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Establishment of hairy root cultures and analysis of rotenoid in Tibetan medicinal plant *Mirabilis himalaica*

Xiaozhong Lan[1, 2], Hong Quan[3], Xinli Xia[1], Weilun Yin[1*]

[1]College of Biological Sciences and Technology, Beijing Forestry University, Beijing 100083, China
[2]TAAHC-SWU Medicinal Plant Joint R&D Centre, School of Food Sciences, Agricultural and Animal Husbandry College, Tibet University, Nyingchi of Tibet 860000, China
[3]Institute of Plateau Ecology, Agricultural and Animal Husbandry College, Tibet University, Nyingchi of Tibet 860000, China

***Corresponding author: weilunyin@163.com**

Abstract

Mirabilis himalaica is an endangered plant species that has been used as an herb by native Tibetan people because of its rotenoid composition. In the present study, the effects of different factors including explants, preculture time and bacterial infection time on the induction rate of hairy roots were investigated. The highest hairy root induction rates were obtained using top leaves as explants, a preculture time of 2 d for young leaves, and *Agrobacterium* infection of young leaves for 20 min. Genomic PCR confirmed that the *rol* genes were integrated into the genome of *M. himalaica*. Different liquid media including MS, 1/2 MS, B5 and 1/2 B5 had significantly different influences on biomass and rotenoid accumulation in hairy root cultures. The hairy root cultures grew better in 1/2 MS and 1/2 B5 than in MS and B5, while a higher level of rotenoid was obtained in hairy root cultures growing in MS and 1/2 MS liquid media. Further, the contents of rotenoid were detected by HPLC in wild roots (0.176 mg.g^{-1} DW), stems (0.168 mg.g^{-1} DW) and leaves (0.157 mg.g^{-1} DW) of *M. himalaica*. In particular, the hairy root cultures had the highest rotenoid content (0.755 mg.g^{-1} DW) in 1/2 MS liquid medium, which was 4.3 times higher than in wild type roots (0.176 mg.g^{-1} DW).

Keywords: *Mirabilis himalaica*; hairy root; culture; rotenoid; induction rate.
Abbreviations: d_day; DW_dry weight; FW_fresh weight; h_hour; MS_ Murashige and Skoog;B5_Gamborg's B5; pRiA4_the plasmid of inducing root from *Agrobacerium* strain A4; qPCR_quantitative PCR; Ri_root induction; vir_ virulence.

Introduction

Mirabilis himalaica, called Ba Zhu in Tibetan, is a traditional Tibetan herbal plant that has been widely used to treat stomach disorders, nephritic edema and gonorrhea by the native Tibetan people since the Tang Dynasty (Zhang et al. 1997). The typical bioactive compounds in *M. himalaica* is rotenoid, which is used as insecticides (George et al. 2000) and were recently found to have anti-cancer activities (Linghu et al. 2014). *M. himalaica* plants (Fig. 1) grow in the very high mountains of the Tibetan Plateau and this makes the yield of this herbal plant very limited. The perennial host roots of *M. himalaica* are the only parts used in the pharmaceutical industry, and the cultivation of *M. himalaica* is currently not available. As a result, *M. himalaica* is now a highly endangered plant species. The demand for *M. himalaica* is stably increasing and this has led to more and more serious destruction-of-wild resources. Plant biotechnology might provide an alternative way to solve this problem. Hairy root cultures are a feasible biosystem to produce valuable metabolites because they have hormone autotrophic growth (Bulgakov et al. 2013), rapid accumulation of biomass (Kastell et al. 2013; Sun et al. 2012) and intact biosynthetic pathways (Chang et al. 2014). Hairy root cultures offer a promising method for high production and productivity of valuable secondary metabolites used as pharmaceuticals, pigments and flavors in many plants (Srivastava and Srivastava 2007) To our knowledge, hairy root cultures were established from more than 100 medicinal plant species, including *Taxus species* (Syklowska-Baranek et al. 2009) and *Ginkgo biloba* (Liu et al. 1997) from gymnosperm and *Hyoscyamus niger* (Jaremicz et al. 2014), *Atropa belladonna* (Yang et al. 2011), *Catharanthus roseus* (Hughes et al. 2004), *Artemisia annua* (Kim et al. 2003) from angiosperm and etc. Further, hairy root cultures are the convenient biosystem that can be used for metabolic engineering. The biosynthetic pathways can be genetically modified by overexpressing/suppressing the key enzymes. A lot of successful cases were reported. For instance, in transgenic hairy root cultures of *H. niger*, biosynthesis of tropane alkaloids was dramatically enhanced by overexpressing both putrescine N-methyltransferase and hyoscyamine 6β-hydroxylase (Zhang et al. 2004). Unfortunately, there are no reports on hairy root cultures of *M. himalaica*. In the present study, hairy root cultures of *M. himalaica* were established to investigate rotenoid production.

Results and Discussion

Optimizing the factors affecting hairy root initiation of M. himalaica

There have been no reports on hairy root cultures of *M. himalaica*. We established bacteria-free seedlings of *M. himalaica* (Fig. 2A) and investigated the effects of different types of explants, preculture time of young leaves and bacterial

Fig 1. The flower and the plants of *Mirabilis himalaica* in the wild.

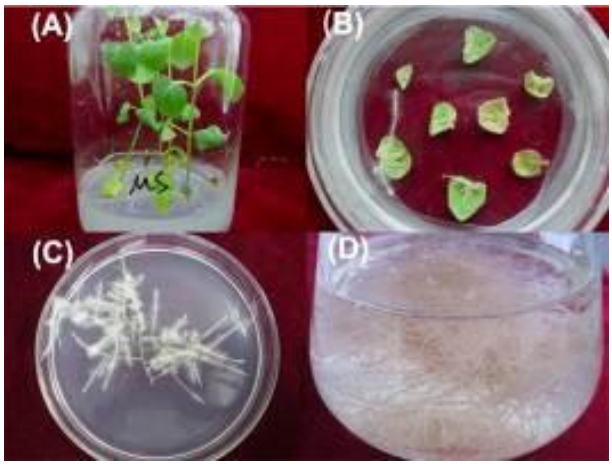

Fig 2. Establishment of *M. himalaica* hairy root cultures. (A) bacteria-free seedlings; (B) hairy roots induced from- wound -sites of leaves; (C) a single hairy root excised from a leaf and subcultured on solid medium; (D) hairy root cultures growing well in liquid medium without plant growth regulators.

infection time on the rate of hairy root initiation. When young leaves, young petioles and young stems were used as initial materials for hairy root initiation, the hairy root induction rates showed significant differences among the three explant types. The highest rooting rate (32.42±8.21%) was obtained from C58C1 (pRiA4)-infected young leaves, while only a few young petioles produced hairy roots (about 5% rooting rate). When stems were used as initial materials, no hairy roots were induced. These results suggested that young leaves were the most suitable initial explants for hairy root initiation (Fig. 3A).
Furthermore, it was found that the preculture time affected the induction rate of hairy roots from young *M. himalaica* leaves. Hairy roots could be induced from leaves without preculture treatment, but at a lower induction rate (10±3.33%). When the leaves were precultured for 2 or 3 d, the hairy root induction rates were 32.22±1.92% and 25.56±5.85%, respectively. When the preculture time was increased, the induction rates decreased significantly. The lowest hairy root induction rate was 6.11±0.96% when the preculture time was 5 d. Therefore, preculturing young leaves for 2 d was optimal for hairy root initiation of *M. himalaica* (Fig. 3B).

Finally, the effects of *Agrobacterium*-infection time on the induction rate of hairy roots were investigated. Young leaves did not generate hairy roots without C58C1 (pRiA4) infection. The hairy root induction rate increased when the infection time of C58C1 (pRiA4) was prolonged. The highest induction rate of hairy roots (34.44±5.09%) was obtained when young leaves were infected by C58C1 (pRiA4) for 20 min (Fig. 3C).
Generally, transformed roots emerged from infected young *M. himalaica* leaves at wound sites after 15 d infection with *Agrobacterium* strain C58C1 (pRiA4) (Fig. 2B). The transformed roots grew very rapidly on hormone-free MS medium, with many lateral branches, abundant root hairs and plagiotropic growth (Fig. 2C). According to the typical morphologies of hairy roots, we concluded that the induced roots were authentic hairy root cultures genetically transformed by the Ri plasmid. Interestingly, not all hairy root lines had the same growth rate, number of branches or root hair numbers. A hairy root line that grew very well with lots of lateral branches and very abundant root hairs was chosen for further study (Fig. 2C and D).

Confirmation of hairy roots of M. himalaica by genomic PCR

Hairy root cultures can be confirmed by genomic PCR. The *rol* gene family in the Ri plasmid is involved in auxin biosynthesis and this is the molecular basis of hairy root formation (Guillon et al. 2006). The *rolB* and *rolC* genes are usually used to confirm the authenticity of hairy roots (Qin et al. 2014; Zhang et al. 2004). When the genomic DNA of *M. himalaica* hairy roots was used as a template, a specific 423-bp *rolB* fragment and a specific 626-bp *rolC* fragment were simultaneously amplified, which was consistent with the PCR-amplified fragments from C58C1 (pRiA4) as the positive control; no fragments were amplified from the wild type *M. himalaica* roots as a negative control (Fig. 4). The results of PCR detection were consistent with many previous reports (Qin et al. 2014; Yang et al. 2011; Zhang et al. 2004). The PCR detection indicated that the Ri plasmid was integrated into the genome of *M. himalaica* and led to hairy root formation. To avoid *Agrobacterium* contamination (false positives), we also detected the *vir* gene of *Agrobacterium*. In the PCR amplification, the *vir* gene fragment was not detected in hairy root cultures or wild type roots of *M. himalaica*, but was detected in *Agrobacterium* C58C1 (pRiA4) (Fig. 3B). This suggested that there was no bacterial contamination.

Growth courses of hairy root cultures of M. himalaica in different liquid media

The composition and type of the culture medium are known to affect the growth and proliferation of hairy roots (Huang et al. 2014). Four types of liquid media, including MS, 1/2 MS, B5 and 1/2 B5, were investigated for their effects on *M. himalaica* hairy root culture growth and rotenoid production. Generally, the hairy root cultures in liquid medium showed an S-shaped growth curve, in which the growth period could be divided into three stages (Fig. 5). In the first stage, from the beginning of liquid culture to 5 d, hairy root biomass was accumulated very slowly. In the following stage from 5–25 d, the hairy root cultures grew very rapidly and the most biomass was accumulated in this stage. Then, in the last stage, after 25 d culture, the hairy root cultures hardly grew and the biomass hardly increased (Fig. 5). However, the final biomass of *M. himalaica* hairy root cultures was significantly different in the different liquid media. In 1/2 B5 and 1/2 MS liquid media, the biomass of hairy root cultures reached 6.30±0.38 g.flask^{-1} FW (fresh weight) and 5.58±0.22 g.flask^{-1} FW, respectively, which was higher than in MS and B5. These results suggested that a lower concentration of salts facilitated hairy root growth and

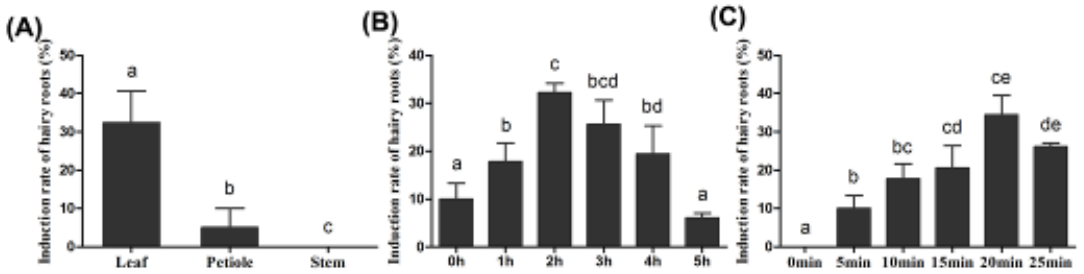

Fig 3. The induction rate of hairy roots from *M. himalaica*. (A) The effects of different explants on the hairy root induction; (B) the effects of preculture time on the hairy root induction rate; (C) the effects of bacterial infection time on the hairy root induction rate. Different letters (a, b, c, d, e) on the top of the bars represent significant difference ($P < 0.05$).

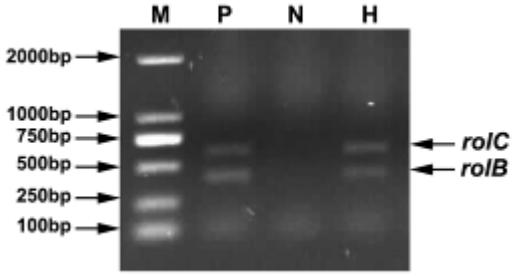

Fig 4. Genomic PCR detection of *rolB* and *rolC*. M: DNA marker DL2000; P: positive control; N: wild type roots; H: hairy root cultures. The arrows indicate the amplified *rolC* (626 bp) and *rolB* (423 bp) gene fragments from the positive control (plasmid pRiA4 as template) and hairy root cultures.

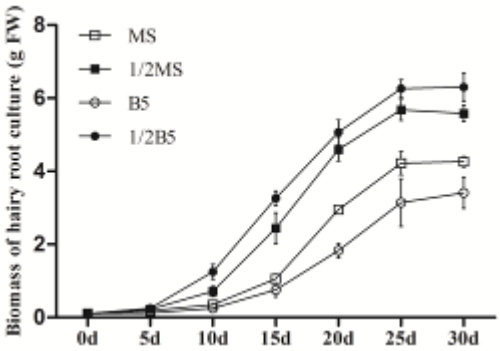

Fig 5. Growth course of hairy root cultures of *M. himalaica* in liquid media.

biomass accumulation. Previous studies showed that high organic nitrogen containing 1/2 B5 or B5 media were more suitable for hairy root growth and survival in *Gentiana scabra* (Huang et al. 2014) and *Catharanthus roseus*.

HPLC analysis of rotenoid in hairy root cultures and wild type roots, stems and leaves of M. himalaica

The composition and type of the culture medium also significantly affects metabolite biosynthesis in hairy root cultures (Giri and Narasu 2000). Hairy root cultures of *Gymnema sylvestre* produced gymnemic acid at much higher levels when cultured in MS liquid medium compared with B5 medium (Nagella et al. 2013). Similar results were also found for metabolite biosynthesis in other hairy root cultures. When hairy root cultures of *Withania somnifera* and *Taxus chinensis* were cultured in MS liquid medium, higher production of withanolide (Murthy et al. 2008) and taxol (Pan et al. 2000) was obtained compared with cultures in B5. However, MS medium does not always facilitate the biosynthesis of metabolites. For example, hairy root cultures of *Scopolia parviflora* produced tropane alkaloids at much higher levels when cultured in B5 liquid medium than in MS liquid medium (Min et al. 2007). Therefore, the most suitable medium for different plant species might be different. In the present study, hairy root cultures of *M. himalaica* in liquid MS and 1/2 MS produced rotenoid at much higher levels, reaching 0.6814±0.0074 and 0.7554±0.0105 mg.g^{-1} DW, respectively, about 3.35 and 3.72 times more rotenoid than cultures growing in B5 liquid medium (Fig. 6). Surprisingly, when hairy root cultures were grown in 1/2 B5 liquid medium, rotenoid were not detected. According to the HPLC analysis of rotenoid, MS and 1/2 MS medium were better for rotenoid production than B5 and 1/2 B5 medium. Finally, the rotenoid contents were also determined in wild type roots, stems and leaves of *M. himalaica*. Rotenoid were detected in wild type roots, stems and leaves but at different levels. The rotenoid contents in wild type roots and leaves were significantly different ($P < 0.05$). The highest rotenoid content was found in wild type roots, at 0.176±0.012 mg.g^{-1} DW; the lowest rotenoid content was in leaves, at 0.157±0.014 mg/g DW (Fig. 7). Comparing the rotenoid contents in hairy root cultures and wild type roots, the hairy root cultures had a much higher capacity for rotenoid biosynthesis.

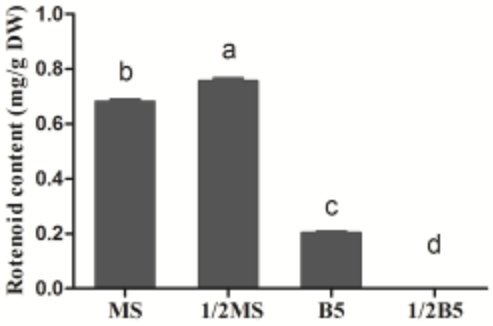

Fig 6. The rotenoid content of hairy root cultures in different types of liquid media. The letters on top of the columns show statistical analysis by Duncan's test. Different letters (a, b, c, d) indicate significant difference at the level of $P < 0.05$.

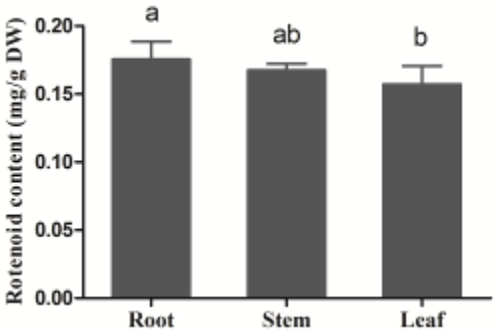

Fig 7. The rotenoid content in different organs of *M. himalaica*. The letters on top of the columns show statistical analysis by Duncan's test. Different letters indicate significant difference at the level of $P<0.05$, and the same letter indicates no significant difference at $P<0.05$.

Materials and Methods

Plant materials

Mature seeds of *M. himalaica* were harvested on the north side of a 4000-meter-high Himalayan mountain in Nyingchi, Tibet in October of 2011. The sterilized seeds were germinated and grown into seedlings on MS solid medium at 20°C under a 16 h photoperiod and 55 μmol m^{-2} s^{-1} light intensity. When the plant seedlings grew to 8–10 cm, the young leaves, petioles and stems were used for genetic transformation (Fig. 2A). The roots, stems and leaves of 3-month-old plants in the field were collected for gene expression and rotenoid analysis. All tissues were frozen in liquid nitrogen for storage.

Establishment of hairy root cultures

To investigate the influence of explant type on the induction rate of hairy roots, top leaves, petioles and stems were used as initial materials for hairy root induction. To study the effect of leaf preculture time on the hairy root induction rate, top leaves were used as initial materials and were precultured for 1–5 d before *Agrobacterium* infection. Finally, the effect of bacterial infection time on the induction rate of hairy roots from top leaves was observed. In each treatment, 13–20 explants were used. Genetic transformation was performed according to Lan et al. (Lan et al. 2013). After the explants were infected by C58C1 (pRiA4) for 25 d, the hairy root induction rate was determined. Roots generated at the cut-edges of leaves four weeks after co-cultivation were excised and cultured on solid hormone-free MS medium. The culture medium contained 250 mg/L carbenicillin to eliminate bacteria. Root culture clones were maintained at 25°C in the dark and routinely subcultured every 30 d. Rapidly growing root clones with no bacterial contamination were used to establish hairy root lines that were confirmed by genomic PCR.

Detection of rolB and rolC gene in hairy root cultures

To confirm the integration of *rol* genes into the genome of *M. himalaica* hairy roots, both the *rolB* (GenBank Accession Number: CAA45540) (forward primer: 5′-GCTCTTGCAGTGCTAGATTT-3′; reverse primer: 5′-GAAGGTGCAAGCTACCTCTC-3′) and *rolC* (GenBank Accession Number: AIY28220) (forward primer: 5′-TAACATGGCTGAAGACGACC-3′; reverse primer: 5′-AAACTTGCACTCGCCATGCC-3′) genes were detected through genomic PCR analysis (Lan et al. 2013). Wild type roots of *M. himalaica* were used as a negative control and C58C1 (pRiA4) as a positive control. To avoid C58C1 (pRiA4) contamination, the *vir* gene was also detected by a pair of primers, forward: 5′-CAGACGATGATAGGCAACAAGA-3′ and reverse: 5′-CAGCGACATAGGAAGTCTCAAT-3′ (Yang et al. 2011).

Culturing hairy roots in liquid media and rotenoid analysis

To investigate the effects of liquid media on biomass and metabolite accumulation in hairy root cultures of *M. himalaica*, a line of hairy roots growing very well with many lateral roots was selected for culturing in MS, 1/2 MS, B5 and 1/2 B5 liquid media. About 20 mg of fresh roots (3 cm in length) were inoculated into 250 mL conical flasks containing 50 mL of liquid medium and maintained on an orbital shaker at 100 rpm and 25℃ in dark. The biomass of the hairy root cultures was weighed at the beginning of the culture and at 5, 10, 15, 20, 25 and 30 d. The rotenoid content was analyzed using hairy root cultures growing in the liquid media for 30 d, and the rotenoid contents in different organs of wild type plants were also analyzed by HPLC (Wu et al. 2011). Firstly, 0.2 g of plant materials were dried at 40℃ and then ground into fine powder. The powders were ultrasonically extracted in 20 ml acetone for 30 min, and then the extracts were kept in dark at room temperature for 48 h. After that, the debris was separated by centrifuge (4000 rmp) and the supernatant was collected and dried by rotary evaporator, followed by being resolved in 1 ml acetone. The acetone solution went through 0.45μm filter film and that could be used for HPLC analysis. 20 μl extract was injected into JPLC for analysis. The HPLC system was Shimadzu CBM-20A and the column was Waters C18. The flowing phase consisted of acetonitrile:water (6:4); the flowing rate was 1.0 $ml.min^{-1}$. The rotenoid was detected on 289 nm by UV SPD-20A detector and the temperature of column oven was 30℃.

Conclusions

To our knowledge, this is the first report on the establishment of hairy root cultures of *M. himalaica*, a traditional Tibetan medicinal plant species. Young leaves of *M. himalaica* were the most suitable explants for hairy root initiation, and a higher hairy root induction rate was obtained when young leaves were precultured for 2–3 d and infected by *Agrobacterium* C58C1 (pRiA4) for 20 min. Liquid media with a low salt concentration, such as 1/2 B5 and 1/2 MS, facilitated the biomass accumulation of hairy root cultures, while MS and 1/2 MS

promoted rotenoid biosynthesis. Because the cultivation of *M. himalaica* has not been available to date, hairy root cultures will provide an alternative source of pharmaceutical metabolites from this plant species.

Acknowledgements

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