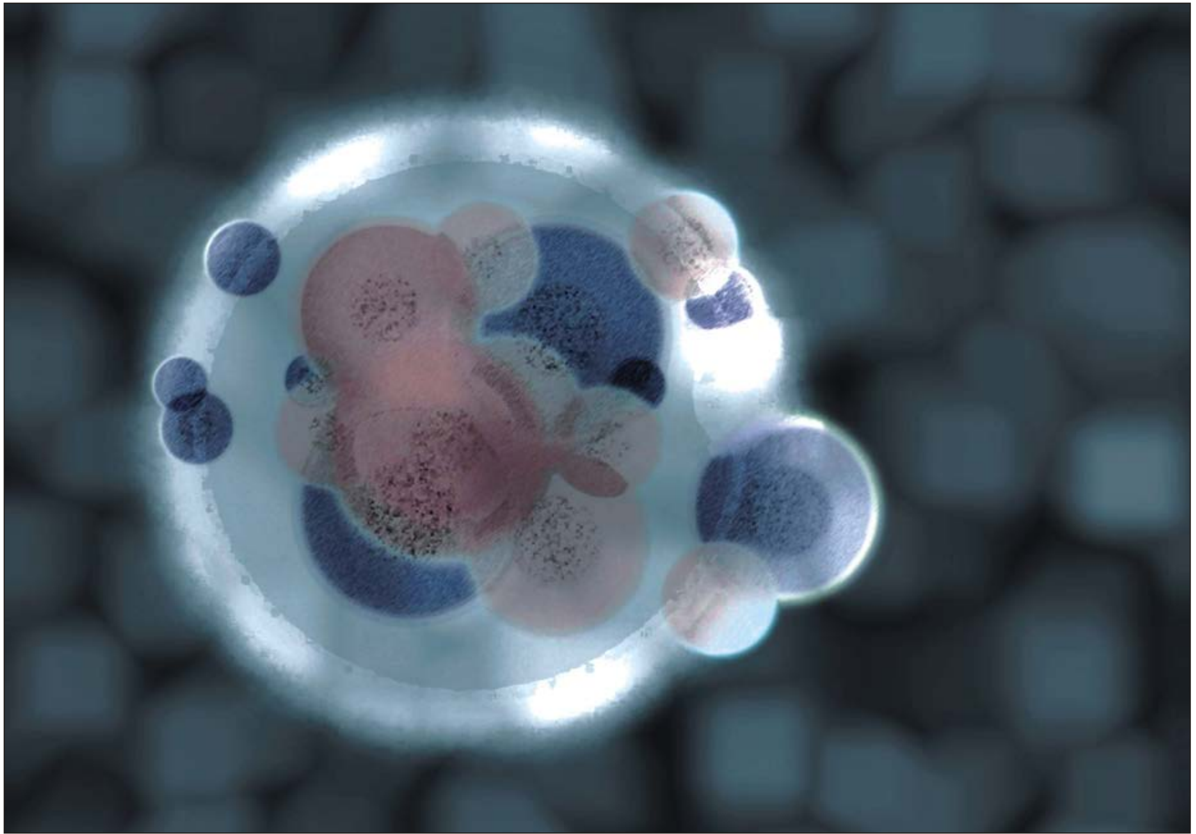

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 6 (1): 87-91, 2006
ISSN 1727-3048
© 2006 Asian Network for Scientific Information

Optimizing Carrot Hairy Root Production for Monoxenic Culture of Arbuscular Mycorrhizal Fungi in Iran

Y.R. Danesh, E. Mohammadi Goltapeh, A. Alizadeh and M. Modarres Sanavy
Department of Plant Pathology, College of Agriculture, Tarbiat Modarres University,
P.O. Box 14115-336, Tehran, Iran

Abstract: This research describes the optimized method of carrot hairy roots production for monoxenic culture of Arbuscular Mycorrhizal Fungi (AMF) in Iran. Inoculation of mature carrot root sections was carried out using four isolates of *Agrobacterium rhizogenes* as well as a mixture of isolates that had been grown in three culture media (LB, YMA and modified medium) with different incubation times (24 and 36 h) before inoculation. Three methods of carrot hairy root initiation were compared in an attempt to optimize hairy root production in terms of the number of root initiations and the health (lack of rotting) of the explants. Results showed that most treatments resulted in hairy root formation, although not equally so. It was shown that the mixture of bacterial strains from three culture media increased the frequency of bacterial colonies with a good transformation characteristic. Also, incubation of inoculated carrot discs for 48-72h in darkness provided a suitable condition for bacterial strains to insert their copies of Ri T-DNA. The source of the carrots was one of the most important factors. With mature, freshly harvested carrots showing better hairy root production. Dissection of the cortex to expose the cambium had a positive effect on hairy root initiation. Increasing bacterial strains incubation time from 24 to 36 h improved transformation efficiency in all treatments but not in method III. Results showed that the best method for initiation of hairy root induction in carrots especially in Iran is the method I.

Key words: Vesicular arbuscular mycorrhiza, monoxenic culture, carrot hairy root, *Agrobacterium rhizogenes*

INTRODUCTION

Root-organ cultures were first developed by White *et al.*[3,4,18]. These authors used excised roots on synthetic mineral media supplemented with vitamins and a carbohydrate source. Great advances have been made in recent years with *in vitro* culture of genetically transformed roots following infection of plant tissues by *Agrobacterium rhizogenes* Conn.[4] by other researchers[5-7]. The bacterium is a soil-borne pathogen causing “hairy root” disease in dicotyledonous plants. This stable transformation[8] 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[8]. *Daucus carota* L. (carrot) and *Convolvolus sepium* L. (bindweed) were among the earliest species to be transformed using this bacterium[9]. These transformed roots have since served in a wide range of fundamental and applied studies. One of the most important of these has been the study of the AM symbiosis[10-13].

Although carrot hairy roots have been used in such studies since 1988, there are no reports of an optimized and reliable method for obtaining these roots especially in Iran. The objective of this study was to compare three different and modified methods for hairy root production in carrot and to set up an optimized system for hairy root production in Iran.

MATERIALS AND METHODS

This study was carried out during the year 2005.

Bacterial strains preparation: Four isolates of *Agrobacterium rhizogenes* (A4s, A4v, AG1 and Arif) were used in this study. A suspension of each strain was prepared by inoculating a loop ful of the colony into the Lauria-Bertani (LB) broth (10 g L^{-1} Bacto tryptone, 5 g L^{-1} Bacto yeast-extract, 5 g L^{-1} NaCl) and overnight incubating on a shaker at 150 rpm at 28°C[10]. This liquid stock culture was used for further subcultures according to standard methods with some modifications[10,14] as follows: A loop ful of each strain suspension was inoculated into the three plates containing LB+agar (15-20 g L^{-1} agar), yeast mannitol agar (YMA) (2 g L^{-1} mannitol, 3 g L^{-1} glucose, 5 g L^{-1} sucrose, 0.5 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4 . 7H_2O$, 0.1 g

Corresponding Author: Y.R. Danesh, Department of Plant Pathology, College of Agriculture, Tarbiat Modarres University. P.O.Box 14115-336, Tehran, Iran

L^{-1} NaCl, 0.05 g L^{-1} $CaSO_4$. $2H_2O$, 0.1gL^{-1} NH_4Cl, 1 g L^{-1} yeast extract, 15-20 g L^{-1}agar) and a modified medium (2 g L^{-1} casein hydrolyzed, 2 g L^{-1} tryptone, 1 g L^{-1} yeast extract, 6 g L^{-1} peptone, 5 g L^{-1} sucrose, 0.5 g L^{-1} $MgSO_4$. $7H_2O$, 15-20 g L^{+} agar). The plates were incubated at 28°C in darkness for 24 and 36 h 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. In this study we used 4 bacterial strains as well as a mixture of them (complex) for inoculation.

Preparation of carrot discs: Freshly harvested carrots were washed thoroughly with water and a detergent (Rika, Darugar co.) were dipped in ethanol (96%) for 30 sec and superficially flamed. Then surface sterilized in 1.5% Sodium hypochlorite for 20 min with occasional stirring on shaker. They were further rinsed three times (5 min each) in sterile distilled water. Each carrot was peeled separately to remove the cortex and to expose the cambium for inoculation. These decorticated carrots were then sliced into several 0.5 cm thick discs and placed onto 0.8% water agar plates with the basal sides faced upwards.

Carrot discs inoculation and maintenance: We used three methods for hairy root induction in carrots. Methods I and II were based on standard method[10] with some modifications. With the first method (I), a loopful of 24 and 36 h colonies of each strain (A4s, A4v, AG1, Arif, Complex) was inoculated on the basal faces of carrot discs and plates were incubated in darkness at 25°C for 48-72 h. Then cultures were inverted onto the MS medium (50% diluted) amended with antibiotics (500 mg L^{-1} cefotaxime) and incubated in moderate light at 25°C till the callus appeared. Two to three successive subcultures were carried out on this medium. In second method (II), the inoculation process were similar to the method I but 48-72 h after inoculation and incubation in darkness at 25°C , the cultures were inverted as in the method I and incubated again in darkness at 25°C till the appearance of callus.

The third method (III) was based on Smith and Dickson[14] with some modifications. For each carrot disc, the basal side of disc was dipped into 24 and 36 h liquid cultures of each bacterial strain in YMA medium for 10 sec. The inoculated discs were t hen removed from the culture medium, dried on a sterile filter paper and placed onto 0.8% water agar with the inoculated sides faced upwards and incubated at 25°C in darkness. Two to three successive subcultures were carried out on MS medium (50% diluted) amended with antibiotics (500 mg L^{-1} cefotaxime).

The proliferating discs should be transferred to the darkness since the light has negative effects on roots 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 mg L^{-1} cefotaxime) and incubated at 25°C. After sufficient growth, the 3 cm long root pieces were cut and transferred onto the MS medium (50% diluted) with 150 mg L^{-1} cefotaxime and incubated at 25°C . Once stability of growth, 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 25°C in darkness.

The pH of all media was adjusted at 5-5.8 before sterilization. Each treatment in this study consisted of 14 replicates. Controls consisted of uninoculated carrot discs. For each treatment, the percentage of carrot discs producing stable hairy roots was calculated by counting the number of plates containing hairy roots after 3-4 weeks. These nonparametric data were then ranked and analyzed in a split-split plot experiments with complete randomized block design (CRBD) using SAS software[15] to determine the best treatment. Means were compared according to Duncan's Multiple Range Test at 5% probability level.

RESULTS

Three methods of carrot hairy root initiation were compared in an attempt to optimize hairy root production in terms of the number of root initiations and the health (lack of rotting) of the explants. In this study, two kinds of roots started forming on the carrot discs after 8-10 days. The first type was delicate, aerial hairy roots without any growth after cutting from the discs and rotted after one week. In contrast, the second type was slightly thicker transformed roots. 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). Hairy root initiation continued to occur from 8-10 days to 3-4 weeks. Approximately all of control discs were still healthy after 4-5 weeks but ultimately began to rot. Two types of rot were observed. The first was due to overgrowth of the *A. rhizogenes* and had a typical slimy appearance. The second type of rot was a general browning and collapse of the carrot discs.

The comparison of the methods I and II led to significant results (Table 1 and 2). Results in Table 1 showed a significant difference between the incubation in moderate light and darkness as well as between the bacterial incubation periods of time (24 and 36 h) before

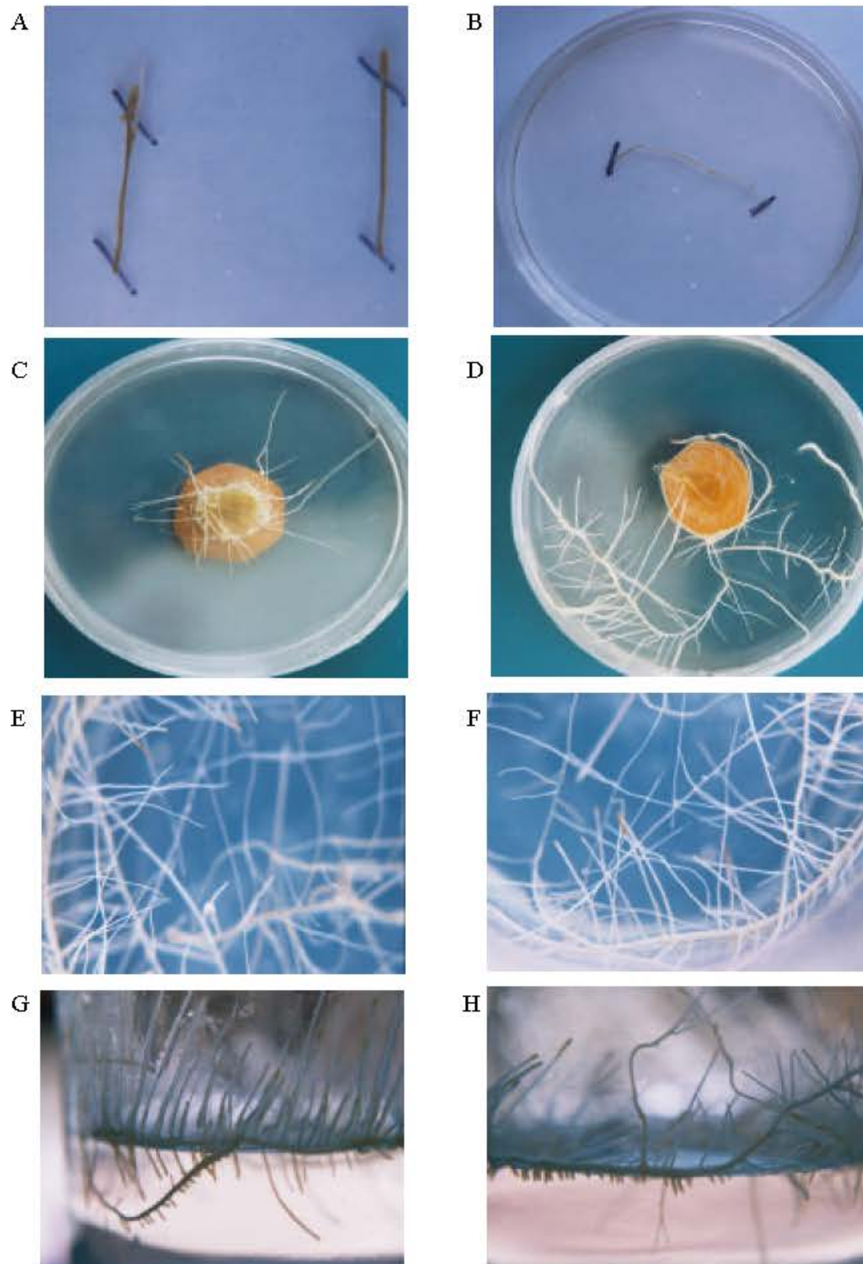

Fig 1: Nontransformed roots (A, B). Transformed roots growth on both sides of discs (C, D). Transformed roots with numerous lateral branches (E, F). Transformed roots with negative geotropism growth (G, H)

inoculation. Effects of bacterial incubation periods of time were more significant on hairy root induction compared with light effects. Also results showed a significant difference among the bacterial strains and the mixture of four bacterial strains (Complex) had the best results (Table 2). Generally it can be concluded that the best treatment is the mixture of bacterial strains in combination with 36 h incubation time and moderate light 48-72 h after inoculation and the method I had more significant effects than the method II on hairy root induction overall. The majority of the rot in this case was due to *A. rhizogenes* overgrowth and was in treatment consisted of A4v strain, 36 h incubation time and incubation in darkness 48-72 h after inoculation. Also the callus was observed in some plates without any root induction even after 5-6 weeks.

Comparison of the methods I and III also had significant results (Table 3). A significant difference

Table 1: Effects of incubation time and light in methods I and II on number of plates containing stable transformed roots

Incubation time (h)**	Light**	Mean
36	ML(I)	5A
	D(II)	3B
24	ML(I)	2BC
	D(II)	1C

**Significant at 1% probability level, D = darkness, ML = moderate light

Table 2: Effects of bacterial strain, incubation time and light in methods I and II on number of plates containing stable transformed roots

Bacterial strain**	Incubation time (h)**	Light**	Mean
Complex	36	ML	11A
		D	7BC
	24	ML	5BCDE
		D	3DEFG
Arif	36	ML	8B
		D	BCDE
	24	ML	3DEFG
		D	2EFG
AGI	36	ML	6BCD
		D	4CDEF
	24	ML	2EFG
		D	2EFG
A4s	36	ML	3DEFG
		D	3DEFG
	24	ML	1FG
		D	0G
A4v	36	ML	2EFG
		D	1FG
	24	ML	1FG
		D	0G
Control	36	ML	0G
		D	0G
	24	ML	0G
		D	0G

**Significant at 1% probability level, D = darkness, ML = moderate

Table 3: Effects of bacterial strain, incubation time and inoculation method on number of plates containing stable transformed roots

Bacterial strain**	Incubation time (h)**	Inoculation method**	Mean
Complex	36	I	11A
		III	1EF
	24	I	5CD
		III	1EF
Arif	36	I	8B
		III	1EF
	24	I	3DE
		III	0F
AGI	36	I	6BC
		III	1EF
	24	I	2EF
		III	0F
A4s	36	I	3DE
		III	0F
	24	I	1EF
		III	0F
A4v	36	I	2EF
		III	0F
	24	I	1EF
		III	0F
Control	36	I	0F
		III	0F
	24	I	0F
		III	0F

**Significant at 1% probability level

between bacterial incubation periods of time was observed for the method I treatments but not for the method III treatments. Effects of inoculation method on hairy root initiation were more significant than those of different bacterial incubation time. As compared with the method III, method I resulted in significantly improved hairy root induction. It can be said that the mixture of bacterial strains (Complex) inoculated by method I in combination with 36 h incubation time led to the best results. It was difficult to control bacterial overgrowth on carrot discs using method III even on media amended with antibiotics. Also, the percentage of the successful hairy root induction was so low (max. 7%) by this method. So it can be concluded that the application of method I had the best results with hairy root induction overall.

DISCUSSION

Carrot is one of the most amenable species of plant for hairy root production[5]. It is therefore not surprising that most treatments resulted in hairy root formation, although not equally so. Although carrot hairy roots have been used to initiate monoxenic AMF culture since 1988, an optimized method for hairy root formation have not been described especially suiting researches in Iran. In this study it was shown that the mixture of bacterial strains from three culture media (LB, YMA and modified medium) increased the frequency of bacterial colonies with a good transformation characteristic. Also, incubation of inoculated carrot discs for 48-72 h in darkness provided a suitable condition for bacterial strains to insert their copies of Ri T-DNA. The carrot source is also an important factor in hairy root induction. Freshly harvested carrots are invariably better at initiating hairy roots overall. This may be due to either the less dormant nature of the carrots. However, cultivar differences cannot be ignored and have been reported as a factor influencing hairy root initiation[16]. Also it was suggested that trimming of the carrot discs to expose the cambium would improve the generation of healthy hairy roots[14].

The transformed hairy roots were better adapted to growth in culture than normal roots and were survived longer periods without subculture[8]. Perhaps the new genome of transformed roots affects its capacity for auxin production.

Bacteria have a higher ability to produce the hairy roots on the basal sides of the slices corresponding to the apical sides because of a higher endogenous auxin level[17]. Increasing bacterial strains incubation time from 24 to 36 h improved transformation efficiency in all

treatments but not in method III. This time indicated the suitable time for exponential phase of bacterial strains for inoculation. As mentioned before, the callus was observed in some treatments without any root induction. May be it was due to instability of the new genome or due to no expression of genes involved in root induction. Proliferating discs should be transferred to darkness since the light imposes negative effects on root growth.

Various culture media have recently been used for growing transformed roots such as Murashige and Skoog (MS) medium[18] and modified white medium (MW)[10]. The last medium was 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 to the 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.

In summary, results showed that the best method for initiation of hairy root induction in carrots especially in Iran is the method I. The use of this method to produce monoxenic cultures of AMF species in Iran will provide a valuable tool for the description and recording the hyphal morphology and branching, sporulation dynamics and spore ontogeny of indigenous strains.

REFERENCES

1. Butcher, D.N., 1980. The Culture of Isolated Roots. In: Tissue Culture Methods for Plant Pathologists. Ingram, D.S. and J.P. Helgelson (Eds.), Blackwell Scientific, Oxford, pp: 13-17.
2. Butcher, D.N. and H.E. Street, 1964. Excised root culture. Bot. Rev., 30: 513-586.
3. White, P.R., 1943. A Handbook of Plant Tissue Culture. J. Cattle, Lancaster, PA, USA.
4. Riker, A.J., W.M. Banfield, W.H. Wright, G.W. Keitt and H.E. Sagon, 1930. Studies on infectious hairy root of nursery apple trees. J. Agric. Res., 41: 507-540.
5. Dhakulkar, S., T.R. Ganapathi, S. Bhargava and V.A. Bapat, 2005. Induction of hairy roots in *Gmelina arborea* Roxb. and production of verbascoside in hairy roots. Plant Sci., 169: 812-818.
6. Fu, C., D. Zhao, X. Xue, Z. Jin and F.S. Ma, 2005. Transformation of *Saussurea involucrata* by *Agrobacterium rhizogenes*: Hairy root induction and syringin production. Process Biochem. (In Press).
7. Van de Velde, W., J. Mergeay, M. Holsters and S. Goormachtig, 2003. *Agrobacterium rhizogenes*-mediated transformation of *Sesbania rostrata*. Plant Sci., 165: 1281-1288.
8. Tepfer, D., 1989. Ri T-DNA from *Agrobacterium rhizogenes*: a source of genes having applications in rhizosphere biology and plant development, ecology and evolution. In: Plant-Microbe Interactions. Kosuge, T. and E.W. Nester (Eds.). McGraw-Hill Publishing, New York, 3: 294-342.
9. Tepfer, D.A. and J. Tempe, 1981. Production d'agropine par des raciness formees sous l'action *d'Agrobacterium rhizogenes*, souche A4. C.R. Acad. Sci. Paris., 292: 153-156.
10. Becard, G. and J.A. Fortin, 1988. Early events of vesicular arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytol., 108: 211-218.
11. Becard, G. and Y. Piche, 1992. Establishment of Vesicular-arbuscular Mycorrhiza in Root Organ Culture: Review and Proposed Methodology. In: Techniques for the Study of Mycorrhiza. (Eds.) Norris, J., D. Read and A. Varma. Academic Press, New York, pp: 89-108.
12. Diop, T.A., G. Becard and Y. Piche, 1992. Long-term *in vitro* culture of an endomycorrhizal fungus, *Gigaspora margarita* on Ri T-DNA transformed root of carrot. Symbiosis., 12: 249-259.
13. Mugnier, J. and B. Mosse, 1987. Vesicular-arbuscular infections in Ri T-DNA transformed roots grown axenically. Phytopathology, 77: 1045-1050.
14. Smith, S. and S. Dickson, 1997. VA Mycorrhizas: Basic research techniques. Cooperative Research Center for Soil and Land Management. Adelaide, Australia, pp: 39
15. SAS Institute Inc., 1992. The SAS System for Windows 3.10, Release 6.08. SAS Institute Inc. Cary, North Carolina.
16. Christey, M.C. and R.H. Braun, 2004. Production of hairy root cultures and transgenic plants by *Agrobacterium rhizogenes* mediated transformation. In: Methods in Molecular Biology. Vol. 286. Transgenic Plants (Ed. L. Pena), pp: 47-60.
17. Ryder, M.H., M.E. Tate and A. Kerr, 1985. Virulence properties of strains of *Agrobacterium* on the apical and basal surfaces of carrot root discs. Plant Physiol., 77: 215-221.
18. Mugnier, J., 1988. Establishment of new axenic hairy root lines by inoculation with *Agrobacterium rhizogenes*. Plant Cell Rep., 7: 9-12.