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Short communication

Antibacterial activity of hairy-root cultures of *Maytenus senegalensis*

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Abstract

Hairy-root cultures were established by infecting sterile root segments of *Maytenus senegalensis* with *Agrobacterium rhizogenes* strains LBA9402 and A4T. Transformed roots produced more dry biomass (strain LBA9402, 0.22 g; strain A4T, 0.18 g) compared to untransformed roots (0.11 g) over a 3-month culture period. This increased 15- to 20-fold over the controls, after a culture period of 6 months. Transformation had no deleterious effect on the antibacterial activity of the root extracts. The minimal inhibitory concentration (MIC) values against *Staphylococcus aureus* of root extracts were 0.65 mg/ml (strain LBA9402), 1.25 mg/ml (strain A4T) and >1.25 mg/ml (untransformed tissues), respectively. Root extracts of both untransformed and transformed root cultures were active against Gram-positive bacterial strains only. Transformation was confirmed using the polymerase chain reaction (PCR) analysis for *rolA* and *rolC* genes. Results of the present study indicate that the induction of hairy roots has the potential to increase the yield of antibacterial compounds of pharmaceutical interest in root cultures of *M. senegalensis*.
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Keywords: *Agrobacterium*; Antibacterial; Hairy roots; Secondary metabolite; Transformation; Traditional medicine

1. Introduction

The large-scale demand for a supply of useful plants has resulted in over-exploitation of natural resources that often leads to the threat of extinction. In order to circumvent this, various approaches serve as alternatives for the production of desirable secondary metabolites from plants. Among these, plant tissue culture techniques have the potential to supplement the commercial production of bioactive plant metabolites (Ramachandra Rao and Ravishankar, 2002).

For plant cell culture techniques to become economically viable, it is important to develop methods that would allow a consistently high yield of products from cultured cells (Berlin and Sasse, 1985). Various strategies have been developed to improve the production of secondary metabolites using plant cell cultures (Ko et al., 1996; Roberts and Shuler, 1997; Lee and Shuler, 2000; Silvestrini et al., 2002). Bioactive secondary metabolites have been produced from root cultures of various plant species such as *Hyoscyamus niger* (Hashimoto and Yamanda, 1986), *Papaver bracteatum* (Kamo and Mahleberg, 1988), *Datura stramonium* (Maldonado-Mendoza et al., 1992) and *Linum flavum* (Van Uden et al., 1992).

The induction of hairy roots following *Agrobacterium*-mediated transformation has enhanced the production of secondary metabolites in many taxa e.g., *Salvia miltiorrhiza* (Chen et al., 1999), *Hyoscyamus muticus* (Jouhikainen et al., 1999) and *Scutellaria baicalensis* (Kuzovkina et al., 2001). Although the ability of plant cell cultures transformed by *rol* genes to increase synthesis of secondary metabolites has been established (Bulgakov et al., 1998; Bonhomme et al., 2000), the activation mechanism of secondary metabolite synthesis in the transformed cultures is still not clear. During the interaction between *Agrobacterium rhizogenes* and plants, partial transfer of the agrobacterial plasmid DNA (Ri-T-DNA) into the plant genome occurs. The plasmid contains the *rolA*, *rolB* and *rolC* genes, each of which is able to induce a neoplastic transformation of plant cells (Spena et al., 1987).

In a previous study, root bark of *Maytenus senegalensis* exhibited moderate antibacterial activity (Matu and Van Staden, 2003). Following this study, root cultures of *M. senegalensis* were established which also exhibited antibacterial activity, although the level of activity was about three times lower than extracts prepared from the root bark of material collected from

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field-grown plants (Matu and Van Staden, 2003; Lindsey et al., 2006). These results prompted the present study in which we attempted to induce hairy roots in root cultures of *M. senegalensis* in an effort to improve the yield of antibacterial compounds in this severely over-exploited traditional medicinal plant.

2. Materials and methods

2.1. Maintenance of root cultures

Established root cultures of *M. senegalensis* (Lam.) Excell were used for the present study (Lindsey et al., 2006). For maintenance of cultures, 10-mm-long root segments including root tips were excised and inoculated into 50 ml Erlenmeyer flasks containing 20 ml of MS (Murashige and Skoog, 1962) liquid medium containing (mg/l): 1-naphthaleneacetic acid (NAA), 0.1; *myo*-inositol, 100; and 3% sucrose. The pH of the medium was adjusted to 5.8 using 1 M KOH or 1 M HCl prior to autoclaving at 121 °C, 103.4 kPa for 20 min. Cultures were kept in the dark at 25±2 °C on a rotary shaker maintained at 120 rpm.

2.2. Growth conditions for Agrobacterium strains

Two strains of *A. rhizogenes* were used. Strain LBA9402 was maintained on YMB medium (all components in g/l): yeast extract, 5; mannitol, 10; $MgSO_4$, 0.1; and KH_2PO_4, 0.1; pH 7.0. Strain A4T was maintained on YMA medium (all components in g/l): yeast extract, 5; casein hydrolysate, 0.5; mannitol, 8; $NH_4(SO_4)_2$, 2; and NaCl, 5; pH 6.6. Frozen cells of both strains were streaked onto agar plates of medium and incubated at 28 °C until the appearance of single colonies. Single colonies of bacteria were inoculated in 3 ml of liquid medium containing (mg/l): rifampicin (Sigma), 150; kanamycin (Sigma), 100; and acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone, Aldrich), 20. They were incubated at 28 °C on a rotary shaker at 60 rpm. Bacterial cells were allowed to grow until the mid-logarithmic phase (OD of 0.6–0.8 at 600 nm). Bacterial cells that had reached the mid-logarithmic phase were centrifuged at 3000 ×g for 10 min in a Sorvall centrifuge using a SS-34 rotor. The supernatant was discarded and the pellet was suspended in 10 ml fresh medium (without antibiotics and acetosyringone). This was re-centrifuged as described and the pellet was suspended in fresh medium to attain an OD of 0.2–0.3 at 600 nm.

2.3. Transformation of root cultures with Agrobacterium strains

Actively-growing root cultures of *M. senegalensis* were used for experiments. Root segments of 10 mm were excised from growing root cultures and incubated in the diluted *Agrobacterium* cell suspension for 30 min at room temperature in sterile 2-ml microfuge tubes. Root segments were carefully removed from the bacterial suspension and spread on 2 layers of sterile filter paper (Whatman No. 1). Following infection, root segments were placed on semi-solid MS medium containing (g/l): sucrose, 30; *myo*-inositol, 0.1; agar, 8; and acetosyringone, 0.02; without antibiotics and incubated in the dark at 22–24 °C for 36 h. Fifteen root segments were placed in each petri plate and 10 plates were prepared for each strain. After 36 h of co-cultivation, a bacterial halo was observed around the root segments. Root segments were removed from the petri plates and washed with sterile distilled water containing 500 mg/l cefotaxime sodium (Claforan, Hoechst Marion Roussel Ltd., Midrand, South Africa), until the turbidity cleared. These were then placed on MS medium containing (g/l): sucrose, 30; *myo*-inositol, 0.1; agar, 8; Na-cefotaxime, 0.5 and kanamycin, 0.1; at 22–24 °C in the dark. The medium was changed every two days and the concentration of Na-cefotaxime was gradually reduced to 125 mg/l, until the *Agrobacterium* was completely eliminated from the root segments. After 3–4 weeks, hairy roots developed on the transformed root segments, which were excised and placed in liquid MS medium (as described above).

2.4. Preparation of root extracts

Root cultures were allowed to grow for 8-weeks and subsequently separated from the medium. Harvested roots were kept in brown paper bags and dried at 50 °C for 72 h. Dried roots (500 mg) were extracted with 20 ml ethanol by sonication in an ultrasound bath for 1 h and left to macerate overnight. Root extracts were filtered through Whatman No. 1 paper into pill vials. Filtrates were taken to dryness in front of a fan to obtain a constant dry weight residue. Residues were dissolved in ethanol to a concentration of 100 mg/ml for antibacterial activity testing.

2.5. Antibacterial activity testing

Extracts from the root cultures were tested against two strains of Gram-positive bacteria (*Bacillus subtilis* ATCC No. 6051, *S. aureus* ATCC No. 12600) and two strains of Gram-negative bacteria (*Escherichia coli* ATCC No. 11775, *Klebsiella pneumoniae* ATCC No. 13883). The microdilution method was used to determine the minimal inhibitory concentration (MIC) values for plant extracts with antibacterial activity (Eloff, 1998). A two-fold serial dilution, starting with 10 mg/ml, was prepared in 96-well microplates, after which 100 µl bacterial culture were added to each well. The antibiotic neomycin was included as reference in each assay and ethanol was used as a blank control. The microplates were incubated overnight at 37 °C. To indicate bacterial growth, 40 µl of 0.2 mg/ml iodonitrotetrazolium chloride (INT, Fluka) was added to the wells and incubated at 37 °C for 30 min. MIC values were recorded as the lowest concentration of the extract that completely inhibited bacterial growth, i.e. a clear well after incubation with INT. Each extract was tested three times.

2.6. Confirmation of transformation by PCR

Transformed and untransformed roots were freeze-dried in liquid nitrogen and stored at −70 °C until further use. DNA was extracted from frozen roots using the protocol described by

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