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PROTOCOL PAPER

Protocol for bonediol production in *Bonellia macrocarpa* hairy root culture

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Abstract

The aim of this protocol was for *Bonellia macrocarpa* hairy root establishment and bonediol production in transformed root cultures. Cotyledons, hypocotyls and roots were used as explants for hairy root induction. Fluorescence microscopy was used for detected tandem dimer of the tomato protein (TDT). A PCR analysis for rolC was used to confirm the presence of these genes. Identification and quantification of bonediol was made for HPLC. Hypocotyls showed greater transformation efficiency (25%). Fluorescence microscopy and PCR analysis confirm genetic transformation. Bonedio production was higher in hairy roots than in complete plants.

Keywords PCR analysis · Fluorescence microscopy · Bonediol production · Hairy roots

Introduction

Aerial parts and root bark extracts of *Bonellia macrocarpa* possess antimicrobial, cytotoxic and antiproliferative activities against various cancer cell lines (Vila-Luna et al. 2017). Bonediol cannot be commercially produced because *B. macrocarpa* is a wild plant and there is no a protocol for organ nor tissue culture. Hairy root cultures have been investigated due to its potential to produce the valuable metabolites that are present in wild type roots (Guillon et al. 2006). The aim of this protocol is to describe the establishment of hairy root of *B. macrocarpa* induced by infection with *Agrobacterium rhizogenes* ATCC15834/pTDT and bonediol production in transformed root cultures.

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Methods

Seedling growth (6–10 weeks)

1. Wash 50 seeds in a laminar air flow cabinet with liquid soap for 15 min.
2. Rinse the seeds three times in sterile water.
3. Add 200 mL of 70% ethanol for 3 min, discard the ethanol and repeat step 2.
4. Add 200 ml of 30% sodium hypochlorite for 20 min, discard supernatant and repeat step 2. CRITICAL STEP (CS): make the solution immediately before the experiment.
5. Incubate seeds on MS with 30 g/L sucrose, 5 mg/L gibberellic acid and 2.5 g/L Phytagel ®, pH 5.6.
6. Place seeds in a dark growth chamber at 25 ± 2 °C for 10 weeks, then transfer to continuous light. CS: darkness stimulates *B. macrocarpa* germination.

Agrobacterium rhizogenes growth (2 days)

1. Inoculate *A. rhizogenes* ATCC15834/pTDT in 20 mL of YMB medium 28 °C, 180 rpm. CS: always use an inoculum directly from a glycerol stock.
2. Incubate 24 h in darkness at 28 °C, 180 rpm. CS: the light affects motility and infectivity of *Agrobacterium*.
3. Subculture 1 mL of bacterial suspension (step 2) in 20 mL YMB and add 200 µM acetosyringone, incubate in darkness 8 h at 28 °C 180 rpm for *Agrobacterium*-mediated transformation. Culture until it reaches OD_{600} 0.4. CS: 100 mM acetosyringone solution prepared with DMSO and sterilized by filtration. Acetosyringone is termolabile.

Agrobacterium rhizogenes maintenance by criococonservacion (1 day)

1. Place 7 mL of the grown culture [see step 1 in "*Agrobacterium rhizogenes* growth (2 days)"] with 3 mL of glycerol in 300 mL sterile glass jar. Mix and aliquot 500 µL in Eppendorf tubes. CS: place them in liquid nitrogen and store immediately at − 80 °C.

Induction of hairy roots by *Agrobacterium rhizogenes*-mediated transformation (7–11 weeks)

1. Hypocotyls, roots and cotyledons from 10 week-old seedlings are harvested in order to provide explants for *Agrobacterium* transformation. CS: explants superficially and longitudinally wounded with a scalpel promote infection of *Agrobacterium*.
2. Place explants in a 300 mL sterile glass jar and add 20 mL of bacterial culture [see step 1 in "*Agrobacterium rhizogenes* growth (2 days)"], immediately place in a glass jar in desiccator and apply vacuum for 10 min. CS: shorter or longer time could affect the frequency of transformation.
3. Transfer the infected explants on MS with 30 g/L sucrose, 200 mg/L glutamine, 200 µM acetosyringone and 2.5 g/L Phytagel, pH 5.6. CS: make sure of accurate sterilization conditions because the glutamine and acetosyringone could be loss.
4. Culture explants in a dark growth chamber at 25 ± 2 °C for 3 days.
5. After 3-days of cultivation, wash the explants with liquid MS with 250 mg/L cefotaxime. CS: be sure to decrease the bacterial load to avoid hypersensitive response in explants.
6. Transfer the disinfected explants on MS with 30 g/L sucrose, 200 mg/L glutamine, 200 mg/L cefotaxime and 2.5 g/L Phytagel, pH 5.6. CS: transfer the explants to fresh medium every 20 days.
7. Culture transformed explants in a growth chamber under darkness at 25 ± 2 °C. CS: keep explants under darkness to avoid oxidative stress.

Hairy root culture (10 weeks)

1. Place emerged roots of 3 cm long from infected explants in 25 mL liquid MS with 250 mg/L cefotaxime, pH 5.6. CS: transfer the roots to fresh medium every 10 days to induce hairy root growth and eliminate the residual bacteria.
2. Culture roots at 25 ± 2 °C and 100 rpm under continuous light.

Verification of the transgenic nature of hairy roots (2–3 h)

1. Finely cut one sample of cultivated root (1–2 mm thick). CS: use a microtome.
2. Place sample on a slide, add one drop of glycerol.
3. Slide and excite with a single beam excitation at 488 nm and emission at 520 nm with a confocal laser scanning microscope. Use a negative tissue specimen as control of autofluorescence.

Gene integration analysis (6–8 h)

1. Isolate DNA of *A. rhizogenes*, uninfected and infected roots according Kit Termo Scientific Phire Plant Direct ®. CS: strictly follow the manufacture's indications.
2. For PCR reactions, use 1 µL of DNA and 10 µL 2X Phire Plant Direct PCR Master Mix, 0.5 µM of each primer (Primers for *rolC* gene: F5′-TGTGACAAGCAG CGATGAGC-3′ and R5′-GATTGCAAACTTGCACTC GC-3′; and for *VirD*$_2$ gene: F5′ATGCCCGATCGAGCT CAAGT-3′ and R5′-CCTGACCCAAACATCTCGGCT GCCCA-3′), and sterile water to a final volume of 20 µL.
3. Run PCR for *rolC*, initial denaturation at 98 °C for 5 min, 40 cycles of amplification (98 °C 5 s, 54.8 °C 5 s and 72 °C 20 s) and 72 °C for 1 min; for *virD*$_2$, follow the same program but annealing temperature at 56 °C.
4. Visualize by gel electrophoresis stained with SYBR Green. CAUTION: SYBR Green is carcinogenic.

Identification and quantification of bonediol (3 days)

1. Dry hairy roots of 10 weeks old in a freezer dryer.

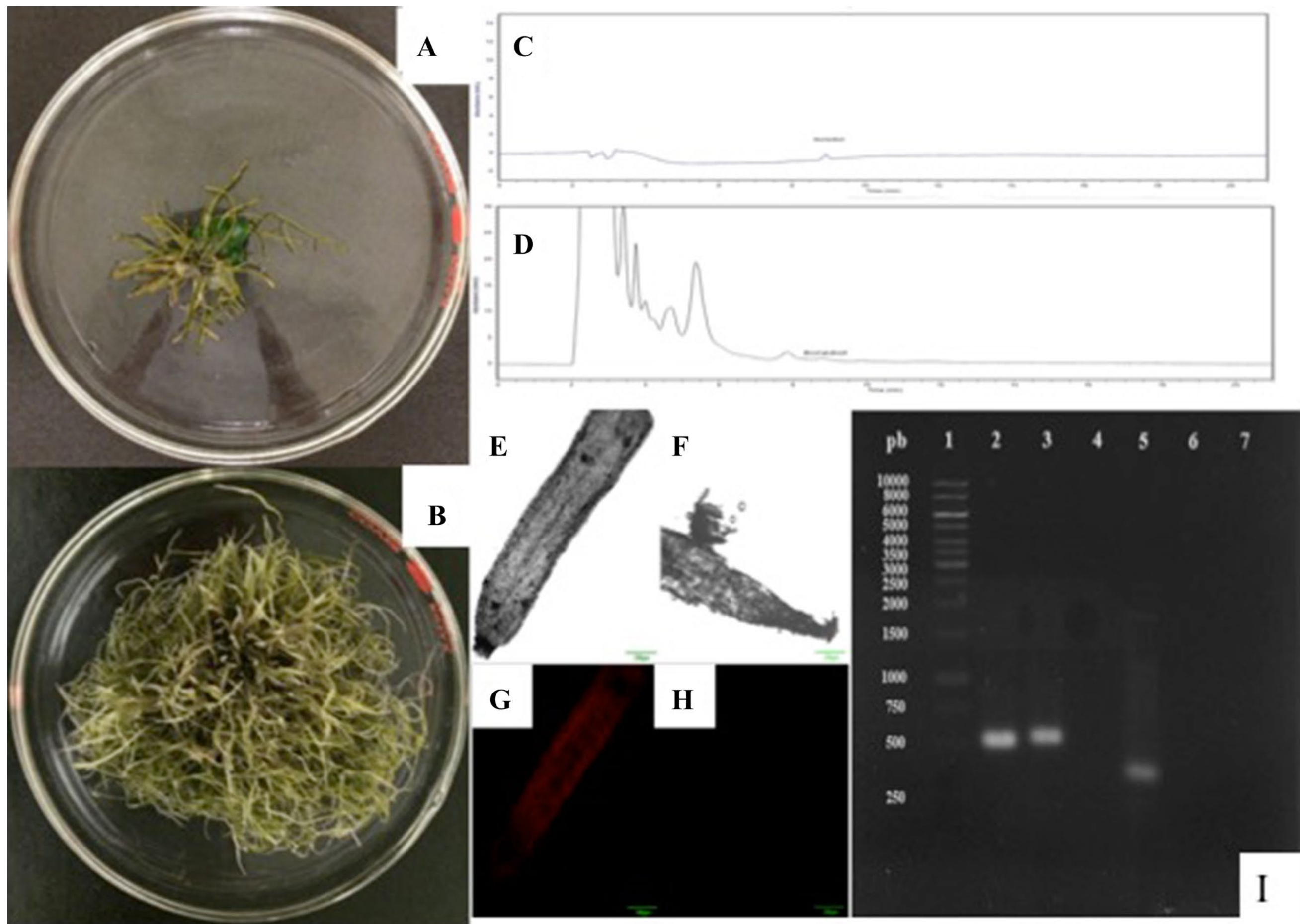

Fig. 1 Hairy roots of *B. macrocarpa* induced by *A. rhizogenes* ATCC15834 pTDT. Induction of the hairy roots from cotyledons of *B. macrocarpa*. **a**, 4 weeks old hairy roots; **b**, 10 weeks old hairy roots without plant growth. Quantification of bonediol in the methanol extracts of hairy roots of *B. macrocarpa* by HPLC. **c**, external standard of bonediol used for the identification; **d**, methanolic extract of culture of hairy roots after 10 weeks of culture. Light and fluorescence microscopy of hairy roots of *B. macrocarpa*. **e** and **g**, root explant from a hairy root; **f** and **h**, root explant form plantlet as negative control. PCR analysis of hairy roots of *B. macrocarpa*. **i** Lane 1, molecular weight marker; lanes 2 and 5, amplification of *A. rhizogenes*; lanes 3 and 6, transformed root; lanes 4 and 7, untransformed root; lanes 2–4 amplification with *rolC* primers; lanes 5–7, amplification with $virD_2$ primers

2. Macerate dried hairy roots (3 g) at room temperature with 450 mL methanol for 36 h (Caamal-Fuentes et al. 2011). CS: macerate every 12 h until reaching 450 mL methanol.
3. Evaporate the methanol with a rotary evaporator at 45 ± 1 °C and with vacuum; and resuspend dry extract in 10 mL methanol grade HPLC. CS: keep the extract in refrigeration.
4. For the standard curve, prepare stock standard solution of 1 mg of bonediol/mL in methanol; and prepare a series of standard solutions of 0.05, 0.1, 0.3, 0.5, 0.7 and 0.9 mg/mL.
5. Quantify bonediol with a Model Flexar HPLC and inject samples into a Zorbax ODS column (5 µm x 250 mm x 4.6 mm).
6. Use deionized water added with 0.05% of formic acid (A) and acetonitrile with 0.05% formic acid 60/40 (v/v) as the mobile phases (B). Start with 30% A, change from 30 to 5% A in 5 min and constant 5% A for 15 min more at a flow rate of 0.8 mL/min. Detection is carried out at 260 nm. 20 µl of the sample is injected and the column is maintained at room temperature (23 °C). CS: a post-run of 1 min of 100% B for cleaner the column between analyses.

Results and discussion

Different explants such as cotyledons, hypocotyls and roots of plantlets were used for hairy root induction in *B. macrocarpa*. The emerged roots were visible 4 weeks

Table 1 Troubleshooting for bonediol production in *Bonellia macrocarpa* hairy root culture

Section	Problem	Possible reason	Solution
Seedling growth (6–10 weeks)	Low % of germination	The seeds are old	Use fresh seeds
		The seeds are immature	Add gibberellic acid to culture medium
		The seeds were not correctly stored	Store the seeds at 4 °C
		Exceeded the time of sterilization method	Sterilize the seeds at the indicated time and rinse with excess water
Agrobacterium rhizogenes growth (2 days) to *Agrobacterium rhizogenes* maintenance by criococonservacion (1 day)	Low virulence of *Agrobacterium rhizogenes*	*Agrobacterium* culture was 2 or more days old	Keep the *Agrobacterium* strain in cryopreservation by glycerol stocks
		Motility and infectivity of *Agrobacterium* strain	Keep *Agrobacterium* culture under darkness and add acetosyringone in culture medium
Induction of hairy roots by *Agrobacterium rhizogenes*-mediated transformation. (7–11 weeks)	Low frequency of transformation	*Agrobacterium* cell concentration	Be sure that *Agrobacterium* cell concentration is around 0.4 OD_{600}
		Reduced or exceeded the time of vacuum	Apply vacuum to explants according the indicated time
		Hypersensitive response of explants	Keep the *Agrobacterium* cell concentration below 0.6 OD_{600} before the infection step Add an antioxidant as glutamine Keep the infect explants under darkness Reduce the time of cocultivation
Hairy root culture (10 weeks)	Low growth rate of hairy roots	Growth of *Agrobacterium* strain in culture medium	Increase the cefotaxime concentration
		Low concentration of nutrients and oxygen	Transfer the hairy roots to new culture medium every 10 days
Verification of the transgenic nature of hairy roots (2–3 h)	False positives or negatives in report gene assay	Inhibition of report gene or expression of a similar gene to the report gene	Select an adequate reporter gene
Gene integration analysis (6–8 h)	Negative result	Isolation of deficient DNA	Be sure to strictly follow the indications of the product
		Disintegration of reactive	Keep the reactive on ice and check expiration dates
		Contamination of samples	Use sterile new material each preparation and work a laminar flow air cabinet
Identification and quantification of bonediol (3 days)	The quantification of bonediol is not possible	The concentration of Bonediol in the sample is very low	Use culture of 7 weeks or more Use more plant material for extraction

after inoculation. No roots emerged in uninfected control explants. The transformation frequency depends of the type of explant, 7.14% cotyledons, 24.99% hypocotyls and 10.71% roots. Low transformation frequency could be due to hypersensitive response because is the first defence responses of plants, and generally characterized by a rapid and localized cell death around the site of infection, due to the accumulation of antimicrobial agents or the production of reactive oxygen species (Pitzschke 2013).

The transgenic nature of the roots emerging was confirmed trough tomato protein (TDT). Infected explants with *A. rhizogenes* pTDT showed red fluorescence, while that red fluorescence was not detected in control (Fig. 1a).

Bonediol was produced in the cultures of transformed roots. In the extract obtained with 10-week culture samples, 2.78 mg bonediol/g dried weight of transformed root were quantified. Presence of bonediol was identified after 7 weeks (Fig. 1c, d). These results suggest the possibility of using massive cultivation of hairy roots of *B. macrocarpa* for the biosynthesis of this metabolite, because the bonediol production is higher in hairy roots that in plants. For troubleshooting advice see Table 1.

Both transformed root from *B. macrocarpa* and *A. rhizogenes* contain *rolC* gene but it was not detected in the untransformed root. Amplification of $virD_2$ gen was only detected in *A. rhizogenes* (Fig. 1b). Light and fluorescence microscopy of hairy roots of *B. macrocarpa* were presented in Fig. 1e, g. Root explant from a hairy root (Fig. 1f) and root explant form plantlet as negative control (Fig. 1h). PCR analysis of hairy roots of *B. macrocarpa* (Fig. 1i).

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