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Hairy Roots Production through *Agrobacterium rhizogenes* Genetic Transformation from *Daucus carota* Explants

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Abstract

We optimized a simple method for hairy roots production from carrot explants which is an important import technology in the view of secondary metabolites production and in-vitro mycorrhiza monoxenic culture development. In our experiment, initially surface sterilized carrot explants were infected with 48 hours old culture of *Agrobacterium rhizogenes* and then incubated under dark conditions at 27^0C for three weeks. Agrobacterium infection and callus formation was observed after 10 days of incubation period followed by hairy roots initiation, proliferation and branching formation after two weeks of incubation period. PCR analysis clearly showed that all produced hairy roots successfully carried root-inducing transferred DNA (Ri T-DNA) in their hairy roots.

Keywords: Hairy roots, *Agrobacterium rhizogenes*, *Daucus carota*, Carrot, Mycorrhiza.

Abbreviations: PCR - Polymerase chain reaction; Ri T-DNA – Root-inducing transferred DNA; YEM - Yeast extract mannitol; CTAB method - Cetyltrimethylammonium bromide method; MS medium - Murashige and Skoog medium; MW medium - Modified white medium

Introduction

Daucus carota L. belongs to family Apiaceae and has been a very popular vegetable food for humans and cultivation worldwide. It is also well known for its medicinal purposes even historically among Greeks and Romans (Kochhar SL, 1998). Carrot became value food because of its rich source of the fat-soluble hydrocarbon, carotene, which is a precursor of vitamin A. Besides the food value, different parts of carrot can be used for different medicinal purposes. Carrot roots are used as refrigerant and seeds are used as aromatic, stimulant and carminative. Carrots are also used in preparation of therapeutic agents for kidney disease, edema, uterine pain, preparation of nervine tonic and also as an aphrodisiac. An infusion of carrot has long been used as a folk remedy for threadworms. Carrot increases the quantity of urine and large amount of carrot to the diet has a favorable effect on the nitrogen balance.

Agrobacterium rhizogenes (updated scientific name: *Rhizobium rhizogenes*) is a gram negative soil bacterium that produces hair root disease in dicotyledonous plants. *Agrobacterium rhizogenes* induces the formation of proliferative multi-branched adventitious roots at the site of infection called 'hairy roots' (Baranski R, 2008). In this study, we optimized a simple method for hairy roots production from carrot explants which is an important import technology in the view of secondary metabolites production and in-vitro mycorrhiza monoxenic culture development.

Materials and Methods

Obtaining of *Agrobacterium rhizogenes* culture and culture growth conditions

Agrobacterium rhizogenes was procured from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India (*Rhizobium rhizogenes* = *Agrobacterium rhizogenes* MTCC NO.2364 and equivalent No. DSM30200). *Agrobacterium rhizogenes* culture was transferred into YEM (Yeast Extract Mannitol) broth at pH 7.2 and incubated at 28 ^{0}C, 100 rpm in an orbital shaker/incubator for 72 hours to obtain optimum growth (OD 0.5-0.6).

Collection and surface sterilization of carrot explants

For our study, fresh carrot explants were procured from local market area based on their tenderness, smoothness and freshness. Selected explants were thoroughly washed under tap water for five minutes to remove soil particles. These carrot explants were then transferred into a beaker containing five drops of a disinfectant (Dettol) solution and were washed thoroughly for five minutes. This was followed by three instance of five-minute interval rinsing with sterile distilled water. Carrot explants were then transferred into a sterile beaker containing 0.1% of mercuric chloride solution and washed thoroughly for ten minutes and followed by three instance of five-minute interval rinsing with sterile autoclaved distilled water. Finally, carrot explants were dipped in 70% ethanol for 30 seconds, exposed short time for flame, peeled out and then sliced into 0.5 cm thick discs to be infected with with *Agrobacterium rhizogenes*.

Infection of *Agrobacterium rhizogenes* culture on carrot explants

In our study, we used freshly harvested carrot explants for efficient genetic transformation. This was based on the results from a previous study where authors observed the better hairy roots initiation with fresh carrot explants (Christey MC, Braun R, 2005). The 0.5 cm surface sterilized carrot explant discs were transferred onto ½ Murashige and Skoog (MS) medium (Murashige T, Skoog F, 1962) and incubated at 28 ^{0}C for 48 hours under dark conditions (Butcher DN, 1980). After 48 hours of incubation period, the carrot explants were infected with two loop full cultures of 72 hours grown *Agrobacterium rhizogenes* (OD=0.6). These infected explants were then incubated at 28 ^{0}C under dark conditions for 2-3 weeks and observed closely for any hairy roots formation.

DNA extraction and PCR analysis

Genomic DNA was isolated from hairy roots of carrot and untransformed roots using cetyltrimethylammonium bromide (CTAB) method as described by Saghai-Maroof et al (Saghai-Maroof MA et al, 1984). PCR was performed to verify the presence of transgenes in the putatively transformed hairy roots. The transgene region was amplified by PCR using gene specific forward 5′CTAGAAACC ATCACGATGCTCTCGC3′ and reverse 5′GGCGTC AACTTACTGG TAGT TGAAC3′primers with initial denaturation at 94 °C for 4 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 59 °C for 45 seconds, extension at 72 °C for 2.20 minutes and final extension at 72 °C for 5 minutes. The amplified fragments were separated by electrophoresis on a 1.2 % agarose gel and detected by staining with ethidium bromide.

Results and Discussion

Agrobacterium infection and hairy root production on carrot explants

Carrot is an important model plant for genetic transformation and for establishing of monoxemic culture of mycorrhiza (Bidondo LF et al, 2012) (Danesh YR et al, 2006) (Dhakulkar S et al, 2005). An effectively optimized and simple procedure for hairy roots production in carrot explants is crucial and can also be very resourceful (Smith S, Dickson S, 1997).

In our study, initially surface sterilized carrot explants were transferred onto MS medium and then infected with two days old cultures of *Agrobacterium rhizogenes* (0.5 to 0.6 OD). After infection, the explants were incubated for 2-3 weeks under dark conditions at 27 ^{0}C. Ridgway et al (2004) achieved hairy roots in carrot explants in a similar manner using two days old culture of *Agrobacterium rhizogenes* (Ridgway HJ, 2004).

We noted that after ten days of incubation period, callus induction was observed followed by hairy roots initiation at the edges of infected carrot discs (Figures 1: A-B). Over the next 2-3 weeks, hairy roots proliferation and formation of lateral branching was observed on carrot disc and hairy roots also showed negative geotropism (Figures 1: B-G). M Srinivasan et al (2014) established similar results of hairy roots production from carrot disc through genetic transformation (M Srinivasan et al, 2014).

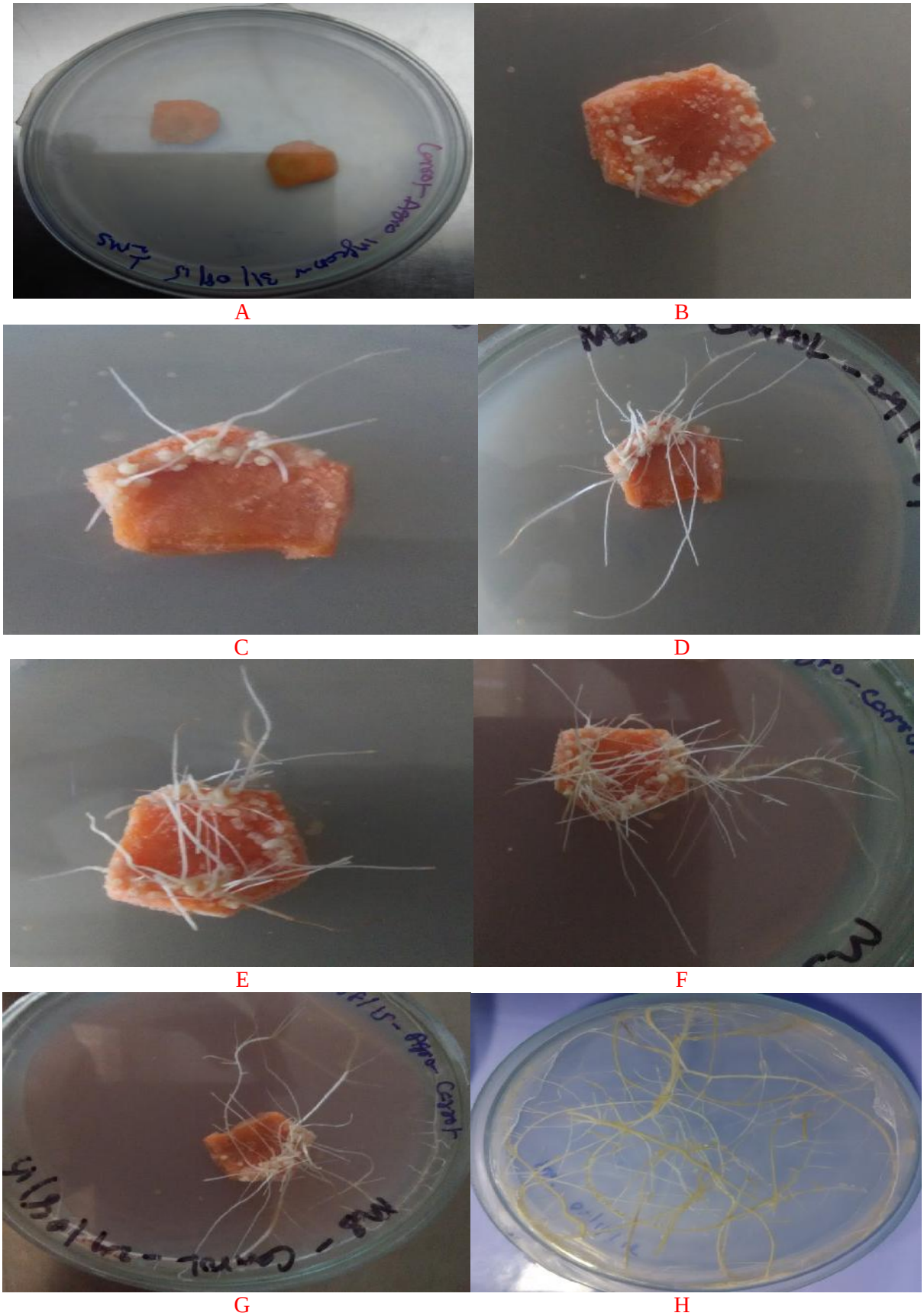

Figure 1: A - Agrobacterium infected carrot explants; B - Infected carrot explants; C - Carrot explants with hairy root initiation; D-G – Hairy roots proliferation and branching formation; H - subculture of hairy roots on antibiotic medium supplemented with cefotaxime.

In our study, we observed the better growth of hairy roots on MS media, so we standardized the MS media for initiation and sub culturing of hairy roots. Some of the previous studies also reported good growth on MS media than modified white (MW) medium (Mugnier J, 1988) (Becard G, Fortin JA, 1988). Hairy roots produced and sub cultured in our study showed much faster growth than normal roots and survived on an average of three months on a one time supplemented nutrient MS agar medium (Tepfer D, 1989).

PCR Analysis of transgenic hairy roots

Total genomic DNA was extracted from untransformed normal carrot roots and five independent transformed hairy roots. This extracted DNA was used for PCR amplification. A 300bp fragment corresponding to the root-inducing (Ri) gene was present in all of the five transformed hairy roots, but was absent in the untransformed control plants (Figure 2). This clearly demonstrated that all produced hairy roots are transformed hairy roots.

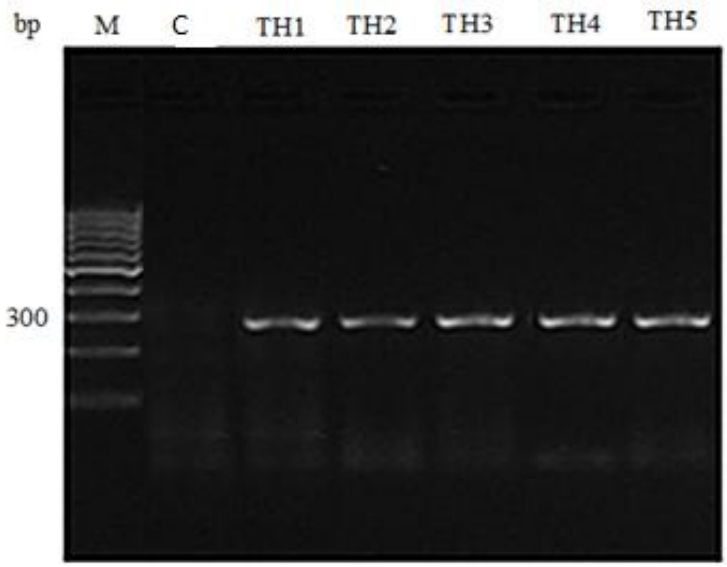

Fig. 2; DNA gel picture containing PCR amplified product of Ri T-DNA gene in transgenic hairy roots (TH). Lane M - Molecular marker; Lane C – Control; Lane TH1 to TH5 - Putative transferred hairy roots.

Conclusion

By this experiment, finally it can be concluded that the 0.6 OD of *Agrobacterium rhizogenes* culture induced high branching hairy roots in sterile carrot explants after two weeks incubation period under dark condition at 26 ^{0}C.The produced carrot hairy roots have multiple industrial applications in view of secondary metabolites (β-carotene) production and *in-vitro* mycorrhizal spores (root symbiotic fungi involved in nutrients absorption from soil) development.

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Conflict of Interest: The author has no conflicts of interest to disclose.

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