# Hairy root culture of white mulberry (*Morus alba* L.) for a source of tyrosinase inhibitors

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## Abstract

White mulberry (*Morus alba* L.) root was reported to contain potential tyrosinase inhibitors such as norartocarpetin, streppogenin, oxyresveratrol (5, 7, 2′, 4′-tetrahydroxy-flavavone) and 2-arylbenzofuran. This study aims at investigating *Morus alba* L. hairy root culture to create a source of tyrosinase inhibitors for skin-whitening application.

*Morus alba* L. leaf (including mesophyll and petiole) and stem explants were randomly wounded and inoculated with *Agrobacterium rhizogenes* ATCC 15834 in 10-40 minutes, and co-culturing for 4 days. After inoculation, *Agrobacterium rhizogenes* were removed by using MS medium adding 200mg/L cefotaxime as antibiotics. Then, fresh inducted hairy roots were grown in non-phytohormone liquid medium (MS, White, Gamborg B5 or WPM) on a shaker (80rpm) at 25 ±1°C. Inducted hairy roots were tested for the present of *rolB* by PCR analysis and its effect on tyrosinase activity using a 96-well plate reader.

The suitable explants for hairy root induction are leaves, especially petioles in 10 minutes (root induction frequency: 83%). After induction, hairy roots on MS was grown more efficiently (increased 3.933 times in FW) than on WPM, B5 and White medium. Inducted hairy roots were successfully transgenes by the present of *rolB* in their genome and had the same anti-tyrosinase activity as the natural roots without significance differences (IC50: 3.860 and 3.597 µg/mL, respectively).

Cultured *Morus alba* L. hairy roots are potential source of tyrosinase inhibitors for future skin-whitening ingredient.

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Key words: hairy root; white mulberry *Morus alba* L.; *Agrobacterium rhizogenes*.

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## Introduction

*Morus alba* L. is widely used in Vietnam and other Asian countries' folk medicines for respiratory disease, skin disease, type 2 diabetes, wound healing, influenza, insomnia, and especially in skin care.[1] The white mulberry is not only an abundant source of vitamin C (almost present in a reduced form) but also contains a lot of natural antioxidants such as carotenoids, quercetin, tannin, flavonoids and saponin[2]. Some articles show that several biochemical compounds isolated from this plant play a pivotal role in pharmaceutical industry such as albafuran, moranoline, kuwanol, morusin, calystegin, albanol and hydroxymoricin.[2] It is interesting that the popular bioactives of *Morus* being found in recent days are skin whitening factors. Two examples of isolated polyphenols are mulberroside F (moracin M-6, 3′-di-*O*-*β*-glucopyranoside) and norartocarpetin (5, 7, 2′, 4′-tetrahydroxyflavone) which have anti-tyrosinase activity higher than kojic acid. Particularly, mulberry root parts contain high potential polyphenols as tyrosinase inhibitors including norartocarpetin, streppogenin and oxyresveratrol (5, 7, 2′, 4′-tetrahydroxy-flavavone).[3-7] Moreover, 2-arylbenzofuran has been recently found as one of the abundant constituents in *Morus alba* root and potent tyrosinase inhibitor.[8] For root biomass and secondary compounds production, hairy roots from genetic transformation using natural vector *Agrobacterium rhizogenes,* have long been identified as one of the most effective methods. With many outstanding advantages, hairy root culture has been applied in several fields such as manufacturing secondary compounds or studying biosynthesis pathways of active substrate.[9] There are some articles about root transformation in mulberry,[10-12] however, these studies are still not approach to tyrosinase inhibition activity. Besides, there have not been any official paper on mulberry hairy root culture in Vietnam. Therefore, this study was carried out to investigate *Morus alba* L. hairy root culture to create a source of tyrosinase inhibitors for skin whitening application.

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## Materials and Methods

White mulberry (*Morus alba* L.) seeds were supplied by Viet Hung Sericulture Research Center (Viet Hung, Vu Thu Dist., Thai Binh Province, Viet Nam). All *in vitro* plant culture medium including MS (Murashige and Skoog, 1962), B5 (Gamborg, 1968), White (White, 1963), and WPM (Woody Plant Medium, Lloyd and McCown, 1981) were supplemented with vitamin Morel, 30g/L sucrose, 8g/L agar, adjusted pH to 5.8 and sterilized by autoclaving at 121°C, 1 atm, in 20 minutes. *Agrobacterium rhizogenes* strain ATCC 15834 was provided by RIKEN Bio-Resource Research Center through the project of MEXT, Japan. The Nutrition Broth (NB) medium (beef extract 3g/L, peptone 5g/L) was used to grow *Agrobacterium rhizogenes* ATCC 15834 supplemented with 8g/L agar and adjusted pH to 7.0.

### Preparation of *Agrobacterium rhizogenes*

*Agrobacterium rhizogenes* was activated and cultured in NB medium for 48h on a shaker (110 rpm) at 30°C to gain a suitable cell density ($OD_{600nm}$=0.5).

### Plant sterilization

Basing on the mirco-propagation of *Morus alba* process of Anis *et al.* (2003),[13] white mulberry seeds were washed with tap water and then decontaminated by shaking in alcohol 70% (1 minute) and the mixture of 1 javel: 1 water (10 minutes), respectively. The seeds were cultured in glass bottles containing solid MS medium after rinsed 3 times with sterile distilled water. These bottles were placed under controlled conditions at 25°C, 2500 lux light with 16h photoperiod.

### Hairy root induction

Basing on the hairy root induction process in peanut seedlings,[14] leaf (including mesophyll and petiole) and stem explants from 2-week-old plantlets were randomly wounded with sterile blade and inoculated with *Agrobacterium rhizogenes* ATCC 15834 for 10 -40 minutes. Then they were briefly dried with steriled blotting paper and cultured in solid co-cultivation medium for 4 days. Finally, these explants were transferred to MS medium adding 200mg/L cefotaxime after 48 hours to remove the remaining *Agrobacterium rhizogenes*. After 30 days, the number of responsive explants and number of hairy roots per explant were recorded and the data were calculated.

### Detecting the rooting genes (PCR analysis)

DNA from *in vitro* hairy roots and seedling roots (as negative control) were isolated by using CetylTrimethyl Ammonium Bromide (CTAB) method.[15] Besides, Ri-plasmid of *Agrobacterium rhizogenes* was isolated for positive control.[16] Primers amplifying *rolB* gene were F-*rolB* (5'- GCTCTTGCAGTGCTAGATTT-3') and R-*rolB* (5'-GAAGGTGCAAGCTACCTCTC-3'). The expected amplified fragment sizes were 423 bp for *rolB* gene. Primers (5'-GGCTTCGCCAACCAATTTGGAGAT-3') and (5'-TTTTGCTCCTTCAAGGGAGGTGCC-3') were used to amplify a 1009 bp fragment of *virG*.

The reactions were performed in total volume of 25µL containing 100ng of plant DNA (or 40ng of Ri-plasmid DNA), 0.5µM of each primer, 5µL 5X MyTaq Reaction Buffer, and 1U MyTaq DNA polymerase (Mytaq$^{TM}$ DNA Polymerase, BIO-21105, Bioline). PCR condition followed initial denaturation program at 95°C for 5 minutes, 35 cycles of amplification (95°C for 30s, 54°C for 30s and 72°C for 60s) and 5 minutes' extension at 72°C (PCR Eppendorf, Mastercycler gradient). PCR products were visualized by agarose electrophoresis (Wealtec, Elite 600).

### Hairy root propagation

Fresh inducted hairy roots (0.5 g) were transferred into 250mL Erlenmeyer flask containing 20mL different liquid medium (MS, White, Woody Plant Medium – WPM and B5), cultured in shaker at 80rpm and 25 ± 1°C every 30 days. The Fresh Weight (FW) was determined every 5 days to establish time course.

### Tyrosinase activity assay

Tyrosinase activity assay was carried out with mushroom tyrosinase enzyme (Sigma T3824-25KU) and measured by using a 96-well plate reader (Perkin Elmer 2030 Elisa reader). The reaction mixture, 30µL 50Mm potassium phosphate buffer (pH 6.8) containing 343U/mL mushroom tyrosinase, was pre-incubated for 15 minutes and added 110µL 2mM L-tyrosine (Sigma T3754). Then, the reaction was carried out at room temperature and incubated for other 15 minutes before measuring $OD_{490nm}$. The reaction mixture without enzyme was served as blank. The reaction mixture with the corresponding solvents (without plant material) was used as control. Kojic acid (Sigma, 95197) is the positive control. The percentage inhibition of tyrosinase was calculated as (1):

$$\% \text{ Inhibition} = 1 - \frac{(\text{Ad-Ab})}{\text{Ac-Aa}} \text{x} 100\% \quad (1)$$

Aa: $OD_{490nm}$ of reaction mixture without sample and enzyme; Ab: $OD_{490nm}$ of reaction mixture without enzyme; Ac: $OD_{490nm}$ of reaction mixture without sample; Ad: $OD_{490nm}$ of full reaction mixture.

### Data Processing and Statistical Analysis

Analysis of variance were performed using SAS GLM Procedure (SAS Institute, 1989)

## Results and Discussion

### Plant sterilization

The results showed that 80% of decontaminant mulberry seeds germinated. After 2 weeks, they became seedlings and have 4 – 6 leaves. These seedlings were used to hairy root inducing with *Agrobacterium rhizogenes* ATCC 15834 (Figure 1).

### Hairy root induction

The effect of explants types and incubation time into *Agrobacterium rhizogenes* on hairy root induction frequency was shown as Table 1. Leaf were more effective inducing than stem in all of incubation times. It is interesting to note that the wound in the petiole formed more roots than in the leaf blade (Figure 2). The results can be explained by the location of the wounds be near or far from vascular system carrying endogenous auxin. The incorporation of *rolB* genes from *Agrobacterium rhizogenes* into DNA enhances the endogenous auxin biosynthesis in plant, and hairy root induction still requires the presence of this auxin. Therefore, the wound in the petiole are easier to induce than in leaf blade. Although having vascular system, the stem is the most difficult tissue to induce due to its thick bark. This partly explains about the

hairy root inducing mechanism that genetic transformation probably effects only on endogenous auxin level but not on auxin storage sites and transportation.

Besides, an optimal induction time that enough for transferring target genes without destroying explants was observed. The longer incubation time is; the weaker explants are. The results show that 10 minutes is the most adequate time for incubation with 83% of the leaf explants produced hairy roots (Figure 3, Table 1).

**Figure 1. Two-week-old white mulberry seedlings.**

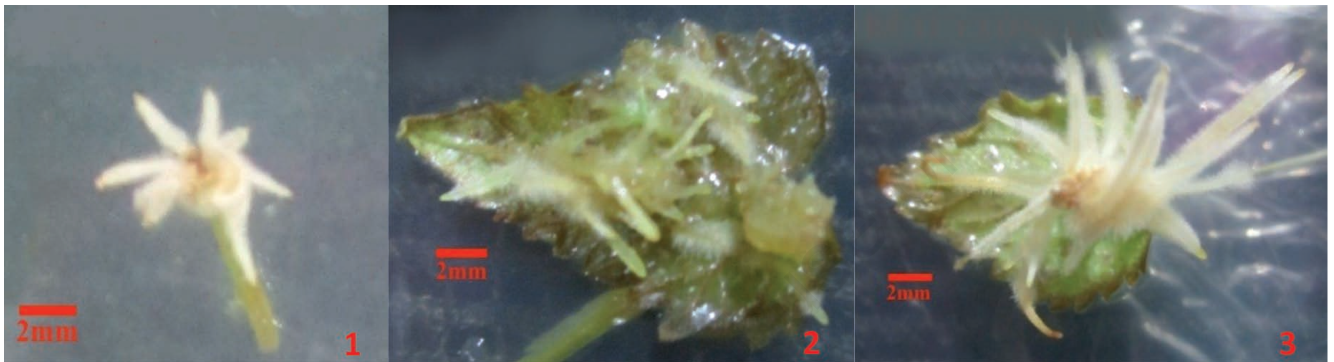


**Figure 2. Hairy roots induction with *Agrobacterium rhizogenes* after 3 weeks.** 1, from stems; 2, from leaves; 3, from petioles.

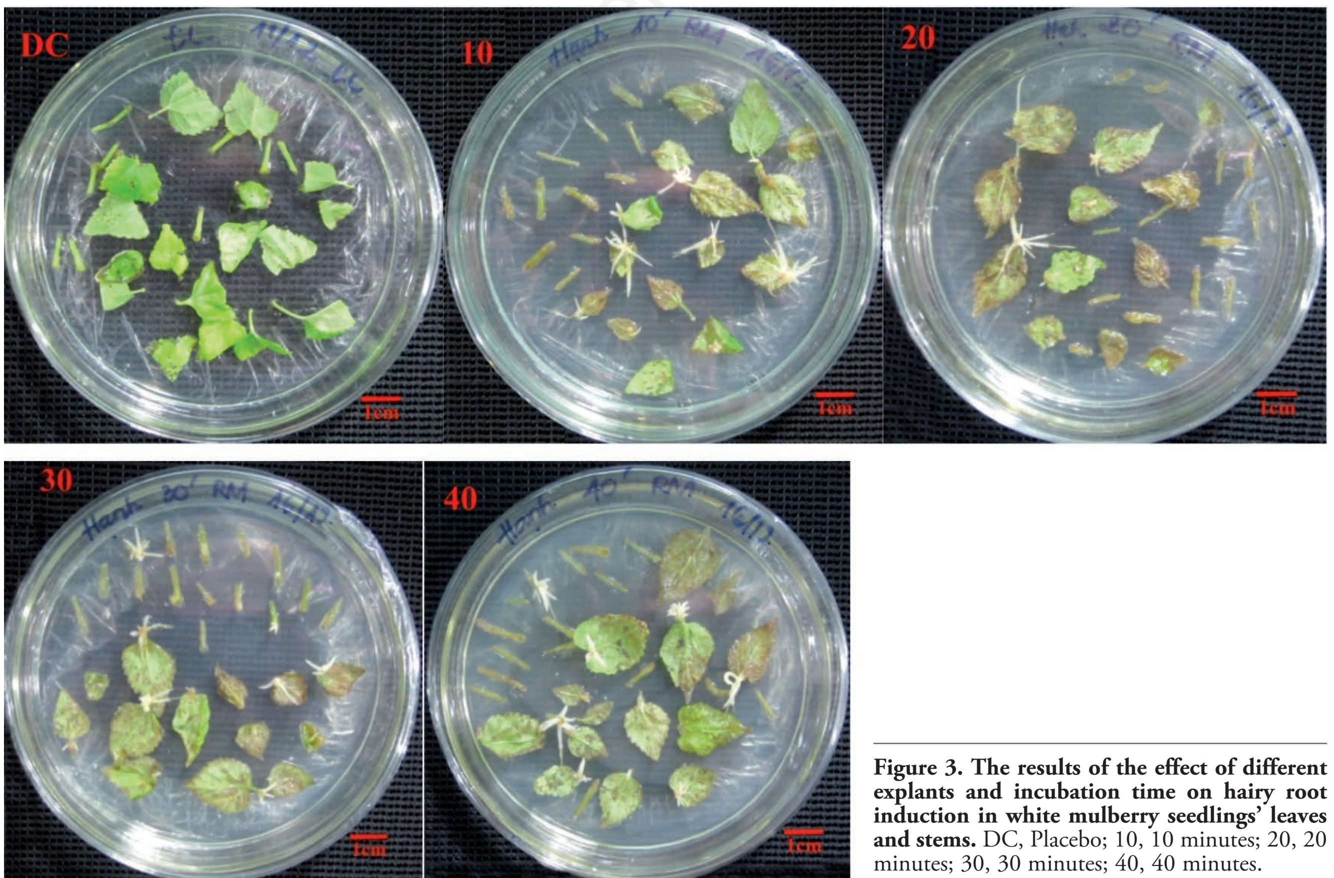


**Figure 3. The results of the effect of different explants and incubation time on hairy root induction in white mulberry seedlings' leaves and stems.** DC, Placebo; 10, 10 minutes; 20, 20 minutes; 30, 30 minutes; 40, 40 minutes.

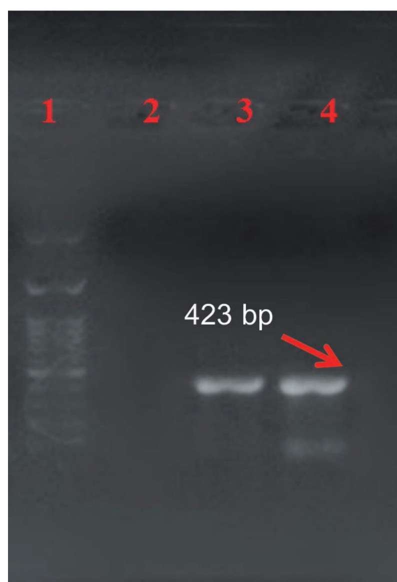


**Figure 4. The PCR detection and amplification of *rolB*.** 1, ladder; 2, placebo; 3, PCR product of *Agrobacterium rhizogenes*; 4, PCR product of hairy root.

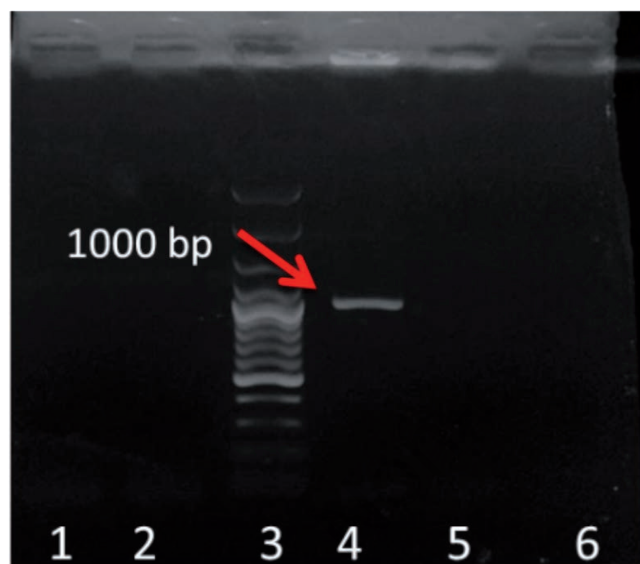


**Figure 5. The PCR detection and amplification of *virG*.** 3, ladder; 4, PCR product of *Agrobacterium rhizogenes*; 5, PCR product of hairy root.

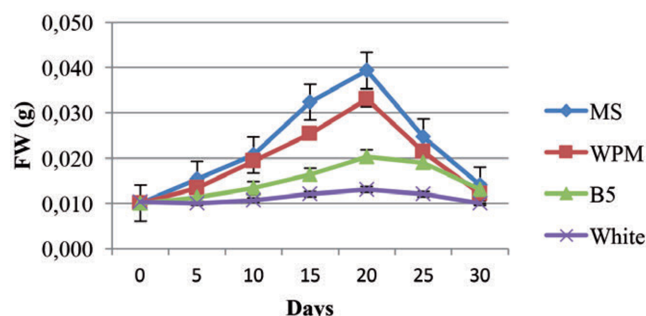


**Figure 6. Growth curves of hairy root on different media.**

## Detecting gen *rolB* in hairy root inducted by *Agrobacterium rhizogenes ATCC 15834*

The PCR product of *Agrobacterium rhizogenes* colony with *rolB* primers and hairy root are both having 423 bp bands (Figure 4). This proves that the PCR product of hairy root containing *rolB*. It means *rolB* from bacteria was successfully transferred to plant DNA. *VirG* was not detected in hairy roots, indicating the completed elimination of *Agrobacterium rhizogenes* in these samples (Figure 5).

## Hairy root propagation

Hairy root induced explants were then cultivated in liquid medium. The growth curves for hairy roots show that there are 4 phases of growth (adaptive phase: 0 – 10$^{th}$ day, early exponential phase: 10 - 20$^{th}$ day, stationary phase: 20 – 25$^{th}$ day, and decline phase: after the 26$^{th}$ day) prolonging approximately 30 days. However, the characteristics of transformed roots and production of biomass are different among media (Figure 6). Figure 6 shows the culturing of mulberry hairy roots on MS was more efficient than on WPM, B5 and White medium. At the end of exponential phase, the hairy roots Fresh Weight (FW) on MS were got 3.933 times comparing to baseline. The lowest is on White medium (1.258 times).

## Tyrosinase inhibition assay of hairy root

The tyrosinase inhibition of *in vitro* hairy root extracts were summarized and compared with natural root extracts in Table 2 and Table 3. The hairy root had strong inhibitory activity and - displayed the approximate $IC_{50}$ value as the natural root (Table 4).

**Table 1. Effect of different explants and incubating time on hairy root induction frequency.**

| Explants | Incubating time (minutes) | Root induction frequency (%) |
|---|---|---|
| Leaf (*including mesophyll and petiole*) | 0 | 0 |
| | 10 | 83.396 |
| | 20 | 77.732 |
| | 30 | 52.735 |
| | 40 | 41.781 |
| Stem | 0 | 0 |
| | 10 | 0 |
| | 20 | 21.438 |
| | 30 | 29.371 |
| | 40 | 12.323 |

**Table 2. Ethanol extract yield of root.**

| Pattern | Natural roots | Hairy roots |
|---|---|---|
| Dry weight (g) | 146.82 | 0.991 |
| Ethanol extract (g) | 17.837 | 0.030 |
| Extract yield (%) | 8.231 | 3.027 |



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**Table 3. Tyrosinase inhibition of *in vitro* hairy root and natural root extracts.**

| Concentration (µg/mL) | I (%) | |
|---|---|---|
| | Natural roots | Hairy roots |
| 50.00 | $97.275^a \pm 0.606$ | $99.243^a \pm 0.152$ |
| 25.00 | $96.871^a \pm 1.667$ | $97.881^a \pm 0.303$ |
| 12.50 | $97.276^a \pm 2.116$ | $96.973^a \pm 0.303$ |
| 6.25 | $86.580^b \pm 1.224$ | $84.864^b \pm 3.330$ |
| 3.12 | $43.895^c \pm 1.667$ | $39.303^c \pm 0.455$ |
| 1.56 | $15.843^d \pm 9.711$ | $12.059^d \pm 6.509$ |

Mean ± SD (n = 3 samples). Values that are statistically different are marked with different letters at $p < 0.05$.

**Table 4. $IC_{50}$ of *in vitro* hairy root and natural root extracts.**

| Samples | $IC_{50}$ (µg/mL) |
|---|---|
| Kojic acid | $19.199^a \pm 1.967$ |
| Natural roots | $3.597^b \pm 0.103$ |
| Hairy roots | $3.860^b \pm 0.030$ |

Mean ± SD (n = 3 samples). Values that are statistically different are marked with different letters at $p < 0.05$.

This result displays that hairy root culture system is a potential alternative method to obtain tyrosinase inhibitors of mulberry in short time. This is the first report in Vietnam.

## Conclusions

This research shows that hairy roots of *Morus alba* L. were successfully induced by *Agrobacterium rhizogenes* ATCC 15834 without exogenous hormones. Hairy root biomass was approximated 3.933 times (compare to baseline) after 20 days by MS liquid medium. Inducted hairy roots had the same anti-tyrosinase activity as the natural roots (IC50: 3.860 and 3.597 µg/mL, respectively). This is a positive result for commercial production of mulberry roots as a source of future whitening ingredients.

## References


1. Syvacy A, Sokmen M. Seasonal changes in antioxidant activity total phenolic and anthocyanin constituent of the stems of two Morus species (*Morus alba* L. and *Morus nigra* L.). Plant Growth Regulation 2004;44:251–6.
2. Devi B, Sharma N, Kumar D, Jeet K, *Morus alba Linn*: a phytopharmacological review. Int J Pharm Pharm Sci 2013;5:14-8.
3. Shin NH, Ryu SY, Choi EJ, et al. Oxyresveratrol as the potent inhibitor on dopa oxidase activity of mushroom tyrosinase, Biochem Biophys Res Commun 1998;243:801-3.
4. Lee SH, Choi SY, Kim H, et al. Mulberroside F isolated from the leaves of *Morus alba* inhibits melanin biosynthesis. Biol Pharm Bull 2002;25:1045-8.
5. Ryu YB, Ha TJ, Curtis-Long MJ, et al. Inhibitory effects on mushroom tyrosinase by flavones from the stem barks of *Morus lhou* (S.) Koidz. J Enzyme Inhib Med Chem 2008;23:922-30.
6. Chang TS. An updated review of tyrosinase inhibitors. Int J Mol Sci 2009;10,6:2440-75.
7. Jeong SH, Ryu YB, Curtis-Long MJ, et al. Tyrosinase inhibitory polyphenols from roots of *Morus lhou*. J Agric Food Chem 2009;57:1195-203.
8. Pradeep P, Su HS, Aditi W, Byung SM, Hyun AJ, Jae SC. Antioxidant and anti-browning property of 2-arylbenzofuran derivatives from *Morus alba* Linn Root Bark. Food Chem 2020;309:125739.
9. Giri A, Narasu M L. Transgenic hairy roots: recent trends and applications. Biotechnol Adv 2000;18:1-22.
10. Oka S, Tewary PK. Induction of hairy roots from hypocotyls of mulberry (*Morus indica* L.) by Japanese wild strains of *Agrobacterium rhizogenes*. J Seric Sci Jpn 2000;69:13-9.
11. Kong W, Yang J, Lu C. Establishment of *Agrobacterium rhizogenes*-mediated hairy roots inducement system for mulberry. Acta Botanica Boreali-Occidentalia Sinica 2010;30:2317-20.
12. Jin-hong Y. Establishment of Plantlet Regeneration Conditions for Mulberry Hairy Root. J Henan Agricultural Sci 2013;42:113-5.
13. Anis M, Faisal M, Singh S K. Micropropagation of mulberry (*Morus alba* L.) through *in vitro* culture of shoot tip and nodal explants. Plant Tissue Cult 2003;13:47-51.
14. Hoang TTM, Quach NDP, Bui VL. Hairy root culture of peanut (*Arachis hypogaea* L.) explants by *Agrobacterium rhizogenes* ATCC 15834 for resveratrol production. Vietnam Academy Sci Technol 2011;49:33-40.
15. Stewart CN Jr, Via VE. A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications Biotechniques 1993;14:748–51.
16. Currier TC, Nester EW. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal Biochem 1976;76:431-41.


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