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Response of Different *Agrobacterium Rhizogenes* Strains for *in vitro* Hairy Root Induction and Accumulation of Rosmarinic Acid Production in *Agastache Rugosa*

[1]Woo Tae Park, [1]Thanislas Bastin Baskar, [1]Sun Kyung Yeo, [2]Nam Il Park, [3]Jong Seok Park and [1]Sangun Park

[1]Department of Crop Science, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon, 34134, Korea
[2]Deptartment of Plant Science, Gangneung-Wonju National University, Gangneung-si, Gangwon-do 25457, Korea
[3]Department of Horticultural Science, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon 34134, Korea

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Corresponding Author:
Sangun Park
Department of Crop Science,
Chungnam National University,
99 Daehak-ro, Yuseong-gu,
Daejeon, 34134, Korea
Email: supark@cnu.ac.kr

Abstract: In this study a total of seven different *Agrobacterium rhizogenes* strains were evaluated for their ability to transform the plant *Agastache rugosa* and to produce the secondary metabolite rosmarinic acid. All the strains of *A. rhizogenes* i.e., 13333, 15834, A_4, LBA9402, R1000, R1200 and R1601 strains tested here in this study, were able to induce hairy root formation in leaf tissue explants. The strain A_4had the highest rate of infection (94.1±8.3%) and the strain R1000 had the lowest rate (88.6±6.9%). The highest frequency of hairy roots per explant (13.6±1.4) was found for strain R1601 and the tallest root length (20.4±1.7 mm) was found for strain 13333. We also evaluated dry weight and level of rosmarinic acid in the hairy roots and found that the highest growth (310.1±14.6 mg/flask) was occurred after infection with strain R1200, while the highest production of rosmarinic acid (68.2±3.8 mg/g dry weight) was noted using strain 13333. Our study showed that *A. rhizogenes* strain 13333 was the most effective of the 7 tested strains for production of transformed root cultures as well as rosmarinic acid in the hairy roots.

Keywords: *Agastache Rugosa*, Hairy Root, Rosmarinic Acid, Strain of *Agrobacterium Rhizogenes*

Introduction

The ornamental plant *Agastache rugosa* (family Lamiaceae), also known as Korean mint, is mainly found in Eastern Asia and is widespread in Korea, Japan and China as reported by (Kang *et al*., 2013; Zielińska and Matkowski, 2014). In recent years, *A. rugosa* has become one of the species of choice for medical research due to its interesting physiological and pharmacological properties (Wilson *et al*., 1992; Song *et al*., 2001; Shin and Kang, 2003; Jun *et al*., 2010) and also have anti-inflammatory and anti-a the rogenic properties (Min *et al*., 1999). *A. rugosa* possesses properties of potentially great importance for development of drugs against diseases such as cholera and for other medical conditions such as vomiting and for the treatment of disorders in intestine (Kim *et al*., 2001; Chae *et al*., 2005; Lee *et al*., 2008; Li *et al*., 2013).

A. rugosa also has use in food industries as it can act as a flavoring agent and add a spicy flavoring to food (Hong *et al*., 2001). Presently, there is considerable interest in Korea on exploiting native plant species like as persimmon, hardy rubber and wild mulberry used as efficient foods (Han, 2010; Park *et al*., 2012). *A. rugosa* leaves may possibly be of use in high-standard useful foods especially for tea, cakes and so on. The leaves can also be used to extract a fragrance reagent for perfumes. Thus, *A. rugosa* exhibits a variety of valuable properties with economic and social benefits.

Agrobacterium rhizogenes (family Rhizobiaceae) is a well-known gram-negative bacterium, can induce hairy root at the place of contamination in plants. By using hairy Root Inducing (Ri)-plasmids, *A. rhizogenes* transmitted T-DNA into plant cells to bring into beinghairy root diseases (Hamill *et al*., 1987). Now a day *A. rhizogenes* has been used to induce hairy roots

to a vast number of plant species (Guillon *et al*., 2006). There is a considerable interest in hairy root cultures for its quick growth, genetic and biochemical stability as well for their ability to produce higher amount of secondary metabolites (Christey and Braun, 2005; Georgiev *et al*., 2007; Srivastava and Srivastava, 2007).

There is a continuing demand for production of rosmarinic acid from different plant sources because of their various pharmacological uses (Petersen and Simmonds, 2003; Gao *et al*., 2005; Swarup *et al*., 2007). Rosmarinic acidhas a wide range of health benefited biological properties i.e., anti-inflammatory, antioxidant and so on those were reported by (Domitrović *et al*., 2013; Rocha *et al*., 2015).

Hairy root cultures from *A. rugosa* can be used to achieve production of rosmarinic acid. Elicitors upon contact with the cells of higher plants trigger increased production of secondary metabolites in different plant species (Uddin *et al*., 2010; Kim *et al*., 2013; Bong *et al*., 2015). Optimization of secondary metabolite production could be achieved not only by elicitors or phytohormones but also could be done by using bioinformatics methods (Tambunan *et al*., 2014).

Here we investigated both the induction of hairy roots and the levels of rosmarinic acid accumulation from *A. rugosa* using different *A. rhizogenes* strains and compare the outcome with the previously reported work on another strain of *A. rhizogenes* (Li *et al*., 2010).

Materials and Methods

Procedure of Sterilization and Germination of Seeds

Before placing seed germination, seeds of *A. rugosa* were sterilized with 70% (v/v) ethanol for 1 min and then 4% (v/v) sodium hypochlorite solution was used for 10 min and finally seeds were rinsed three times with sterilized water.

Six seeds were kept on a 25 mL^{-1} of agar-solidified culture medium having the size of ×15 mm petri dishes for germination. The basal MS medium contained salts and was solidified by using 0.8% (w/v) agar following the techniques described by (Murashige and Skoog, 1962.) The pH of the medium was attuned to 5.8 before adding agar and there after the medium was sterilized through autoclaving maintaining temperature of 121°C for a period of 20 min. For germination the seeds were kept in a growth chamber following a temperature of 25°C, a flux rate of 35 µmol s^{-1} m^{-2} and a 16-h photoperiod under standard cool white fluorescent tubes.

Growth of Agrobacterium Rhizogenes

Cultures of *A. rhizogenes* strains used in this study i.e., 13333, 15834, A_4, LBA9402, R1000, R1200 and R1601were instigated from glycerol stocks. They were allowed to grow in a liquid Luria-Bertani medium, to mid-log phase (OD_{600} = 0.5) overnight maintaining the temperature of 28°C with shaking at 180 rpm. *A. rhizogenes* were cultured for a period of 10 min through centrifugation at 224×*g* and then suspended again in the MS medium containing 30 g L^{-} sucrose. The cell density of *A. rhizogenes* was maintained at 600 nm of 1.0 for inoculation through a spectrophotometer absorption unit.

Establishment of Hairy Root Cultures

Leaves of *A. rugosa* were sampled for establishing hairy roots, from the plants those were grown *in vitro* condition and were cut at the ends with a size of 7×7 mm. The cut pieces of leaves were put into the culture of *A. rhizogenes* strains of 13333, 15834, A_4, LBA 9402, R1000, R1200 or R1601 in liquid inoculation medium for 10 min and then these samples were dry on sterile filter paper and finally incubated in dark condition on MS medium containing agar-solidified at 25°C. After 2 days of co-cultivation the explants were moved to a hormone-free medium, where medium contained a combination of MS salts and vitamins, 30 g L^{-} sucrose, 500 mg L^{-} cefotaxime and 8 g L^{-} agar. Within 2 weeks many hairy roots were initiated from the injured sites of the explants. The hairy roots those initiated from explants were separated and then they were cultured again in the dark condition keeping them on agar-solidified MS medium at 25°C. Immediately after repeated transfers to fresh medium rapidly growing hairy root cultures were found. An amount of 0.5 g [DW/l]l of isolated roots were moved to a 30 mL^{-1} MS liquid medium (30 g L^{-} sucrose), in 100 mL^{-1} flask. Root cultures keeping in 100 mL^{-1} flasks were moved in a growth chamber puting them in a gyratory shaker (100 rev/min) at 25°C with a flux rate of 35 µmol $s^{-1}m^{-2}$ and a 16-h photoperiod under standard cool white fluorescent tubes. Hairy roots were allowed to grow for 21 days and then they were harvested and finally from that samples dry weights as well as level of rosmarinic acid contents were measured. Three flasks were used for each culture and experiments were repeated thrice.

HPLC Analysis of Rosmarinic Acid

For rosmarinic acid analysis in HPLC, 1 g of collected hairy roots were frozen in liquid $N_2$and then were ground using a mortar and pestle to a fine powder and finally extracted using 10 mL^{-1} methanol for two times for a period of 24 h at 25°C. After drying under vacuum the crude extracts were achieved and before placing in HPLC analysis it was finally dissolved in methanol. Rosmarinic acid was quantified using a

System Gold 126 HPLC and 128 photodiode array detector manufactured by Beckman-Coulter, Mississauga, Canada under a C18 reverse phase column having 4.6 mm internal diameter, 250 mm length; Ultra sphere, Beckman-Coulter at room temperature. A solvent gradient was used as in the mobile phase by mixing of 70% solvent A (3% acetic acid in water) to 30% solvent B (methanol); where the solvent gradient reached 100% solvent Bafter 50 min. The solvent flow rate was maintained in a constant at 1.0 ml/min. Samples were detected at 280 nm wavelength.

Data Analysis

The data are reported as means±standard deviation.

Results

A. rugosa leaf explants showed susceptible for infection by all the strains of *A. rhizogenes* used in this study. No significant differences were observed in the morphologies for the hairy roots production in either of the strains used in this study. The rates of hairy root formation produced by the different *A. rhizogenes* strains were: A_4 (94.1±8.3%), 13333 (93.2±8.4%), R1200 (92.7±7.4%), LBA9402 (91.6±9.1%), 15834 (91.5±7.8%), R1601 (89.3±8.2%) and R1000 (88.6±6.9%). These results were shown in Fig. 1.

The numbers of hairy roots per explant at 30 days after inoculation were as follows: 13.6±1.4 for strain R1601; 13.0±1.7 for 13333; 12.3±0.8 for R1601; 11.7±0.8 for A_4; 10.6±1.3 for LBA9402); 9.7±1.3 for 15834; and 9.6±1.3 for R1000. The hairy root lengths in well-developed roots for each strain were as follows: 20.4±1.7 mm for strain 13333; 19.4±1.4 mm for R1200; 18.2±1.3 mm for LBA9402; 17.4±1.1 mm for R1601; 16.5±1.4 mm for A_4; 16.2±1.1 mm for 15834; and 15.8±0.8 for R1000, results were shown in Table 1.

Hairy root cultures from each of the seven strains were subculture in a fresh medium for a period of two to three months and there after transferred into liquid medium. In the liquid culture, MS medium was used and then kept them to grow for 21 days. At this level dry weight of hairy roots and level of rosmarinic acid s were evaluated in response to different strains (Fig. 2). The highest growth was found in R1200 strain with 310.1±14.6 mg/flask. For the other strains, we found: 306.8±16.4 mg/flask for strain 13333; 296.3±12.1 mg/flask for A_4; 292.5±18.8 mg/flask for LBA9402; 291.3±12.0 mg/flask for R1000; 283.2±21.9 mg/flask for 15834; and 278.8±23.8 mg/flask for R1601. The levels of rosmarinic acid were (Fig. 3) 68.2±3.8 mg/g DW for strain 13333; 67.7 ±4.4 mg/g DW for R1200; 60.8±3.3 mg/g DW for R1000; 56.4±4.4 mg/g DW for R1601; 55.6±4.1 mg/g DW for LBA9402; 52.0±4.6 mg/g DW for 15834; and 51.8±5.3 mg/g DW for A_4.

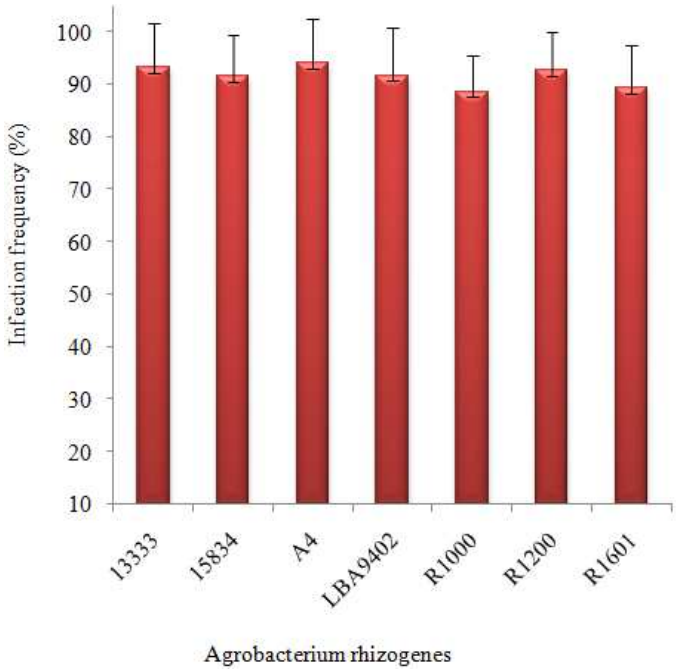

Fig. 1. Effect of strains of *A. rhizogenes* on the infection frequency of *A. rugosa*hairy root cultures (The values represent the mean±SD of three independent measurements)

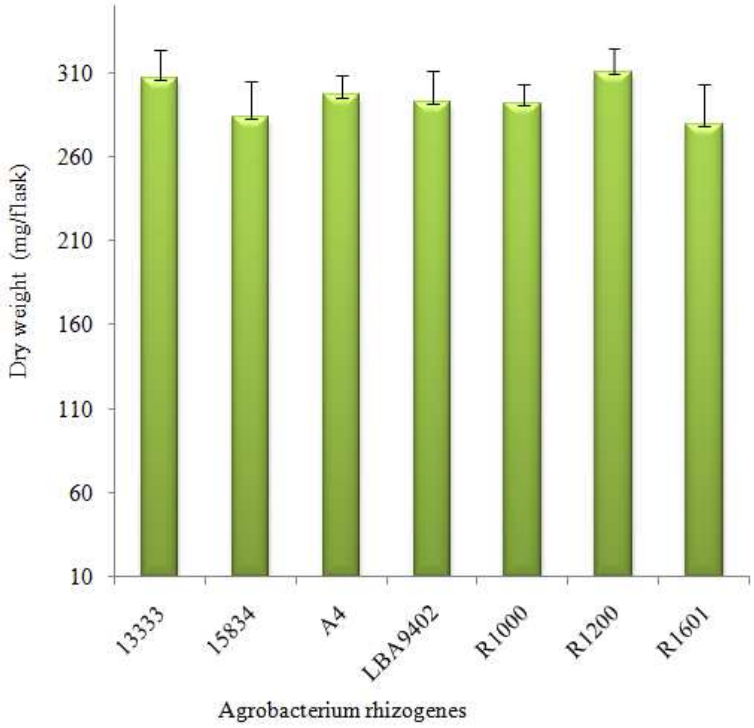

Fig. 2. Effect of strains of *A. rhizogenes* on the growth of *A. rugosa*hairy root cultures (The values represent the mean±SD of three independent measurements)

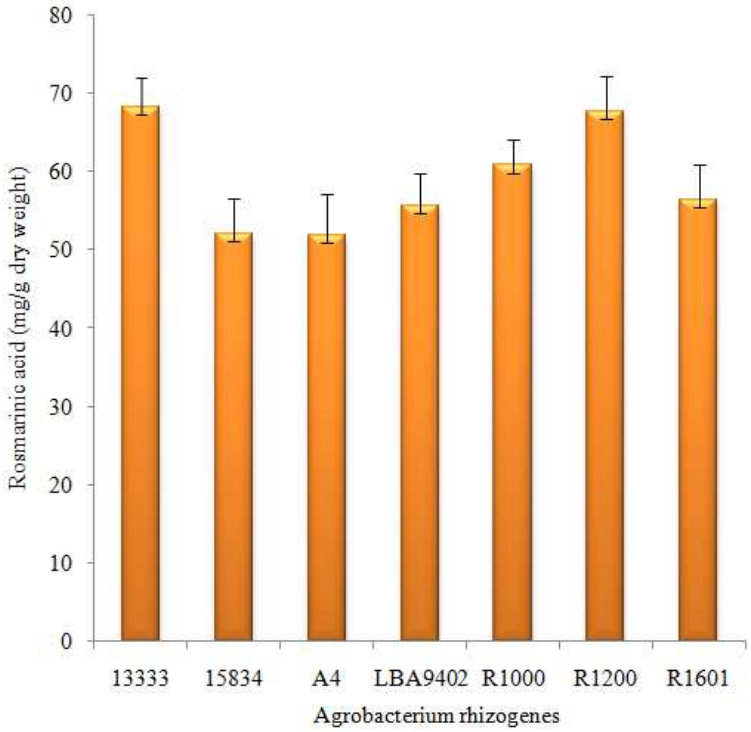

Fig. 3. Effect of *A. rhizogenes* on rosmarinic acid production in *A. rugosa*hairy root cultures (The values represent the mean±SD of three independent measurements)

Table 1.Influence strains of *A. rhizogenes*forthe growth of *A. rugosa*hairy root cultures

Agrobacterium strains	Number of hairy roots	Root length (mm)
13333	13.0±1.7	20.4±1.7
15834	9.7±1.3	16.2±1.1
A_4	11.7±0.8	16.5±1.4
LBA9402	10.6±1.3	18.2±1.3
R1000	9.6±1.3	15.8±0.8
R1200	13.6±1.4	19.4±1.4
R1601	12.3±0.8	17.4±1.1

The values represent the mean±SD from three independent measurements

Discussion

It is well reported that *A. rhizogenes* strains showed positive response for hairy root development and also responded highly showing vigorous growth behavior and finally a wide range variation of accumulation of secondary metabolites. From a previous study which was reported a long time before where different *A. rhizogenes* strains were used to examine rooting ability, accumulation of saponin and amount of astragal sides in *Astragalusm on gholicus* (Ionkova *et al*., 1997). The hairy roots in *Capsicum* species was initiated through *A. rhizogenes* strains has also been reported (Setamam *et al*., 2014). From the research findings of (Vanhala *et al*., 1995; Mateus *et al*., 2000) where they reported that *Agrobacterium* infection was shown to increase root growth and higher accumulation of alkaloids and tropane content in *Hyoscyamus muticus* transgenic root cultures. *Gentiana macrophylla*hairy root cultures were evaluated after infection with four *A. rhizogenes* strains,where different responses were observed in each hairy root linefor their root growth and also for accumulation of secoiridoid glucoside, gentiopicroside (Tiwari *et al*., 2007). The selection of an effective *Agrobacterium* strain is highly responsible for the production of transformed root cultures on types of plant species and it could t be determined empirically.

The comparative abilities of the *A. rhizogenes* strains like 13332, 15834, R1000, R1200 and R1601 were studied in *Rubiaakane* to check the ability of hairy root formation and level of anthraquinones production (Lee *et al*., 2010). It is reported that different *A. rhizogenes* strains (i.e., A_4, 15834, K599, LBA 9402, 9365 and 9340) helped to induce transformed hairy roots in shoot tip meristem explants of *Artemisiaannua* Giri *et al*. (2001).

Rosmarinic acid is found in a variety of plant species as an ester of caffeic acid. Previous studies reported that hairy root cultures *A. rugosa* produces rosmarinic acid by inducing *A. rhizogenes* strain R1000 from the hairy root of the mint family of many species like *Ocimum basilicum* (Tada *et al*., 1996), *Salvia miltiorrhiza* (Chen *et al*., 2001), *Coleus forskohlii* (Li *et al*., 2005) and *Salvia of ficinalis* (Grzegorczyk *et al*., 2006). rosmarinic acid was also found. The technique of hairy roots induction could be a best planting material for the accumulation of secondary metabolites. Here in this study, we compared the abilities of seven different *A. rhizogenes* strains to induce hairy roots and identified different responses in terms of infection frequency, number of roots, root lengths, dry weights and rosmarinic acid production in *A. rugosa*. We found that *Agrobacterium rhizogenes* strain 13333 was best for accumulation of rosmarinic acid in the hairy roots of *A. rugosa.*

Conclusion

In conclusion, this report describes a quick and effective protocol of *A. rhizogenes* mediated transformation for the enhancement of hairy root cultures and accumulation of a vital secondary metabolite like rosmarinic acid in *A. rugosa* which can easily compare the effectiveness of *A. rhizogenes* strains 13333, 15834, A_4, LBA9402, R1000, R1200 and R1601. Strain 13333 showed the best characteristics to initiate hairy root and of production of rosmarinic acid. All seven strains induced hairy root formation and production of rosmarinic acid but at different levels. From our observations, we conclude that *A. rugosa* is a promising and suitable candidate for accumulation of rosmarinic acid in the in vitro root cultures.

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Author's Contributions

Woo Tae Park, Thanislas Bastin Baskar, Sun Kyung Yeo and Jong Seok Park: Conduction of experiments and analyzed the data

Nam Il Park: Planning the idea of the study and preparation of manuscript

Sangun Park: Planning, coordination and editing the manuscript.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of

the other authors have read and approved the manuscript and no ethical issues involved.

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