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The study on callus induction from different explants of *Myrica nana* A. Chev.

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ABSTRACT. *In order to establish the suspension cell line of Myrica rubra to extract the effective components such as polyphenols, flavonoids, quercetin and dihydromyricetin. Effect of different plant growth regulator on root, stalk and leaf callus induction was studied in this paper. It showed that optimal root callus induction culture medium was MS + 0.2 mg $\cdot L^{-1}$ 6-BA + 1mg $\cdot L^{-1}$ 2,4-D ,optimal stalk callus induction culture medium was MS + 0.6 mg $\cdot L^{-1}$ 6-BA + 1mg $\cdot L^{-1}$ 2,4-D, Optimal stalk callus induction culture medium was MS + 0.2 mg $\cdot L^{-1}$ 6-BA + 3mg $\cdot L^{-1}$ 2,4-D + 0.2mg $\cdot L^{-1}$ NAA. Root, stalk and leaf callus induction rate were 100%,78.67% and 21.3% in their optimum culture conditions respectively.*

KEYWORDS: *Waxberry; Callus; Induction*

1. Introduction

Waxberry, a species of Myricaceae[1], is one of the most famous fruit trees in China. It is also a common economic and ecological tree species in southern China. Waxberry is native to the provinces and regions of Southeast China and the Yunnan-Guizhou Plateau, and it has wild species in mountainous areas[2]. There are 6 kinds of waxberry in China: hairy waxberry, green waxberry, dwarf waxberry, whole leaf waxberry and large waxberry. The wild waxberry in Yunnan is dwarf waxberry[3,4], which is our specialty of subtropical fruit tree, evergreen small tree or shrub. Its content of vitamin C is higher than cultivar, and the content of acid is also higher than cultivar[5]. Ten phenolic compounds including myricetin, myricetin 3-O-α-L-alapiran glycoside, myricetin 3-O-β-D-galactoside, myricetin (i.e. myricetin 3-O-α-L-rhamnoside), kaempferol 3-O-β-D-glucoside, epigallocatechin 3-O-gallate, profenoxin B-2, profenoxin B-23-O-gallate and gallic acid can be isolated from fresh leaves of wild waxberry in Yunnan Province[5], so it has high development value. The deep utilization of waxberry is gradually deepening, and the varieties with good quality are more and more valued by fruit processing, breeding and cultivators[6]. The water decoction or extract from the leaves of wild waxberry in Yunnan province has bacteriostatic effect on G + Staphylococcus aureus and

Bacillus subtilis, and the bacteriostatic effect of wild waxberry plants in Yunnan is very different[7,8]. The leaves of wild waxberry in Yunnan have quercetin, a flavonoid compound, which has the functions of expectoration, cough relief and free radicals resistance. It also has strong pharmacological activities in the prevention and treatment of cancer, anti-bacterial, anti-inflammatory and the prevention and treatment of cardiovascular diseases[9]. The research on tissue culture and artificial cultivation of waxberry will play an important role in protecting wild resources and germplasm resources of waxberry. At present, the researches on waxberry at home and abroad mainly focus on cultivation techniques, but the rapid propagation and callus are seldom mentioned[10,11]. In this paper, the effects of different plant growth regulators on callus induction from different explants of waxberry have been studied in order to obtain callus. By the method of suspension culture, high redifferentiation and considerable number of cells could be obtained in a relatively short time, so as to obtain secondary metabolites of wild waxberry in Yunnan. Try to explore a new way for improving the multi-channel utilization and sustainable development of waxberry.

2. Materials and methods

2.1 Materials

(1) Explant

The experimental materials were collected from Dabaoshan, Shui Zishu village, Kuang Shan town, Huize county, Qujing city, Yunnan province. Mature and full seeds were selected to grow into sterile seedlings by sterile inoculation in college of horticulture and gardening, Southwest Forestry University. The roots, stems and leaves of the healthy and aseptic waxberry's seedlings were taken as explants.

(2) Culture medium

At the time of rooting induction, 8mg·L^{-1} concentration of PVP was added to the MS basic medium. Callus induction medium is MS basic medium with different concentrations of 6-BA, 2,4-D, NAA. Different culture medium types as shown in table 1, add agar 0.7%, sucrose 3%, pH 5.9, packed separately and sterilized at 121℃ for 20 minutes. The chemical reagent used in the experiment was analytically pure.

Table.1 Different medium types

Medium code	Explant type	Hormone /mg·L^{-1}		
		6-BA	2,4-D	NAA
Root-1	Root	0.2	1	-
Root -2	Root	0.2	2	-
Root -3	Root	0.3	1	-
Root -4	Root	0.3	2	-
Root -5	Root	0.4	1	-

Root -6	Root	0.4	2	-
Root -7	Root	1.00	-	0.10
Root -8	Root	1.50	-	0.05
Root -9	Root	0.50	-	0.15
Stalk-1	Stalk	-	1	0.15
Stalk -2	Stalk	-	1	0.3
Stalk -3	Stalk	-	1	0.45
Stalk -4	Stalk	-	1	0.6
Stalk -5	Stalk	-	1.5	0.15
Stalk -6	Stalk	-	1.5	0.3
Stalk -7	Stalk	-	1.5	0.45
Stalk -8	Stalk	-	1.5	0.5
Stalk -9	Stalk	-	2	0.15
Stalk-10	Stalk	-	2	0.3
Stalk -11	Stalk	-	2	0.45
Stalk -12	Stalk	-	2	0.6
Stalk -13	Stalk	-	2.5	0.15
Stalk -14	Stalk	-	2.5	0.3
Stalk -15	Stalk	-	2.5	0.45
Stalk -16	Stalk	-	2.5	0.6
Leaf-1	Leaf	0.2	1	0.1
Leaf -2	Leaf	0.2	1.5	0.1
Leaf -3	Leaf	0.2	2	0.1
Leaf -4	Leaf	0.4	2.5	0.2
Leaf -5	Leaf	0.4	3	0.2
Leaf -6	Leaf	0.4	3.5	0.2
Leaf -7	Leaf	0.6	4	0.3
Leaf -8	Leaf	0.6	4.5	0.3
Leaf -9	Leaf	0.6	5	0.3

2.2 Methods

(1) Callus induction

Select the sections of robust root and stem, cut into 1 cm of small segments, inoculated on the MS culture medium containing different plant growth regulator, They were cultured in dark at 25 ℃. Each group was inoculated with 30 bottles and 4 explants in each bottle. After 20 days, the callus growth and growth were recorded. Each experiment was repeated three times.

(2) Statistics of experimental results

Callus induction rate = the number of explants forming callus/ the number of inoculated explants*100%.

(3) Data analysis and processing

SPSS17.0 was used for statistical analysis.

3. Results and analysis

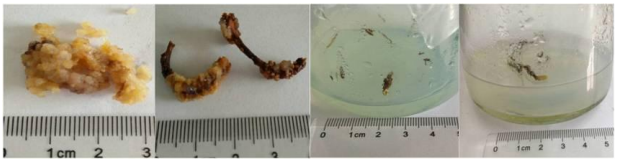
3.1 The effects of plant growth regulators on callus induction of arbutus root

Table.1 The effect of different combinations of various hormone concentration on root callus induction

Medium code	Hormone /mg·L^{-1}			Explant number	Induction number of callus	Induction rate of callus (%)	Culture days (d)	Color of callus	Texture of callus	Callus growth
	6-BA	2,4-D	NAA							
Root-1	0.2	1	-	50	50±0	100±0f	20	yellow	Loose,mud	+++
Root -2	0.2	2	-	50	49±1	98±2f	20	yellow	Loose,mud	+++
Root -3	0.3	1	-	50	46±2	92±4e	20	yellow	granular,loose	++
Root -4	0.3	2	-	50	50±0	100±0f	20	yellow	granular,loose	++++
Root -5	0.4	1	-	50	33±1.15	66±2.31d	20	tawny	compact	++
Root -6	0.4	2	-	50	28±2	56±4c	20	tawny	compact	++
Root -7	1.00	-	0.10	50	19±1.53	39±3.06b	20	green	compact	+++
Root -8	1.50	-	0.05	50	12±1.73	24±3.46a	20	green	compact	+
Root -9	0.50	-	0.15	50	34±2.65	68±5.29d	20	green	compact	++

Note: The dedifferent lower case in a same column means significant difference, besides, the same mean no significant difference, the same below. Dedifferentiation ability of callus and callus proliferation capacity.+ is poor; ++ is general; +++ is good; ++++ is best.

In the callus induction experiment of Bayberry root, 9 kinds of media were used, and the callus was cultured on MS medium containing different plant growth regulators for about 15 days. The callus could be seen at the incision, but the color became darker with the prolongation of culture time. After 20 days of culture, the callus induction rate was statistically analyzed. There were 4 kinds of culture medium that could make the callus induction rate of arbutus root reach more than 90%.In medium 1 and medium 4, callus induction rate of arbutus root reached 100%. Intuitive analysis showed that the optimal ratio of plant growth regulators for callus induction was 0.2 mg·L^{-1} 6-BA,1 mg·L^{-1} 2,4- D or 0.3 mg·L^{-1} 6-BA,2 mg·L^{-1} 2,4-D, with the induction rate reaching 100%. 2, 4-d had significant effect on callus induction of arbutus root. The induction rate of medium with 2, 4-d was above 50%, and the induction rate of medium without 2, 4-d was not ideal. The lowest induction rate is below 30%. NAA had no significant effect on the induction rate of arbutus root.



A.Yellow mud callus B. Yellow granule callus C. Tawny compact callus D. Tawny compact callus

Figure. 2 The effect of different combinations of various hormone concentration on root callus induction

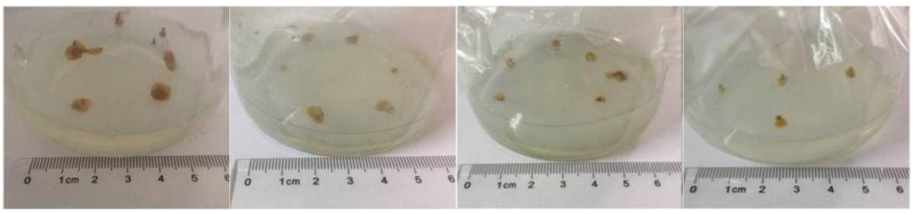
3.2 Effects of plant growth regulators on stalk callus induction

Table. 3 The effect of different combinations of various hormone concentration on stalk callus induction

Medium code	Hormone //mg·L^{-1}		Explant number	Induction number of callus	Induction rate of callus (%)	Culture days (d)	Color of callus	Texture of callus	Callus growth
	2,4-D	6-BA							
Stalk -1	1	0.15	50	18±1.53	35.3±3.06de	20	tawny	granular,loose	++
Stalk -2	1	0.3	50	30±2.08	60.67±4.16g	20	tawny	granular,loose	+++
Stalk -3	1	0.45	50	21±1.53	42.67±3.06f	20	tawny	granular,loose	++
Stalk -4	1	0.6	50	39±1.53	78.67±3.06h	20	yellow	loose,mud	++++
Stalk -5	1.5	0.15	50	11±1.53	22.67±3.06a	20	黄白色	compact	+
Stalk -6	1.5	0.3	50	20±1.15	39.34±2.31ef	20	tawny	compact	++
Stalk -7	1.5	0.45	50	30±1.53	60.67±3.06g	20	yellow	loose,mud	+++
Stalk -8	1.5	0.5	50	16±1.53	32.67±3.06cd	20	tawny	granular,loose	++
Stalk -9	2	0.15	50	20±1.53	39.34±3.06ef	20	tawny	compact	++
Stalk-10	2	0.3	50	16±0.58	32.66±1.15cd	20	黄白色	compact	+
Stalk-11	2	0.45	50	13±0.58	25.32±1.15ab	20	yellow	compact	+
Stalk-12	2	0.6	50	14±1.53	28.67±3.06bc	20	tawny	compact	++
Stalk-13	2.5	0.15	50	20±1.53	39.34±3.06ef	20	tawny	loose,mud	++
Stalk-14	2.5	0.3	50	20±0.58	39.34±1.15ef	20	tawny	compact	++
Stalk-15	2.5	0.45	50	18±1.15	35.33±2.31de	20	tawny	granular,loose	++
Stalk-16	2.5	0.6	50	29±2	58±4g	20	yellow	compact	++

Note: The dedifferent lower case in a same column means significant difference, besides, the same mean no significant difference, the same below. Dedifferentiation ability of callus and callus proliferation capacity.+ is poor; ++ is general; +++ is good; ++++ is best.

In the experiment of stalk callus induction, 16 kinds of medium were used. It was cultured on MS medium containing different plant growth regulators for about 15 days, and the callus was visible at the incision. After 20 days of culture, the callus induction rate was statistically analyzed. The highest induction rate was medium No.4, about 78.67%. The lowest induction rate was medium No.5, only 22.67%. According to the analysis, the optimum ratio of plant growth regulators for stalk callus induction was 1 mg·L^{-1} 2,4-D,0.6 mg·L^{-1} 6-BA. The induction rate reached 78.67% by adding 1 mg·L^{-1} 2,4-D and 0.6 mg·L^{-1} 6-BA to MS basic medium. The induction rate of stalk callus induction was generally low, and the induction rate of medium 1, 3, 5, 6, 8, 9, 10, 11, 12, 13, 14 and 15 was below 50%. The induction rate of medium 2, 4 and 16 was more than 50%.



A. Yellow muddy callus B. Yellow granule callus C. Tawny compact callus D. Yellow brown loose callus

Figure.3 The effect of different combinations of various hormone concentrations on stalk callus induction

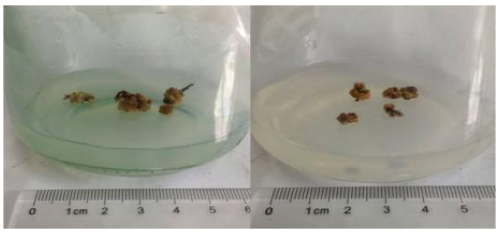
3.3 Effects of plant growth regulators on callus induction of arbutus leaves

Table. 4 The effect of different combinations of various hormone concentration on leaf callus induction

Medium code	Hormone /mg·L^{-1}			Explant number	Induction number of callus	Induction rate of callus (%)	Culture days (d)	Color of callus	Texture of callus	Callus growth
	6-BA	2,4-D	NAA							
Leaf -1	0.2	1	0.1	50	3±0.58	5.33±1.15a	20	tawny	compact	+
Leaf -2	0.2	1.5	0.1	50	3±0.58	6.67±1.15ab	20	tawny	compact	+
Leaf -3	0.2	2	0.1	50	4±1	8±2abc	20	tawny	granular,loose	+
Leaf -4	0.4	2.5	0.2	50	6±0.58	11.33±1.15bc	20	yellow	compact	+
Leaf -5	0.4	3	0.2	50	11±2.08	21.33±4.16d	20	yellow	granular,loose	++
Leaf -6	0.4	3.5	0.2	50	10±2.65	20±5.29d	20	yellow	granular,loose	+
Leaf -7	0.6	4	0.3	50	6±1.53	12.67±3.06c	20	tawny	granular,loose	+
Leaf -8	0.6	4.5	0.3	50	4±1.53	7.33±3.06abc	20	tawny	granular,loose	+
Leaf -9	0.6	5	0.3	50	3±1	6±2ab	20	tawny	compact	+

Note: The dedifferent lower case in a same column means significant difference, besides, the same mean no significant difference, the same below. Dedifferentiation ability of callus and callus proliferation capacity.+ is poor; ++ is general; +++ is good; ++++ is best.

Nine kinds of medium were used in the callus induction experiment of arbutus leaves. It was cultured on MS medium containing different plant growth regulators for about 20 days, and the callus can be observed at the incision. The callus induction rate was calculated after 20 days. The medium with the highest induction rate was No.5, reached 21.33%. The medium with the lowest induction rate was No.1, only 5.33%. According to the analysis, the optimal ratio of plant growth regulators in the callus induction of arbutus leaves was 0.4 mg·L^{-1} 6-BA,3 mg·L^{-1} 2,4-D and 0.2 mg·L^{-1} NAA, that is, adding 0.4 mg·L^{-1} 6-BA,3 mg·L^{-1} 2,4-D and 0.2 mg·L^{-1} NAA into the basic MS medium, the induction rate reached 21.33%. The callus induction rate of arbutus leaves was very low, and the induction rate of all medium was lower than 30%. The highest was 21.33%, and the lowest was 5.33%.



A. Yellow granule callus B. Tawny compact callus

Figure.4 The effect of different combinations of various hormone concentrations on stalk callus induction

4. Conclusion and discussion

The results showed that callus could be induced from different explants, roots, stems and leaves of Myrica rubra. The callus of Myrica rubra root was most easily induced by using these nine kinds of media, and the induction rate was high, reaching 100%. However, the callus of waxberry leaves was difficult to induce by using these nine kinds of media, and the highest induction rate was only 21.33%. And the growth is poor. In this experiment, 16 kinds of media were used for the stalk callus induction. The induction rate was between the two, and the growth was general. There was no significant difference in the color and texture of callus produced by each explant, but there were some differences in growth. The callus of waxberry root grew best. The results showed that 2,4-D had a significant effect on the callus induction of waxberry root.Different explants contain different components, which will lead to differences in callus induction. In addition, the concentration of different hormones and the combination of different hormones can also affect the induction of callus.

At present, there are few reports on tissue culture of waxberry. Because woody plants contain more phenolic substances, when the explants are cut, the cells near the incision are very vulnerable to injury, leading the phenolic compounds and polyphenol oxides spilling out to the outside. The spilled phenolic compounds are easily oxidized into dark brown phenolic substances and water. Phenolic substances, under the action of tyrosinase, cause protein aggregation of the explants that can result in medium browning. It can affect the normal growth of explants and even lead to the death of explants. The browning of callus could be significantly reduced by timely bottle rotation, fresh medium replacement and low temperature treatment of explants. In the previous experiments, the browning of Myrica rubra explants was serious. Therefore, adding PVP to the culture medium, the browning was relieved to some extent, but there were still some explants browning. Further experiments were needed to completely eliminate the effect of browning. In the subsequent experiments, the optimal medium for inducing the proliferation of Myrica rubra aseptic seedlings was also suitable for the callus induction of explants after adding different concentrations of hormones, but the effects of different plant growth regulators on callus induction were different. There were differences not only in

callus induction rate but also in callus texture and callus growth. Different explants contain different components, which can also lead to the differences in callus induction. In addition, the concentration of different hormones and the combination of different hormones also affect the induction of callus. Therefore, the effects of 2,4-D on callus induction of Myrica rubra roots can be further explored from the perspective of physiology and biochemistry. The components contained in different explants can be analyzed. Continue to optimize the combination and concentration of hormone to improve the induction rate of callus and the quality of callus. To provide suitable experimental materials for the study of the cell suspension culture of arbutus. At present, the study on biosynthesis of plant secondary metabolites by suspension culture of plant cells is an important part in the field of biotechnology. The excellent germplasm sources of plant suspension culture cells are mostly obtained by callus induction and isolation and screening of the single cell. Therefore, the appearance and physiological state of callus directly affect the quality of subsequent cell suspension system. The looser the callus is, the more dispersed the cells are. Therefore, the selection of white or yellowish callus with fine grain, loose and fragile, moist and bright appearance is conducive to the induction of suspension cell lines. Studying the physiological and biochemical characteristics of Myrica rubra and exploring the differentiation rule of its callus have important theoretical significance and application value for the establishment of cell suspension system of Myrica rubra, and contribute to the effective development and sustainable development of Myrica rubra resources.

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