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Hairy root induction of *Momordica cochinchinensis* (Lour.) Spreng.

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Abstract

Momordica cochinchinensis is a highly nutritious plant, with lycopene and beta-carotene in the seed coat. These compounds are secondary metabolites that plants create to protect themselves from environmental stresses. Due to the number of extracts from naturally grown plants are often unstable and uncertain. Also, they may be insufficient quantity for market demand. Hairy root culture technique can increase productivity. This research interested in hairy root culture, therefore, this study aimed to investigate hairy root induction of *M. cochinchinensis* by using *Agrobacterium rhizogenes* strain ATCC 15834 and TISTR 511. Two strains of bacteria could induce hairy roots in *M. cochinchinensis* which roots emerged from a node and stem of explants. *RolB* genes of hairy roots were detected by PCR analysis. The length of the *rolB* genes was 700-800 bp. After investigation of hairy roots growth for a while, they stopped growing and turned brown until dead. However, this study can be used as basic information for the improvement of hairy root induction from *M. cochinchinensis.*

Keywords: *Momordica cochinchinensis* (Lour.) Spreng., *Agrobacterium rhizogenes*, Hairy root culture, Secondary metabolite

Introduction

Gac fruit (*Momorodica cochinchinensis* (Lour.) Spreng) in Thailand is known as "Fakkao" is a species of ancient Asian. It is found in southern Asia especially Southeast Asia. It belongs to the Cucurbitaceae family (Toan et al., 2017). Gac seed has been used as traditional Chinese medicine because of interesting composition (Ishida et al., 2004). Recently, a pentacyclic triterpenoid ester was isolated from the gac seeds and used as a medicine (Shan et al., 2001). Gac aril contains many important substances, including lycopene, β-carotene, lutein and other phenolic compounds such as myricetin, apigenin, gallic acid, ferulic acid and p-

hydroxybenzoic acid. These substances are bioactive compounds that possess antioxidant properties and beneficial for health (Kubola and Siriamornpun, 2011). Lycopene has been shown the correlation of reduced risk of certain cancers, such as prostate and lung (Gerster, 1997; Giovannucci, 1999; Michaud et al., 2000; Giovannucci et al., 2002). Vuong et al. (2003) found that gac aril contained 802 µg of lycopene and 175 µg of β-carotene/g of fresh weight. Gac fruit has been used for food supplements, cosmetic ingredients and dermatitis treatment (Putiyanan et al., 2009). However, the amount of extracts from the naturally growing plants is often uncertain. Its quality is not stable because it depends on the season and the cultivated area. Besides, it may be an insufficient quantity to supply market demand (Chaudhuri et al., 2005).

Plant tissue culture techniques such as callus culture and cell suspension culture have been used for secondary metabolites production. The external stress signals (e.g., pathogen elicitors, oxidative stress, wounding, etc.) use to induce plant secondary metabolism (Efferth, 2019). The limitations of these techniques are unsuccessful in an industrial scale due to contamination. Hence, hairy root inductions by *Agrobacterium rhizogenes* are an alternative method. Hairy root cultures have been widely studied for the production of secondary metabolites in many plant species because of the high growth rate and the cultures do not require phytohormones. Secondary metabolites of transformed roots produce sustained generations without losing genetic or biosynthetic stability (Giri and Narasu, 2000). The strong correlation between morphological differentiation and secondary metabolite production gives more interested in the application of organized cell cultures for the large-scale production of many phytochemical compounds (Filová, 2014).

Hairy roots from medicinal plants such as *Panax notogiseng* *Coptis chinensis* and *Savia miltiorrhiza* can produce more secondary metabolites than their callus (Gaosheng and Jingming, 2012). In addition, the quantity of flavonoids (catechin myricetin and quercetin) was found in hairy root of *Momordica charantia* higher than the control (Thiruvengadam et al., 2014). Ginsenoside was also produced in 5 L stirred tank bioreactor using adventitious root culture (Jeong et al., 2008). Thus, the purpose of this research was a preliminary study of hairy root induction from Gac fruit using *A. rhizogenes* for becoming a source of bioactive compounds production in the future.

Research Objective

To induce hairy root from Gac fruit using *A. rhizogenes* strains ATCC 15834 and TISTR 511

Materials and Methods

Explants preparation

M. cochinchinensis were obtained from the pergola in Kalasin Province, Thailand. Young shoots (5 cm from the tip) of a 3-month-old plant were sterilized with 20% Clorox for 10 mins, followed by sterilization with 10% Clorox for 10 mins. The sterilized shoots were rinsed five times with sterile distilled water then they were cut into small parts (leaves (5 x 5 mm), nodes and stems) with a sterile blade. These explants were used for the experiment.

Bacterial culture

Agrobacterium rhizogenes strains ATCC 15834 and TISTR 511 obtained from TISTR Culture Collection, Thailand were used for induction. Bacteria were cultured on YEB agar medium (Vervliet et al., 1975) overnight at 26 ± 2 °C. The single colony was transferred into 50 ml YEB liquid medium and cultured at 26 ± 2 °C to reach OD_{600} nm approximately 0.6-1.0. The bacterial suspension was used for infection.

Induction of hairy roots

M. cochinchinensis explants were immersed in the bacterial suspension of *A. rhizogenes* for 30 mins and then blotted dry on sterile Whatmann No. 1 filter paper. After that, the infected explants were placed on hormone–free MS medium containing 100, 150 and 200 mg/l acetosyringone and incubated at 26 ± 2 °C for 3 days in the dark. They were then transferred to hormone–free MS medium containing 250 mg/l cefotaxime and cultured at the same conditions to investigate apparent hairy roots.

Amplification of *rolB* gene by polymerase chain reaction (PCR)

The DNA was extracted from transformed and non-transformed roots of *M. cochinchinensis* using GF-1 Plant DNA Extraction Kit (Vivantis, Malaysia), as described by the supplier. PCR was performed using 2X Taq Master Mix (Vivantis, Malaysia) following the manufacturer's instructions. The pair of primer for the *rolB* gene was forward 5′-ATGGATCCCAAATTGCTATTCCCCCACGA-3′ and reverse 5′-TTAGGCTTCTTTCATTCGGTTTACTGCAGC-3′ (Panda et al., 2017). The PCR conditions were as follows: 94°C for 2 min, then 35 cycles of 94°C for 30 s, 53°C for 30 s and 72°C for 1 min; followed by elongation at 72°C for 5 min. The PCR products were separated on 1% agarose gels stained

with ethidium bromide and the gels were exposed under UV light to detect the presence of amplified products.

Results

Induction of hairy roots

Non-*In vitro* explants of *M. cochinchinensis* were used for induction of hairy roots by *A. rhizogenes* strains ATCC 15834 and TISTR 511. At a concentration of 200 mg/ l acetosyringone resulted in hairy roots formation. Roots emerged from a node and stem of explants that infected with *A. rhizogenes* ATCC 15834 and TISTR 511 respectively (Fig. 1). Also, the node had become callus before roots with numerous hairs appeared. However, after 2 months, all hairy roots attained a length of 1 cm and stopped growing later.

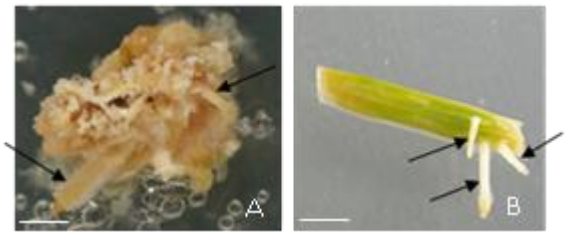

Figure 1 Hairy roots of *M. cochinchinensis* were induced by *A. rhizogenes* ATCC 15834 (A) and *A. rhizogenes* TISTR 511 (B) (Bar: 5 mm)

PCR analysis of *rolB* gene

The *rolB* gene of hairy roots was detected by PCR analysis. Transformed roots, which were induced by *A. rhizogenes* TISTR 511 and ATCC 15834, showed the presence of 800 bp and 700 bp *rolB* genes respectively (Fig. 2). Non-transformed roots also showed negative results.

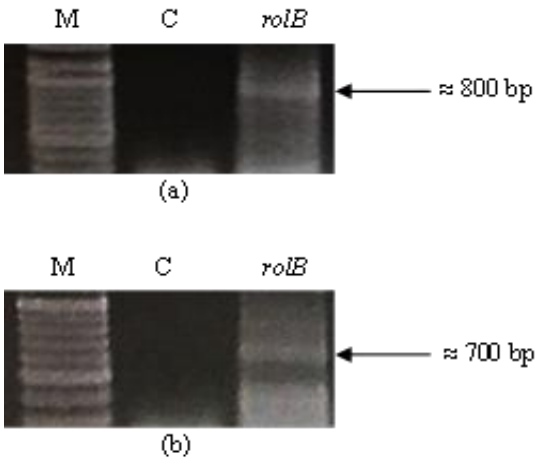

Figure 2 *RolB* gene fragment of transformed and non-transformed roots (a: Induction by *A. rhizogenes* TISTR 511; b: Induction by *A. rhizogenes* ATCC 15834; M: Maker; C: Non-transformed roots)

Discussion

Usually, hairy roots are fast growth and able to grow on hormone-free medium (Sevón et al., 2002). The explants from *in vitro* seeding of Indian and Korean cultivars of *M. charantia* were inoculated with *A. rhizogenes* strains (KCTC 2703 and KCTC 2704). Roots emerged within 2–5 days at the wounded edges of explants, and hairy roots developed within 20 days of inoculation (Thiruvengadam et al., 2014). Similarly, after 10 days of inoculation of *M. charantia* with *A. rhizogenes* wild type strain MTCC 532 showed small hairy roots protruded from leaf and cotyledon explants and the roots developed within 21 days (Swarna and Ravindhran, 2012). However, in this experiment, hairy roots of *M. cochinchinensis* exhibited small roots emerged from only one node and stem of all explants and stopped growing afterward although acetosyringone was added into the medium. Acetosyringone, which released by wounded plant cells, has been reported that it used to activate *vir* genes and enhance agrobacterium-mediated transformation of plant species (Kumar et al., 2006). The addition of 150 µM acetosyringone was able to increase the transformation frequency of carrot hairy roots (Srinivasan et al., 2014). Formation of hairy roots on muskmelon cultivar 'Birdie' was also enhanced when inoculated by *A. rhizogenes* grown in medium containing 20 µM of acetosyringone (Mohiuddin et al., 2011). Wahyuni et al. (2017) had studied the effect of various density of *A. rhizogenes* strain LB510 on hairy roots induction of gandarusa (*Justicia*

gendarussa Burm. f.) leaf plant. Various density of OD_{600} = 0.1, 0.2, 0.3, 0.4 and 0.5 were examination. The density of OD_{600} 0.2 was the best treatment.

Various factors influence on hairy root induction by *A. rhizogenes* such as bacterial cell density (Wahyuni et al., 2017), concentration of acetosyringone, infection method, co-cultivation time (Swain et al., 2012), light- dark incubation (Nourozi et al., 2016), concentrations of salts in culture media (Bivadi et al., 2014), types of explants and *A. rhizogenes* strains (Setamam et al., 2014). Nourozi et al. (2016) determined the effects of light and dark conditions on hairy root induction of *Agastache foeniculum* by *A. rhizogenes* strain A4. The highest hairy root induction was obtained under light conditions compared with dark conditions. Mohiuddin et al. (2011) studied the hairy root induction in Thai melon using 5 strains of *A. rhizogenes,* 8196, 15834, 9402, A4 and A105. *A. rhizogenes* 15834 was the best for hairy root induction from the melon stem. Callus occurred in day 7 after induction and then hairy roots appeared in 4 days later.

Unless the above factors, which affect the hairy root production, the other factors have also an effect on growth and secondary metabolite production such as nutrients, light, and temperature. In this research, the hairy roots grew slowly due to inappropriate culture conditions. In 1997, Cooker and Webb studied effects of temperatures (15, 25 and 35°C) and sucrose concentrations (0.5, 3, 6 and 9% (w/v)) on the growth of hairy root from *Lotus corniculatus*. The highest growth was achieved at 25 °C and 3% w/v sucrose concentration. The hairy root of *Solanum aviculare* also had the highest specific growth rate when cultured at 25°C. However, if the temperature was above 25°C, the specific growth rate decreased (Yu et al., 1996). The culture medium is another factor affecting the growth of hairy root. Rosic et al. (2006) found that WPM medium gave the highest growth in hairy root from *Rhamnus fallax* while the hairy root of *Solanum khasianum* Clarke had the highest growth rate in MS medium (Jacob and Malpathak, 2005).

The *rol* genes located on the T-DNA of Ri plasmid. They are considered essential for the hairy root initiation based on transposon “loss-of-function” analysis (White et al., 1985). *RolB* gene size ranging 700 to 850 bp length depending on the strain and present in all Ri plasmids with approximately 60 % identity between strains. It encodes approximately 230–280 amino acids which have a molecular weight of 30 kDa localized in the plasma membrane (Ozyigit et al., 2013). Bellincampi et al. (1996) report that the *rolB* may have a critical role in the early steps of hairy-root induction.

Conclusion

A. rhizogenes strain ATCC 15834 and TISTR 511 could induce hairy roots from node and stem of *M. cochinchinensis* although their growth rate was not achieved. However, *rolB* genes were observed in the transformed roots. This study can be probable as basic information for the improvement of hairy root induction from *M. cochinchinensis* in the future.

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