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Production of Hairy Roots by Two Different Strains of *Agrobacterium rhizogenes* in Castor (*Ricinus communis* L.) Plant

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Abstract:

Two different strains of Agrobacterium rhizogenes both were tested to investigate their ability for production of hairy roots in Ricinus communis L. . Booth strains used in this study were able to induce hairy roots. The strain R1000 recorded the highest transformation frequency 90.7% along with the high number 7.0 of hairy roots per explant and root length 5.0 cm . The results indicate that direct injection method was the efficient by taking bacteria from the dish and two longitudinal scratches made on hypocotyls using needle injury.

Key words: Agrobacterium rhizogenes strain, hairy roots, Ricinus communis

Agrobacterium rhizogenes is a genus of gram-negative soil bacteria belonging to the *Rhizobiaceae*, responsible for hairy root formation at the site of infection. This bacteria can donate T-DNA, excised from Ri(root inducing)-plasmids in hundreds kb in size, to the plant cell (Hooykass and Schilperoort,1992).

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Hairy root cultures have been produced in different plants using the strain R1601 including Carrot (Al-Mallah and Mohammed, 2012), Sugar beet (Al-Mallah and Al-Ne'ma, 2012), Fenugreek (Al-Mahdawe *et al.,*2013) and Cowpea (Rashed and Abdullah, 2013), and by using strain R1000 on plants of Tomato and Potato (AL-Mallah and Masyab, 2014), Common Madder (Sook *et al.,*2010), Mugwort (Sujatha *et al.,*2013), Wishbone flower (Tao and Li, 2006) and Chinese foxglove (Young *et al.,* 2012). *Agrobacterium rhizogenes*-mediated gene transfer is being widely used to obtain hairy root lines with high production of useful metabolites (Canel *et al*., 1998; Ali *et al*., 2012). *Agrobacterium*-mediated hairy roots are fast-growing, genetically stable which can also be successfully cultured in large scale bioreactors and have been intensively utilized to produce a stable and high production of secondary metabolites in several medicinally important plants (Sevon and Oksman-Caldentey, 2002).

Castor (*Ricinus communis* L.) belongs to the *Euphorbiaceae* family and is one of the important medicinally oil seed crop (Kumari *et al.*, 2008).

The aim of this study was concentrated on the production of hairy roots by the strains R1601 and R1000 using direct injection and Cocultivation methods.

Materials and Methods

Seed sterilization and germination

Seeds were harvested from mature castor (*Ricinus communis* L.) plants of variety Zanzibariensis cultivated in the field. The seeds were firstly soaked in distilled water for 24 h and surface-sterilized with 70% (v/v) ethanol for 1 min and 5% (v/v) sodium hypochlorite solution for 10 min, then rinsed three times in sterilized water. Two seeds were placed on 25 ml of 0.8% agar-solidified MS medium (Murashige and Skoog, 1962) in 100 ml

glass bottle. The medium was adjusted to pH 5.8 before adding agar, and then sterilized by autoclaving at 121 ^{o}C for 20 min.

Preparation of hypocotyls

The germinated seedlings were maintained under standard cool white fluorescent tubes at 16-h photoperiod. The hypocotyls of 3-4 cm length were cut from 10 days old seedlings by sterile blade and placed on autoclaved filter paper to remove the medium and water. These explants were used in bacterial inoculation experiments.

Preparation of *A. rhizogenes*

A. rhizogenes strain R1000 (supplied by Professor P. Urwin, Faculty of Biological Science, Leeds Univ., UK) and strain R1601 (supplied by Professor E.W. Nester, Washington Univ., USA) was grown to mid-log phase (OD A600 =0.5) at 28°C on a shaker at 180 rpm in liquid Luria Bertani medium (1% tryptone, 0.5% yeast extract, and 1%NaCl, pH 7.0) with addition of 50 µg/ml Kanamycin for strain R1000 and /100 µg/ml Kanamycin + 100 µg/ml Carbenicillin/ for strain R1601. The bacterial cells were collected by centrifugation for 10 min at 2000 rpm, and resuspended to cell density of OD A600 = 1.0 in the liquid inoculation medium /MS salts and vitamins containing 30 g/L sucrose/ (Young *et al.,* 2012).

Bacterial – hypocotyl co-cultivation

Excised hypocotyls of *R. communis* were used as explant material for co-cultivation with each of *A. rhizogenes* R1000 and R1601. The excised hypocotyls were incubated with *A. rhizogenes* suspension for 15, 30, 45 and 60 min, blotted dry on sterile filter paper, they placed vertically in sterilized agar solidified MS medium, and incubated in the dark at 25±2°C. Two days of co-cultivation, the explants were transferred to hormone-free medium containing MS salts and vitamins, 30 g/L sucrose, 250 mg/L cefotaxime, and 8 g/L agar (Al-Mallah and

Mohammed, 2012). After two weeks, 2-3 hairy roots developed at the wound sites. These hairy roots were excised aseptically and subcultured in the dark at 25±2°C on MS medium containing 1.0 mg/L NAA and 250 mg/L cefotaxime. After repeated transfers to fresh medium, rapidly growing hairy root cultures were obtained.

Bacterial direct injection of hypocotyls

The hypocotyls excised from 10 days *in vitro* grown seedlings were used for direct injection with each of *A. rhizogenes* R1000 and R1601. Excised hypocotyls (3-4 cm) were inoculated by using insulin 1.0 ml syringe to take a swap of *A. rhizogenes* culture growing in Petri dish. Then two longitudinal scratches (2 cm) were made by this syringe and then placed on dry sterile filter paper. Hypocotyls were cultured vertically in 30 ml sterilized MS solid medium using 100 ml glass bottle, then incubated in dark at 25±2°C. After two days the explants were transferred to hormone-free fresh medium containing MS salts and vitamins, 30 g/L sucrose, 250 mg/L cefotaxime, and 8 g/L agar. When the hairy roots initiate from wound sites after 5 days, the explants were placed horizontally on the medium surface to give free space for hairy roots growth. Numerous hairy roots were emerged at the wound sites after 10 days.

Hairy roots cultures establishment

The hairy roots were separated from each explants and subcultured in the dark at 25±2°C on MS medium containing 1.0 mg/L NAA and 250 mg/L cefotaxime (Iftekhar *et al.*, 2010) . After repeated transfers on fresh medium, rapidly growing hairy root cultures were obtained.

Results and Discussion

The data in table (1) expressed the ability of both strains R1000 and R1601 to produce hairy roots from Castor hypocotyls by co-

cultivation method and the suitable period was 45 min for both strains.

Table.1 Effect of co-cultivation period on production of hairy roots from hypocotyls of castor (*Ricinus communis* L.) plant by *Agrobacterium rhizogenes* strain R1000 and strain R1601.

Co-cultivation period (min)	strain R1000			strain R1601		
	No. of survival explants	No. of explants produced H.R.	Transformation frequency (%)	No. of survival explants	No. of explants produced H.R.	Transformation frequency (%)
Control	32[a]	0	0.0	27	0	0.0
15	30	11	36.6	31	4	12.9
30	28	18	64.2	32	11	34.3
45	29	19	65.5	31	13	41.9
60	31	20	64.5	28	10	35.7

[a] Total number of 35 explants were used / treatment for each strains

Again, the results indicate that strain R1000 was more efficient than strain R1601 in producing hairy roots and recorded high transformation frequencies. This could be due to the growth pattern, infectivity and plasmid copy number (AL-Mallah and Masyab, 2014).

Concerning direct injection method the results in table (2) exhibit that both strains inducing hairy roots formation. Again strain R1000 was more efficient in parameter of transformation frequency.

Table.2 Effect of direct injection on production of hairy roots from hypocotyls of castor (*Ricinus communis* L.) plant by *Agrobacterium rhizogenes* strains R1000 and R1601.

Bacterial Strain	No. of Inoculated Explants	No. of Survival Explants	No. of explants Produced H.R.	Transformation frequency (%)
R1000	60	54	49	90.7
R1601	60	56	19	33.9

The high efficiency of strain R1000 perhaps due to the *rol* genes (*rol* A, B, and C) of Ri-plasmid of *A. rhizogenes* which is

responsible for the induction of hairy roots in plants (Young *et al.,* 2012). Recent study confirmed strongly the transfer of these genes into genome of root cells (Mohammed, 2013).

The observations that strain R1000 was generally induced numerous of hairy roots by direct inoculation method compared with strains R1601(table 3).

Table.3 Differences in numbers and length of hairy roots produced from hypocotyls after 10 days of inoculation in castor (*Ricinus communis* L.) plants using two different inoculation methods by strains R1000 and R1601 of *Agrobacterium rhizogenes*.

Bacterial strain	Inoculation method	Average No. of hairy roots / explant	Average length of hairy roots (cm)
R1000	Co-cultivation	4.0	4.0
R1000	Direct injection	7.0	5.0
R1601	Co-cultivation	1.0	3.0
R1601	Direct injection	3.0	3.5

These results indicates that the inoculated hypocotyls by direct injection which placed vertically in the solid medium (Fig.1.a) induced hairy roots 5 days after inoculation (Fig.1.b) and numerous of hairy roots were formed after 10 days of inoculation which were off-white color with 3-5 cm length (Fig.1.c). Dense accumulation of hairy roots occur within two weeks of culture (Fig.1.d) .

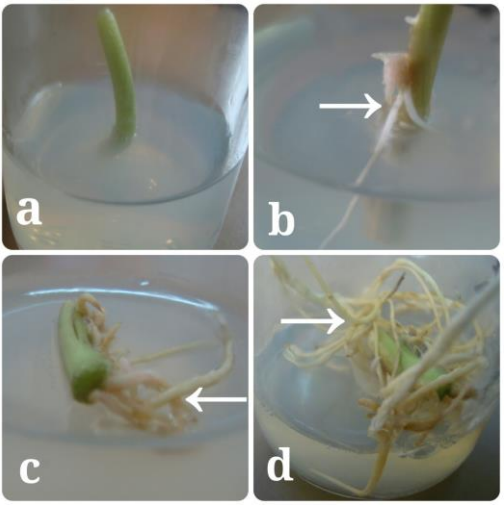

Fig. 1. Hairy root cultures of *Ricinus communis* L. using direct injection by *Agrobacterium rhizogenes* strain R1000. (a) Hypocotyl of 10 days seedling cultured on hormone-free medium containing MS salts and vitamins, 30 g/L sucrose, 250 mg/L cefotaxime, and 8 g/L agar (b) Hairy roots (arrowed) initiation in (a) after 5 days of inoculation (c) Numerous hairy roots (arrowed) produced after 10 days of inoculation (d) Accumulation of hairy roots (arrowed) of two weeks old culture .

The observation that R1000 was more capable in hairy root induction probably due to the *rol* genes present on Ri-plasmid of this strain which is responsible for the induction of hairy roots in plants (Young *et al.,* 2012). Also the specificity of *Agrobacterium* transformation is closely connected with the age and hormonal balance of the host tissue (Nin *et al.*,1997). In this study the high efficiency of strain R1000 compared with strain R1601 coincide with results of other studies (Sujatha *et al.,*2013 ; Tao and Li, 2006 ; Tiwari *et al.*, 2007). This results could possibly be explained by the different plasmids contained in the strain (Nguyen, *et al.,*1992), in which genes of Ri T_L-DNA direct the synthesis of a substance induces the cells to differentiate into root formation under the influence of endogenous auxins (Ooms, *et al.,*1986) and/or Ri T_R-DNA containing *tms* loci which directly synthesize auxin (Capone, *et al.,*1989) which induce hairy root formation. The superiority of

direct injection method for bacterial inoculation perhaps due to the insurance of bacterial penetration inside the tissues and to the fact that wound response was the most important factor for the successful transformation (Potrykus, 1990).

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