Optimizing hairy root production from explants of *Phyllanthus hainanensis*, a shrub used for traditional herbal medicine

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Abstract *Phyllanthus hainanensis* is a shrub that has been used in traditional herbal medicine. It has great pharmaceutical potential for treating diseases such as cancer and diabetes. As a prerequisite for propagation of this species on a large scale, hairy roots in *P. hainanensis* were induced using *Rhizobium rhizogenes* and various factors affecting hairy root induction and growth evaluated. Seven factors were tested: (1) type of explant, (2) type of culture medium, (3) duration of pre-culture, (4) *R. rhizogenes* inoculum cell density, (5) duration of infection, (6) acetosyringone concentration in the culture medium, and (7) duration of incubation. The optimal protocol for hairy root induction and growth was: young shoots, pre-cultured in Y1 for 2 d, inoculated with *R. rhizogenes* broth with an OD_{600} of 0.6 for 20 min, and incubated for 3 d. Putative transgenic hairy roots were initially identified by morphology and then confirmed by polymerase chain reaction. Successful and optimal production of hairy roots is a critical prerequisite for industrial scale clonal propagation of *P. hainanensis*. Being able to cultivate the plant on a large scale will provide rapid and ready supply of the plant materials that can be used in herbal medicine and in scientific and industrial exploitation.

Keywords hairy root induction, medicinal plant, MS growth medium, *Phyllanthus hainanensis*, *Rhizobium rhizogenes*

1 Introduction

Phyllanthus (Euphorbiaceae) is a genus well known for its use in traditional herbal medicine for treating a variety of diseases such as cancer, diabetes, hyperlipidemia, asthma, ulcer, hepatitis and malaria. For example, *Phyllanthus* spp. have traditionally been used as a therapeutic alternative for the treatment of malaria and hyperlipidemia in South America[1]. In China and South East Asia, both *Phyllanthus amarus* and *P. urinaria* have been used to treat diabetes and diarrhea[2–4]. More recently, *P. niruri* was found to be an effective treatment for kidney stones[5,6]. Bioactive compounds extracted from fruits of *P. emblica* exhibit antidiabetic activity[7] and root extracts of *P. amarus* are hepatoprotective and able to induce apoptotic cell death in human breast cancer cells[8], and induce antitumor immune responses against tumor antigen[9]. A variety of secondary metabolites with antioxidant, anticancer and antimicrobial properties extracted from *Phyllanthus* have been identified, including diterpenoids, polyphenols, alkaloids, flavonoids, terpenoids and lignans[3,7,10–12].

Among the *Phyllanthus* spp., the shrub, *P. hainanensis*, has become a focus due to the recent discovery of phainanoids in extracts of this species[13,14]. Phainanoids, a class of metabolites with immunosuppressive properties, have great potential in treating diseases such as breast cancer, diabetes, hyperlipidemia and hypertension. Six phainanoids, A–F, have been isolated from root extracts of *P. hainanensis* and shown to exhibit potent immunosuppressive activities[13,14]. The species has a tropical and subtropical distribution, occurring in parts of Hunan, Guangxi and Hainan of China[15]. *P. hainanensis* has been used as herbal medicine for treatment of ophthalmic and inflammatory diseases[4]. There is no reported cultivation of the species. The growing demands for *P. hainanensis* materials as herbal medicine and for scientific and pharmaceutic exploitation have exerted pressure on the wild populations and caused local extinctions. This species is found only in small isolated populations in mountainous areas within its range. In addition, collection of

P. hainanensis from the wild is labor intensive and expensive. Commercial cultivation of the species will help conserve wild populations and should make its commercial utilization more cost effective. As a critical prerequisite for industrial scale clonal propagation of this important medicinal plant, we undertook experiments to develop a protocol for production of hairy roots in *P. hainanensis.*

Hairy root is a phenotype of T-DNA caused by *Rhizobium rhizogenes* (syn. *Agrobacterium rhizogenes*) infection of plant tissues followed by the insertion of the root-inducing plasmid (Ri plasmid) carried by the bacterium into the host nuclear genome[16]. Hairy roots are characterized by numerous branches, ageotropism, fast growth, hormone independence and high level production of secondary metabolites[17,18]. Induction of hairy roots has been attempted in a large number of medicinal plant species, including *Cannabis sativa*[19], *Scutellaria ocmulgee*[20], licorice (*Glycyrrhiza* spp.)[21], *Rhaponticum carthamoides*[22], *Panax ginseng*[17] and *Sphagneticola calendulacea*[23].

In this study, *R. rhizogenes* strain K599 was used to induce hairy roots in *P. hainanensis*. To optimize hairy root production, tests included different types of explant, including leaves, young shoots and roots, and various growth media and cultural conditions for hairy root production and growth. Of the explant types tested, it was found that young shoots were most suitable for hairy root induction. The production of hairy roots was identified by root morphology and confirmed by PCR analysis.

2 Materials and methods

2.1 Starter cultures

R. rhizogenes strain K599, from cultures preserved at −80°C, was grown on sterilized LB agar (pH 7.2) at 28°C for 48 h in the dark. A single bacterial colony was excised from the agar and placed in a 10 mL centrifuge tube containing 3 mL LB broth. The tube was placed on an orbital shaker at 200 r·min^{-1} for 20 h at 28°C in the dark. The broth was opaque white-yellow after 20 h. This broth

was used as the starter for all subsequent experiments.

2.2 Plant material

Seedlings of *P. hainanensis* were collected from a mountainous area of Changjiang County, Hainan, China and were grown in a greenhouse at Huazhong Agricultural University. A healthy plant was selected and propagated by tissue culture as part of a separate study. These clonal plants were potted and grown in the greenhouse. All plant materials used in the present study were derived from these plants.

2.3 Surface sterilization of plant materials

All plant material (leaves, young shoots and roots) were cleaned and surface sterilized before use. Plant material was first soaked in saturated detergent solution for 20 min, then cleaned with a soft brush and finally rinsed in running water for about 1 h. After cleaning, the material was surface sterilized in a laminar flow cabinet; first it was soaked in 75% alcohol for 30 s, then rinsed with sterile distilled water three times (2 min each), soaked in 0.1% mercuric chloride solution for 10 min with frequent stirring, rinsed with sterile distilled water five times, and finally blotted dried with sterile filter paper. Surface sterilized plant material was used in all experiments.

2.4 Experimental design

To determine the optimal protocol for hairy root production for *P. hainanensis*, a series of experiments were conducted to evaluate the effects of (1) type of explant, (2) type of culture medium, (3) duration of pre-culture, (4) *R. rhizogenes* inoculum cell density, (5) duration of infection, (6) acetosyringone (AS) concentration in the culture medium, and (7) duration of incubation. Details of the experiment design are presented in Table 1.

2.5 Effect of explant type and pre-culture medium

Experiment 1 was designed to study the effects of explant (leaves, young shoots and roots) and the pre-culture

Table 1 Experimental design for testing factors affecting hairy root production of *Phyllanthus hainanensis*

Factor	Level
Type of explant	Leaves, young shoots and roots
Type of culture medium*	MS and Y1
Duration of pre-culture	0, 1, 2, and 3 d
Inoculum cell density (OD$_{600}$)	0.2, 0.4, 0.6, 0.8, and 1.0 in MS + 100 µmol·L^{-1} AS broth
Infection time	5, 10, 15, 20, 25, and 30 min at OD$_{600}$ of 0.5–0.6
Concentration of acetosyringone (AS) in the culture medium	50, 100, 150, and 200 µmol·L^{-1}
Incubation time	0, 1, 2, 3, 4, and 5 d

Note: *, MS medium, Murashige and Skoog basal medium; Y1 medium, MS + 2,4-D (0.5 mg·L^{-1}) + NAA (0.25 mg·L^{-1}) + 6-BA (0.5 mg·L^{-1}).
2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, naphthaleneacetic acid; 6-BA, 6-benzylaminopurine; OD$_{600}$, optical density at 600.

medium (MS vs. Y1, see Table 1 for details), using a two-factor and unequal-level complete combination design for a total of six treatment combinations.

Explant pre-culture: three to four small puncture wounds were made in the leaves made with a sterile inoculation needle; young shoots and roots were cut to about 10 mm long. The explants were then transferred to the MS and Y1 growth plates and pre-cultured for 2 d at 25°C in dark. For each treatment, a total of 12 explant pieces were placed on each of three replicate plates.

R. rhizogenes inoculation: the pre-cultured explants were immersed in a culture of *R. rhizogenes* (MS + 100 μmol·L⁻¹ AS) with OD_{600} of 0.5–0.6 for 20 min with occasional gentle stirring to ensure even and complete coverage of the explants by the bacterium. The explants were then removed from the *R. rhizogenes* broth, blotted dry with sterile filter paper, placed on MS + 100 μmol·L⁻¹ AS agar, and incubated for 3 d at 25°C in the dark.

Hairy root production: after incubation, the explants were transferred to MS + 100 mg·L⁻¹ cefotaxime (Cef) agar for hairy root culture. The addition of Cef to the growth medium was to inhibit the growth of any residue *R. rhizogenes* on the explants. After 30 d, the number of main roots, number of hairy roots, root length and number of explants with necrotic/browning spots were recorded.

Of the six treatments, young shoots pre-cultured in Y1 gave the highest rate of hairy root production and fastest root growth (Table 2). The timing of the first hairy root emergence also differed between the types of explant. Hairy roots were first observed after 5 d for root explants and 7 d for young shoot explants; but hairy roots from root explants were usually small and with limited branching compared to those from young shoots. In contrast, it took 15 d for the first hairy root to emerge from leaf explants. Also, of the types of explant, a higher percentage of leaves suffered from bacterial contamination, evidenced by necrotic spots or browning, and died within the 30 d experimental period. Thus, in all subsequent experiments, only young shoots were used as explants.

2.6 Effect of pre-culture time

To determine the effects of pre-culture time on hairy root development, surface sterilized shoots were pre-cultured on Y1 plates for 0 (no pre-culture), 1, 2 and 3 d at 25°C in dark. The pre-cultured shoots were then inoculated, incubated and assessed as in Experiment 1.

2.7 Effects of *R. rhizogenes* culture cell density

R. rhizogenes culture cell density, measured as OD_{600}, estimates the number of bacteria the explants are exposed to when inoculated. If the culture density is too high, it can cause severe browning and necrotic lesions, and reduce the success rate of hairy root production. However, if the culture density is too low, there may not be sufficient bacteria to infect the explants and induce hairy root production. To determine the optimal *R. rhizogenes* culture density for hairy root development, the pre-cultured explants were immersed in broth with OD_{600} of 0.0 (control), 0.2, 0.4, 0.6, 0.8 or 1.0 for 20 min. The inoculated explants were then incubated and assessed as in Experiment 1.

2.8 Effect of infection time

Infection time refers to the length of time the explants are immersed in *R. rhizogenes* broth. To study the effects of infection time on hairy root production, the pre-cultured shoot explants were immersed in broth (OD_{600} of 0.5–0.6) for 5, 10, 15, 20, 25 or 30 min. The inoculated explants were then incubated and assessed as in Experiment 1.

2.9 Effect of acetosyringone concentration

The phenol, AS, has the ability to facilitate the release of signaling molecules of Ri plasmid of *R. rhizogenes* and transfer into the host plant nuclear genome, hence increasing the success rate of hairy root production. Thus, AS was added to the MS broth for *R. rhizogenes* culture and to the MS agar medium for incubation culture.

Table 2 Effect of explant type and pre-culture growth medium on hairy root development of *Phyllanthus hainanensis*

Growth medium	Explant type	Hairy root induction rate/%	Number of roots/Explant	Average root length/mm
MS	Mean	19.4±4.8 b	1.2±0.3 a	48±10 a
	Leaf	2.8±2.8 c	0.3±0.3 b	9±9 b
	Young shoot	33.3±4.8 ab	1.6±0.1 a	69±6 a
	Root	22.2±2.8 b	1.8±0.4 a	66±5 a
Y1	Mean	29.6±6.7 a	1.5±0.2 a	59±9 a
	Leaf	8.3±4.8 c	0.8±0.4 b	32±16 b
	Young shoot	50.0±4.8 a	1.9±0.1 a	74±6 a
	Root	30.6±7.3 b	1.9±0.2 a	70±7 a

Note: To compare MS and Y1, all measurements from the three explant types are pooled. Comparisons between different explant types are conducted separately for MS and Y1. Means (*n* = 3) followed by the same letters are not significantly different (*P* = 0.05). MS and Y1 indicate the same meanings as Table 1.

The optimal concentration of AS varies with plant species. To determine the optimal AS concentration for *P. hainanensis* hairy root induction, various amounts of AS (50, 100, 150 and 200 µmol·L^{-1}) were added to the culture broth and in the incubation medium and the effect on hairy root development evaluated. The pre-culture, inoculation, incubation and assessment were as in Experiment 1.

2.10   Effect of incubation time

Incubation time refers to the duration the explants were cultured on the solid medium immediately after inoculation with *R. rhizogenes*. To study the effects of incubation time, shoot explants were incubated for 0, 1, 2, 3, 4, and 5 d at 25°C in the dark. After incubation, the explants were transferred to the solid growth medium of MS + Cef for hairy root culture. After 30 d assessment were made as in Experiment 1. Figure 1 comprises selected photographs of hairy root development from various explants and growing conditions.

2.11   Confirmation of Ri plasmid by PCR analysis

R. rhizogenes transforms plant tissue by inserting the Ri plasmid into the host nuclear genome which results in hairy root production[16]. To verify the presence of Ri plasmid in hairy roots, a PCR was performed on root DNA. Total DNA of hairy and normal roots was extracted separately using the CTAB method[24]. After purification, the total DNA from the hairy roots was used as the template for PCR amplification; the total DNA of the normal roots was used as a negative control and *R. rhizogenes* K599 as a positive control. Specific primers of *RolA* and *RolC* designed for PCR amplification are given in Table 3.

PCR reaction system (10 µL): DNA template or K599 liquid culture 0.5 µL, sense and antisense primers (Tian Yihui Pty Ltd., Guangzhou, China) each 0.5 µL, ddH$_2$O 3.5 µL, 2 × Es Taq MasterMix (Dye) (Kang Wei Century Co., Beijing, China) 5 µL. The PCR procedure was pre-denaturation at 94°C for 5 min followed by 30 cycles of denaturation (94°C for 30 s), annealing (55°C for 30 s) and extension (72°C for 45 s) followed by further extension at 72°C for 3 min and preservation at 4°C. After the reaction, 6 µL of the PCR products were electrophoresed on 1% agarose gel in the presence of 5 µg·L^{-1} of ethidium bromide. DNA bands were observed and photographed by automatic gel imaging system JY04S-3E (Biobase Co., Jinan, China).
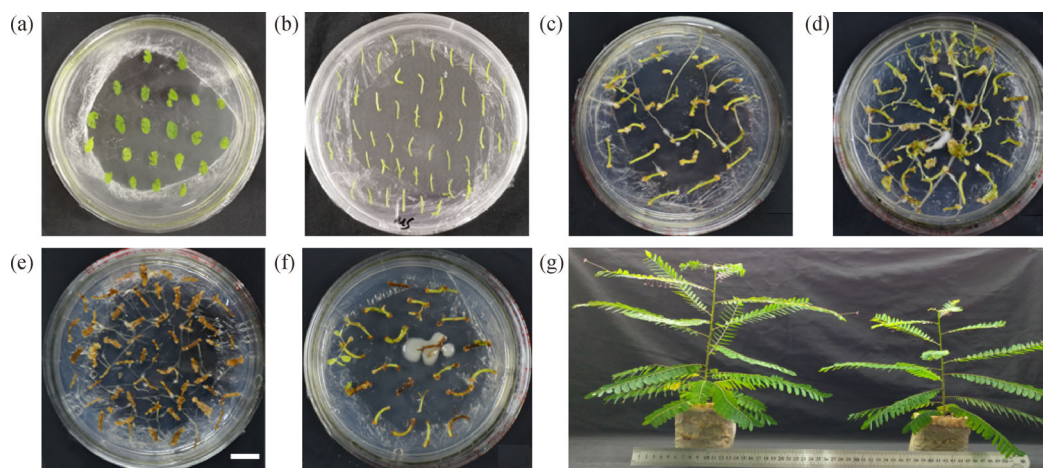

Fig. 1   The effects of explants, growth medium, and *Rhizobium rhizogenes* incoulum cell density on hairy root development of *Phyllanthus hainanensis*. (a) Leaf explants, day 0; (b) young shoot explants, day 0; (c) hairy roots from young shoot explants inoculated with *R. rhizogenes* OD$_{600}$ of 0.6 and incubated on MS medium, day 30; (d) hairy roots from young shoot explants are inoculated with *R. rhizogenes* OD$_{600}$ of 0.6 and incubated on Y1 medium, day 30; (e) hairy roots from root explants are inoculated with *R. rhizogenes* OD$_{600}$ of 0.6 and incubated on Y1 medium, day 30; (f) hairy roots from young shoot explants are inoculated with *R. rhizogenes* OD$_{600}$ of 1.0 and incubated on Y1 medium, day 14, showing browning of explants and bacterial contamination; (g) *P. hainanensis* plants of 3 months old growing in pots. Scale bar = 10 mm.

Table 3   Primers for PCR amplification

Gene	P1 (5′–3′)	P2 (5′–3′)
RolA	GCT CGT TGT CTC CGA CCT AT	GGT CTG AAT ATT CCG GTC CA
RolC	ATG GCG GAA TTT GAC CTA TG	TTA GTT CCA TCT GCC CAT CC

Note: Primer design follows that of Cao & Shockey[25].

2.12　Data processing

The rate of contamination, i.e., the percentage of explants that had necrotic lesions and/or browning spots, was calculated as:

Rate of contamination (%) = the number of contaminated explants/the total number of inoculated explants × 100

The induction rate of hairy roots, i.e., the percentage of explants that produced hairy root, was calculated as:

Induction rate of hairy root (%) = number of explants producing hairy roots/total number of inoculated explants × 100

ANOVA was used to compare differences in induction rate of hairy root, contamination rate, number of roots produced and root length between treatments. For comparison between MS and Y1 (Experiment 1), all measurements from the three explant types were pooled. Comparisons between different explant types were conducted separately for MS and Y1. All analyses were conducted using SPSS 22. Statistical significance threshold was set at $P < 0.05$.

3　Results and discussion

3.1　Effect of explant type

Explant type plays an important role in hairy root development and growth. Hairy root induction rate, the percentage of explants that successfully develop hairy roots, varied greatly between different types of explant and between different plant species. For example, *Vitis vinifera* internode explants were superior to leaf and node explants in the number of hairy roots produced, hairy root growth rate and time of first hairy root emergence[26]. Even for the same type of explant, development of hairy roots varies with explant age. For example, 40-d-old leaf explants of *Echinacea angustifolia* were the most suitable for hairy root induction and growth[27]. In the present study, young shoots were superior to roots and leaves in hairy root induction rate (Table 2). For explants pre-cultured in Y1, hairy root induction rate of young shoots was 19.4% and 41.7% higher than those of roots and leaves, respectively ($P < 0.05$). Furthermore, young shoots produced more and longer hairy roots than those produced by leaves ($P < 0.05$); but the number of hairy roots and the average

hairy root length produced by young shoots and roots were similar ($P > 0.05$). Similar patterns in hairy root induction rate, average number of roots produced and average root length were observed for the explants pre-cultured in MS (Table 2).

3.2　Effect of pre-culture time and medium

Pre-culture explants prior to inoculation of *R. rhizogenes* can improve the rate of hairy root production, number of roots produced and root growth rate. Pre-culture facilitates the release of signal molecules and reduces contamination of the explants, thus increasing hairy root induction rate[19,20]. The type of pre-culture media effect hairy root development and growth. Of the two pre-culture growth media tested in the present study, Y1 was superior to MS basal medium with 10.2% more explants pre-cultured in Y1 producing hairy roots than those pre-cultured in MS (Table 2, $P < 0.05$). However, explants pre-cultured in Y1 did not have significantly different ($P > 0.05$) number or length. There were large variations between the types of explant, thus masking possible differences between MS and Y1.

Pre-culture of the explants for 1 d increased hairy root induction from 11.1% to 22.2%. The optimal pre-culture time was 2 d, with the hairy root induction rate increasing by 2.5 times compared to the 0-d controls (Table 4, $P < 0.05$). No significant differences were recorded for the number of roots produced and average root length (Table 4, $P > 0.05$). These results are similar to species such as *Psammosilene tunicoides*[28] and *Ardisia crenata*[29], which had a 2-d optimal pre-culture time.

3.3　Effect of *R. rhizogenes* culture cell density

Hairy root is a pathological reaction of host plant caused by *R. rhizogenes* infection[30] so inoculum cell density is important. The cell density of the *R. rhizogenes* inoculum culture can have a profound influence on hairy root induction and growth. The optimal culture OD_{600} varies from 0.2 for *Justicia gendarussa*[31], 0.6 for *Bacopa monnieri*[32] and 0.8 for *V. vinifera*[26]. In the present study, hairy root induction rate and hairy root growth had a distinct unimodal pattern as the OD_{600} of the *R. rhizogenes* culture changed from 0.0 (control) to 1.0 (Fig. 2(a)). No hairy roots were recorded for explants in the control. The optimal culture OD_{600} was 0.6, at which 36.1% of the

Table 4　Effect of pre-culture time on hairy root induction in explants of *Phyllanthus hainanensis*

Pre-culture time/d	Hairy root induction rate/%	Mean number of root	Average root length/mm
0	11.1±2.8 c	1.7±0.3 a	25±2 a
1	22.2±7.3 bc	1.8±0.1 a	27±2 a
2	38.9±5.5 a	2.1±0.1 a	32±3 a
3	30.5±2.8 ab	1.9±0.2 a	29±3 a

Note: Means ($n = 3$) followed by the same letters are not significantly different ($P \geqslant 0.05$).

explants produced hairy roots, 8.3% higher than an OD_{600} of 0.4 ($P < 0.05$) and 9.4% higher than an OD_{600} of 0.8 ($P < 0.05$). However, no significant effect on root length between OD_{600} of 0.4 and 0.8 was recorded (Fig. 2(b), $P > 0.05$). Also, the average number of roots produced per explant were similar between explants inoculated with cultures of different optical density.

Contamination rate showed a linear increase as *R. rhizogenes* culture cell density increased from OD_{600} of 0.2 to 1.0, increasing from 8.2% to 58.3%, a 7-fold increase (Fig. 2(a)). This large increase in contamination rate associated with increasing cell density may have been responsible for the decline in hairy root induction rate in these treatments.

3.4 Effect of infection time

The infection time, or the duration explants were exposed to *R. rhizogenes* broth, affects the transfer of the Ri plasmid genes to host genomes and the infection of other host tissues by the bacterium. Short infection times may result in incomplete transfer of the Ri plasmid genes to host genomes, thus reducing the hairy root induction rate. Whereas, prolonged infection time may increase the risk of explant tissues being contaminated by the bacteria, which causes local necrotic lesions or death of the explant. The optimal infection time varies between 10 min for *B. monnieri*[32], 20 min for *V. vinifera*[26] and 30 min for *Semecarpus anacardium*[33]. Our results showed that hairy root induction had a unimodal pattern with infection time with maximum induction rate (38.9%) recorded at 20 min (Fig. 3(a)). A 5-min difference in infection time (either plus or minus) reduced the induction rate by more than 10%. For instance, when infection time was reduced from 20 to 15 min, hairy root induction rate was reduced by 11.1% to 27.8% ($P < 0.05$); at 25 min, the induction rate was 16.7% lower than at 20 min ($P < 0.05$). As infection time increased, contamination rate of explants increased. The

death of these contaminated explants reduced the hairy root induction rate as no hairy root was produced on dead explants.

In contrast to hairy root induction rate, the number of roots produced per explant and average root length were not significantly affected by infection time (Fig. 3(b), $P > 0.05$).

3.5 Effect of acetosyringone concentration

During hairy root induction, AS facilitates the release and transfer of the Ri plasmid to the host. It has been reported for a number of species that adding AS to the growth medium increases hairy root induction rate[34,35]. The effective concentration of AS ranges from 20 to 200 $\mu mol \cdot L^{-1}$, depending on the plant species. To determine the optimal AS concentration for hairy root induction in *P. hainanensis*, 0 (control) to 200 $\mu mol \cdot L^{-1}$ AS was added to the growth medium, and its effects on hairy root induction and development were assessed. The hairy root induction rate responded to changes in AS concentration in a similar way to the response to *R. rhizogenes* culture optical density (Fig. 3(a); Fig. 4(a)). When no AS was added (control), only 19.4% of the explants produced hairy roots. The percentage increased to 30.6% and 47.2% ($P < 0.05$), when 50 and 100 $\mu mol \cdot L^{-1}$ of AS were added, respectively; hairy root induction rate declined when AS concentration was 150 $\mu mol \cdot L^{-1}$ or above. In contrast, the number of hairy roots per explant and average root length gave no significant response to different AS concentrations (Fig. 4(b), $P > 0.05$).

3.6 Effect of incubation time

After inoculation, an incubation period is necessary for the Ri plasmid of the bacterium to fully integrate into the genome of the host. The optimal incubation period varies from 2 d for *V. vinifera*[26] to 4 d for *Semecarpus*
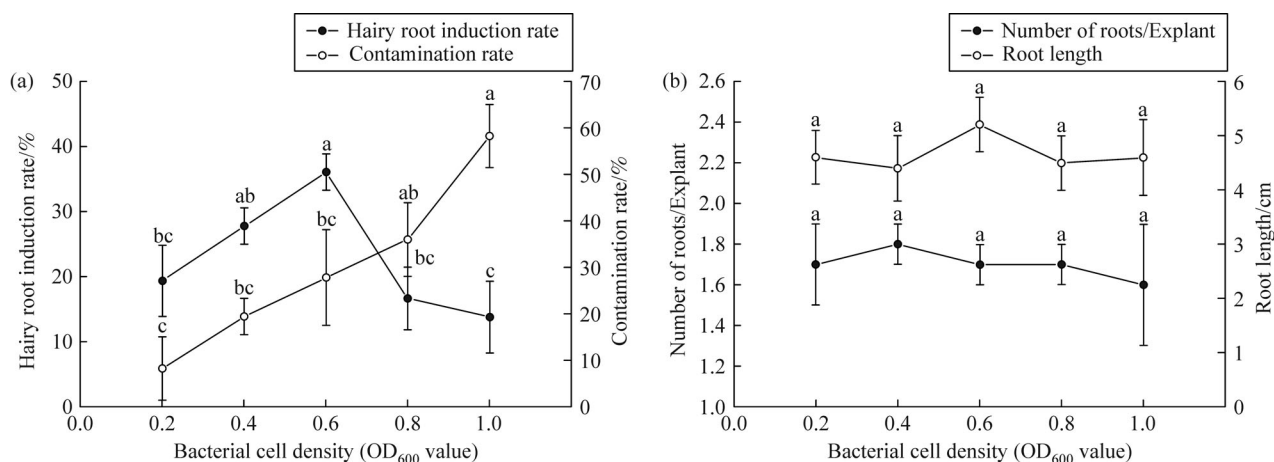

Fig. 2 Effect of cell density of *Rhizobium rhizogenes* broth concentration on hairy root induction rate (a) and growth (b) of *Phyllanthus hainanensis*. Means ($n = 3$) with the same letter are not significantly different ($P \geqslant 0.05$).
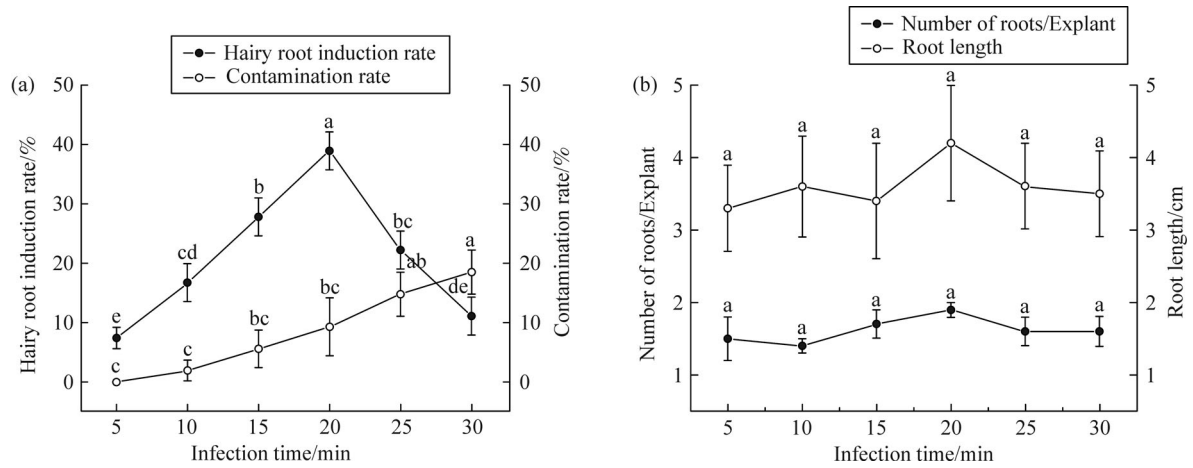
Fig. 3 Effect of infection time on (a) hairy root induction rate and contamination rate and (b) hairy root development of *Phyllanthus hainanensis*. Means ($n = 3$) with the same letter are not significantly different ($P \geqslant 0.05$).
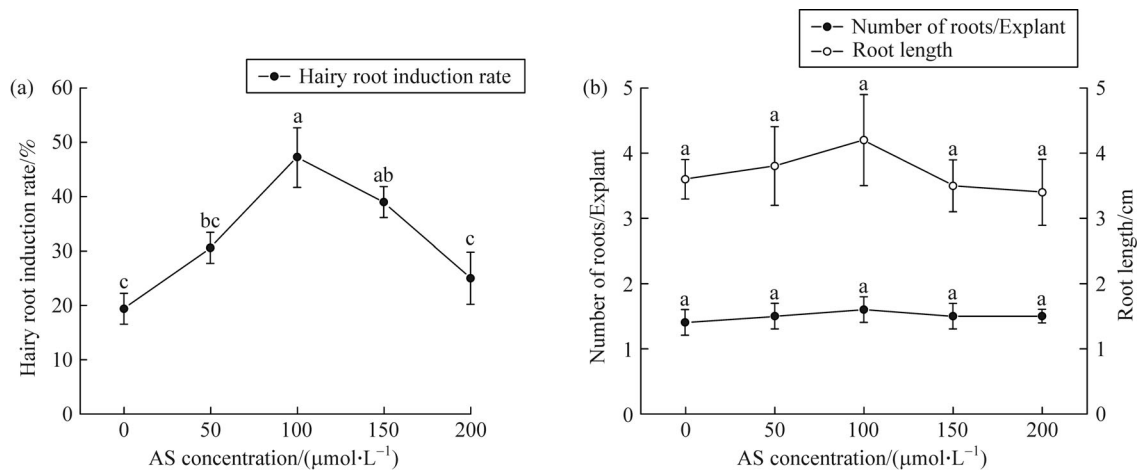

Fig. 4 Effect of acetosyringone (AS) concentration on (a) hairy root induction rate and (b) hairy root development of *Phyllanthus hainanensis*. Means ($n = 3$) with the same letter are not significantly different ($P \geqslant 0.05$).

anacardium[33]. In *P. hainanensis*, incubating the inoculated explants for 0 (control) to 5 d resulted in up to a 14-fold variation in hairy root induction rate between treatments (Fig. 5(a)). When the inoculated explants were transferred directly to the MS + Cef agar without incubation, only 2.8% produced hairy roots. The percentage increased steadily as incubation time increased from 1 to 3 d, and reached 38.9% when incubated for 3 d. The percentage decreased as incubation time was > 3 d and declined to 5.6% when incubated for 5 d. By comparison, incubation time had no statistically significant effect on the number of hairy root produced and average root length (Fig. 5(b)) with the exception of the control, which produced one or two less roots per explant ($P < 0.05$) and average root length was more than 20 mm shorter than in the incubated treatments ($P < 0.05$).

3.7　PCR detection of Ri plasmid in hairy root

Hairy roots induced by *R. rhizogenes* are characterized by considerable branching, ageotropism, rapid growth, hormone independence and increased secondary metabolites concentrations[17,18]. Compared to normal roots, hairy roots can be identified by their increased branching and rapid growth in hormone-free MS medium. In *P. hainanensis*, hairy roots showed the typical characteristics of increased branching. The PCR conducted in the present study confirmed that *R. rhizogenes* DNA fragments of 215 and 433 bp, corresponding to the *RolA* and *RolC* amplicons, respectively, was present (Fig. 6). These results thus confirmed that T-DNA of the Ri plasmid of *R. rhizogenes* K599 was integrated in genome of the hairy root.
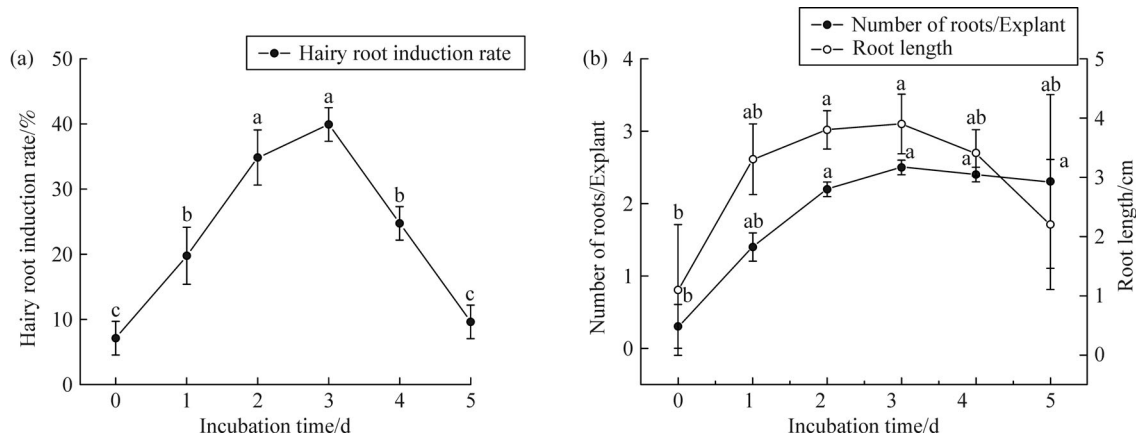
Fig. 5 Effect of incubation time on (a) hairy root induction rate and (b) hairy root development of *Phyllanthus hainanensis.* Means (n = 3) with the same letter are not significantly different ($P \geqslant 0.05$).
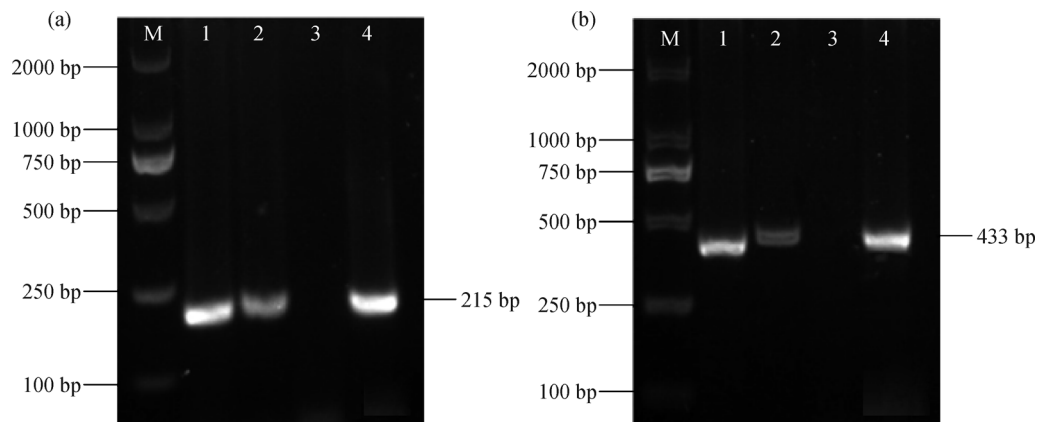

Fig. 6 PCR gels for hairy roots of *Phyllanthus hainanensis* with Ri plasmid of *Rhizobium rhizogenes* strain K599. (a) *RolA*-specific primer; (b) *RolC*-specific primer. M, DL 2000 DNA marker; 1–2, hairy roots; 3, normal roots (negative control); 4, *R. rhizogenes* K599 (positive control).

4　Conclusions

This study demonstrated that *R. rhizogenes* K599 can be used successfully for hairy root induction in *P. hainanensis*. Hairy root induction and growth in *P. hainanensis* are affected by a number of factors, including explant type, culture medium, and infection and incubation time. The young shoot explants gave the best results in terms of hairy root induction rate, the number of hairy roots produced per explant and optimal root growth. Other factors such as pre-culture time, cell density of bacterial culture, culture medium, infection and incubation time were evaluated for their effects on hairy root induction and growth. The optimal protocol for hairy root induction and growth for *P. hainanensis* was found to be: young shoots, pre-cultured in Y1 for 2 d, inoculated by submergence in *R. rhizogenes* broth with an OD_{600} of 0.6 for 20 min and incubated for 3 d. This is the first attempt to induce hairy roots in

P. hainanensis. The successful production of hairy roots and optimization of the production protocol is a critical prerequisite step for industrial scale clonal propagation of this important medicinal plant. In addition, hairy roots are good source of extractable secondary metabolites. Being able to cultivate the plant on a large scale will provide rapid and ready supply of the plant materials that can be used in herbal medicine and in scientific and industrial exploitation, thus diminishing the demand for *P. hainanensis* from wild populations and help the conservation of this species.

Compliance with ethics guidelines　Zhaogui Yan, Shengyu Liu, Junlian Zhang, Guan Huang, Lijun Duan, and Yaomei Ye declare that they have no conflicts of interest or financial conflicts to disclose.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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