Study on the growth patterns of transformed carrot hairy roots in an optimized system

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Danesh, Y.R., Mohammadi Goltapeh, E. and Alizadeh, A. (2006). Study on the growth patterns of transformed carrot hairy roots in an optimized system. Journal of Agricultural Technology 2(1) 89-97.

Growth patterns of transformed carrot hairy roots in an optimized system of root induction were studied. Inoculation of mature carrot root sections was carried out using a mixture of four isolates of *Agrobacterium rhizogenes* that had been grown in three culture media (LB, YMA and modified medium) with 36 hours incubation time before inoculation. Results showed that most assays resulted in hairy root formation, although this was not equal. The source of the carrot was one of the most important factors, with mature, freshly harvested carrots showing better hairy root production. These roots had the ability to quickly form numerous lateral roots as well as a negative geotropic growth habit. Lateral roots were initiated from the both sides of the carrot discs and occurred from 8-10 days to 3-4 weeks. Root growth on MW medium followed a distinct pattern over 20 days and root elongation rates did not have a regular exponential trend over time.

Key words: *Agrobacterium rhizogenes*, carrot hairy root, growth patterns, Iran

Introduction

Great advances have been made in recent years with *in vitro* cultivation of isolated roots, especially the culture of genetically transformed roots following infection of plant tissues by *Agrobacterium rhizogenes* Conn. (Riker *et al*., 1930; Dhankulkar *et al*., 2005; Fu *et al*., 2005). This bacterium is a soil-borne pathogen causing "hairy root" disease in dicotyledonous plants. This stable transformation (Tepfer, 1989) produces Ri (Root inducing) T-DNA transformed plant tissues that are morphogenetically programmed to develop as roots. Their modified hormonal balance makes them particularly vigorous and allows profuse growth on artificial media (Tepfer, 1989). *Daucus carota* L. (carrot) and *Convolvolus sepium* L. (bindweed) were among the earliest species to be transformed using this bacterium (Tepfer and Tempe, 1981).

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These transformed roots have since served in a wide range of fundamental and applied studies. One of the most important has been the study of AM symbiosis (Mugnier and Mosse, 1987; Becard and Fortin, 1988; Becard and Piche, 1992; Diop *et al*., 1992). Although carrot hairy roots have been used in studies since 1988, there are no comprehensive reports of the growth patterns of these roots in an optimized condition. The objective of this study was to show the growth pattern of these transformed roots in an optimized system in Iran.

Materials and methods

Bacterial strain preparation

Four isolates of *Agrobacterium rhizogenes* (A4s, A4v, AG1 and Arif) were used in this study in 2005. A suspension of each strain was prepared by inoculating a loop of the colony into the Lauria-Bertani (LB) broth ($10gL^{-1}$ Bacto tryptone, $5gL^{-1}$ Bacto yeast-extract, $5gL^{-1}$ NaCl) and incubating overnight on a shaker at 150 rpm at 28ºC (Becard and Fortin, 1988). This liquid stock culture was used for further subcultures according to standard methods with some modifications (Becard and Fortin, 1988; Smith and Dickson, 1997) as follows: A loop of each strain suspension was inoculated into the three plates containing LB+Agar (15-20 gL^{-1} agar), yeast mannitol agar (YMA) ($2gL^{-1}$ mannitol, $3gL^{-1}$ glucose, $5gL^{-1}$ sucrose, 0.5 gL^{-1} K_2HPO_4, $0.2gL^{-1}$ $MgSO_4$. $7H_2O$, $0.1gL^{-1}$ NaCl, $0.05gL^{-1}$ $CaSO_4.2H_2O$, $0.1gL^{-1}$ NH_4Cl, $1gL^{-1}$ yeast extract, 15-20 gL^{-1} agar) and a modified medium ($2gL^{-1}$ casein hydrolysed, $2gL^{-1}$ tryptone, $1gL^{-1}$ yeast extract, $6gL^{-1}$ peptone, $5gL^{-1}$ sucrose, 0.5 gL^{-1} $MgSO_4$. $7H_2O$, 15-20 gL^{-1} agar). The plates were incubated at 28ºC in darkness for 36 hours before inoculation on carrot discs. After this time, growing colonies of each strain on the above mentioned media were collected by a sterilized spatula and mixed with each other for inoculation.

Carrot discs preparation and inoculation

Carrot discs were prepared using the optimized method (Danesh *et al*., 2006). For inoculation, a loop of 36 hour old bacterial mixture was inoculated on the base of carrot discs and plates were incubated in darkness at 25ºC for 48-72 hours. Cultures were inverted onto the MS medium (50% diluted) amended with antibiotics (500 mgL^{-1} cefotaxime) and incubated in moderate light at 25ºC till the callus appeared. Two to three successive subcultures were carried out on this medium (Danesh *et al*., 2006).

Root segments maintenance

The proliferating discs should be transformed to the darkness since the light has negative effects on root growth. Proliferating discs with 5-7 cm long roots were selected and these root segments aseptically excised, put on MS medium (50% diluted) amended with antibiotics (300 mgL^{-1} cefotaxime) and incubated at 25ºC. After sufficient growth, the 3 cm long root pieces were cut and transferred onto the MS medium (50% diluted) with 150 mgL^{-1} cefotaxime and incubated again at 25ºC. Once growth had stabilized, cloned cultures were initiated by aseptically excising 1 cm long root pieces and transferring them into the glass jars containing modified White medium (MW) and kept at 25ºC in darkness. The pH of all media was adjusted at 5.5-5.8 before sterilization (Danesh *et al*., 2006).

Growth pattern of roots in modified white medium (MW)

The elongation rate as well as the growth pattern of roots were studied by placing ten 1 cm tips of lateral roots on modified white medium (MW) for 20 days in inverted Petri plates and incubated at 25ºC in darkness. The linear elongation (cm) of each individual root was measured every 2 days and used to calculate the growth rate of the corresponding tissue (cmd^{-1}) and to establish a growth pattern during the culture. The treatment in this study was the time (2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 days) and each treatment consisted of 10 replicates. The data (root elongation and root elongation rate) were then analyzed in complete randomized block design (CRBD) using SAS software. Means were compared according to Duncan's Multiple Range Test at 5% probability level. Regression models of root elongation as well as root elongation rate were also obtained.

Results

In this study, two kinds of roots started forming on the carrot discs after 8-10 days. The first type was a delicate, aerial hairy root without any growth and after cutting from the discs they rotted after one week. The second type was a slightly thicker transformed root. One desirable characteristic of these transformed roots was their ability to quickly form numerous lateral roots. Another characteristic observed in transformed roots was the inversion of their geotropic habit of growth. Roots were initiated from the both sides of the discs (Fig. 1).

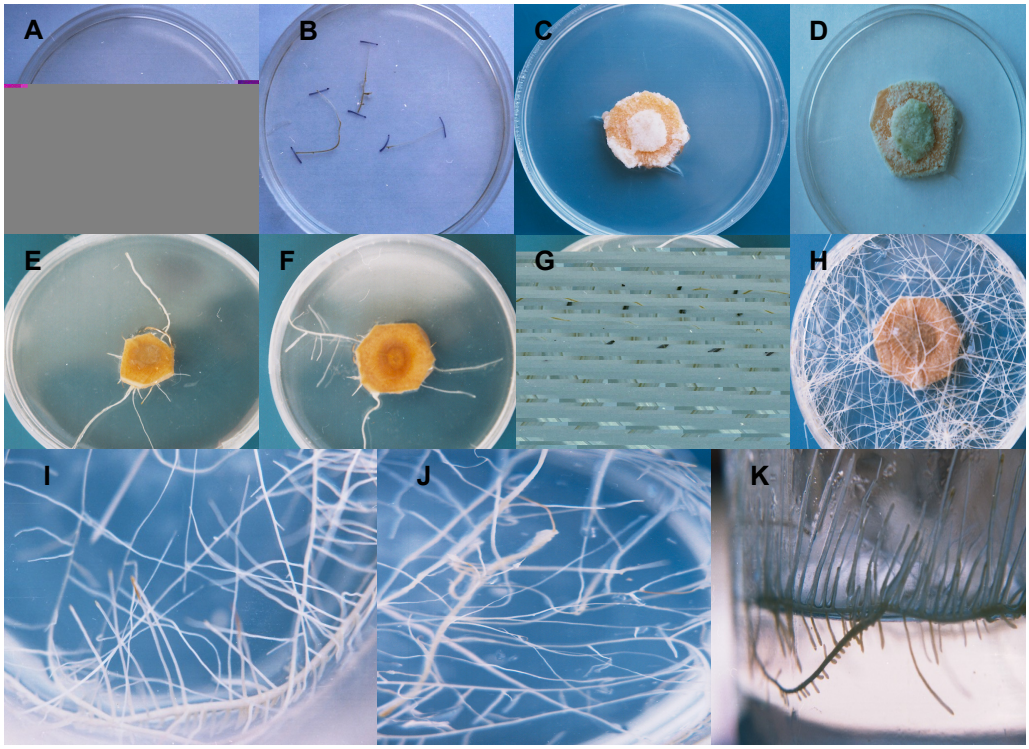

Fig 1. Non-transformed roots (A, B). Callus formation on carrot discs (C, D). Initiation and growth of transformed roots on carrot discs (E-H). Lateral branches of Transformed roots (I, J). Negative geotropism of transformed roots (K).

Hairy root initiation continued to occur from 8-10 days to 3-4 weeks. In some plates, the callus was observed without any root induction. This may be due to instability of the new genome or due to no expression of genes involved in root induction. After 20 days of culture on MW medium, individual roots were very similar, showing a mean length of 18.4 cm with a low coefficient of variation 2.04%. The results of root elongation analysis (Table 1) showed that there were significant differences among the root elongation means in different days. Also, the trend of root elongation (Fig. 2) followed a distinct pattern over the 20 days of culture. The root elongation mean was 0.26cmd^{-1} between days 2 and 4. Then, it was 0.83cmd^{-1} and 1.3cmd^{-1} between days 4 to 12 and 12 to 20, respectively. The suitable regression model for root elongation is as follows:

$$Y = -0.0011X^3 + O.O66X^2 + O.87$$
$$R^2 = 99\%$$

Since the root elongation means data were cumulative, we decided to use a root elongation rate index. The results of this index analysis (Table 2) showed that there were significant differences among the root elongation rate means. Also, the trend of root elongation rate (Fig. 3) was not regular and constant. As shown in Table 2, the greatest amount of root elongation rate was on day 14 and the least on day 2. Therefore, there was not any exponential trend in root elongation rates over the 20 days. The suitable regression model for the root elongation rate is as follows:

$$Y = -0.00024X^4 + 0.011X^3 - 0.17X^2 + 1.23X - 1.94$$
$$R^2 = 90\%$$

Table 1. Comparison the root elongation means over 20 days culture on MW medium.

Day**	Root elongation mean (cm)
0	1J
2	1.04J
4	1.55I
6	3.21H
8	4.97G
10	6.52F
12	8.14E
14	10.79D
16	13.36C
18	15.98B
20	18.41A

** Significant at 1% probability level

Discussion

Carrot is one of the most amenable species of plant for hairy root production (Dhankulkar *et al*., 2005). It is therefore not surprising that most treatments resulted in hairy root formation, although not equally. Carrot hairy roots have been used to initiate monoxenic culture since 1988, but detailed studies on growth patterns of these roots has not been carried out especially in Iran. The carrot source is an important source in hairy root production.induction. Freshly harvested carrots are invariably better at initiating hairy roots overall. This may be due to the active nature of the carrots. However, cultivar differences cannot be ignored and have been reported as a factor influencing hairy root initiation (Christey and Braun, 2004). The transformed roots were better adapted to growth in culture than

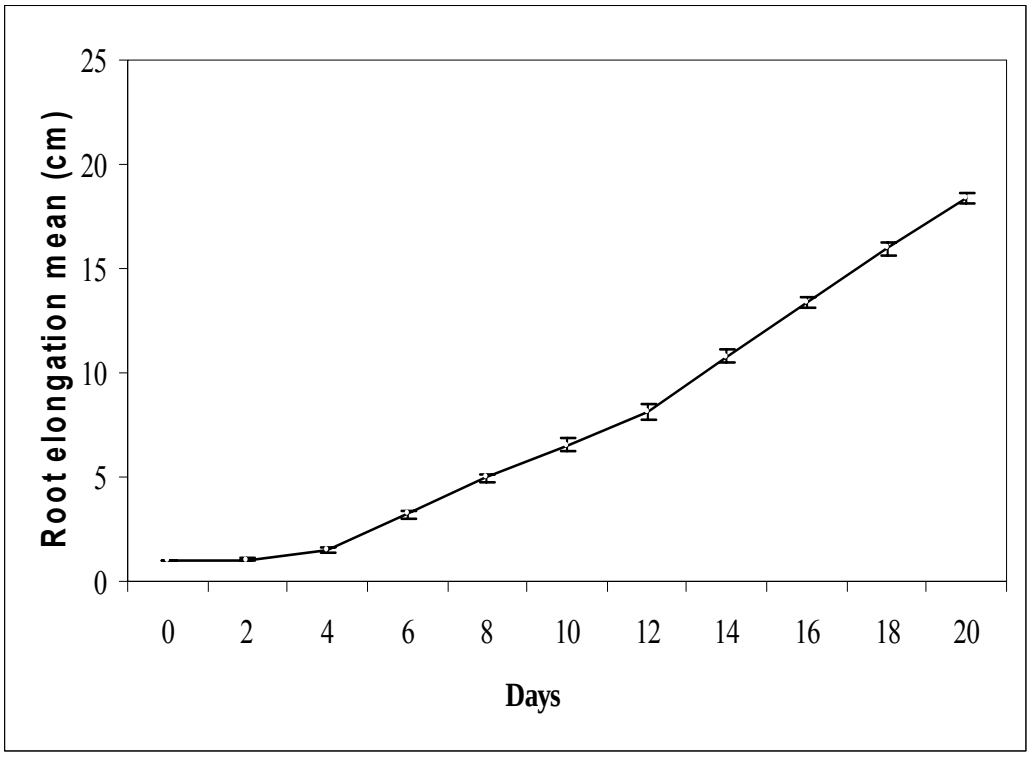

Fig 2. Trend of root elongation over 20 days culture on MW medium.

Table 2. Comparison of the root elongation rate means over 20 days culture on MW medium.

Day**	Root elongation rate mean (cm)
14	2.65A
18	2.62A
16	2.57A
20	2.43B
8	1.76C
6	1.66CD
12	1.62D
10	1.55D
4	0.51E
2	0.04F

** Significant at 1% probability level

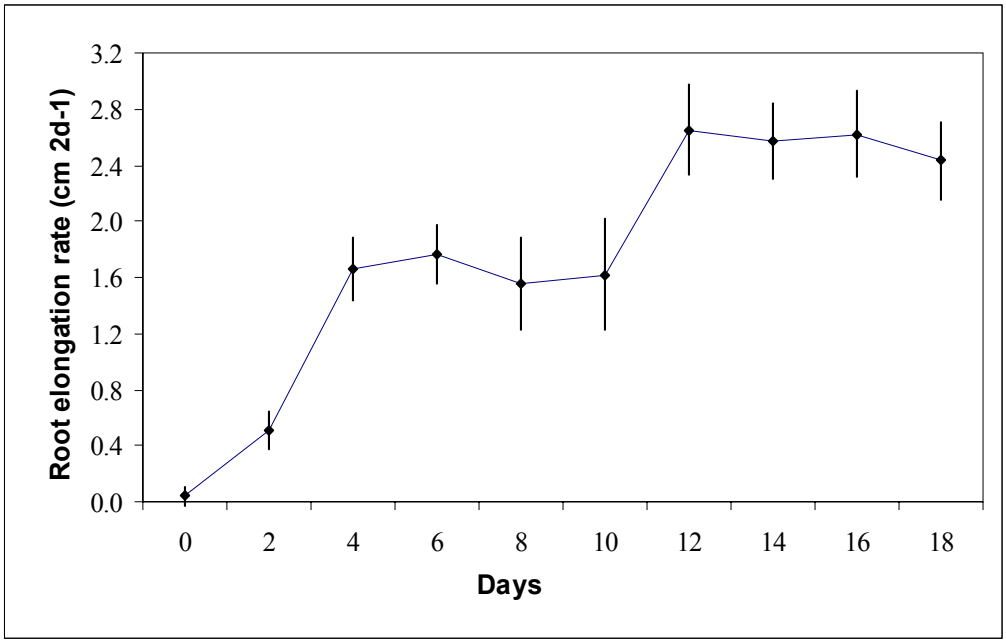

Fig 3. Root elongation rates during 20 days of culture. Each point is a mean value of 10 root elongation rates measured every 2 days on MW medium. Vertical lines indicate sample standard deviations.

normal roots and survived longer periods without subculture (Tepfer, 1989). Their hormonal balance is modified, allowing profuse proliferation on synthetic media. It is generally accepted that this modification induces the production of growth hormones in the roots, thereby eliminating the necessity of incorporating of plant hormones into the culture medium. Stability of the transformation over time is dependent on the host cultivar and bacterial strain combination (Labour *et al*., 2003). In addition, depending on the gene incorporated into the plant genome, the root can have a change of geotropism. Some hairy roots show a highly negative geotropic nature, some only have a slightly negative geotropic behaviour, while others keep their positive geotropism. This is due to a change of auxin sensitivity and the redistribution of this hormone after root transformation (Legue *et al*., 1996). Roots with a negative geotropism should be inoculated in an inverted position, to make the roots grow inside the medium. As mentioned before, the callus was observed in some treatments without any root induction. This may be due to instability of the new genome or due to noexpresion of genes involved in root induction.

Various culture media have recently been used for growing transformed roots such as Murashige and Skoog (MS) medium (Mugnier, 1988) and modified White medium (MW) (Becard and Fortin, 1988). The latter medium

is preferred to MS, even when diluted, because it allows significantly better growth of the roots. Specifically the presence of ammonium in MS medium causes a rapid (less than two weeks) drop in the pH of the culture medium that is important for root growth. On White medium, nitrogen exclusively in the form of nitrate is assimilated, which counteracts the acidification of the culture medium following root growth. In this way, the culture medium is buffered and maintains pH at 6 for several months. It was shown that the trend of root elongation followed a distinct pattern over the 20 days and the growth means were not similar in different days. The trend of root elongation rate was not regular and did not follow a constant exponential trend.

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(Received 7 April 2006; accepted 12 May 2006)