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Research article

Study the effect of hairy root transformation on rapid growth (growth morphology) of *Nepeta cataria in vitro* cultures

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Abstract

In the current study, an efficient protocol for in vitro plant regeneration and genetically transformed root induction by *agrobacterium rhizogenes* strain *A4* was developed. Powerful response for hairy roots induction was achieved using stem explants cultured on co-cultivation half strength MS-medium containing 500 mg/l cefotaxime. Contrary leaf explants does not have any response for hairy roots induction. Maximum shoot number (23.601 ± 2.073) and root number (13.604 ± 1.673) were investigated in the transformed plants with lengths 21.611 ± 2.073 cm and 10.923 ± 0.741cm, respectively. The highest value of hairy growth (1.506 ± 0.104 g) was attained after 15 days of Culture. Hairy root lines were successfully confirmed by PCR using the *rolB* gene. Collectively, our results demonstrate that we have successfully used the methodology of transformation to induce hairy root culture of *N. cataria* and facilitate a valuable alternative approach for regeneration a genetically transformed roots.

Key words: *Nepeta cataria*, Seed germination, *Agrobacterium rhizogenes A4*, Hairy root transformation, PCR analysis.

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1. Introduction

Catinp (*Nepeta cataria* L.) is a perennial herb as a member of the mint family (Labiatae), has a considerable folkloric reputation. Catnip has a lot of traditional properties such as sedative, carminative, and antispasmodic. Moreover that *N.cataria* has been used to treat various diseases (insomnia, flatulence, upset stomach colds, flu, and fevers) [1, 2]. Catinp involved several classes of secondary metabolites essential oils including monoterpenes and terpenoids, sterols, flavonoids and phenolic compounds, [3, 4, 5, 6, 7]. Genetically

engineered root cultures have been used as a model system to study various aspects of the metabolic and molecular regulation of several natural product pathways [8]. Major interest in the transformation study is due to the possibilities in genetic manipulation of plant cells offered by the system [9]. Genetic transformation protocol has a high potential to be used in medicinal plant, established a simple, rapid and reliable protocol for plant regeneration and hairy root induction. The use of fast-growing hairy roots could have immense potential in investigating the molecular regulation of genes encoding biosynthetic enzymes of important medicinal compounds [10].

Well know that transformed root is highly differentiated and can cause stable and extensive production of secondary metabolites, whereas other plant cell cultures have a strong tendency to be genetically and biochemically unstable and often synthesize very low levels of useful secondary metabolites [11]. Hairy root-infected *A. rhizogenes* is characterized by a high growth rate and genetic stability. During agrobacterial infection, the *rolA*, *rolB*, and *rolC* genes of the plant pathogen *A.rhizogenes* are transferred into the plant genome, causing tumor formation and hairy root disease. These genes are activators of secondary metabolism in transformed cells in different plant families [12, 13]. *Agrobacterium* introduces several oncogenic genes into the host plant, leading to formation of tumors [14], and in the laboratory this microorganism is used widely for plant genetic engineering [15, 16]. Transformation by *agrobacterium* infection requires the presence of two genetic components located on the bacterial tumor-inducing (Ti) plasmid: the transferred DNA (T-DNA), which is introduced into the plant cell genome, and the virulence (*vir*) region composed of seven loci—*virA*, *virB*, *virC*, *virD*, *virE*, *virG*, and *virH* encoding most components of the protein apparatus for T-DNA transfer. In addition, several bacterial chromosomal virulence (*chv*) genes participate in the early stages of *Agrobacterium* attachment to the plant cells [17, 18, 19, 20]. Currently, one of the developing trends is hairy root culture techniques that established by transformation of plant cells with *Agrobacterium rhizogenes* and enable high production of these secondary metabolites for extensive industrial applications [21, 22, 23] and useful as pharmaceuticals, cosmetics, and food additives [24, 25, 26]. Hairy root cultures have many advantages, including biochemical and genetic stability, independence from seasonal and geographical conditions, rapid growth comparable to plants grown naturally [27, 28, 29]. The objective of our research was to provide information on the establishment of hairy roots for *Nepeta cataria* in both of their leaf and stem explants by *Agrobacterium rhizogenes* (A4) and induction efficiency.

2. Material and method

Seed sterilization and germination

The experiments of the present study were carried out in Plant Biotechnology Department, Genetic Engineering and Biotechnology Division, National Research Centre, Giza, Egypt. Seeds of *Nepeta cataria* were obtained from Aroma and Medical Plant Department, National Research Center, Giza, Egypt and stored at 4°C. The seeds were washed by tap water and soap for 3 hours to decrease the microbes loading. Under aseptic conditions were surface sterilized by placing them in 70% ethanol for 30 sec, then washing by sterile distilled water for

three times. Seeds were immersed in 75% Clorox (5.25% sodium hypochlorite) containing two drops of Tween 20 per 100ml for 20 min with frequent agitation. Then they were washed three times for at least 2 min in sterile distilled water. Five seeds were placed on jar containing solidified MS-media of Murashige and Skoog 1962. pH of the used medium in our work was adjusted to 6 using 0.1N of either KOH or HCL prior autoclaving which was at 121˚C for 23 min at pressure 1.2Kg/cm^2. The seeds were incubated in a culture room at 24 ± 2˚C and were kept under 16h photoperiod of fluorescent 45µ mol cool white light tubes and 8h dark.

Sterile explants preparation and pre-culturing

After 30 days of seeds cultivation, seedlings were cut into two segments (leaf and stem) about 0.5-1 cm long for each before culture. Both of leaf and stem were infected by *Agrobacterium rhizogenes* strain (A4) which be prepared according to Lee SY et al. [30].

***Agrobacterium rhizogenes* culture**

A. *rhizogenes* strain A4 was initiated from glycerol stock and maintained as follow: The culture of *A*. *rhizogenes*strain A4 was initiated from glycerol stock and maintained on YEP medium (10 g peptone, 10 g yeast extract, and 5 g NaCl in 1 L dis.H20) solid medium for 48 h at 28°C in the dark, single clone was grown for 48 h in YEP liquid medium at 28°C on a rotary shaker at 100 rpm in the dark.

Establishment of hairy root cultures

Leaves and stems of *Nepeta cataria* were taken from plants grown *in vitro* (30 days old). Excised leaves and stems were dipped into *A. rhizogenes* culture in liquid inoculation medium for 10 min, blotted dry on sterile filter paper to remove excess bacteria before culture on agar-solidified hormone free full strength MS-medium, and incubated in the dark at 25°C for 2 days. After 2 days of co-cultivation, the explant tissues were transferred to hormone-free half strength MS-medium containing 500 mg/L cefotaxime to eliminate bacteria and then incubated in the dark, then incubated in growth chamber at 25 ± 2°C and under complete dark conditions. Numerous hairy roots were emerged from the wound sites within 6 weeks. After repeated and transfer to fresh medium, rapidly growing hairy root cultures were obtained. Isolated roots (300 mg) were transferred to 30 ml of MS liquid medium, containing 30 g/l sucrose, in 200 ml flasks. Root cultures were maintained at 25°C on a rotary shaker at 100 rpm in growth chamber in the dark. One explant per flasks and 30 explants were used for each culture and the experiments were repeated twice. Hairy root growth was investigated by harvesting five flasks at intervals of 3 days during 21 days culture period. Data were non-determined in non-transgenic cultures.

Statistical analysis

All analyses were performed in triplicate and data reported as mean ± standard deviation (SD).

PCR detection

Plant DNA for polymerase chain reaction (PCR) analysis was extracted as described by Edwards K et al. [31]. The tissue (50 mg fresh weight) was homogenized in 200 l of extraction buffer (0.5% SDS, 250m M NaCl, 100m M Tris-HCl pH 8 and 25 m M EDTA pH 8) and centrifuged at 14,000 rpm for 5 min. The supernatant was transferred to a new tube and an equal volume of isopropanol was added. The sample was incubated on ice for 5 min and then centrifuged for 10 min at 14,000 rpm. The pellet was dried at 60oC for 5-

10 min and then resuspended in 100 µl of TE (10m M Tris-HCl pH 7.4 and 1m M EDTA pH 8). Plant genomic DNA was extracted by using DNA-Kit (Wizard R genomic DNA purification kit, A1120, Promega, USA) according to Hamill JD et al. [32]. Genomic DNA used as template on PCR was isolated from roots excised from infected and uninfected plants (control). The primer used for amplification of *rolB* gene was 5' ACTATAGCAAACCCCTCCTGC-3' and 5'-TTCAGGTTTACTGCAGCAGGC-3', with product size 652 bp (biomers. net GmbH, Ulm, Germany). The amplification cycle consisted of denaturation at 95 °C for 1 min, primer annealing at 55 °C for 1 min, and primer extension at 72 °C for 1 min. After 30 repeats of the thermal cycle and final extension 72 °C for 5 min, amplification products were analyzed on 1.5% agarose gel.

3. Results and Discussion

Seeds germination

Young seedlings of *Nepeta cataria* were obtained from the germinated seeds grown on MS-basal medium after one month of the culture, the *in vitro* seedlings were used as plant materials, it is clear that these seedlings suffer from the stunting as shown in Figure 1.

Figure 1. *In vitro* seedlings of *Nepeta cataria* after 30 days of cultivation on MS-basal medium

Effect of explants type on hairy root induction

In this research, hairy root formation and culture system of *N. cataria* was established using *A. rhizogenes A4* strain, two different explants (leaf and stem), were infected with *A. rhizogenes* to investigate the hairy root induction. Both of leaf and stem explants of *N. cataria* after their inoculation with *A. rhizogenes* and incubation for two days on full strength MS-medium were transferred to half strength -MS medium containing 500 mg/l cefotaxime to remove the excess of *A. rhizogenes*. In this study, it is appeared that half strength medium was chosen to be the optimal co-cultivation medium for re-culture the infected explants, where major nutrients prevent relatively *A. rhizogenes* transformation. This achievement was in accordance with Sharafi A et al. and Sharafi A et al.[33, 34] who proved that macro elements in inoculation and co-cultivation media have inhibitory effects on *A. rhizogenes* mediated transformation, as the transformation efficiency was drastically increased by removing some major mineral components, for that they used ½ MS for inoculation and co- cultivation medium instead of full strength MS medium.

Data in Figure 2 illustrates the growth in both of stem and leaf explants of *N. cataria* after were infected with *A. rhizogenes* for six weeks of inoculation, where stem began to grow more rapidly forming intact plantlets while, leaf stop to grow recording negative response for shoot elongation.

From our results, it could be advised to use stem as the better explant of *N. cataria* to ensure the success of transformation more than the leaf explants. Our investigation agree with Valimehr S et al. [35] who reported that stem explants were the best explants for *A. rhizogenes*

mediated transformation in *Nepeta pogonosperma* while leaf explants showed a low rate of hairy root induction. But at the same condition, our results are in disagreement with the investigations had been achieved by Lee SY et al. [30] who reveal that leaf explant of *N. cataria* was chosen as the optimal explant tissue for co-cultivation with *A. rhizogenes* more than those derived from stem explants. Moreover that, it could be concluded that the explants type play a role in hairy root induction, this is investigated by Md Setmam NM [36] who reported that one of the main factors that contribute to achieving hairy root induction is the type of explants used. Also, several studies on hairy roots in *Capsicum* species have used various explants such as hypocotyls [37], cotyledons [38], leaves [39] and mesophyll protoplasts [40].

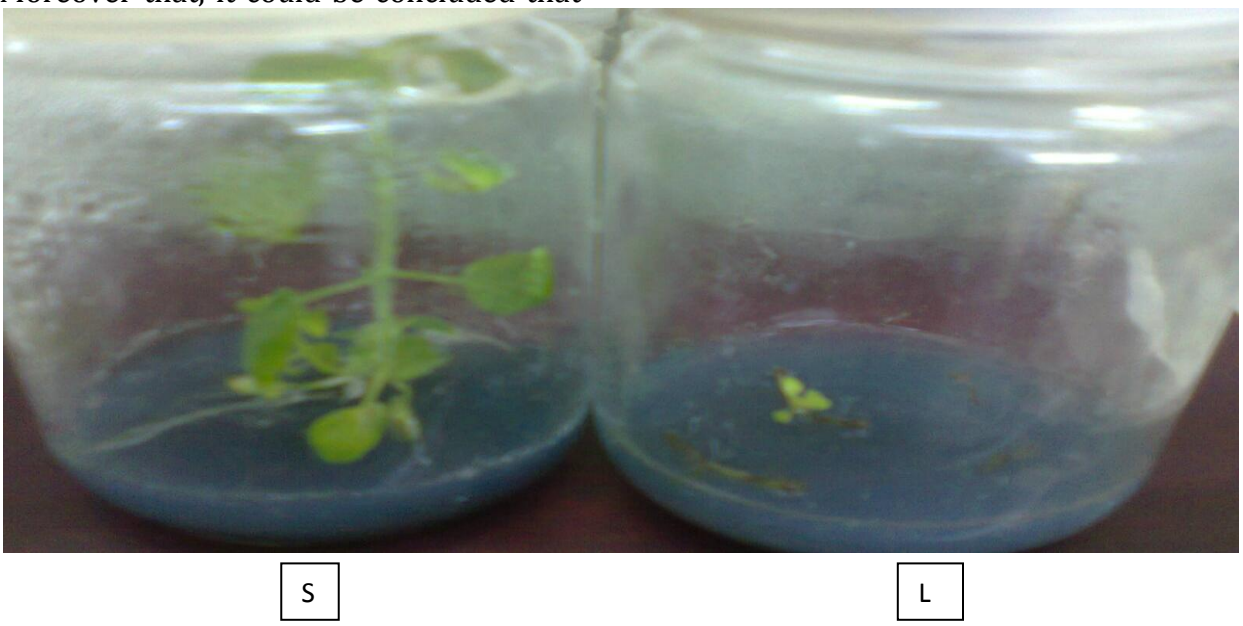

Figure 2. *N. cataria* explants, stem (S) and leaf (L) co-cultured with *A.rhizogenes* (A4) strain after 6 weeks of the infection

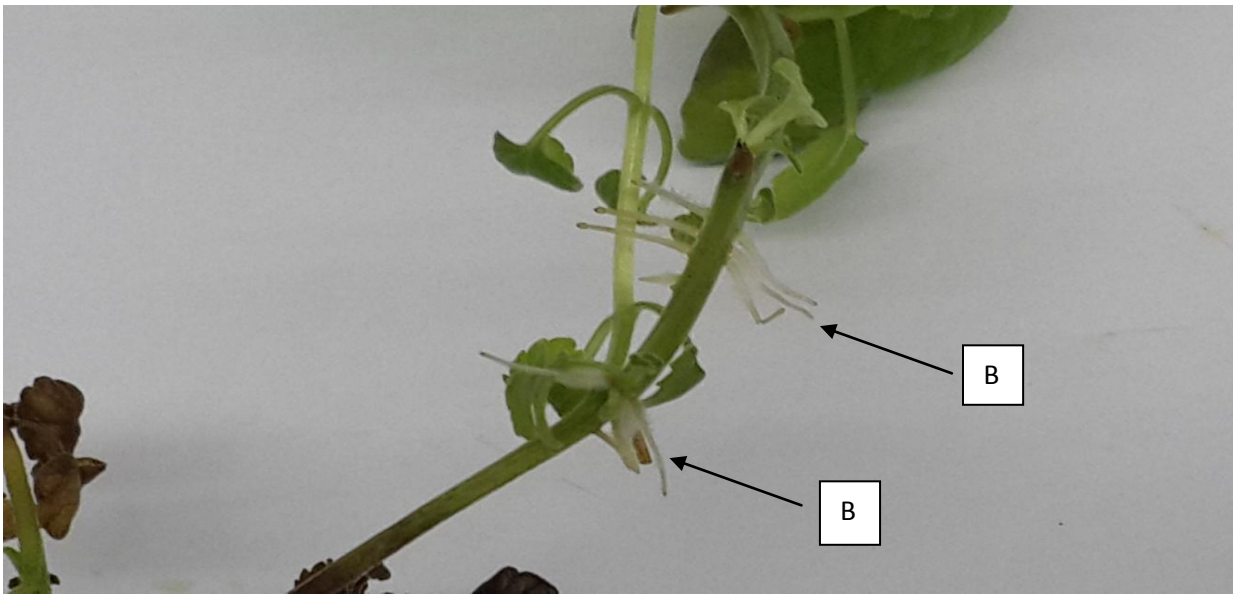

Figure 3. Hairy root initials (B) of the infected stem explants during six weeks of culture

In our experiment, it is clear that, the hairy root initials derived from the infected stem explants during six weeks of inoculation with *A. rhizogenes* were emerged from wound sites (Figure 3). Those are in accordance to Porter JR et al. and Winans SC [41, 42] who reported that the wound sites are a common location for hairy root induction since they serve as a genetic transfer point for *A. rhizogenes*.

Effect of infection by *Agrobacterium rhizogenes* A4 strain on stem explants vitality for growth development of *Nepeta cataria*

In this study, it is manifested that the growth of the infected stem by *A4* agrobacterium, transgenic plant (A) forming intact plantlets had better than that of the non-infected stem by *A4* agrobacterium, non-transgenic plant (B). Therefore it could be concluded that transformation by A4 bacterial strain enhance more growth efficiency of the infected stem explants to elongate and enhance more shoots and roots forming intact plantlets, contrary the non infected stem explants which grow slightly as illustrate in Figure 4.

A **B**

Figure 4. Development of transgenic (A) and non transgenic (B) plants of stem explants of *Nepeta cataria* after inoculation with *A. rhizogenes (A4)* strain within 6 weeks of the infection.

These results are in line with the mentioned investigation mentioned in Figure 1 that intact plantlets were donated by seeds grown on MS-basal medium were suffer from stunting and recorded slight germination. For this, our obtained results reveal that the use of gene, A4 agrobacterium is not limited to induce hairy roots only but also, to increase the growth efficiency for the cultured explants. These investigations are resemble with the obtained results by Hashem [43] who proved that, in hairy root cultures, the active expression of *rol*

genes due to the presence *vir* genes on the *A. rhizogenes* *Ri*-plasmid may cause extreme synthesis of endogenous auxin and cytokinin in the host cells. Also, it is observed from data in Figure 4 that *N. cataria* plants transformed with *A. rhizogenes* strain A4 (A) were morphologically similar to *N. cataria* plants non- transformed (B). The obtained investigation is matched with both of Sukhapinda et al. [44] who revealed that *M. Sativa* plants transformed with *A. rhizogenes* strain A4 were phenotypically normal except for extensive and shallow roots, and Tabaeizadeh Z [45] who reported that regenerated *Lotus corniculatus* plants transformed with wild-type *A. rhizogenes* showed no morphological difference with respect to leaf shape or plant height when compared to seed grown plants.

Four parameters were used to estimate full growth efficiencies of the infected stem explants of *Nepeta cataria*. These parameters were number of leaves and plant height for shoots, number of roots and root lengths per single explants (Table 1). Overall, the presented results showed that the parameter values of the transgenic plants by *A4* agrobacterium were higher than those for the non-transgenic plants. The derived results from Table 1 reveal that shoots number recorded maximum value, 23.601 ± 2.073 in transgenic plants with height, 21.611 ± 2.073 cm (Figure 5) compared with non-transgenic plants which recorded less values in both of shoots number, 8 ± 1.414 and plant height, 4.513 ± 0.790 cm. Roots number recorded high values, 13.604 ± 1.673 in transgenic plants with lengths, 10.923 ± 0.741 cm (Figure 5), contrary non-transgenic plants which recorded negative response for roots induction.

In addition to these obtained results, Figure 5 shows different root morphologies between hairy roots (A) and normal roots (B) of the transgenic plants. The induced hairy roots were emerged on wound sites against geotropic resemble white fluff, compared with normal roots which emerged at the end of the plantlets with the geotropic as brown long growths with a smooth surface. Our obtained results are largely in line with Md Setmam NM et al. [36] who approved that the successfully induced hairy roots of *Capsicum* species appeared as whitish fungus needle-like structures and follow geotropic patterns while those normal roots can be described as yellowish long rods with a smooth surface in a plageotropic response. Our investigations were discussed by Karmarkar and Kim et al. [46, 47] who reveal that the plageotropic growth of hairy roots in plants is possibly due to the lack of amyloplasts in the starch grains of hairy roots, sensory balance receptors called statoliths lead to the diverse hairy root growth directions.

Table 1. Number and length, cm (mean ± standard deviation) of both of shoot and root for transgenic and non- transgenic plants from stem explants of *Nepeta cataria* after 6 weeks of cultivation

Explants	Parameters	Non-transgenic plant	Transgenic plant
Shoots	No. of leaves	8 ± 1.414	23.601 ± 2.073
	Plant height (cm)	4.513 ± 0.790	21.611 ± 2.073
Roots	No. of roots	N.D	13.604 ± 1.673
	Root lengths (cm)	N.D	10.923 ± 0.741

N.D: No Data

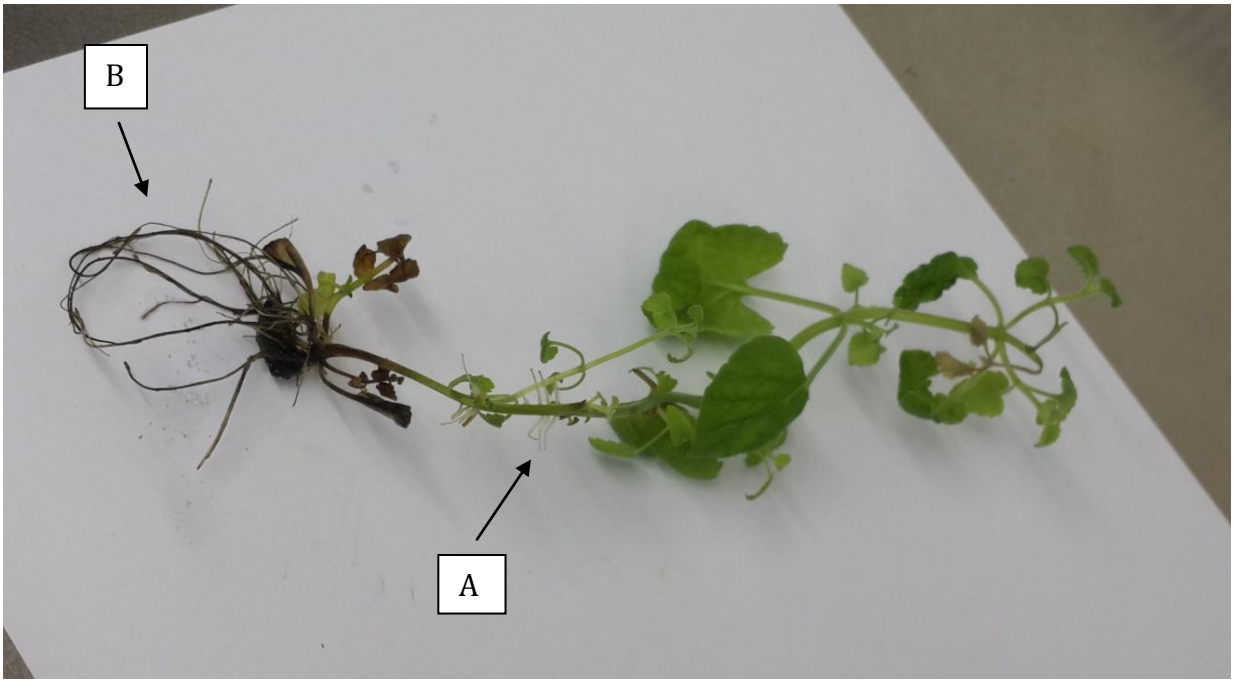

Figure 5. Hairy root induction (A) and normal roots (B) of the infected stem explants of *Nepeta cataria* within six weeks of exposure to *Agrobacterium rhizogenes*.

Hairy roots growth (yield) for transgenic and non transgenic plants of *N. cataria*

After 6 weeks of culture, hairy roots began to grow more rapidly. Rapidly growing hairy roots were excised from the intact plantlets and subculture on fresh MS liquid medium, containing 30 g/l sucrose. Hairy roots generally increased in weight as investigated in Table 2**.**

Fresh weight (g) of these hairy roots was recorded at intervals 3 days within 21 days for both of transgenic and non-transgenic plants. Continuously, hairy roots fresh weight in transgenic plants increase intervals 3 days till for 21 days of culture. Furthermore, it is observed that the growth of hairy roots was slight from 3 days (0.354 ± 0.008 g), 6 days (0.503 ± 0.016) till 9 days (0.709 ± 0.012 g) of culture, then began more rapidly from 12 days (1.021 ± 0.102 g) till end of 15 days (1.506 ± 0.104 g) of culture, follow that re-deficiency rate of the growth from 18 (1.761 ± 0.106 g) to 21 (1.863 ± 0.109 g) days of culture.

Table 2. Hairy root growth (fresh weight/g) per single explant (mean ± standard deviation) for non-transgenic and transgenic plants of *N. cataria* during 21 days of cultivation.

Culture period /day	Hairy root fresh weight/g	
	Non – Transgenic	Transgenic
0	N.D	0.311 ± 0.007
3	N.D	0.354 ± 0.008
6	N.D	0.503 ± 0.016
9	N.D	0.709 ± 0.012
12	N.D	1.021 ± 0.102
15	N.D	1.506 ± 0.104
18	N.D	1.761 ± 0.106
21	N.D	1.863 ± 0.109

Therefore, it is advised to transfer the isolated hairy roots of *N*. *cataria* on another fresh MS liquid medium each 15 days of culture to ensure the success of subculture process to get as much of the hairy roots growth in a good state. Our results are in accordance with Lee SY et al. [30] who proved that the dry weight of *N*. *cataria* hairy roots increased from the original inoculum level of 0.5 g dry wt/l to attain 11.7 g dry wt/l, the maximum growth (11.2 g dry wt/l) was attained after 15 days of culture, whereas, after 18 days of culture the hairy roots turned brown and a brown pigment was released into the culture medium.

DNA and PCR analysis for *rolB* gene in transgenic and non-transgenic plants of *Nepeta cataria*

In this work, the positive results obtained in the amplification of the *rolB* gene in the transformed plants can be attributed to true transformation events rather than to residual Agrobacterium. For that to investigate presence of the *rolB* gene transferred from Ri plasmid to the infected plants, PCR analysis was performed. PCR analysis led to amplification of *rolB* gene in the transgenic hairy root line. Figure 6 shows PCR analysis for detection of the *rolB* gene in the obtained hairy root line of transgenic plants (T) and non transgenic plants (C) of *N. cataria* by PCR amplification. The amplification of *rolB* genes expressed as single band in the transgenic plants (T) and disappeared in the non transgenic plants (C). The amplification of *rolB* genes results revealed that the transgenic plants (T) contained *rolB* genes from the Ri-plasmid. The *rolB* genes of the Ri-plasmid are responsible for the induction of hairy roots by *Agrobacterium rhizogenes* [8]. Also, Tepfer [48] proposed that Ri T-DNA might be used to improve the rooting ability of root stocks or to create root systems.

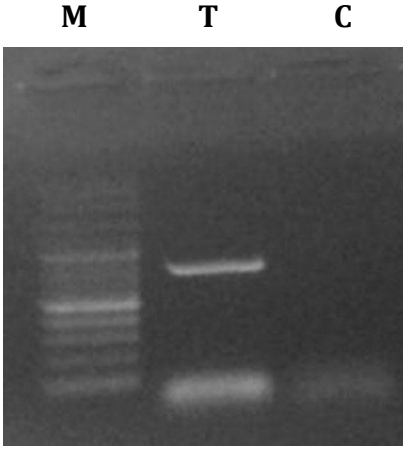

Figure 6. Molecular analysis of hairy roots; a PCR analysis for detection of the rolB gene (652 bp) in hairy root line of N. cataria; M: DNA ladder (100 –1500); T: transformed plants, C: (control) non transformed plants.

Conclusion

In the present study we have demonstrated an efficient *Agrobacterium rhizogenes* mediated transformation protocol for establishment of *N. cataria* hairy root cultures, and a valuable alternative approach for the hairy roots induction from *N. cataria*. It should be pointed out that our studies of *Agrobacterium rhizogenes* transformation have been succeeded. Our results indicate that the possibilities for hairy roots induction, roots formation and obtaining improved plants drastically of *N.cataria* rather than using the traditional methods of cultivation. Therefore, further work, including expression of trans-genes in *N. cataria* for improvement the regeneration of the plants including high production of secondary metabolites in their hairy root cultures for extensive industrial applications.

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