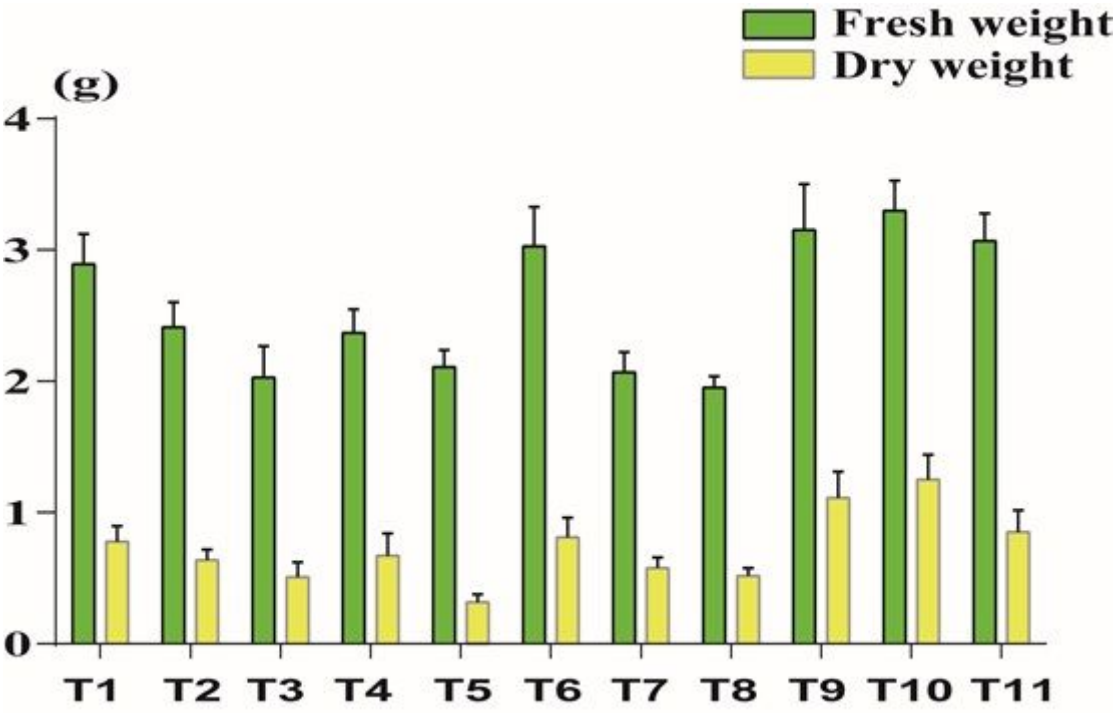

Figure 1

The effect of different hormone combinations and concentrations on the fresh weight and dry weight of callus, and the difference in each treatment represents the growth of callus.

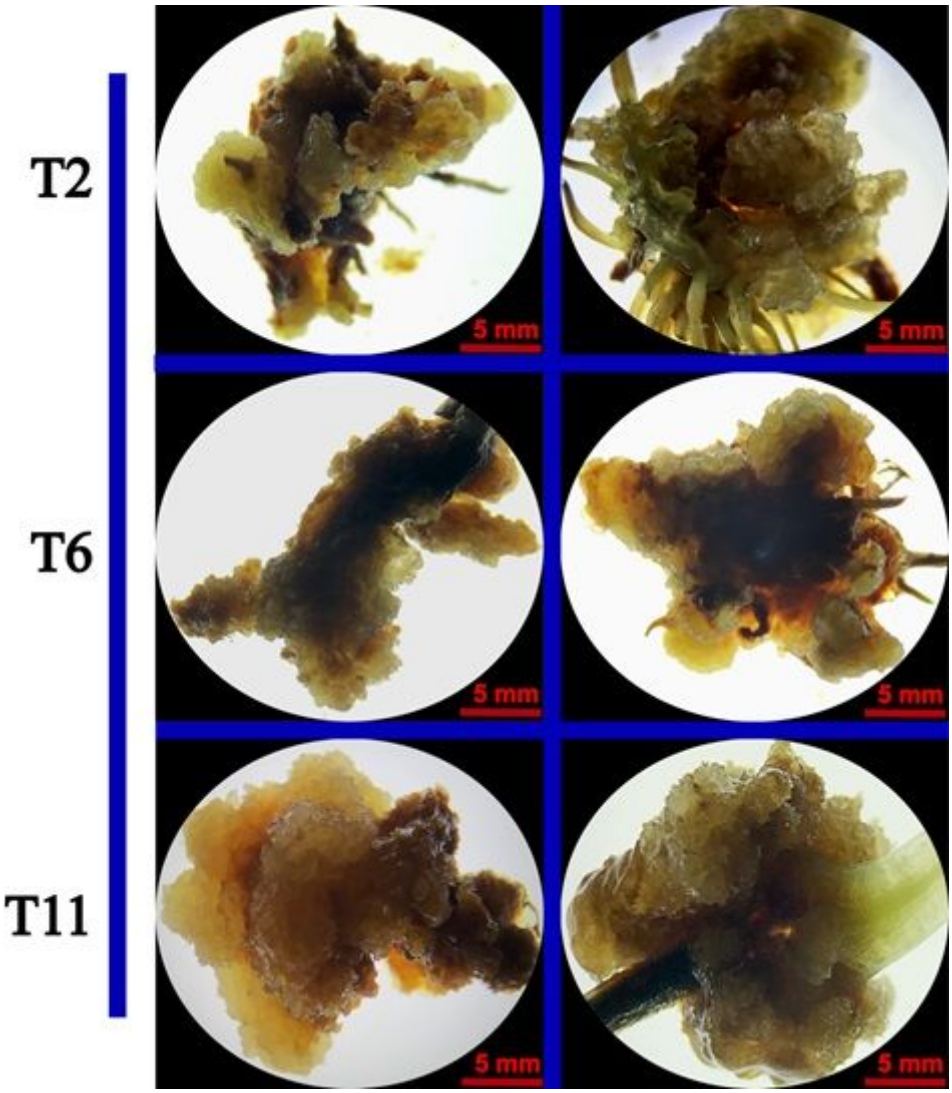

Figure 2

The characteristics of callus formation were observed through an optical microscope. Several combinations with higher induction rates (T2, T6, T11) were selected for investigation, and it was found that the callus formed in T2 was light yellow in color, loose in texture and abundant in small vacuoles, so it was suitable for differentiation culture. Scale bar: 5 mm.

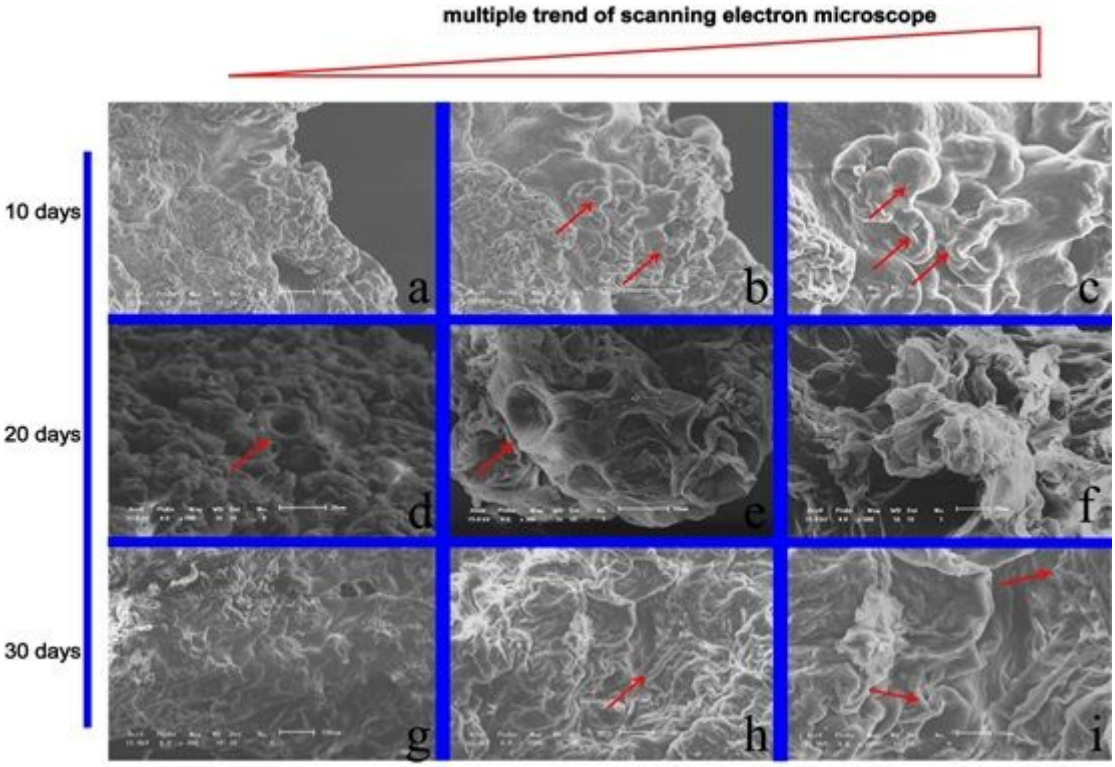

Figure 3

The surface structure of Larch callus was analyzed by scanning electron microscope. a-c The callus cultured for 10 days had obvious bulges on the surface and cracks on the bulging surface; d-f After 20 days of culture, the surface of the callus began to bulge and began to dent, and the arrangement became dense; g-i After 30 days of continuous culture, the structure of embryoid body will appear at the crack, and the shape and size of the embryoid structure are not much different, which was related to the development stage of callus.

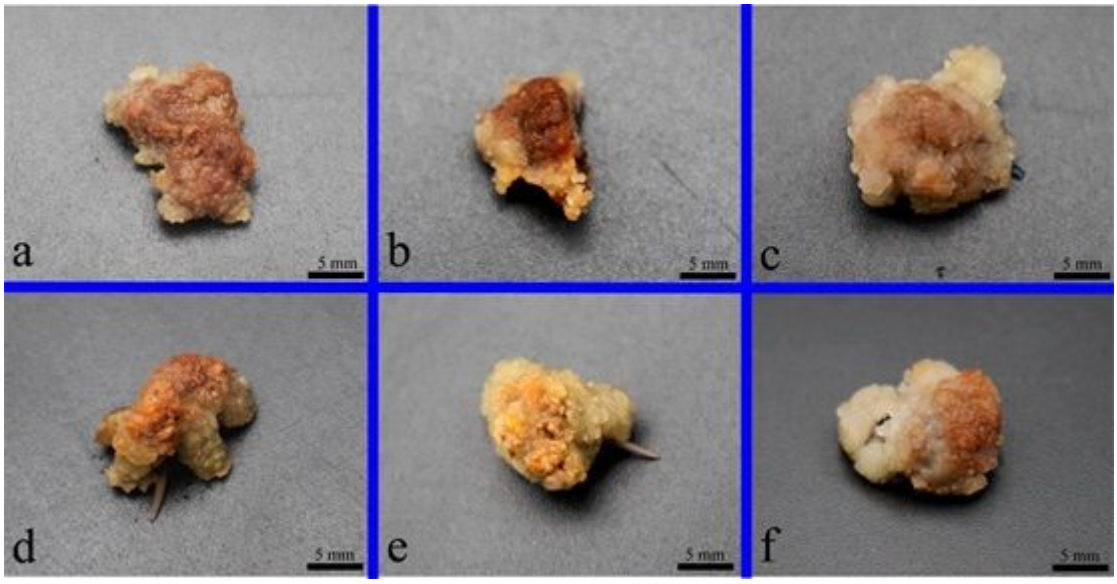

Figure 4

Effect of photoperiod on callus morphology of larch. a,b 24 h/d dark culture, most of the callus showed brown to brown and hard texture; c,d 24 h/d light culture, the callus was mostly light yellow brown, and the surface color of callus was gray; e,f 12 h/d light culture callus was light yellow, yellow-green, and texture was relatively tight. Scale bar: 5 mm.

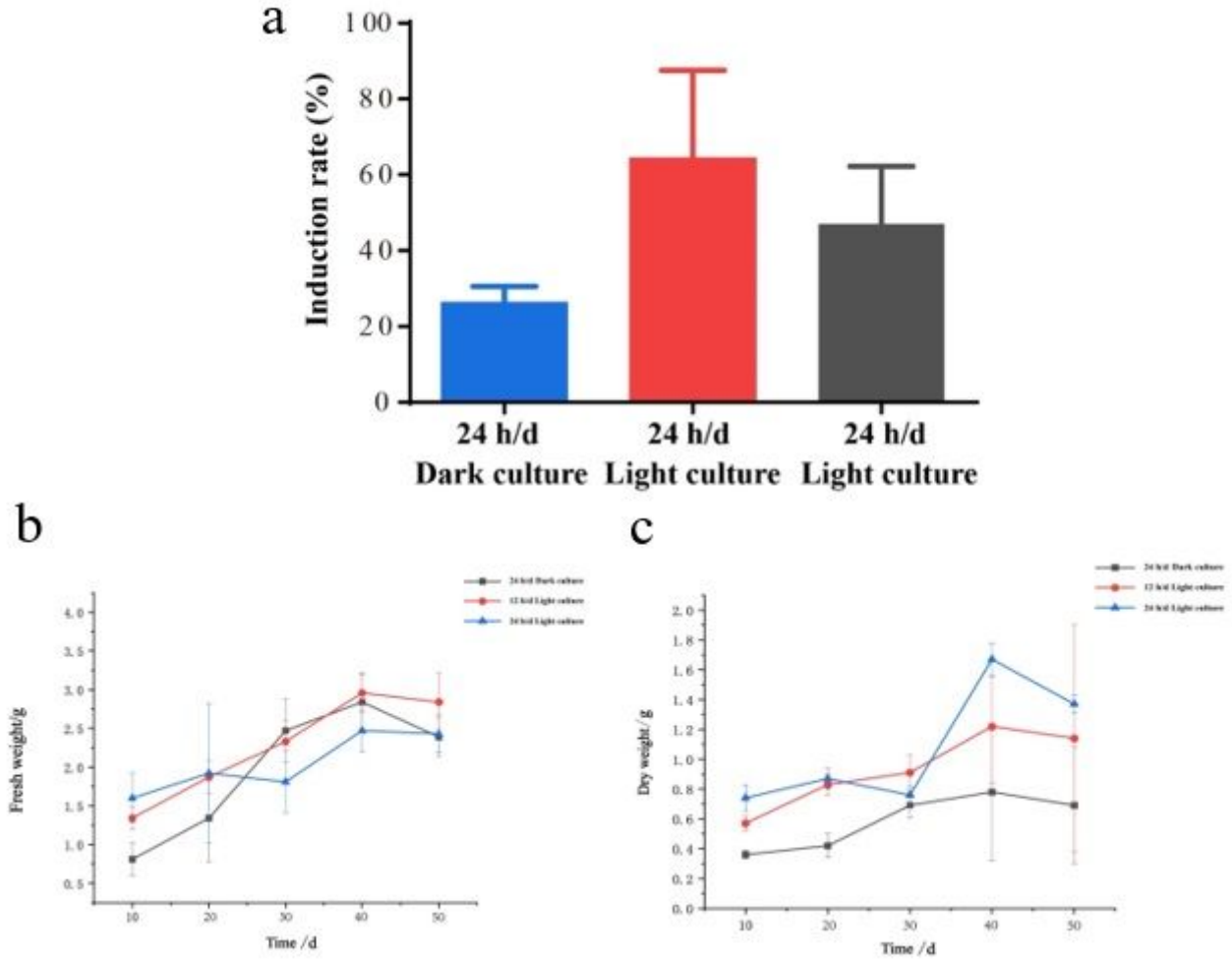

Figure 5

The effect of different photoperiod treatments on the callus induction rate, fresh and dry weight. a Compared with other photoperiod treatments, the callus induction rate of 12 h/d light culture was the highest 64.4%; b The fresh weight of three photoperiod treatments showed a steady upward trend, and the fresh weight reached the maximum of 2.94 ± 0.62 g after 40 days of 12 h/d light culture; c The dry weight reaches a maximum of 1.67±0.22 g under 24 h/d light culture conditions.

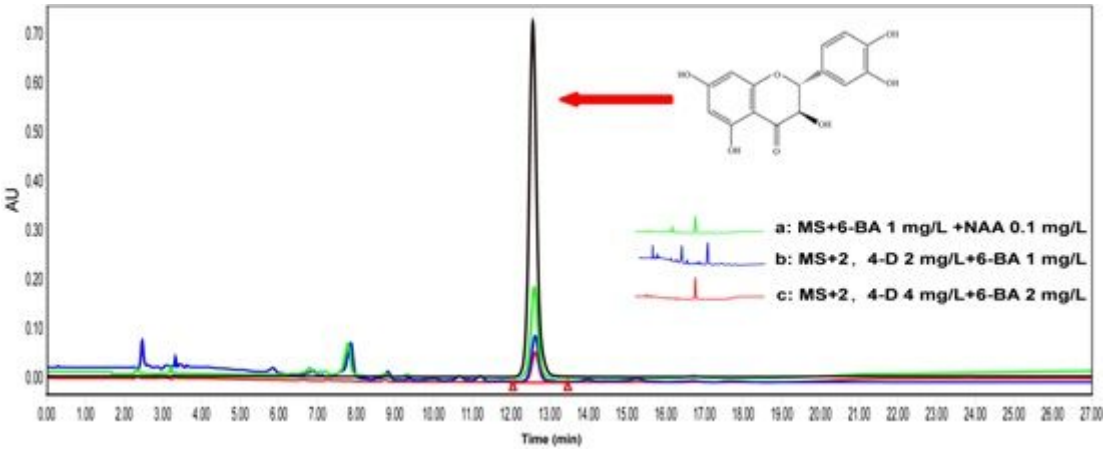

Figure 6

The results showed that the content of taxifolin was determined by HPLC. a Spectrogram of standard substance; b Inducing condition: 1 mg / L of 6-BA + 0.1 mg / L of NAA; c Inducing condition: 2 mg / L of 2,4-D + 1 mg / L of 6-BA; d Inducing condition: 2,4-D 4 mg / L + 6-BA 2 mg / L.