Study on Tissue Culture of Japanese Honeysuckle (*Lonicera japonica*)

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Abstract [**Objectives**] This study was conducted to increase the reproduction coefficient of Japanese honeysuckle (*Lonicera japonica*) to keep the character of optimal benign. [**Methods**] The young leaves of medicinal Japanese honeysuckle were selected as explants, and MS was used as the basic culture medium. Suitable culture concentrations and conditions were screened through different concentration gradients of growth regulators and cytokinin. [**Results**] As the concentration of 6-BA in the culture medium increased, the browning rate increased, and the browning phenomenon occurred earlier. On the contrary, a lower concentration of 6-BA was suitable for the differentiation and growth of young leaves, and the browning response was slow. However, if the cultivation time was too long and the materials were not transferred in a timely manner, browning would also occur. The optimal combination of levels was obtained through a 3 × 3 orthogonal experiment (three parallel groups for each of 6-BA and NAA). The culture conditions included a constant temperature of 26 ℃ and light intensity of 1 200 lx. The optimal medium for inducing callus proliferation was MS + 6-BA 0.5 mg/L + NAA 0.5 mg/L; and the optimal medium for inducing bud differentiation was MS + 6-BA 1.0 mg/L + NAA 0.1 mg/L. [**Conclusions**] This study provides a theoretical basis for accelerating the development of the honeysuckle industry.

Key words *Lonicera japonica*; Explants; Growth regulator; Cytokinin
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Generation Situation of *Lonicera japonica*

Morphological and biological characteristics

Japanese honeysuckle (*Lonicera japonica*), also known as Rendong, Jinyinteng, Yinteng, Ersehuateng, Erbaoteng, Youzhuanteng, Zifengteng and Yuanyangteng, is an angiosperm of the plant kingdom. It belongs to *Lonicera* of Caprifoliaceae in Dipsacales of Metachlamydeae.

Japanese honeysuckle is a semi-evergreen vine. The young branches are clean reddish brown, densely covered with yellowish-brown spreading hard straight rough hairs, glandular hairs, and pubescence, and the lower part is often hairless. The leaves are papery, ovate to oblong-ovate, sometimes ovate-lanceolate, or obovate. The total peduncle is usually solitary in the upper leaf axil of small branches, equal in length or slightly shorter than the petiole, densely covered with short and soft hairs mixed with glandular hairs. The bracts are large, leafy, ovate to elliptical in shape, and have both surfaces pubescent or sometimes nearly hairless. The tip of the bracteoles is circular or truncate. The corolla is white, sometimes slightly red on the sunny side at the base, and turns yellow later. Both stamens and styles are higher than the corolla. The fruit is round, blue black when ripe, and shiny. The seeds are ovoid or elliptical, brown, and have shallow horizontal grooves on both sides.

Growth habits

Japanese honeysuckle prefers a mild and humid climate, ample sunlight, and is tolerant to cold, drought, and waterlogging. Its suitable temperature for growth is in the range of 20 – 30 ℃, and it has no strict requirements for soil due to good tolerance to salt and alkali. However, it is advisable to cultivate Japanese honeysuckle in humus soil with deep and loose soil layers. Japanese honeysuckle is mainly propagated using seeds and cuttings.

Chemical composition

Japanese honeysuckle contains more than 30 kinds of volatile oils, flavonoids, organic acids, triterpenoid saponins and up to 15 kinds of inorganic elements.

Disease and pest control

The diseases include brown spot. In addition to reducing the source of the disease and strengthening management, 3% Jinggangmycin 5×10^{-5} (50 ppm) solution can be used in the early stage of the disease by spraying continuously for 2 – 3 times. The pest, *Amphicercidus sinilonicericola* Zhang, can be controlled by chemical agents. *Xylotrechus grayii* White can be controlled in the field from July to August on sunny days with temperatures above 25 ℃ by releasing *Scleroderma guani* Xiao et Wu, with good results. Inchworms can be controlled with chemicals during their infancy.

Pharmacological effects

Within safe concentration ranges, flavonoid extracts of both Japanese honeysuckle and *Lonicera hypoglauca* have significant antiviral effects. *In vitro*, they have significant blocking, inhibiting, and neutralizing effects on PRV, respectively. Fan Hongwei and other experts found that organic acid compounds contained in Japanese honeysuckle can inhibit rabbit platelet aggregation induced by ADP (adenosine diphosphate), and the greater the concentration and dosage of the compounds, the more obvious the effect. The dose and effect are positively correlated. Japanese honeysuckle can strengthen immune function, promote lymphocyte transformation, and enhance phagocytic function of white blood cells.

Current supply and demand situation of the Japanese honeysuckle market

In the pharmaceutical industry, the usage of Japanese

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honeysuckle is increasing year by year. Taking a pharmaceutical production enterprise in Jiangsu with a large usage of honeysuckle as an example, the demand was 40 t in 2004, and the budget usage in 2010 was 110 t. According to the company's product sales plan, the expected usage would reach 130 t by 2013, with a demand increase of about 20%. Wang Laoji alone had an annual demand for 3 000 t of Japanese honeysuckle, and it was expected that the demand would double to 6 000 t by 2013. The demand for Japanese honeysuckle in various industries across the country is approximately 20 000 t. Due to the strong and deep development of Japanese honeysuckle in various industries in recent years, as well as the maintenance of the current situation in planting methods and scales in various regions, the price of Japanese honeysuckle has been consistently rising[2].

Chinese medicinal materials required abroad are of standard quality, with clear chemical composition, and are extracted and high-tech products. The production, processing and packaging quality of medicinal materials at home are not high. The GC-MS method has been applied to analyze the specific composition of the volatile oil from Japanese honeysuckle, and there are only four types of acids, with a relative content of 48.24%[1]. Among them, chlorogenic acid and isochlorogenic acid are one of the basic ingredients of traditional Chinese medicine for clearing away heat and toxic materials and sterilization. At present, about 80% of the prescriptions of traditional Chinese medicine for clearing away heat and toxic materials and sterilization contain Japanese honeysuckle. Japanese honeysuckle has a certain effect on drug resistance caused by the abuse of chemical antibiotics and is non-toxic. Therefore, it is known as the "antibiotic" in the plant kingdom. With the opening of the international market and the standardization of planting, the demand for Japanese honeysuckle and its health products is likely to increase. Common health products produced from honeysuckle in the market include honeysuckle wine, honeysuckle tea, honeysuckle cola, honeysuckle soda, honeysuckle candy, and Yinxian toothpaste. Honeysuckle flower distillate made from Japanese honeysuckle is refreshing. Yinxian toothpaste, which is mainly made of it, also has good effects in preventing and treating oral diseases[3].

Materials and Methods

Inoculation and cultivation of explants

Selection and disinfection of explants Young stems and leaves growing newly in the year were collected from the Japanese honeysuckle base in Xinzuotang, Boluo County, Huizhou, Guangdong Province as explants. The female parent was disease-free, insect-free and grew normally. The stem segments were rinsed with clean water, and the young leaves were cut off. The stem segments were put into 300 ml wide-mouth bottles with a stopper. An ultra-clean workbench was opened, and an alcohol lamp, a wide-mouth bottle for 75% alcohol and cotton balls, matches, various forceps for sterilization, inoculation needles, scalpels, surgical scissors and culture media required for inoculation were placed in a sterile room or an inoculation box. The ultraviolet lamp was turned to irradiate for 30 – 45 min for sterilization. The leaves were first sterilized with 75% alcohol for 45 s, then with 0.15% $HgCl_2$ for 8 min, and finally rinsed with sterile water 4 – 5 times. The water was then sucked dry with sterile filter paper.

Explant inoculation and primary culture Hands and the workbench were wiped with alcohol cotton balls, and the inoculation tools were burnt with the flame of the alcohol burner, and then cooled for later use. The sterilized leaves were cut under sterile conditions into 2 cm × 2 cm small pieces, which were quickly placed into culture media at different ratios, with four explants per bottle and three bottles per parallel group, totaling 12 explants. During inoculation, each conical flask should be inclined to the flame, and the inoculation operation was carried out near the flame of the alcohol burner. After material inoculation, the mouth of the conical flask was burnt on the flame of the alcohol burner while rotating it, and then covered with the bottle cap, and the name of the material and the date of inoculation were recorded. After the materials were inoculated, they were cultured in a constant-temperature incubator at a temperature of 24 – 26 ℃ under light conditions of 12 – 16 h/d. The growth status of callus tissue was observed after about 30 d of cultivation.

The primary culture medium was MS + 6-BA + NAA + sucrose 3% + agar 0.72%, with a pH of 5.8. The ratios of plant growth regulators are shown in Table 1.

Table 1 Ratios of plant growth regulators in various groups of culture media mg/L

No.	6-BA	NAA
1	0.1	0.1
2	0.1	0.5
3	0.1	1.0
4	0.5	0.1
5	0.5	0.5
6	0.5	1.0
7	1.0	0.1
8	1.0	0.5
9	1.0	1.0

Proliferation of callus tissue Callus subculture media were prepared according to Table 2. Callus tissues, which were light green, relatively large, and almost grew all around, were selected and inoculated on the MS medium with the best growth in the primary culture. The original culture medium adhering to calli was cut off, and the calli were cut using tweezers and a small knife into small pieces of about 0.5 cm × 0.5 cm, which were inoculated onto the subculture media, according to 2 callus tissues per bottle. A total of 15 bottles were obtained, and the inoculated materials were cultured in an illumination incubator at a temperature of 25℃ under light conditions of 12 h/d and a light intensity of 1 500 – 2 000 lx. The growth of callus tissues was observed at any time. The proliferation rate of callus tissue was calculated 15 d later.

Induction of adventitious buds

The induction media for adventitious buds were prepared

according to Table 2, and the proliferated calli were inoculated into the media numbered 1 – 9 in Table 2. The callus tissues with vigorous growth after proliferation were inoculated into MS basic culture medium with different concentrations of 6-BA and NAA. They were cultured with 12 h of light a day at a temperature of about 28 ℃. The growth of callus tissues was observed at any time. The differentiation rate of adventitious buds was calculated 30 d later.

Table 2 Ratios of plant growth regulators in various groups of culture media mg/L

No.	6-BA	NAA
1	0.5	0.05
2	1.0	0.05
3	1.5	0.05
4	0.5	0.10
5	1.0	0.10
6	1.5	0.10
7	0.5	0.15
8	1.0	0.15
9	1.5	0.15

Results and Analysis

Primary culture

The cultivation results are shown in Table 3. After 5 d of primary culture, 7 bottles of culture medium showed microbial infection, and 1 bottle had black colonies in the middle of 1 explant without hyphae. Because colonies appeared in a short period of time and there were no hyphae, it might be melanomyces. Moreover, in all 6 bottles, small white spots appeared on the edges or middle of explants, with filaments. Due to its long reproductive cycle and the presence of hyphae, it might be an actinomycete. The possible reason for the high contamination rate was that there was hair on the leaves of honeysuckle, which was difficult to clean; the mercury disinfection time was short or the concentration of mercury was insufficient; and the operation might be improper, resulting in contamination during the inoculation process. Thirteen explants died, all starting from the edge and gradually spreading to the entire explant. Possible causes of death included prolonged exposure to mercury or alcohol poisoning, or failure to rinse thoroughly with water after mercury and alcohol poisoning; or the explants were burned by being too close to the outer flame of the alcohol burner during inoculation. There were 5 explants that did not grow, which might be due to that the concentrations of cytokinin and auxin were too low to play a promoting role. There were 17 browned callus tissues.

The total number of explants was 108, of which 8 explants were contaminated. A total of 7 bottles were inoculated, and the contamination rate was 7.4%. There were 13 dead explants, showing a mortality rate of 12.04%. Seventeen explants were browned, showing a browning rate of 15.74%. Six explants did not grow. Fifty two explants dedifferentiated into calli, with a callus induction rate of 48.15% (Fig. 1).

After 20 d of primary culture, the leaves curled up around, and some callus tissues grew, becoming tender green, with a dense texture (Fig. 2). Some grew in a square shape with a square hollow center, and showed light green loose callus tissue. Some had a small amount of white loose callus tissue at the edge. Some showed light yellow loose callus tissue.

Table 3 Induction rate of calli by NAA x mg/L +6-BA y mg/L

NAA x mg/L + 6-BA y mg/L	Number of bottles inoculated	Total number of explants	Total number of explants forming calli	Growth vigor	Induction rate // %
1	3	12	2	+	16.67
2	3	12	4	+	33.33
3	3	12	2	+	16.67
4	3	12	5	+ +	41.67
5	3	12	8	+ + + +	66.67
6	3	12	7	+ + +	58.33
7	3	12	5	+ +	41.67
8	3	12	5	+ +	41.67
9	3	12	4	+	33.33

(– death; + poor; + + general; + + + good; + + + + vigorous).

After 30 d of primary culture, there was also callus growing in the middle part of those showing a square shape with a square hollow center (Fig. 3). Explants in MS +0.5 mol/L 6-BA +0.5 mol/L NAA had the most vigorous growth of callus tissue at the edge, and there were also many callus tissues growing in the middle, with a loose texture and a green color.

In summary, MS +0.5 mol/L 6-BA +0.5 mol/L NAA was the optimal medium for inducing callus proliferation. The calli presented a loose structure, a bright color, and a vigorous growth state, and should belong to type II callus proliferation, excited division type[15].

The dense and slow growth of several callus tissues with MS +0.1mol/L 6-BA +0.1mol/L NAA might be caused by low hormone concentrations. The calli might belong to the conservative division type[15].

As the concentration of 6-BA increased, browning became more severe, and as the concentration of NAA increased, the growth of callus tissue became better and better. The proliferation of calli in MS +1.0 mol/L 6-BA +1.0 mol/L NAA showed a decaying type, *i.e.*, structural relaxation and slow growth[15].

Therefore, excessive concentration of 6-BA could promote callus browning, and relevant reports indicate that 1% of activated carbon can effectively alleviate browning phenomenon, as activated carbon has the ability to adsorb toxic metabolites from cells.

Moreover, browning also increased with time, possibly because that over time, a large amount of nutrients were consumed in the culture medium, resulting in an increase in the concentration of secondary metabolites and toxic substances, leading to an increase in browning.

Following three groups, MS +0.5 mol/L6-BA +0.1 mol/LNAA, MS +0.5 mmol/L 6-BA +1.0 mol/L NAA, MS +1.0 mol/L 6-BA +0.1 mol/LNAA, showed growth of loose callus tissues, which were white, bluish white, and yellowish white. A large number of studies believe that such white loose granular calli growing along the leaf incision is a single embryonic cell derived from the somatic cell embryo in the calli, which belongs to single cell origin[16].

In primary culture, high pollution and browning rates led to low callus induction rate, which poses a challenge to the tissue culture of Japanese honeysuckle. Therefore, thorough sterilization is necessary, and 1% activated carbon can be added to the culture medium.

Fig. 1 Primary culture at 10 d

Fig. 2 Primary culture at 24 d

Fig. 3 Primary culture at 30 d

Subculture and proliferation of callus tissue

MS + 0.5 mmol/L 6-BA + 0.5 mol/L NAA was the optimal medium for inducing proliferation of callus tissues, and the callus tissues were yellow green and had a loose and swollen structure. Therefore, the subculture medium for inoculation was MS + 0.5 mol/L 6-BA + 0.5 mol/L NAA. After 10 d of subculture, white plaques and hyphae appeared on the surface of three callus tissues, indicating that they should be actinomycetes. After 30 d, most of the callus tissues grew well, with the main colors being light green and yellow green, and the tissues were loose (Fig. 4). In specific, there were 30 explants in total, 3 of which were contaminated, showing a contamination rate of 16.67%, and 3 died, showing a mortality rate of 10%, and 19 survived, standing for a callus induction rate of 63.33%. The contamination and mortality rates were still very high, which was probably caused by inexperienced operating techniques.

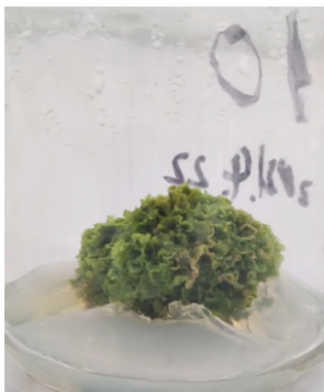

Fig. 4 Subculture at 30 d

Induction of adventitious buds

The results are shown in Table 4. There were totally 54 callus tissues, of which 3 callus tissues were contaminated, showing a contamination rate of 5.56%, and 13 callus tissues suffered from browning, showing a browning rate of 5.56%. The callus induction rate was 77.78%, and the bud differentiation rate was 68.52%.

Table 4 Induction rate of adventitious buds by NAA *x* mg/L + 6-BA *y* mg/L

NAA *x* mg/L + 6-BA *y* mg/L	Number of bottles inoculated	Total number of explants	Number of adventitious buds	Growth vigor	Induction rate//%
1	3	12	2	+	16.67
2	3	12	4	+	33.33
3	3	12	2	+	16.67
4	3	12	5	+ +	41.67
5	3	12	8	+ + + +	66.67
6	3	12	7	+ + +	58.33
7	3	12	5	+ +	41.67
8	3	12	5	+ +	41.67
9	3	12	4	+	33.33

(− death; + poor; + + general; + + + good; + + + + vigorous).

The number of clustered buds varied for each callus at different growth regulator concentrations, and the calli differentiated at 1.0 mol/L 6-BA + 0.1 mol/L NAA had the highest number of adventitious buds, which were emerald green in color and grew vigorously (Fig. 5).

Fig. 5 Induction of adventitious buds at 30 d

The experimental results indicated that the optimal growth regulator ratio for differentiation of clustered adventitious buds was MS + 1.0 mol/L 6-BA + 0.1 mol/L NAA.

During the induction process of adventitious buds, the contamination rate and mortality rate were significantly reduced, indicating that the operational ability was exercised and the success rate of the experiment was improved.

Conclusions and Discussion

Conclusions

During the entire process of inducing calli and adventitious buds in Japanese honeysuckle, it could be clearly seen that the formation of explant calli was closely related to the action of exogenous hormones. When multiple plant growth regulators were used comprehensively, their combination ratio and concentration were the most basic conditions for successful tissue culture. MS + 0.5 μmol/L 6-BA + 0.5 μmol/L NAA had the best effect during callus culture. The optimal medium for the cultivation of adventitious buds was MS + 0.1 mg/L NAA + 1.0 mg/L 6-BA, which had the best effect.

Due to the fact that Japanese honeysuckle belongs to woody plants, each stage of its cultivation cycle was relatively long. Moreover, due to the hairy surface of honeysuckle leaves, it was difficult to thoroughly sterilize the materials, resulting in a low survival rate of callus tissue. During the experiment, there were a large number of deaths and browning, so the sterilization time and UV disinfection time should be controlled well. The concentration of mercuric chloride should also be controlled during the treatment of leaves to maintain the activity of leaves and buds and enhance the induction rate and survival rate.

Discussion

Prevention and control of explant browning The browning of explants is the main limiting factor that hinders the induction rate of calli. Three ways are used to control the browning of explant wounds. The first is to utilize antioxidant substances. The second is to utilize the adsorption capacity of adsorbents. The third is to treat explants at a certain low temperature[14].

Although ascorbic acid and citric acid in antioxidants have achieved the purpose of browning control to a certain extent, they have certain effects on the growth of calli and agar coagulation, so the pH value needs to be adjusted before sterilization. Soaking the wound with 350 – 800 PPM DL-cysteine sterile solution can effectively control early browning, but this effect will be weakened with time due to limited amount of dipping . It often fails before calli grow, and the wounds still exhibit varying degrees of browning.

Activated carbon has a certain ability to control browning. However, due to poor selective adsorption, active ingredients of some substances in culture media are reduced, leading to a low callus induction rate and poor development, and the effective utilization time of culture media is reduced, resulting in high waste. PVP (polyvinylpyrrolidone) is used as an adsorbent to control browning for a long time, but its effect is lower than that of antioxidants. After inoculation with explants, keeping them at a low temperature of 2 – 6 ℃ can reduce enzyme activity and phenol secretion, thereby achieving the effect of controlling browning. However, the time required for this treatment should not be too long, as the vitality of the plant body will be damaged after 72 h.

On the basis of above experiments, a comprehensive method was adopted, that is, 350 – 300 ppm DL-cysteine was used to treat the wounds before inoculation, and 150 – 300 mg/L ascorbic acid + 150 – 300 mg/L citric acid + 5 g/L PVP was added to the culture medium, and the low-temperature treatment at 2 – 5 ℃ for 4 – 8 h after inoculation had a very high control effect on wound browning, and the browning rate was reduced to below 10%.

Selection of sterilizing agents During the process of tissue culture, the conventional disinfection method for explants is usually using 75% ethanol combined with 0.1% mercuric chloride for disinfection. Due to the presence of sparse pubescence and glandular hairs on the surface of honeysuckle plants, it is difficult to completely kill microbes using conventional disinfection methods. Research has shown that disinfecting stem segments with axillary buds using 75% ethanol combined with 0.1% – 0.2% mercuric chloride has a high contamination rate and a low survival rate. Some studies also reported that sodium chlorate and calcium hypochlorite were used instead of mercury chloride to sterilize honeysuckle explants, but the sterilization effect was not as good as mercury chloride. There also have been studies showing that using 75% alkaline ethanol instead of 75% ethanol for surface sterilization could achieve good sterilization effects. The reason may be OH^- in alkaline ethanol can change the nature of charges carried on the surface of the cell membrane and increase the permeability of the membrane, thereby improving the bactericidal effect of ethanol and mercury chloride[17].

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(Continued on page 18)

times dilution, 250 g/L azoxystrobin suspending agent 1 200 times dilution, 500 g/L fluazinam suspending agent 1 500 times dilution and 50% prochloraz wettable powder 1 500 – 2 000 times dilution, can be sprayed for control of anthracnose.

⑤ Aphids: For aphid control, 2% matrine water aqua 1 500 times dilution, or 10% imidacloprid wettable powder 2 000 times dilution, 10% flonicamid water dispersible granule 1 000 – 1 500 times dilution and 30% pymetrozine · clothianidin suspending agent 2 000 times dilution can be sprayed.

⑥ Thrips: Thrips are controlled by spraying 16% acetamiprid · novaluron emulsifiable concentrates 1 500 – 2 000 times dilution, 25% spinetoram water dispersible granule 1 000 – 1 500 times, *etc.*

⑦ Red spiders: Red spiders are controlled by spraying 0.1% *Veratrum nigrum* rhizome extract soluble solution 350 times dilution, 0.5% ivermectin emulsifiable concentrates 500 – 1 000 times dilution, 43% bifenazate suspending agent 1 500 times dilution and 110 g/L etoxazole suspending agent 1 500 times dilution in the early stage of mites, while focusing on the back of leaves.

Attention should be paid to the use of cross rotation of pesticides to reduce the occurrence of drug resistance. Prohibited (restricted) pesticides mustn't be used, and the application of pesticides should strictly follow pesticide safety intervals.

Harvesting, Storage and Transportation Techniques

Harvesting

When the maturity of fruit reaches 8 – 9 (2 – 3 circles of seeds on the shaded side of 'Kaorino' fruit do not turn red), harvesting is carried out. The fruit should be graded according to their size and placed in corresponding sponge mats, which are then neatly placed in harvesting baskets.

Storage and transportation

Short-distance (<50 km) or short-term (≤24 h) sales can be carried be using simple containers. For long-distance (>50 km) or long-term (>24 h) sales, fixed storage devices should be used and the fruit should be pre-cooled for transportation in a timely manner. The fruit should be stored in an environment at 0 – 1 ℃ with a relative humidity of 90% – 95%. During transportation, cold chain transport vehicles should be used to control the temperature within 2 – 6 ℃[9].

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