






THE EFFECT OF DIFFERENT CONCENTRATIONS OF ACETOSYRINGONE ON TRANSFERRING *rol* GENES TO SOME CITRUS ROOTSTOCKS VIA *Agrobacterium rhizogenes*

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ABSTRACT. Genetic engineering technologies have been taken part in the breeding strategies of fruit crops. Several biotechnological methods can be applied to plant to have better ones in the process of fruit breeding. Among these methods, *Agrobacterium*-mediated gene transfer is frequently used. Citrus species are the most widely grown fruit crops. Despite substantial genetic diversity and interspecific fertility, the genus Citrus includes some of the most difficult species to breed. This is due to several obstacles for conventional breeding. For this reason, biotechnological breeding methods are used in citrus breeding. Acetosyringone is one of the phenolic compounds secreted by injured plant cells and affects the transformation efficiency. This study aims to determine the effect of different acetosyringone concentrations (0, 25, 50, 100 and 200 µM) on the transformation of *rol* genes in citrus genotypes and check whether the plantlets obtained after transformation carry the *rol* genes by molecular methods. Tuzcu 3131 sour orange, Gou Tou sour orange, Carrizo citrange, Rough lemon, Volkameriana, Trifoliolate orange were used in this study. As a result, the highest shoot formation was obtained at 200 µM acetosyringone concentration and the highest root formation was obtained at 25 µM acetosyringone concentration.

Keywords: hairy root, dwarf rootstock, *Agrobacterium*-mediated transformation, *rolABCD*

INTRODUCTION

Genetic transformation is used as a tool for the genetic improvement of many economically important agricultural products because it allows the incorporation of interesting traits into the genotype without other traits. Citrus fruits are among the important plant species that are economically produced. The development of citrus fruits with classical breeding methods is difficult due to reasons such as the long juvenile period, often within and between species incompatibility, and polyembryony [1]. Therefore, genetic engineering methods are used in citrus breeding. Different methods are used in genetic transformation studies for citrus, including particle bombardment, PEG, macroinjection, microinjection and transformation via *Agrobacterium*. However, the most effective method among these methods is *Agrobacterium*-mediated transformation [2].

Agrobacterium-mediated transformation is an effective and universal tool for broad-spectrum genetic modification of plant species. *A. tumefaciens* and *A. rhizogenes* species are used for *Agrobacterium*-mediated gene transfer.

A. rhizogenes is a gram-negative soil bacterium responsible for the development of hairy root disease in dicotyledon plants and some monocotyledon plants. After a plant infected with this bacterium, some changes occur in its growth and development. The

most typical of these changes is the shortening of the internode distance and the suppression of the apical dominance. Genes found in the plasmid DNA of the bacterium and called "rol (root loci) genes-rolABCD" play a role in these changes. Among these genes, *rolA* and *rolC* genes cause dwarfing in plants, while the *rolB* gene causes hairy root formation [3]. Both features are desired in rootstock breeding.

A. rhizogenes carrying the Ri plasmid has some advantages. Plant cells transformed by *A. rhizogenes* are easily separated from the transformed tissues by hairy root formation and explants develop in a hormone-free environment [4, 5]. Thus, it provides the development of transgenes through marker-free selection using hairy root morphology as the first indicator of transformation [6].

Citrus species are the most widely grown fruit crops. In citrus, juvenile periods are often very long, self- and cross-incompatibility and pollen and/or ovule sterility are relatively common, and the presence of adventitious somatic embryos in the nucellus greatly limits hybrid production [2]. For this reason, biotechnological breeding methods are used in citrus breeding.

Until today, some studies have been carried out to obtain dwarf rootstock through genetic engineering. Dwarf transgenic plants were obtained by transferring the *rol* genes to *Antirrhinum majus* L. [7], *Atropa belladonna* [8], *Malus domestica* [9], *Actinidia deliciosa* [10], *Juglans regia* [11], *Populus* [12], *Solanum ariculare* [13], *S. tuberosum* [14], *Citrus trifoliata* [15, 16], Troyer citrange [17, 18].

Acetosyringone is one of the phenolic compounds secreted by injured plant cells. The acetosyringone compound secreted from injured plant cells attracts the attention of *A. rhizogenes* bacteria through virulence genes located in the Ri plasmid. When there is no acetosyringone in the environment, the T-DNA region in *Agrobacterium* may not be transferred to the plant cell.

This study aims to determine the effect of different acetosyringone concentrations on the transformation of *rol* genes in citrus genotypes and check whether the plantlets obtained after transformation carry the *rol* genes by molecular methods.

MATERIALS AND METHODS

Plant Material

Tuzcu 3131 sour orange (*C. aurantium* L.), Gou Tou sour orange (*C. aurantium* L.), Carrizo citrange (*Poncirus trifoliata* Raf. X *C. sinensis* Osb. var. "Carrizo"), Rough lemon (*C. jambhiri* Lush.), Volkameriana (*C. volkameriana* Tan. and Pasq.), Trifoliolate orange (*C. trifoliata* L.) were used in this study. Seeds were obtained from Çukurova University, Agriculture Faculty, Horticulture Department, Tuzcu Citrus Variety Collection, Adana, Turkey.

Bacterial Strain and Plasmid

A. rhizogenes ATCC15834 (LGC Promochem, Boras, Sweden) was used for induction of hairy roots. This wild-type *A. rhizogenes* strain ATCC15834 has been used in molecular breeding studies to produce Ri plants [6, 19, 20] and this strain contains the agropine-type plasmid pRi15834. This strain was cultured in liquid MYA medium [21] and shaken at 300 rpm for 48 h in darkness at 28 °C.

Transformation Assays

Hypocotyl, cotyledon, leaf and internodal stem explants were used in transformation studies. Explants were inoculated with *A. rhizogenes* suspension (OD600 = 0.3) and shaken at 80 rpm for 30 min. Different concentrations of acetosyringone (0, 25, 50, 100 and 200 µM) were added to the inoculation fluid to determine the effect of acetosyringone in transformation studies. Then the explants were blotted onto sterile filter paper and further co-cultivated with *A. rhizogenes* on MS [22], in petri dishes for 2 days in darkness at 24 °C. After co-cultivation, the explants were rinsed in sterile water, blotted onto sterile filter paper and cultured on MS containing 500 mg/l Cef [6].

All cultures were kept in the dark at 26 °C for 4 weeks. After the formation of callus/shoot from the cuts of the segment, the explants were transferred onto MS medium including 1 mg/l BA and the cultures were kept under light conditions with 16 hours photoperiod. In all transformation assays MYA liquid medium without bacteria was applied to the explants as a control.

PCR analysis

Polymerase chain reaction (PCR) was used to confirm the integration of T-DNA into the plant genome. DNA was extracted from shoots of candidate Ri-lines and control plants following CTAB protocol [23]. *rol* genes were amplified with a specific primer set shown in Table 1.

Table 1. Specific primer set for *rol* genes

Primer Sets	Genes	Primer Sequences (5'-3')	Amplification Length (bp)	Annealing temperature (°C)
1	<i>rolA</i>	F: ACGGTGAGTGTGGTTGTAGG R: GCCACGTGCGTATTAATCCC	403	55.0
2	<i>rolC</i>	F: CTAGACACATGAAGAACGCC R: GAAAAAACCTGATGCTTGCC	272	56.0
3	<i>rolC</i>	F: TGTGACAAGCAGCGATGAGC R: AAACCTTGCACTCGCCATGCC	480	55.0
4	<i>rolD</i>	F: AACTTTTCCAGCCCCAAAC R: CACTATCAAACCTCGATATCC	402	55.0

The following temperature programme was applied for amplification in the DNA thermal cycler (AB Applied Biosystems ® Gene Amp ® PCR System 2700): 95 °C for 3 min, followed by 35 cycles 95 °C for 30 s (denaturation), primer set depending on temperature (Table 1) for 30 s (annealing) and 72 °C for 1 min (elongation), with a final 5 min elongation at 72 °C. The amplified fragments sequences were fractionated in 1% TAE agarose gel [6].

Statistical Analysis

Experiments were performed in a completely randomized design with three replicates. All data were calculated as percentages and arcsine-transformed. Means were separated by analysis of variance and the LSD test was performed. Data analysis was performed by using the JMP® software (SAS Institute, Cary, NC) ver. 8.00 and significance were considered at P < 0.05.

RESULTS AND DISCUSSION

Thirty days after *A. rhizogenes* infection, putatively transformed roots and shoots were scored on the injured surface of the explants. Root formation was obtained from leaf (at all acetosyringone concentrations) and internodal stem explants, shoot formation was obtained from internodal stem explants in Gou Tou sour orange. Root and shoot growth was not observed from hypocotyl and cotyledon explants in Gou Tou sour orange. The highest root growth was obtained from leaf explants (63.16%) at 25 μM acetosyringone concentration. The highest shoot formation (7.69%) was obtained from 25 μM acetosyringone concentration in internodal stem explants.

Most root growth was obtained from Tuzcu 31-31 orange (34.88%) at 25 μM acetosyringone concentration. Roots and shoots were obtained from stem explants at all acetosyringone concentrations except 100 μM . The highest shoot growth from stem explants was obtained at 200 μM acetosyringone concentration (31.25%), and the highest root growth at 25 μM acetosyringone concentration (15%) in Tuzcu 31-31 orange.

In Volkameriana; the highest shoot formation from hypocotyl explants was obtained at 25 μM acetosyringone concentration (9.52%). Roots were obtained from leaf explants at all acetosyringone concentrations except at 0 μM acetosyringone concentration. Shoot development was not observed from leaf explants. The highest shoot growth from stem explants was at 17.39% in 200 μM acetosyringone concentration, while the highest root growth was obtained at 100 μM acetosyringone concentration (52.63%). Images of roots obtained from stem explants as a result of transformation studies using different acetosyringone concentrations in Volkameriana genotype are presented in Fig 1.



Fig. 1. Roots developing from the stem explants of Volkameriana

In Trifoliate orange, root and shoot emergence from only stem explants was observed. Root and/or shoot growth was not observed from hypocotyl, leaf and cotyledon explants.

Root and shoot emergence from stem explants were observed in Carrizo citrange. Only root growth was observed from hypocotyl and leaf explants. Root and/or shoot growth

was not observed from the cotyledon explants. Shoots were obtained at 100 and 200 μM acetosyringone (Fig 2) and roots were obtained at all acetosyringone concentrations from stem explants. The highest shoot formation from stem explants was obtained at 100 μM acetosyringone concentration (25%), while the highest root formation was obtained at 50 μM acetosyringone concentration (36.84%). Root growth was observed from leaf explants only at 100 μM acetosyringone concentration and root growth percentage was determined as 18.75.



Fig. 2. Shoot obtained as a result of transformation in Carrizo citrange

Root and shoot emergence from stem explants was observed in the Rough lemon. While only root development was observed from hypocotyl explants, root and/or shoot growth was not obtained from cotyledon and leaf explants. Shoot at 0 and 200 μM acetosyringone concentration and root at all acetosyringone concentrations were obtained from stem explants. Shoot formation from stem explants was determined as 20% at both 0 and 200 μM acetosyringone concentration, while the highest root formation was obtained at 0 μM acetosyringone concentration (50%). Obtaining shoots at 0 μM acetosyringone concentration from stem explants is thought to be due to the presence of growth nodes in stem explants. It can be considered that the amount of acetosyringone secreted from injured tissue is sufficient to attract the interest of the bacteria.

Data on shoots developed from explants for all genotypes are given in Table 2a-f, and data on root formation are given in Table 3a-f. The graphic of shoot and root formation is given in Fig 3.

Table 2a. Data on shoots developed from explants for Carrizo citrange

Genotype	Acetosyringone (µM)	Explant	Genotype* Acetosyringone* Explant	Genotype* Acetosyringone	Genotype Mean
Carrizo citrange	0	Hypocotyl	0.00p (0.00)	0.00m (0.00)	1.83D (2.41)
		Cotyledon	0.00p (0.00)		
		Internodal stem	0.00p (0.00)		
		Leaf	0.00p (0.00)		
	25	Hypocotyl	0.00p (0.00)	0.00m (0.00)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	0.00p (0.00)		
		Leaf	0.00p (0.00)		
	50	Hypocotyl	0.00p (0.00)	0.00m (0.00)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	0.00p (0.00)		
		Leaf	0.00p (0.00)		
	100	Hypocotyl	0.00p (0.00)	6.25ef (7.15)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	25.00c (28.62)		
		Leaf	0.00p (0.00)		
	200	Hypocotyl	0.00p (0.00)	2.94h (4.90)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	11.76g (19.63)		
		Leaf	0.00p (0.00)		

Table 2b. Data on shoots developed from explants for Volkameriana

Genotype	Acetosyringone (µM)	Explant	Genotype* Acetosyringone* Explant	Genotype* Acetosyringone	Genotype Mean
Volkameriana	0	Hypocotyl	0.00p (0.00)	1.39l (3.36)	2.75C (5.06)
		Cotyledon	0.00p (0.00)		
		Internodal stem	5.56m (13.46)		
		Leaf	0.00p (0.00)		
	25	Hypocotyl	9.52i (17.68)	2.38i (4.42)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	0.00p (0.00)		
		Leaf	0.00p (0.00)		
	50	Hypocotyl	0.00p (0.00)	0.00m (0.00)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	0.00p (0.00)		
		Leaf	0.00p (0.00)		
	100	Hypocotyl	3.85o (11.23)	3.58de (7.43)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	10.50h (18.49)		
		Leaf	0.00p (0.00)		
	200	Hypocotyl	8.33jk (16.53)	6.43a (10.12)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	17.39f (23.95)		
		Leaf	0.00p (0.00)		

Table 2c. Data on shoots developed from explants for Rough lemon

Genotype	Acetosyringone (µM)	Explant	Genotype* Acetosyringone* Explant	Genotype* Acetosyringone	Genotype Mean
Rough lemon	0	Hypocotyl	0.00p (0.00)	5.00g (6.42)	2.00D (2.57)
		Cotyledon	0.00p (0.00)		
		Internodal stem	20.00e (25.70)		
		Leaf	0.00p (0.00)		
	25	Hypocotyl	0.00p (0.00)	0.00m (0.00)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	0.00p (0.00)		
		Leaf	0.00p (0.00)		
	50	Hypocotyl	0.00p (0.00)	0.00m (0.00)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	0.00p (0.00)		
		Leaf	0.00p (0.00)		
	100	Hypocotyl	0.00p (0.00)	0.00m (0.00)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	0.00p (0.00)		
		Leaf	0.00p (0.00)		
	200	Hypocotyl	0.00p (0.00)	5.00g (6.42)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	20.00e (25.70)		
		Leaf	0.00p (0.00)		

Table 2d. Data on shoots developed from explants for Trifoliolate orange

Genotype	Acetosyringone (µM)	Explant	Genotype* Acetosyringone* Explant	Genotype* Acetosyringone	Genotype Mean
Trifoliolate orange	0	Hypocotyl	0.00p (0.00)	7.14cd (7.71)	4.48B (5.42)
		Cotyledon	0.00p (0.00)		
		Internodal stem	28.57b (30.84)		
		Leaf	0.00p (0.00)		
	25	Hypocotyl	0.00p (0.00)	0.00m (0.00)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	0.00p (0.00)		
		Leaf	0.00p (0.00)		
	50	Hypocotyl	0.00p (0.00)	4.46g (6.06)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	17.86f (24.27)		
		Leaf	0.00p (0.00)		
	100	Hypocotyl	0.00p (0.00)	4.54g (6.12)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	18.18f (24.49)		
		Leaf	0.00p (0.00)		
	200	Hypocotyl	0.00p (0.00)	6.25ef (7.20)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	25.00c (28.80)		
		Leaf	0.00p (0.00)		

Table 2e. Data on shoots developed from explants for Tuzcu 31-31 sour orange

Genotype	Acetosyringone (µM)	Explant	Genotype* Acetosyringone* Explant	Genotype* Acetosyringone	Genotype Mean
Tuzcu 31-31 sour orange	0	Hypocotyl	0.00p (0.00)	5.77f (6.90)	4.31A (5.69)
		Cotyledon	0.00p (0.00)		
		Internodal stem	23.08d (27.63)		
		Leaf	0.00p (0.00)		
	25	Hypocotyl	18.18f (24.49)	5.79b (9.27)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	5.00n (12.60)		
		Leaf	0.00p (0.00)		
	50	Hypocotyl	0.00p (0.00)	2.17j (4.22)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	8.70j (16.88)		
		Leaf	0.00p (0.00)		
	100	Hypocotyl	0.00p (0.00)	0.00m (0.00)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	0.00p (0.00)		
		Leaf	0.00p (0.00)		
	200	Hypocotyl	0.00p (0.00)	7.81c (8.07)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	31.25a (32.29)		
		Leaf	0.00p (0.00)		

Table 2f. Data on shoots developed from explants for Tuzcu 31-31 Gou Tou sour orange

Genotype	Acetosyringone (µM)	Explant	Genotype* Acetosyringone* Explant	Genotype* Acetosyringone	Genotype Mean
Gou Tou sour orange	0	Hypocotyl	0.00p (0.00)	0.00m (0.00)	0.69E (1.50)
		Cotyledon	0.00p (0.00)		
		Internodal stem	0.00p (0.00)		
		Leaf	0.00p (0.00)		
	25	Hypocotyl	0.00p (0.00)	1.92jk (3.97)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	7.69k (15.88)		
		Leaf	0.00p (0.00)		
	50	Hypocotyl	0.00p (0.00)	1.56kl (3.57)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	6.25l (14.31)		
		Leaf	0.00p (0.00)		
	100	Hypocotyl	0.00p (0.00)	0.00m (0.00)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	0.00p (0.00)		
		Leaf	0.00p (0.00)		
	200	Hypocotyl	0.00p (0.00)	0.00m (0.00)	
		Cotyledon	0.00p (0.00)		
		Internodal stem	0.00p (0.00)		
		Leaf	0.00p (0.00)		

LSD genotype: 0.17***, LSD acetosyringone: 0.15***, LSD explant: 0.14***, LSD genotype*acetosyringone: 0.39***, LSD genotype*explant: 0.34***, LSD explant*acetosyringone: 0.32***, LSD genotype*explant*acetosyringone: 0.78***

Table 3a. Data on roots developed from explants for Carrizo citrange

Genotype	Acetosyringone (µM)	Explant	Genotype* Acetosyringone* Explant	Genotype* Acetosyringone	Genotype Mean
Carrizo citrango	0	Hypocotyl	0.00x (0.00)	8.39mn (8.37)	9.28B (10.26)
		Cotyledon	0.00x (0.00)		
		Internodal stem	33.58ijk (33.51)		
		Leaf	0.00x (0.00)		
	25	Hypocotyl	16.66p (23.43)	11.30f (13.56)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	28.57l (30.84)		
		Leaf	0.00x (0.00)		
	50	Hypocotyl	0.00x (0.00)	9.05klm (8.71)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	36.21gh (34.87)		
		Leaf	0.00x (0.00)		
	100	Hypocotyl	0.00x (0.00)	8.85g (12.07)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	16.67p (23.42)		
		Leaf	18.75o (24.88)		
	200	Hypocotyl	0.00x (0.00)	8.82lmn (8.60)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	35.29hi (34.40)		
		Leaf	0.00x (0.00)		

Table 3b. Data on roots developed from explants for Volkameriana

Genotype	Acetosyringone (µM)	Explant	Genotype* Acetosyringone* Explant	Genotype* Acetosyringone	Genotype Mean
Volkameriana	0	Hypocotyl	88.24a (59.57)	22.06e (14.89)	17.07A (16.19)
		Cotyledon	0.00x (0.00)		
		Internodal stem	0.00x (0.00)		
		Leaf	0.00x (0.00)		
	25	Hypocotyl	0.00x (0.00)	9.84k (9.11)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	0.00x (0.00)		
		Leaf	39.39f (36.45)		
	50	Hypocotyl	0.00x (0.00)	4.34n (8.22)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	12.5r (20.27)		
		Leaf	4.88w (12.63)		
	100	Hypocotyl	19.23o (25.20)	26.51a (25.42)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	52.63c (42.65)		
		Leaf	34.21ij (33.85)		
	200	Hypocotyl	16.67p (23.42)	22.62b (23.31)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	26.09m (29.43)		
		Leaf	47.73e (40.41)		

Table 3c. Data on roots developed from explants for Rough lemon

Genotype	Acetosyringone (µM)	Explant	Genotype* Acetosyringone* Explant	Genotype* Acetosyringone	Genotype Mean
Rough lemon	0	Hypocotyl	0.00x (0.00)	12.5i (10.36)	6.44D (7.58)
		Cotyledon	0.00x (0.00)		
		Internodal stem	50.00d (41.45)		
		Leaf	0.00x (0.00)		
	25	Hypocotyl	16.66p (23.42)	7.29h (10.92)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	12.5r (20.26)		
		Leaf	0.00x (0.00)		
	50	Hypocotyl	0.00x (0.00)	8.00n (8.17)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	32.00k (32.69)		
		Leaf	0.00x (0.00)		
	100	Hypocotyl	0.00x (0.00)	2.77q (4.77)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	11.11s (19.11)		
		Leaf	0.00x (0.00)		
	200	Hypocotyl	0.00x (0.00)	1.66r (3.69)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	6.67v (14.79)		
		Leaf	0.00x (0.00)		

Table 3d. Data on roots developed from explants for Trifoliate orange

Genotype	Acetosyringone (µM)	Explant	Genotype* Acetosyringone* Explant	Genotype* Acetosyringone	Genotype Mean
Trifoliate orange	0	Hypocotyl	0.00x (0.00)	0.00s (0.00)	4.90E (4.95)
		Cotyledon	0.00x (0.00)		
		Internodal stem	0.00x (0.00)		
		Leaf	0.00x (0.00)		
	25	Hypocotyl	0.00x (0.00)	8.33mn (0.35)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	33.33jk (33.40)		
		Leaf	0.00x (0.00)		
	50	Hypocotyl	0.00x (0.00)	0.00s(0.00)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	0.00x (0.00)		
		Leaf	0.00x (0.00)		
	100	Hypocotyl	0.00x (0.00)	6.81o (7.52)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	27.27lm (30.11)		
		Leaf	0.00x (0.00)		
	200	Hypocotyl	0.00x (0.00)	9.39kl (8.88)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	37.50g (35.52)		
		Leaf	0.00x (0.00)		

Table 3e. Data on roots developed from explants for Tuzcu 31-31 sour orange

Genotype	Acetosyringone (µM)	Explant	Genotype* Acetosyringone* Explant	Genotype* Acetosyringone	Genotype Mean
Tuzcu 31-31 sour orange	0	Hypocotyl	0.00x (0.00)	9.06g (11.67)	7.13B (10.34)
		Cotyledon	0.00x (0.00)		
		Internodal stem	7.69u (15.85)		
		Leaf	28.57l (30.83)		
	25	Hypocotyl	0.00x (0.00)	14.13c (17.77)	
		Cotyledon	6.67v (14.75)		
		Internodal stem	15.00q (22.15)		
		Leaf	34.88hij (34.19)		
	50	Hypocotyl	0.00x (0.00)	5.94j (9.79)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	8.7t (16.89)		
		Leaf	15.09q (22.28)		
	100	Hypocotyl	0.00x (0.00)	1.66r (3.68)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	0.00x (0.00)		
		Leaf	6.67v (14.75)		
	200	Hypocotyl	0.00x (0.00)	4.85klm (8.77)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	6.25v (14.27)		
		Leaf	13.16r (20.81)		

Table 3f. Data on roots developed from explants for Tuzcu 31-31 Gou Tou sour orange

Genotype	Acetosyringone (µM)	Explant	Genotype* Acetosyringone* Explant	Genotype* Acetosyringone	Genotype Mean
Gou Tou sour orange	0	Hypocotyl	0.00x (0.00)	7.29h ₁ (10.64)	8.07C (9.52)
		Cotyledon	0.00x (0.00)		
		Internodal stem	8.33tu (16.36)		
		Leaf	20.83n (26.21)		
	25	Hypocotyl	0.00x (0.00)	17.69d (15.79)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	7.69u (15.85)		
		Leaf	63.08b (47.34)		
	50	Hypocotyl	0.00x (0.00)	9.89g (11.89)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	6.25v (14.20)		
		Leaf	33.33jk (33.37)		
	100	Hypocotyl	0.00x (0.00)	3.84p (5.62)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	0.00x (0.00)		
		Leaf	15.38q (22.48)		
	200	Hypocotyl	0.00x (0.00)	1.64r (3.65)	
		Cotyledon	0.00x (0.00)		
		Internodal stem	0.00x (0.00)		
		Leaf	6.59v (14.27)		

LSD genotype: 0.20***, LSD acetosyringone: 0.18***, LSD explant: 0.16***, LSD genotype*acetosyringone: 0.46***, LSD genotype*explant: 0.54***, LSD explant*acetosyringone: 0.38***, LSD genotype*explant*acetosyringone: 0.93***

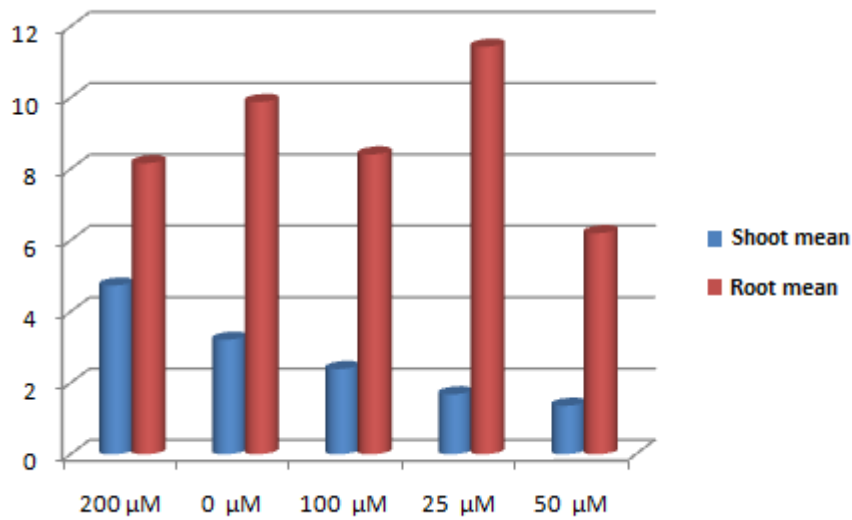


Fig 3. The graph of shoot and root formation

The control of whether the shoots and roots carry the *rol* genes was carried out by PCR analysis (Fig 4).

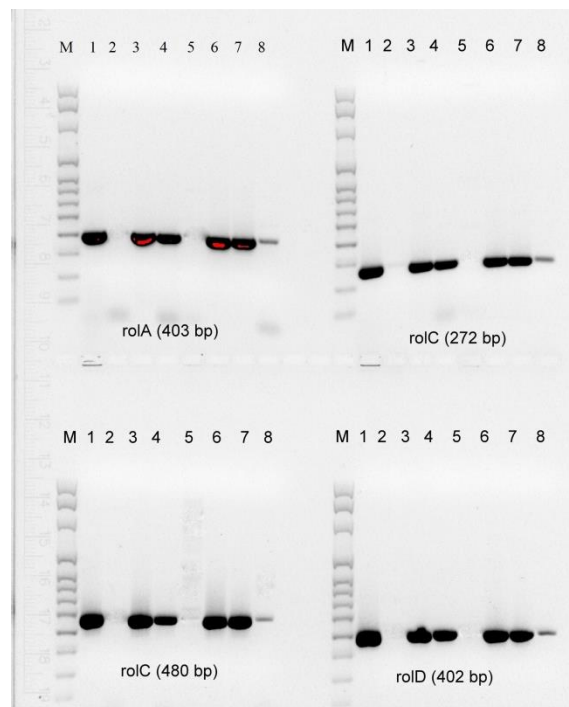


Fig.4. M: Marker. 1: Bacteria DNA, 2: Carrizo citrange control DNA, 3- 4: Putative shoots DNA of Carrizo citrange, 5: Tuzcu 31-31 sour orange control DNA, 6-7-8. Putative roots DNA of Tuzcu 31-31 sour orange

Transformation efficiency is depend on gene and explant type, co-cultivation conditions and *Agrobacterium* strain [24]. Also, the concentration of acetosyringone used in *Agrobacterium*-mediated gene transfer is very important in terms of transformation efficiency. Therefore, it is very important to determine the appropriate acetosyringone concentration in transformation studies. So, researchers have tried to determine the optimum acetosyringone concentration in different plant species to increase

transformation efficiency. It was investigated different acetosyringone concentrations (50-500 μM) and callus age to optimize *Agrobacterium*-mediated gene transfer in paddy. EHA 105 strain containing the binary vector p35SGUSINT of *A. tumefaciens* was used in that study. The most transformation efficiency was obtained from 35-day callus with 350 μM acetosyringone [25]. Godwin et al. [26] were investigated transformation efficiency in 5 plant species (*Allium cepa*, *Antirrhinum majus*, *Brassica campestris*, *Glycine max*, and *Nicotiana tabacura*) using three *A. tumefaciens* strains (N2 / 73, A281 and Ach5) in the presence and absence of 200 μM acetosyringone. The presence of acetosyringone during co-cultivation increased transformation efficiency. When *N. tabacum* was infected with Ach5 strain, transformation efficiency was higher in the absence of acetosyringone. These findings suggest that acetosyringone may suppress virulence genes in some strain/plant species interactions. In our study, different acetosyringone concentrations were used during the inoculation of explants belonging to citrus genotypes to increase transformation efficiency. The optimum acetosyringone concentration was different for each genotype and explant used in the assays. The highest root formation from stem explants in Gou Tou sour orange and Rough lemon, highest root formation from hypocotyl explants in Volkameriana genotype, and highest shoot formation from stem explants in Trifoliate orange were obtained from acetosyringone-free trials. These findings show the importance of the effect of acetosyringone concentration/bacterial strain/plant species/explant type interaction on the activation of virulent genes in *Agrobacterium*-mediated gene transfer.

CONCLUSION

Plant breeding studies are long-term studies that can keep for many years. With the rapid development of biotechnological methods in recent years, these breeding processes have been shortened by adapting biotechnological tools to plant breeding programs [27]. In this study, the effect of different acetosyringone concentrations on obtaining dwarf rootstock, which is very important for citrus, was investigated. In conclusion, the optimum acetosyringone concentration was different for each genotype and explant used in the assays. In general, the highest shoot formation was obtained at 200 μM acetosyringone concentration and the highest root formation was obtained at 25 μM acetosyringone concentration.

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