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Original article

Hairy root induction and plant regeneration of tobacco (*Nicotiana tabacum* L.)

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Abstract

The present study showed the morphogenic potential of an important aromatic and industrial tobacco plants for the development of transgenic plants through *Agrobacterium rhizogenes* (strain 15834) for *rolA, rolB, and rolC* genes. Leaf explants were inoculated with 1/25, 1/30, and 1/50 diluted bacterial culture (OD600= 0.8) for 30 min. containing different acetosyringone doses 250 µM, 200 µM, and 150 µM respectively. The highest number of hairy roots was obtained by 1/25 containing 250 µM acetosyringone was 100%. These hairy roots were then transferred to media containing different combinations of plant growth hormones 0.5, 1.0, and 2.0 mg/L of BAP and NAA. The nutrient medium consisting of MS vitamins supplemented with 2 mg/L BAP and 0 mg/L NAA 91.7 had the highest callus formation. However, the highest callus weight was detected in 0.5 mg/L BAP and 1 mg/L NAA medium, while the highest shoot formation explant rate was 56.7% in 2 mg/L BAP and 1 mg/L NAA medium. Number of shoots per explant was detected in 1 mg/L BAP medium. Putative transgenic plants identified by PCR analyzes. As a result of the study, out of 54 putative transgenic plants 37 samples were confirmed by PCR for primers rolA, rolB, and rolC.

Keywords

A. rhizogenes, hairy roots, *Nicotiana tabacum* L, Ri plasmid, rol genes

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Introduction

A. rhizogenesis belonging to the family *Rhizobiaceae* is a gram-negative bacterium holding a hairy root-inducing plasmid (Ri-plasmid). Hairy roots are capable of exogenous hormone-independent growth, high lateral branching and root development, therefore, called genetically engineered root. In comparison with normal root, hairy roots grow faster than the adventitious roots (K.Y. PAEK & al [1]; K.W. Yu & al [2]) and accumulate higher levels of certain valuable compounds compared with adventitious roots and plant-grown roots (G.P. MIAO & al [3]; X. HAO & al [4]; M. SHI & al [5]). They are usually non-chimeric and similar in phenotype and structure to wild-type root. These kinds of roots can be identified by a reporter gene through PCR analysis (N.I. PARK & al [6]). DNA is transferred and incorporated in the genome (Ri plasmid) of wounded and infected plant explants. The development of hair roots depends on the expression of the *rolA, rolB, rolC,* and *rolD* genes in the T-DNA region leads to the formation of hairy roots emerging at the wounded surface of explants (M.C. CHRISTEY & al [7]). Therefore, *A. rhizogenes*-mediated transformation is used to induce many secondary metabolites and for recombinant protein production, metabolic engineering studies, and the large-scale production of bioactive compounds with medicinal properties (K.D. DEBOER & al [8]; N.N. ONO & al [9]; A. WIŚNIEWSKA & al [10]; C. THILIP & al [11]; R. XUE & al [12]; N. YANG & al [13]). Mechanism of the hairy root production has been carried out a broad spectrum of plant species via *A. rhizogenesis* for many years. Different factors such as genotype, co-cultivation temperature, inoculation time, co-cultivation time, vacuum infiltration, pH, constructs model, age and type of explant and bacterial cell density may affect the transformation frequency of *Agrobacterium* - mediated transformation in plants (S.N. SHEIKHOLESLAM & al [14]; S. URANBEY & al [15]; H.D. JONES & al [16]; B.W. TAGUE & al [17]; M.J. KOETLE & al [18]; E. ANAYOL & al [19]; H.A.A. AHMED & al [20]; H.A.A. AHMED & al [21]; H.A.A. AHMED & al [22]). Transformation frequency is also considerably affected by media composition containing different compounds (S. BARPETE & al [23]). Asetosyringone (AS) was also used as an additive to increase the transformation efficiency of gene transfer by *Agrobacterium* - mediated (T. KUMAR & al [24]; H.A.A. AHMED & al [20]; M. BALASUBRAMANIAN & al [25]; N. ZARE & al [26]). This compound (AS) has a special role as a signal attracting and transforming unique, oncogenic bacteria in the genus *Agrobacterium*. The virA gene on the Ti plasmid of *A. tumefaciens* and the Ri plasmid of *A. rhizogenes* is used by these soil bacteria to infect plants, via its encoding for a receptor for acetosyringone and other phenolic phytochemicals exuded by plant wounds (B. SCHRAMMEIJER & al [27]). AS widely is used on *Agrobacterium* - mediated plant transformation for vir gene induction (K. OZAWA [28]; B. SCHRAMMEIJER & al [27]; M.K. FERIZ & al [29]; T. THOMAS [30]; E.E. Uchendu & al [31]; A. PAUL & al [32]; A.M.P. JONES & al [33]). The efficiency of AS on transfer of T- DNA by inducing Vir genes largely depends on genotype, explant type, culture conditions, and concentrations of AS. Therefore, these types of compounds are important to enhance *Agrobacterium* - mediated genetic transformation efficiency in plants (S.N. SHEIKHOLESLAM & al [14]). The majority common *A. rhizogenes* strains which demonstrated by Ri plasmids are agropine-type: pRi15834, pRi1855, pRiLBA940, pRiHRI and pRiA4, mannopine-type: pRi8196, cucumopine type: pRi2659 and mikimopine-type pRi1724. Even though mikimopine and cucumopine are stereo-isomers, there is no homology between opining biosynthetic genes on the nucleotide equality (A. OUARTSI & al [34]; V. VEENA & al [35]; I.I. OZYIGIT & al [36]). The T-DNA are under the control of the virulence (vir) genes, which function in the process of T-DNA excision. This induction process is driven by the genes virA and virG (S.E. STACHEL & al [37]). VirA gene mainly drives expression of the vir genes encoding a membrane-bound kinase that perceives chemical signals, such as phenolic inducers as acetosyringone (AS), from wounded plant cells (S.E. STACHEL & al [38]; H. CHO & al [39]; J. XI & al [40]). In this study, improvisation of culture media, various combinations of cytokinin/auxin and AS was used for inducing transgenic hairy root on leaf-derived calli of tobacco.

Materials and Methods

Plant material

Turkish local commercial variety of tobacco (*Nicotiana tabacum* L.) cv. Samsun was used for development and optimization of regeneration of *A. rhizogenes*-mediated transformation. The seeds were obtained from Department of Field Crops, Faculty of Agriculture, Ankara University, Ankara, Turkey. This study was conducted in Department of Field Crops, Faculty of Agriculture, Ankara University, Turkey during 2018 and reconfirm in 2021 for protocol reproducible.

In vitro culture conditions

The Tobacco seeds were kept soaked in 70% ethyl alcohol for 3 min. followed by 1% sodium hypochlorite for 15

min. and then rinsed with double distilled water three times for 5 min. each. Then, the seeds were cultured on MS medium (T. MURASHIGE & al [41]) with 30 g/L sucrose that incubated under 24°C for 16/8 h photoperiod at 16000 lux light intensity Plantlets with a height of 12-14 cm and 6-8 leaves were obtained after four weeks.

A. rhizogenes strain and culture conditions

Wild-type agropine strain of *A.*ATCC 15834 (American Type Cultures Collection, Manassas, USA) were used for plant transformation studies. The bacterial plasmid DNA carries rol genes (*rol ABC*). *A. rhizogenes* strain was inoculated in NA nutrient agar medium (3 g/L yeast extract, 5 g/L peptones, and 1 g/L agar) and grown at 28°C overnight. A single-cell bacterial colony was initiated from the nutrient agar and cultured in 10 ml liquid NB (nutrient broth, Micropoli) medium at 28ºC on a rotary shaker (130 rpm) until OD600 reached around 0.8-1. Thereafter, 10 µl of the fresh culture of *A. rhizogenes* was used for inoculation.

Co-cultivation and genetic transformation

Three different protocols were used for optimization of transformation via *A. rhizogenes*. The leaf discs explants were inoculated with *A. rhizogenes* culture diluted 1:25, 1:30, and 1:50 in liquid MS medium containing 250, 200 and 150 µM acetosyringone respectively for 30 min. After bacterial infection, the explants were transferred to the co-cultivation gelrite solidified MS media for 72 h under dark condition. After co-cultivation, explants were washed with sterile MS medium containing 250 mg/L Cefotaxime to prevent overgrowth of the bacteria. The washed explants were cultured on MS media containing 300 mg/L ticarcillin disodium/clavulanate potassium (Duchefa Biochemie, Hofmanweg, Haarlem, Netherlands). These vessels were incubated at 24±1°C under 16/8 h at 16000 lux light photoperiod condition.

Regeneration of plants from putative transgenic shoots

For callus induction and putative transgenic shoot, hairy roots, were cut into 15-25 mm pieces then cultured on MS media supplemented with different combinations of PGR 0.5, 1.0 and 2.0 mg/L of BAP and NAA, 30 g/L sucrose, 7 g/L agar, and 300 mg/L ticarcillin disodium/clavulanate potassium. All cultures were incubated in growth chamber room at 24ºC under 16/8 h (light/dark) photoperiod cycle.

Rooting and acclimatization of putative transgenic plantlets

Well-developed shoots (5-6 cm length,) grown on selective medium containing ticarcillin disodium/clavulanate potassium were rooted on a selective MS medium containing 0.2 mg/L IBA, 300 mg/L ticarcillin disodium/clavulanate potassium. The rooted plantlets were washed with tap water for 10-15 min. and transferred to pots containing mixture of peat moss, perlite, and soil (1:1:1). The pots covered with polythene bags for 1 week for creating humidity and adaptation purposes then polythene were removed as they adapted to greenhouse conditions.

Molecular screening of rol abc genes by PCR

The presence of *rol* genes in plants was confirmed by PCR analysis *(Phire Plant Direct PCR Kit, Thermo Scientific*) according to manufacturer's instructions. Phire Plant Direct PCR Kit allows use of fresh tissue instead of pure DNA. Approximately 100 mg leaf sample was collected from each putative transgenic plant and grinded with a spatula. A total of 10 µl 2x Phire Plant PCR Buffer (dNTP and $MgCl_2$), 10 pmol Primer mix and 0.4 µl Phire Hot Start DNA II polymerase was added to 0.5 µl leaf extract and final volume of 20 µl done by analyzed grade distilled water. For amplification of the *rolA gene*, the forward 5-GTTAGGCGTGCAAAGGCCAAG-3 and reverse 5-TGCGTATTAATCCCGTAGGTC-3, product length 239-bp, *rolB gene*, the forward 5- AAAGTCTGCTATCATCCTCCTATG-3 and reverse 5-AAAGAAGGTGCAAGCTACCTCTCT-3 product length 348-bp. For amplification of the *rolC gene,* the forward 5-AAATGCGAAGTAGGCGCTCCG-3 and reverse 5-TACGTCGACTGCCCGACGATGATG-3 and the product length 190-bp primers were used as described by S. ZDRAVKOVIC-KORAC & al [42] and A. DI GUARDO & al [43]. Reactions were amplified using the following program: an initial denaturation at 98ºC for 5 min.; 40 cycles at 98ºC for 5 s, 60ºC for 15 s, 72ºC for 30 s; a final extension at 72ºC for 3 min. The amplification products were separated by electrophoresis on 1.5 % agarose gels.

Experimental Design and Statistical Evaluation

The *experimental design used in the study* was completely randomized *with three* replications. The data consisted of the averages of independent measurements. Data given in percentage were subjected to arcsine (√X) transformation (G.W. SNEDECOR & al [44]) before statistical analysis and all data were analyzed with one-way analysis of variance (ANOVA) according to (O. DÜZGÜNEŞ & al [45]) and the differences were compared by Duncan's multiple range test using SPSS programme.

Results and Discussion

Genetic transformation of tobacco leaves

Tobacco (*Nicotiana tabacum* L.) cv. Samsun were used as initial source of leaf explants for gene transformation.

Table 1. Effect *A. rhizogenesis* dilutions and AS on hairy root formation from leaf explant of tobacco

***A. rhizogenes* dilutions and AS**	**Root-forming explant rate (%)**		**Number of hairy roots per explant**	
Control	83.3	b	1.2	c
1:25 dilution with 250 µM AS	100.0	a	3.0	a
1:30 dilution with 200 µM AS	56.7	c	1.4	bc
1:50 dilution with 150 µM AS	86.7	b	1.6	b

Values within a column followed by the different letters are significantly different at the 0.05 level of significance using Duncan test

For hairy root formation, explants were inoculated in three different bacterial dilutions of *A. rhizogenes* (1:25, 1:30, and 1:50) supplemented with different levels of acetosyringone concentration (250 µM, 200 µM and 150 µM) for 30 min. to increase gene transformation frequency. Hairy root formation was not seen on non-inoculated control explants (Fig. 1A). The first hairy root structures were observed from the scar tissue of the leaf explants after 6 day of culture inoculation (Fig. 1B). On the other hand, well-developed hairy roots on leaf explants were formed after two weeks of culture inoculation (Fig. 1C and D). The results of the study were statistically significant at the 5% level for *Agrobacterium* and explant type Table 1. The root formation and number of roots per explants ranged from 56.7 to 100% and 1.2-3.0 respectively. *A. rhizogenes* culture diluted to 1:25 containing 250 µM acetosyringone gave the maximum hairy root formation Table 1. The genetic transformation frequency is crucially based on genotype dependent reported by H.A.A. AHMED & al [21] and H.A.A. AHMED & al [22]. Whereas, efficiency of infection was highly influenced by strain of *A. rhizogenes* that plays a significant role in transformation P. TAVASSOLI & al [46]. However, the *A. rhizogenes* host strain ATCC15834 has been commonly used for hairy root culture in Hypericum perforatum, Salvia wagnerian and Lavandula angustifolia M. TSURO & al [47]; B. RUFFONI & al [48]; J. HENZELYOVÁ & al [49].

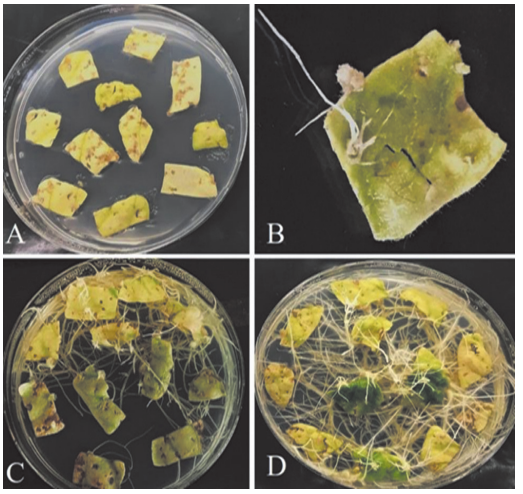

Figure 1. Development of transgenic hairy roots from *N. tabacum* leaf explants by *A. rhizogenes* strain 15834. (A) control (non-inoculated leaf disc), (B) hairy roots formation on leaf explants after 6 day of inoculation, (C & D) hairy roots formed after two weeks and six weeks respectively.

Development of putative transgenic hairy root clones

Putative transgenic hairy roots (by cutting about 20 mm) were cultured on MS medium with different growth regulators combination of BAP (0, 0.5, 1 and 2 mg/L) and NAA (0, 0.5, 1, 2 mg/L) and containing 300 mg/L ticarcillin disodium/clavulanate potassium to eliminate bacteria Table 2 and reliable regeneration. A total number of sixteen growth regulator combinations were used to identify the suitable explant growth. The each treatment was replicated thrice and each Petri dishes contained 20 explants. The culture explants were subcultured to a fresh nutrient medium every two weeks. Non-transgenic control explants was unresponsive for shoot and root regeneration on MS medium containing 300 mg/L ticarcillin disodium/clavulanate potassium after 8–10 weeks (Fig. 2A). Indirect shoot organogenesis was observed on cut surface of explants inoculated with *A. rhizogenes* harboring rol A,B,C genes after four weeks of culture inoculation (Fig. 2B, C, D, E and F). The calli induction ranged from 0.00 to 91.7 % and more than 90% callus induction was observed on MS medium supplemented with 1 mg/L BAP and 2 mg/L BAP devoid with NAA. Table 2. It seems that NAA is inhibiting the callus induction in the culture media. No callus growth was observed on controle medium. The fresh callus weight ranged from 0.00 to 1.264 g, whereas, highest weight was obtained with 2 mg/L BAP and 1 mg/L NAA Table 2. Shoot initiation and formation on putative transgenic hairy roots were developed after 5-6 weeks that ranged from 0.0 to 56.7%. The highest shoot formation was recorded on MS medium containing 2 mg/L BAP and 1 mg/L NAA. (Fig. 2D). In addition, the number of shoot per explant ranged from 0.5 to 2.4 shoot per explant.

Table 2. Influence of different BAP and NAA concentrations on callus and of putative transgenic shoot formation from hairy roots

No	BAP mg/L	NAA mg/L	Regenerative Callus formation explant rate (%)		Callus weight per explant (g)		Shoot induction explant rate (%)		Number of shoot per explant	
1	0.0	0.0	0.0	d	0.000	g	0.0	c	0.0	c
2		0.5	50.0	c	0.418	efg	10.0	bc	0.7	abc
3		1.0	50.0	c	0.419	efg	26.7	abc	1.3	abc
4		2.0	50.0	c	0.236	fg	6.7	bc	0.7	abc
5	0.5	0.0	71.7	b	0.385	efg	55.0	a	2.0	ab
6		0.5	50.0	c	0.995	bc	30.0	abc	2.0	ab
7		1.0	50.0	c	1.658	a	8.3	bc	0.5	bc
8		2.0	50.0	c	1.177	bc	6.7	bc	1.4	abc
9	1.0	0.0	90.0	a	0.232	fg	41.7	ab	2.5	a
10		0.5	50.0	c	0.847	bcde	53.3	ab	1.7	abc
11		1.0	50.0	c	0.946	bcd	30.0	abc	1.3	abc
12		2.0	50.0	c	0.874	bcde	15.0	abc	1.3	abc
13	2.0	0.0	91.7	a	0.486	defg	38.3	ab	2.4	ab
14		0.5	50.0	c	1.221	ab	21.7	abc	1.4	abc
15		1.0	50.0	c	1.264	ab	56.7	a	1.9	abc
16		2.0	50.0	c	0.694	cdef	16.7	abc	1.1	abc

Values within a column followed by the different letters are significantly different at the 0.05 level of significance using Duncan test.

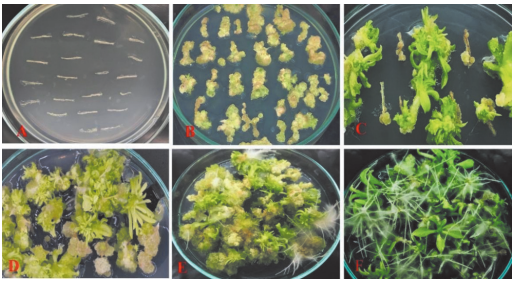

Figure 2. Callus formation and shoot regeneration development in medium control (A), callus indution (B), shoot inition (C), the 5th combination (0.5 mg/L BAP and 0 mg/L NAA) (D), the 9th combination (1 mg/L BAP). and 0 mg/L NAA) (E), The highest callus formation rate in the 13th combination (2 mg/L BAP and 0 mg/L NAA) (F)

Rooting and adaptation of putative transgenic plantlets

The 4-5 cm long shoots developed on hairy roots were cut and rooted on MS medium containing different combination of BAP (0, 0.5, 1, and 2 mg/L), NAA (0, 0.5, 1, 2 mg/L) and 300 mg/L ticarcillin disodium/clavulanate potassium (Fig. 3A and B). The root induction ranged from 0.00 to 58.2% and highest root induction was noted on MS medium containing 1 mg/L NAA Table 3. The well rooted shoots were transferred to pots contained potting mixture (peat and perlite 1:1) for acclimatization in growth chamber (Fig. C and D). The acclimatization rate of putative plants ranged from 0.00 – 65.9% Table 3.

Confirmation of rol gene integrations

A. rhizogenes infection was removed by frequent subcultures with antibiotics in the culture medium. The putative transgenic plants T0 and T1 tobacco plants was confirmed by PCR amplification of the *rol A,B,C* genes. The putative transgenic plants obtained and checked by molecular method whether they carry the role genes. The PCR prim-

Figure 3. Rooting of transgenic putative shoots grown on (1 mg/L NAA) medium (A,B) adaptation of rooted plants in greenhouses using a mixture of peat moss, perlite, and soil (1:1:1).

Table 3. Influence of BAP and NAA on rooting of putative transgenic shoots and adaptation to the external environment

No	BAP mg/L	NAA mg/L	Number of rooted shoots	Rate of putative transgenic plants acclimatized (%)
1	0.0	0.0	0.0 b	0.0 d
2		0.5	22.2 ab	15.0 bcd
3		1.0	58.2 a	54.8 abc
4		2.0	20.8 ab	16.9 bcd
5	0.5	0.0	37.1 ab	65.9 a
6		0.5	49.7 a	51.7 abc
7		1.0	14.3 ab	18.3 bcd
8		2.0	11.9 ab	13.1 cd
9	1.0	0.0	30.7 ab	58.1 ab
10		0.5	24.9 ab	25.9 abcd
11		1.0	31.9 ab	31.9 abcd
12		2.0	25.4 ab	31.9 abcd
13	2.0	0.0	50.5 a	47.2 abc
14		0.5	28.1 ab	37.4 abcd
15		1.0	41.1 a	46.9 abc
16		2.0	44.6 ab	34.2 abcd

Values within a column followed by the different letters are significantly different at the 0.05 level of significance using Duncan test.

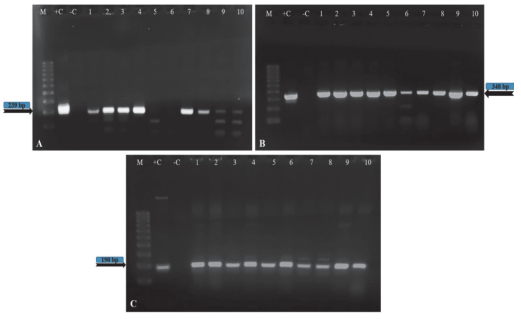

Figure 4. Confirmation of putative transgenic plants by PCR. (A) PCR amplification of the 239 bp rolA gene, (B) 348 bp rolB gene and (C) 190 bp rolC gene in individual transgenic plants developed from hairy root lines transformed by *A. rhizogenes* strain 18455. -C, control (non-transformed roots); +C, positive control (pRi 15834); M, molecular weight 100 bp DNA marker Ladder.

ers previously designed for role genes were used. The Polymerase Chain Reaction (PCR) showed the amplification of band sizes of 239 bp, 348 bp and 190 bp sizes for *rol A, B, C* genes respectively (Fig. 4 A, B and C). These results showed the Ri T-DNA integration into the putative regenerated plant genome. The results obtained specify the previous reports of B. LI & al [50] and Y.A. MOGHADAM & al [51]. Among the genes of rol, rolB plays a critical role in pathogenicity, while rolA and rolC subscribe to the root induction G. SUJATHA & al [52].

Conclusions

Putative transgenic hairy roots were obtained by transferring the rol genes A,B,C to tobacco cv. Samsun via *A. rhizogenes*. The in vitro regenerated explants (leaves) were used as initial explants for culture. The highest hairy root formation (100%) was obtained from 1:25 *A. rhizogenes* dilution containing 250 µM AS. In many plants species, acetosyringone has been reported to increase the rate of transformation with *Agrobacterium* that may be related to the activation of vir genes, which are necessary for the transfer of Ri T-DNA to plant tissue. As a result of the experiment, the highest shoot regeneration achieved in MS media containing 0.5 mg/L BAP, 1 mg/L BAP, and 2 mg/L BAP concentrations 71.7, 90.0, and 91.7 respectively. The putative transgenic plants were confirmed by PCR. As results of PCR, out of 54 putative transgenic plants 37 samples were confirmed by PCR for primers rolA, rolB, and rolC. The *rolA* and *rolC* in bacteria cause stunting and the *rolB* gene causes hairy roots in the plants. *In vitro* regeneration of transgenic hairy roots will result with different physiological and morphological characteristics containing role genes. Moreover, this kind of transgenic plants will play an economically important role in the production of beneficial secondary metabolites through hairy root culture. Due to the role of genes in transgenic plants with Ri lines, it is expected that with the increase of hairy roots, the plant will better adhere to the soil, increase

efficiency in water and mineral substance uptake, and be effective in drought tolerance.

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