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Short Communication

Resveratrol production in hairy root culture of peanut, *Arachis hypogaea* L. transformed with different *Agrobacterium rhizogenes* strains

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Five different strains of *Agrobacterium rhizogenes* differed in their ability to induce peanut (*Arachis hypogaea* L.) hairy roots and also showed varying effects on the growth and resveratrol production in hairy root cultures*. A. rhizogenes* R1601 is the most effective strain for the induction (75.8%), growth (7.6 g/l) and resveratrol production (1.5 mg/g) in hairy root of peanut. Our results demonstrate that the use of suitable strains of *A. rhizogenes* may allow study of the regulation of resveratrol biosynthesis in hairy root cultures of *A. hypogaea.*

Key words: *Arachis hypogaea* L., *Agrobacterium rhizogenes*, hairy root, peanut, resveratrol.

INTRODUCTION

Peanut (*Arachis hypogaea* L), belonging to the Leguminosae family, is an annual oil seed and a legume native to South America but now grown in diverse environments in all over the world (Sharma and Bhatnagar-Mathur, 2006).

Resveratrol (trans-3, 5, 4'-trihydroxystilbene) is found in a wide variety of plants and peanut is one of the potent natural sources of phenolic antioxidant (Burns et al., 2002; Wenzel and Somoza, 2005). Resveratrol is a potent chemical and studies show it has anti-inflammatory, antioxidant, anti-infective properties, and has also promising therapeutic activity in various cancers, including breast, prostate, and neuroblastoma (Laux and Aregullin, 2004; Aggarwal et al., 2004; King et al., 2005). In many plant species, hairy root cultures have widely proven to be an efficient alternative production system for secondary metabolites because of their genetic and biochemical stability, rapid growth rate and ability to synthesize natural compounds at levels comparable to *in vivo* grown plants (Giri and Narasu, 2000; Guillon et al., 2006).

In vitro production of resveratrol from the callus and hairy root culture of *A. hypogaea* has been reported (Ku et al., 2005; Medina-Bolivar et al., 2007). However, no studies have been carried out for the induction of hairy roots in peanut for the production of resveratrol using the different strains of *Agrobacterium rhizogenes.* The ability of transformation and production of secondary metabolites can be enhanced by the use of different *A. rhizogenes* strains. In this study five different strains of *A. rhizogenes* strains viz.15834, A4, R1000, R1200 and R1601 were considered to check their capability to induce the formation of hairy roots on *A. hypogaea* root cultures.

MATERIALS AND METHODS

Plant material

Seeds of *A. hypogaea* were surface-sterilized with 70% (v/v) ethanol for 1 min and 2% (v/v) sodium hypochlorite solution for 10 min, then rinsed three times in sterilized water. Four seeds were placed on 25 ml of agar-solidified culture medium in Petri dishes

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Table 1. Effect of different strains of *A. rhizogenes* on hairy induction from leaf explants of *A. hypogaea.*

Agrobacterium strains	Infection frequency (%)	Number of hairy roots	Root length (cm)
15834	36.8 ± 3.8	2.3 ± 0.3	1.0 ± 0.1
A4	27.1 ± 2.5	1.2 ± 0.1	0.7 ± 0.1
R1000	71.2 ± 6.6	4.1 ± 0.4	1.3 ± 0.2
R1200	58.2 ± 5.7	3.5 ± 0.4	1.2 ± 0.1
R1601	75.8 ± 6.3	4.6 ± 0.5	1.6 ± 0.2

Values represent the mean ± SD of three independent measurements 30 days after inoculation. Approximately 50 seedlings were examined for each measurement.

(100 x 15 mm). The basal medium consisted of salts and vitamins of MS (Murashige and Skoog, 1962) medium and solidified with 0.7% (w/v) agar. The medium was adjusted to pH 5.8 before adding agar, and then sterilized by autoclaving at 121°C for 20 min. The seeds were germinated in a growth chamber at 25°C under standard cool white fluorescent tubes with a flux rate of 35 $\mu mols^{-1}m^{-2}$ and a 16 h photoperiod.

Preparation of *Agrobacterium rhizogenes*

Five *A. rhizogenes* cultures (15834, A4, R1000, R1200 and R1601) were grown to mid-log phase (OD_{600} = 0.5) at 28°C on a gyratory shaker at 180 rpm in liquid Luria-Bertani medium. The bacterial cells were collected by centrifugation for 10 min at 1500 rpm, and resuspended at a cell density of A_{600} = 1.0 in liquid inoculation medium (MS salts and vitamins containing 30 g/l sucrose).

Establishment of hairy root cultures

Excised leaves of *A. hypogaea* from 20-day-old in vitro grown seedlings were used as the explant material for co-cultivation with five *A. rhizogenes* strains. The excised leaves were dipped into the *A. rhizogenes* culture in liquid inoculation medium for 15 min, blotted dry on sterile filter paper, and incubated in the dark at 25°C on Phytagar-solidified MS medium. After two days of co-cultivation, the explant tissues were transferred to hormone-free medium containing MS salts and vitamins, 30 g/l sucrose, 200 mg/l Timentin and 8 g/l Phytagar. Within three to four weeks, numerous hairy roots had emerged from the wound sites. The hairy roots were separated from the explant tissue and sub-cultured in the dark at 25°C on Phytagar-solidified MS medium. After repeated transfer to fresh medium, rapidly growing hairy root cultures were obtained.

Isolated roots (100 mg) were transferred to 30 mL of MS liquid medium, containing 30 g/l sucrose, in 100 ml flasks. Root cultures were maintained at 25°C on a gyratory shaker (100 rpm) in a growth chamber under standard cool white fluorescent tubes with a flux rate of 35 $\mu mol\ s^{-1}\ m^{-2}$ and a 16 h photoperiod. After 3 weeks of culture, hairy roots were harvested and the dry weight and resveratrol content were determined. Each experiment was carried out with 3 flasks per culture condition and repeated twice.

HPLC analysis of resveratrol

Hairy roots were collected and fresh samples were stored in sealed clear polyethylene plastic bags at -80°C until they were used. Collected samples were dried at -80°C in brown paper bags for at least 24 h and dried samples were ground into a fine powder (40-mesh) by mill. Samples (2 g) were extracted with 12 ml of 0.1 N aqueous HCl : acetonitrile (1:5, v/v) mixture at room temperature for 1 h. The extract was filtrated through filter paper (Whatman No. 42) and evaporated (Heidoph VV2011, 40°C). The evaporated extract was resuspended with 10 ml of 80%-MeOH and prepared for HPLC analysis. The standard chemical (*trans*-3, 5, 4'- trihydroxystilbene) was purchased from Sigma.

Resveratrol was quantified by HPLC of a model LC-10A liquid chromatography (Shimadzu Co., Kyoto, Japan) equipped with Shimadzu SPD-10A spectrophotometer operated at the wavelength of 280 nm. The separation of resveratrol was performed on a J'sphere ODS-H80 fractionation column, (S-4 µm 80A, 250*10°C) with a flow rate of 1ml min^{-1}. Gradient elution was performed with solvent A consisting of 2% 0.018 M aqueous ammonium acetate and solvent B comprising 70% solvent A and 30% organic solvent. Organic solvent composed of 82% MeOH, 16% n-butanol, and 2% 0.018 M ammonium acetate. The sample was injected (5 µl) and applied gradient elution was , 0 - 1 min, wash of 90% solvent A; 1 - 21 min, linear gradient from 90 to 75% solvent A; 21 - 36 min, linear gradient from 75 to 55% solvent A; 36 - 56 min, linear gradient from 45 to 100% solvent B; 56 - 82 min, wash of 100% solvent B. Identification and quantification of resveratrol were carried out by comparing the retention times and the peak areas respectively with those of resveratrol standard or by direct addition of resveratrol standard into the sample (spike test). All samples were run in triplicate.

RESULTS AND DISCUSSION

Different strains of *A. rhizogenes* (15834, A4, R1000, R1200 and R1601) were studied for the induction of hairy roots from leaf explants in peanut (*A. hypogaea*). After 2 days of co-cultivation with five strains of *A. rhizogenes*, explant tissues were transferred to agar-solidified MS medium containing 200 mg/l Timentin, to effect killing of *A. rhizogenes.* Our results show that all strains of *A. rhizogenes* induced hairy roots at the site of explant infection after 30 days of inoculation. However, the five strains of *A. rhizogenes* differed in their ability to infect hairy roots, with strains R1601 being the most effective (Table 1). Strains R1000 and R1601 infected more than 70% of explants, induced an average of 4.1 and 4.6 hairy roots per explant, and the mean root length was 1.3 to 1.6 cm. The other three strains of *A. rhizogenes* were less effective in all of the above aspects.

The five strains of *A. rhizogenes* also differed in effects on growth of hairy root culture and production of resveratrol. Hairy root culture infected with strains R1601

Table 2. Effect of different strains of *A. rhizogenes* on growth and resveratrol production in *A. hypogaea* hairy root cultures.

Agrobacterium strains	Dry weight (D.W.) (g /l)	Resveratrol/D.W. (mg/g)
15834	5.4 ± 0.6	1.2 ± 0.1
A4	4.3 ± 0.4	0.8± 0.1
R1000	7.1 ± 0.7	1.3 ± 0.2
R1200	6.8 ± 0.6	1.1 ± 0.1
R1601	7.6 ± 0.7	1.5± 0.2

Values represent the mean ± SD of six independent measurements 3 weeks after culture.

accumulated the greatest dry weight of hairy root and produced the greatest amounts of the compound (Table 2). Hairy root cultures transformed with strain R1601 produced 1.5 mg resveratrol per g of dry weight, almost 1.88 times that of the lowest-producing strain A4.

Previous studies have examined the effect of different strains of *A. rhizogenes* on the growth and production of secondary metabolites in hairy root cultures of numerous medicinal plants, including *Gentiana macrophylla*, *Hyoscyamus muticus*, *Hyoscyamus albus*, and *Astragalus mongholicus* (Ionkova et al., 1997; Zehra et al., 1999; Tiwari et al., 2007). In this study, we demonstrate that inoculation of peanut with *A. rhizogenes* strains R1000 and R1601 effectively induces hairy root formation. Our rapidly growing transformed hairy root cultures produced resveratrol, an important anti-cancer compound. This system could function as an easy, reliable, and well-defined method for the production of secondary metabolite and study of the molecular regulation of genes encoding biosynthetic enzymes of this important plant.

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