Research paper

A Study of Callus Induction and Polysaccharide Contents of *Cyclocarya paliurus* (Batalin) Iljinskaya

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【Summary】

The aims of this study were to determine the optimal medium for callus induction from tender leaves of *Cyclocarya paliurus* (Batalin) Ilijinskaya and determine differences in polysaccharide contents among two types of calli and various types of *C. paliurus* leaves. Using an orthogonal analysis, the optimal conditions for *C. paliurus* callus tissue induction were found to be mainly influenced by the basal medium and the exogenous hormone concentration and ratio. Callus induction and browning rates were influenced by the following, in decreasing order of significance: basal medium, the cytokinin, 6-benzylaminopurine (6-BA), and the auxin, indole-3-butyric acid (IBA). The optimal medium was determined to be NN69 medium supplemented with 1.0 mg L^{-1} 6-BA and 0.05 mg L^{-1} IBA. Next, the polysaccharide content was determined by the phenol-sulfuric acid method. The polysaccharide content in the callus was found to be markedly higher than that in *C. paliurus* leaves collected over different periods, as well as those in commercial and handmade teas. Furthermore, the polysaccharide content in green calli was higher than that in brown calli. The present findings are important in terms of the development, utilization, and industrial applications of *C. paliurus*, particularly *C. paliurus* tea.

Key words: *Cyclocarya paliurus*, tissue culture basal medium, exogenous hormone, callus, polysaccharides.

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研究報告

青錢柳愈傷組織誘導及多糖含量的研究

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摘 要

為增加青錢柳繁殖率，提高青錢柳多糖產出。本研究以青錢柳當年生嫩葉為外植體，對基本培養基、外源激素及其濃度配比等關鍵因素進行正交設計試驗，獲得青錢柳愈傷組織誘導優化培養配方，同時對不同狀態的愈傷組織、不同生長時期嫩葉及不同加工方式青錢柳茶進行多糖含量測定比較。結果顯示：關鍵因素中影響青錢柳嫩葉愈傷組織產生及褐化的主次順序為：基本培養基> 6- 氨基腺嘌呤(6-BA) > 吲哚丁酸(IBA)；最佳愈傷組織誘導配方為：NN69 + 1.0 mg L^{-1} 6-BA + 0.05 mg L^{-1} IBA；綠色愈傷多糖含量大於褐色愈傷，青錢柳8月葉多糖含量高於4月葉，手工制茶多糖含量高於機制茶。

關鍵詞：青錢柳；基本培養基；外源激素；愈傷組織；多糖。。

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INTRODUCTION

Cyclocarya paliurus (Batalin) Iljinskaya is a deciduous tree in the family Juglandaceae. It is one of the most important protected and endangered plants in China. It is mainly distributed in mountainous areas of Jiangxi, Zhejiang, Hunan, Fujian, and Sichuan Provinces (Fang et al. 2007). *Cyclocarya paliurus* leaves were found to contain polysaccharides, amino acids, and flavonoids, which have blood sugar- and blood lipid-lowering effects (Li et al. 2001, He et al. 2012). These leaves therefore have high medicinal value and great potential for various applications, including the herbal preparation called Qing Qian Liu tea. However, the natural growth of *C. paliurus* is restricted by its low sexual reproductive coefficient and difficulty asexually reproducing, which has greatly hindered development of its applications. At present, it is mainly propagated asexually through cuttings as reported by Li et al. (2014a), who optimized key factors such as the matrix ratio, temperature, humidity, and hormones for *C. paliurus* propagation by cuttings. They showed that the highest rooting rate attained was only 35.6%.

Plant tissue culture is an important method to rapidly and efficiently breed desirable varieties, and by the addition of appropriate plant growth regulators, this approach can also aid the production of secondary metabolites (Li et al. 2014b). *Cyclocarya paliurus* tissue culture is increasingly becoming a focus of forestry research. Although there have been many studies on inducing adventitious buds from different explants (Hu et al. 2005, Shangguan et al. 2006, Wang 2008, Wu et al. 2008, Cheng et al. 2009, Hu et al. 2009, Zhang et al. 2010, 2018), polysaccharide content analyses have not been reported for this species. The objectives of this study were to optimize the medium formulation for callus induction from tender leaves of *C. paliurus* and compare and analyze polysaccharide con-

tents of calli, natural leaves, and processed leaves of *C. paliurus*. Through comparative analysis, it was clear that the suitable period of leaf picking for making *C.paliurus* tea and the suitable callus state of *C.paliurus* for extracting polysaccharides. We expect that this study will provide insights for further research and development related to the potential applications of this species.

MATERIALS AND METHODS

Materials for testing and processing

The materials for callus induction were young leaves of a 1-yr-old *C. paliurus* tree, which was about 1 m tall. It had been transplanted from its provenance, i.e., Shaoyang City, Suining County, Hunan Province, China in April 2017 at the germplasm resource nursery of the Central South Forestry University of Science and Technology. Fresh leaves of *C. paliurus* were immediately packed in a bag to maintain their freshness and then stored in a refrigerator at 2.6℃ for 8 h after preservation and anti-browning treatment. Leaves were rinsed in running water for about 15 min, drained, and placed in a jar. The work bench was sterilized with 70% alcohol for 30 s. The material was rinsed with sterile water 3~5 times, soaked in a 0.1% mercury solution for 10 min, and rinsed with sterile water at least 5 times. Leaves were then blotted with sterile filter paper, cut into leaf discs of about 0.5×0.5 cm, and inoculated into pre-prepared sterile medium. Each bottle (240 mL) was inoculated with 3 explants, and 10 bottles were inoculated for each treatment.

The calli used for polysaccharide determination were green calli and brown calli induced after 3 subcultures. New leaves collected in April and August, leaves from a 1-yr-old tree, leaves from a 3-yr-old tree, manually prepared *C. paliurus* tea leaves, and industrially prepared *C. paliurus* tea leaves were obtained from the center of *C. paliurus* culture in Suining County. Fresh leaves were randomly sampled from different parts of the tree. The control hand-made common green teas were randomly purchased from a supermarket. After sample collection, the leaves and calli were subjected to drying at 60℃ for 2 d with a Yiheng air dry oven (DHG-9023A, Shanghai, China). After the samples had dried to a constant weight, the dried samples were ground into powder with a Jiuping stainless steel mill JP-150A (Yongkang, China).

Screening of induction medium

Considering the callus-induction degree, callus-browning degree, and callus growth as evaluation indices, 3 levels were set: basal medium type, the concentration of the cytokinin, 6-benzylaminopurine (6-BA), and the concentration of the auxin, indole-3-butyric acid (IBA). The concentration of sucrose used in the medium was 30 g L^{-1}, and the agar concentration was 6 g L^{-1}. An L9(3^3) orthogonal test was used to study the 3 levels (Tables 1, 2). The incubator temperature was 25±1℃. The light was cool daylight provided by Philips lamps (TLD 36 W/54-765; Shuzhou, China); the light intensity was 50 μmol·m^{-2}·s^{-1}, and the light duration was 12 h d^{-1}. The growth status was recorded after subculturing once every 20 d and thrice thereafter (Tables 1, 2).

Determination of polysaccharide contents of *C. paliurus* leaves

Polysaccharide contents of *C. paliurus* leaves were determined by the phenol-sulfuric acid method (Xie et al. 2010). Sample powder (0.5 g) was accurately weighed, wrapped in fast filter paper, and put in a round-bottom flask bottle, to which 40 mL of 80% ethanol was added and refluxed in a water bath at 90℃ for 1 h. Next, the solvent was drained, and 20 mL dis-

tilled water was added, followed by soaking at 90℃ for 1 h. The extract was poured into a 100-mL volumetric bottle. Extraction was repeated, and the extract was mixed and shaken again in distilled water. Absorption was measured by a standard curve method. Absorbance values were measured with a Jiebosen ultraviolet-visible spectrophotometer UV-7504C (Shanghai, China). The concentration of glucose (ρ) was calculated by a regression equation. The polysaccharide content of *C. paliurus* was calculated by the following formula: polysaccharide % = ρ×V×f/m×100, where ρ is the glucose concentration (mg mL^{-1}), V is the polysaccharide dilution factor (mL), f is the conversion factor (the average conversion factor was 2.828), and m is the sample weight of *C. paliurus* (mg).

Standard curve preparation

Glucose was dried to a constant weight in an oven at 105℃. Dried glucose (0.2003 g) was precisely weighed and placed in a 200-mL volumetric flask to which distilled water was added to dissolve and dilute the solution to prepare a 1.0 mg mL^{-1} standard solution. Then, 2.5, 5, 10, 15, and 20 mL standard solutions were prepared in 100-mL volumetric flasks; shaken evenly; and used to obtain a glucose series at 25, 50, 100, 150, and 200 μg mL^{-1}. One milliliter of each standard solution was put into a test tube, and to each tube, 1 mL of 5% phenol and 5 mL of concentrated sulfuric acid were added. After 10 min, the absorbance was measured at 488 nm, and a standard curve was drawn. The regression equation of the standard curve was calculated.

Table 1. Factors affecting callus induction from excised leaves of *Cyclocarya paliurus*

Factor	Level		
	1	2	3
Basal medium	WPM	NN69	Modified MS
6-BA (mg L^{-1})	0.5	1.0	0.5
IBA (mg L^{-1})	0.01	0.05	0.1

WPM, woody plant medium; NN69, Nitsch and Nitsch medium 1969; MS, Murashige-Skoog medium; modified MS medium: changed from 1650 mg L^{-1} NH_4NO_3 of MS medium to 825 mg L^{-1} and 0.1 mg L^{-1} thiamine hydrochloride to 0.5 mg L^{-1}; 6-BA, 6-benzylaminopurine; IBA, indole-3-butyric acid.

Table 2. Orthogonal design of factors affecting callus induction from excised leaves of *Cyclocarya paliurus*

Group number	Basal medium	6-BA (mg L^{-1})	IBA (mg L^{-1})
1	WPM	0.5	0.01
2	WPM	1.0	0.05
3	WPM	1.5	0.1
4	NN69	0.5	0.05
5	NN69	1.0	0.1
6	NN69	1.5	0.01
7	Modified MS	0.5	0.1
8	Modified MS	1.0	0.01
9	Modified MS	1.5	0.05

WPM, woody plant medium; NN69, Nitsch and Nitsch medium 1969; modified Murashige-Skoog (MS) medium: changed from 1650 mg L^{-1} NH_4NO3 of MS medium to 825 mg L^{-1} and 0.1 mg L^{-1} thiamine hydrochloride to 0.5 mg L^{-1}; 6-BA, 6-benzylaminopurine; IBA, indole-3-butyric acid.

Data analysis

The orthogonal design data of callus induction were analyzed by Orthogonal Design Assistant II v3.1 (http://www.xue51.com/soft/7598.html), and the polysaccharide content data of *C. paliurus* were analyzed by SPSS Statistics 17.0 software (SPSS, Chicago, IL, USA) with a one-way analysis of variance (ANOVA). Each sample had 3 biological replicates. Significant differences among means were assessed using Duncan's multiple comparison at $p < 0.05$.

RESULTS

Intuitive screening of the induction medium

Some explants of young leaves of *C. paliurus* were removed after sterilization and inoculation. After 3 subcultures, 10~15 explants were retained in each combination for the statistical analysis. As shown in Table 3, the basal medium of formulae 7~9 was modified Murashige-Skoog (MS) medium, which yielded poor callus induction, loose calli, low survival rates, and severe browning (Fig. 1A). The basal medium of formulae 4~6 was Nitsch and Nitsch 1969 (NN69), which achieved a 100% callus rate. Numerous light-green, lustrous calli that grew well were obtained (Fig. 1B). The basal medium of formulae 1~3 was woody plant medium (WPM), which yielded a high degree of callus induction, a dark-green color, and a low degree of browning (Fig. 1C, D). Considering the callus rate, browning degree, and callus growth status together, combination 5, namely, NN69 + 1.0 mg L^{-1} 6-BA + 0.1 mg L^{-1} IBA, was found to be the most suitable for further study (Fig. 1, Table 3).

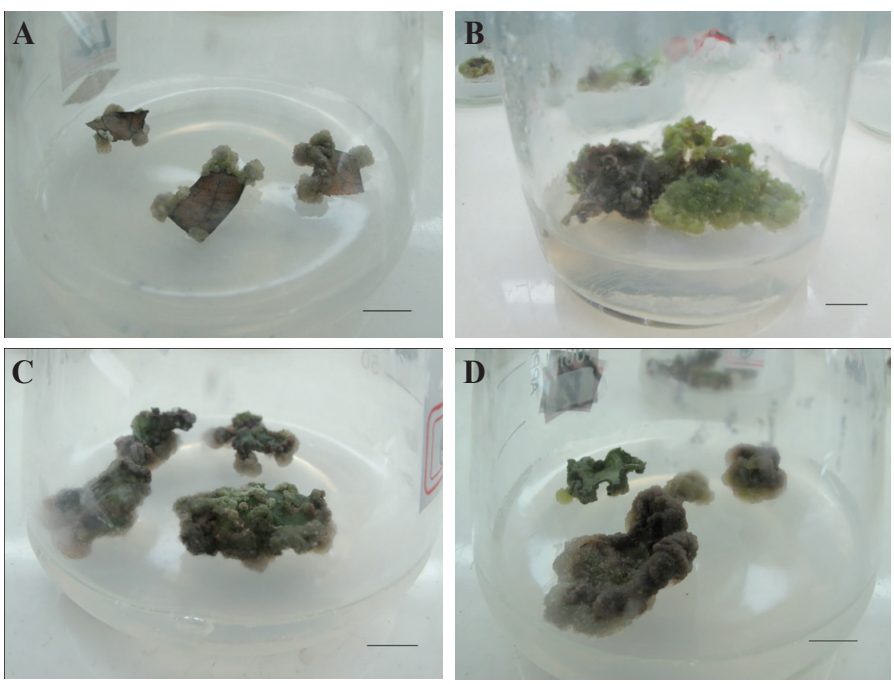

Fig. 1. A: Few brown and white calluses (modified MS medium). B: Considerable number of dense, green and brown calluses (NN69 medium). C: Moderate number of dense, dark-green and brown calluses (WPM). D: Numerous dense, light-green and brown and white calluses (WPM).bar = 1cm

Table 3. Results of the orthogonal experiment

Series no.	Inoculation no.	Callus no.	Callus rate	Callus growth	Browning degree	Callus characteristics characteristics
1	10	10	100%	++	---	Dense, dark-green and brown
2	13	13	100%	++++	--	Dense, dark-green and brown
3	12	10	83.3%	+	---	Slightly dense, light-green with brown and white
4	11	11	100%	+++++	-----	Dense, light-green with brown and white, green with red
5	10	10	100%	++++	-	Dense, green with brown
6	13	13	100%	+++	---	Loose, brown and white
7	15	14	93.3%	+	-----	Dense, brown and white
8	10	10	100%	++	-----	Loose, brown and white
9	12	12	100%	+	-----	Loose, brown and white

Note: The more + symbols, the greater the callus growth; the more - symbols, the more serious the browning degree.

Orthogonal analysis of the best medium for callus induction

A quantitative analysis was carried out using the software Orthogonal Design Assistant II v3.1 (Tables 4, 5). According to data of the degree of callus formation, extreme differences of the 3 factors were as follows: 2.667 for basal medium, 1.666 for 6-BA, and 1.333 for IBA. The greater the extreme difference, the greater the influence. Therefore, the influence of the factors affecting the degree of callus induction was in the following order: basal medium > 6-BA > IBA. According to the browning degree data, the extreme differences were 2.333 for basal medium, 1.666 for 6-BA, and 1.000 for IBA, which indicated that the 3 factors affecting the browning degree of calli were also in the same order as above (Table 4).

Results of the variance analysis according to the degree of callus formation shown in Table 5 were consistent with results of the range analysis, and the difference between basal medium and 6-BA was significant at $p < 0.05$, which indicated that different basal media and different concentrations of 6-BA had significant effects on callus induction from *C. paliurus* leaves. Taking the experimental data together, combined with the range analysis and variance analysis, the best medium formula was theoretically found to be NN69 + 1.0 mg L^{-1} 6-BA + 0.05 mg L^{-1} IBA. Results of the variance analysis based on the browning degree as shown in Table 4 were consistent with results of the range analysis, but there were no significant differences among the basal medium, 6-BA, and IBA, and the range of CK was >1.0. According to data of the browning degree test, together with the range analysis and variance analysis, the best medium formulation for low browning was found to be WPM + 1.0 mg L^{-1} 6-BA + 0.1 mg L^{-1} IBA. However, the confidence of screening the best medium formula according to the browning degree was low, and more callus could producing more polysaccharide, so NN69 + 1.0 mg L^{-1} 6-BA + 0.05 mg L^{-1} IBA is more suitable for callus induction to extract polysaccharide (Table 5).

Analysis of polysaccharide contents of *C. paliurus*

Standard curve preparation

According to the standard curve of glu-

Table 4. Orthogonal range analysis

Serial no.	Basal medium	6-BA (mg L^{-1})	IBA (mg L^{-1})	CK (control settings were 0)	Callus growth	Browning degree
1	WPM	0.5	0.01	1	2	3
2	WPM	1.0	0.05	2	4	2
3	WPM	1.5	0.1	3	1	3
4	NN69	0.5	0.05	3	5	5
5	NN69	1.0	0.1	1	4	1
6	NN69	1.5	0.01	2	3	3
7	Modified MS	0.5	0.1	2	1	5
8	Modified MS	1.0	0.01	3	2	5
9	Modified MS	1.5	0.05	1	1	5
Callus growth						
K1	2.333	2.667	2.333	2.333		
K2	4.000	3.333	3.333	2.667		
K3	1.333	1.667	2.000	2.667		
R	2.667	1.666	1.333	0.334		
Browning degree						
K1	2.667	4.333	3.667	3.000		
K2	3.000	2.667	4.000	3.333		
K3	5.000	3.667	3.000	4.333		
R	2.333	1.666	1.000	1.333		

Note: The degree of callus formation was quantified according to the number of + symbols; the degree of browning was quantified according to the number of - symbols.
WPM, woody plant medium; NN69, Nitsch and Nitsch medium 1969; modified Murashige-Skoog (MS) medium: changed from 1650 mg L^{-1} NH_4NO_3 of MS medium to 825 mg L^{-1} and 0.1 mg L^{-1} thiamine hydrochloride to 0.5 mg L^{-1}; 6-BA, 6-benzylaminopurine; IBA, indole-3-butyric acid.

Table 5. Orthogonal variance analysis

Index	Factor	Sum of squares of deviations	Degrees of freedom	*F* ratio	*F*-critical value	Significance
Callus level	Basal medium	10.889	2	49.050	19.000	*
	6-BA	4.222	2	19.018	19.000	*
	IBA	2.889	2	13.014	19.000	
	Error	0.222	2	1.000	19.000	
Browning degree	Basal medium	9.556	2	3.308	19.000	
	6-BA	4.222	2	1.461	19.000	
	IBA	1.556	2	0.539	19.000	
	Error	2.889	2	1.000	19.000	

Note: * Represents significant difference at $p < 0.05$.
6-BA, 6-benzylaminopurine; IBA, indole-3-butyric acid.

cose measured by the phenol-sulfuric acid method and linear regression, the regression equation was Y = 0.006845x + 0.222204, R^2 = 0.950170 (where Y is the absorbance

value, and X is the glucose concentration). Results showed that the glucose standard had a good linear relationship in the range of 0~0.2 mg mL^{-1}.

Polysaccharide contents in test samples

Polysaccharide contents in samples was measured by the phenol-sulfuric acid method (Table 6). Under natural growth conditions, the polysaccharide content in leaves of *C. paliurus* in August was significantly higher than that in leaves in April. Furthermore, the polysaccharide content in leaves from the 3-yr-old *C. paliurus* tree was higher than that from the 1-yr-old *C. paliurus* tree. There were more green than brown calli in the 3-yr-old *C. paliurus* compared to the 1-yr-old tree and in hand-prepared *C. paliurus* tea leaves compared to mechanically prepared *C. paliurus* tea leaves. However, the polysaccharide content in mechanically prepared *C. paliurus* tea was similar to that in common green tea. Therefore, more active ingredients of *C.paliurus* can be obtained by hand-making with the new leaves of 3-year-old in August (Table 6).

DISCUSSION

The success of plant tissue culture is determined by the medium composition, explant type, type and concentration of exogenous hormones, illumination conditions, and certain inherent biological characteristics of the plant itself, among which the basal medium type and the concentrations and proportions of exogenous hormones play the most important roles (Zhang et al. 2013, Li et al. 2016). The browning of *C. paliurus* leaves is the most serious problem in tissue culture. Cold shock could trigger calcium signaling and directly affect plant defense responses to pathogen infection (Knight et al. 1991, Kudla et al.2010). It has been reported that cold treatment greatly can reduce explants and callus browning (Wan-Jun et al.2013). In this study, explants were pretreated at 2.6℃ for 8 h before inoculation, which effectively reduced the browning rate of explants. Results showed that the browning degree of explants on modified MS basal medium was significantly higher than those on the other 2 basal media. The lowest browning degree was with WPM + 1.0 mg L^{-1} 6-BA + 0.1 mg L^{-1} IBA. This might have been due to high concentrations of inorganic salts and ions in modified MS basal medium, especially nitrate and ammonium-free nitrogen, which directly affect the physiological and biochemical activities of cells. Meanwhile, the concentration of inorganic salts in WPM was lower, promoting the overall coordinated development of plants, and the absorption of nitrate nitrogen was better than that of ammonium nitrogen at low concentrations (Jin et al. 2018).

The proportion of plant growth regulators also had a great influence on the callus induction of *C. paliurus* leaves. Results of the orthogonal design analysis showed that the ef-

Table 6. Polysaccharide contents of test samples

Sample	Polysaccharides (%)	Sample	Polysaccharides (%)
New leaves collected in April	0.88 ± 0.08^{e}	Green calli	3.21 ± 0.09^{a}
New leaves collected in August	1.19 ± 0.04^{d}	Brown calli	2.14 ± 0.06^{b}
Hand-made *C. paliurus tea*	1.22 ± 0.03^{d}	Leaves from 1-yr-old tree	1.87 ± 0.06^{c}
Machine-made *C. paliurus tea*	0.76 ± 0.01^{e}	Leaves from 3-yr-old tree	2.15 ± 0.06^{b}
Hand-made common green tea	0.8 ± 0.07^{e}		

Note: Different lowercase letters after a numeral represent a significant difference at $p < 0.05$.

fect of the cytokinin, 6-BA, was greater than that of the auxin, IBA, in terms of the degree of callus formation and extent of browning. When the concentration of 6-BA was too low, polykaryon formation occurred which prevented cell differentiation, thereby accelerating cell senescence and eventually causing death. However, a high concentration of 6-BA caused sharp shrinkage of the cell volume due to intense division activities; the formed calli cannot grow normally and gradually become brown (Shangguan et al. 2006). We found that the optimum formula for callus induction from tender leaves of *C. paliurus* was NN69 + 1.0 mg L^{-1} 6-BA + 0.05 mg L^{-1} IBA, and the optimum concentrations of 6-BA and IBA were moderate. Higher auxin and lower cytokinin concentrations were found to increase the callus induction rate (Zhang et al. 2012, Mo et al. 2015), unlike our present findings. The callus induction rate from tender leaves of *C. paliurus* was the lowest under higher cytokinin concentrations and lower auxin concentrations. It also reached 83.3% or more, but with fewer subsequent adventitious buds, indicating that the high proportion of cytokinins and auxins has greater impacts on induction of adventitious buds in subsequent calli.

Polysaccharide contents in green calli induced from *C. paliurus* leaves were the highest, followed by brown calli and 3-yr-old natural leaves. Callus induction of *Schisandra sphenanthera* showed similar result, that polysaccharide in green calluses was higher than brown calluses and WPM basal medium was not appropriate for polysaccharide accumulation (Liang et al. 2011). The polysaccharide content in leaves of *C. paliurus* in August was higher than that in April. This indicates that with an increase in tree age and leaf age, organic matter and secondary metabolites in the tissues and organs of *C. paliurus* cumulatively increase. This conclusion is consistent with results of a comparison of polysaccharide contents of *Ginkgo biloba* L. leaves at different ages (Chen et al. 2006). It also has been reported that the content polysaccharide in peach gum increased with the increase of tree age, but the content of polysaccharide in peach gum decreased when the tree entered the senescence stage (Liang et al. 2019). Under tissue culture conditions, explants of *C. paliurus* grew vigorously, metabolized faster than under natural conditions, and accumulated polysaccharides faster (Xiaoxiang et al. 2016). Therefore, polysaccharide contents in calli (both green and brown) were higher than those of other tested materials. Thus, calli might be preferred material for the industrial production of secondary metabolites from *C. paliurus*. However, differences in polysaccharide contents in calli of the same color on different media warrant further study. The polysaccharide content of hand-prepared *C. paliurus* tea was higher than that of industrially prepared *C. paliurus* tea, but the polysaccharide content in industrially prepared *C. paliurus* tea did not significantly differ from that of ordinary green tea. This indicates that the active ingredients in leaves of *C. paliurus* are lost to a greater extent during mechanical processing. Thus, to maximize the benefits of *C. paliurus* tea, the main production processing should be manual.

CONCLUSIONS

Using tender leaves of *C. paliurus*, we cultured green calli with good growth status using NN69 + 1.0 mg L^{-1} 6-BA + 0.05mg L^{-1} IBA medium with 12 h d^{-1} of 50 μmol·m^{-2}·s^{-1} cool daylight at 25±1℃. Polysaccharide levels were higher in the studied pairs as follows: leaves and calli > common green tea; old leaves > tender leaves; calli > leaves; green calli > brown calli; and manually made *C. paliurus* tea > mechani-

cally produced *C. paliurus* tea. These results provide clues for effective large-scale industrial production of *C. paliurus* polysaccharides and highlights the scientific basis for rational sampling and preparation of *C. paliurus* tea.

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