Tr. J. of Botany
23 (1999) 373-377

Research Article

Agrobacterium rhizogenes-mediated Hairy root Formation in Some *Rubia tinctorum* L. Populations Grown in Turkey

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Received: 14.09.1998
Accepted: 29.07.1999

Abstract: *Rubia tinctorum* L. seeds were collected from 4 different regions of Turkey. Plants were grown in greenhouse and cotyledons were inoculated with *Agrobacterium rhizogenes* strains 15834, 2628, R1000 and 9365. *Rubia tinctorum* plants were also grown in the field for comparative analysis of root anthraquinone content.

Agrobacterium rhizogenes strain 2628 induced only callus formation on the cut surface of cotyledon explants while strains 15834, R1000 and 9365 produced hairy roots on the same explants. Anthraquinone content was identified in the extracts of the plant roots grown in the field and in the extracts of the *Agrobacterium rhizogenes*-induced hairy roots. The percentage of anthraquinone present in the plant roots was found to be different from that in the hairy roots.

Key Words: *Rubia tinctorum L., Agrobacterium rhizogenes*, hairy roots, anthraquinone

Türkiye'de Yetişen Bazı *Rubia tinctorum* L. Populasyonların da *Aorabacterium rhizogenes* aracılığı ile Saçak Kök Oluşumu

Özet: *Rubia tinctorum* L. tohumları Türkiye'nin 4 farklı bölgesinden toplanmıştır. Bitkiler serada yetiştirildikten sonra kotiledon yaprakları *Agrobacterium rhizogenes*'in 15834, 2628, R1000 ve 9365 suşları ile enfekte edilmiştir. *Rubia tinctorum* bitkileri köklerindeki anthraquinone içeriğini karşılaştırmak için tarlada da yetiştirilmiştir.

Agrobacterium rhizogenes'in 2628 suşu kotiledon eksplantların kesim yüzeylerinde sadece kallus oluştururken 15834, R1000 ve 9365 suşları da aynı eksplantta saçak kök oluşturmuşlardır. Tarlada yetişen bitki köklerinin ekstraktında ve *Agrobacterium rhizogenes*'in oluşturduğu saçak köklerin ekstraktında anthraquinone içeriği belirlenmiştir. Bitki köklerinde ve saçak köklerde var olan yüzde anthraquinone farklı bulunmuştur.

Anahtar Sözcükler: *Rubia tinctorum L., Agrobacterium rhizogenes*, saçak kök, anthraquinone

Indroduction

Rubia tinctorum L., commonly known as wild madder, is a member of the *Rubiaceae*. It is a herbaceous perennial plant. The roots of the plant have been known for their dyeing and medicinal properties since ancient times. These characteristics are due to the presence of anthraquinones (1). Owing to their heat and light resistant characteristics, anthraquinones have potential use in the food processing industries (2).

A. rhizogenes, the causative agent of hairy root syndrome, is a common soil organism capable of entering a plant through a wound and causing a proliferation of secondary roots (3-5). The underlying mechanism of hairy root formation is the transfer of several bacterial genes to the plant genome. The observed morphogenic effects in the plants after infection have been attributed to the transfer of part of a large plasmid known as the Ri (Root-inducing) plasmid. The symptoms observed with *A. rhizogenes* are suggestive of auxin effects resulting from an increase in cellular auxin sensitivity rather than auxin production (4).

A. rhizogenes – transformed root cultures of *Rubia tinctorum* have already proven to be useful tools in the study of condensed anthraquinone biosynthesis. A number of plant species have been transformed with *A. rhizogenes,* including *R. tinctorum,* and the established root cultures have been shown to produce target secondary compounds (6).

In this paper, the induction and growth of hairy roots from *Rubia tinctorum* using *Agrobacterium rhizogenes* is presented. The purpose of this study is to investigate the interaction between *Rubia tinctorum* populations collected from different regions of Turkey, and various *A. rhizogenes* strains.

Materials and Methods

Plant Material

The madder (*Rubia tinctorum*) seeds were collected from Izmir (Çesme), Antalya (Bademagaci), Usak and Konya. After germination in a greenhouse, cotyledon explants were excised from 14-day old seedlings, sterilized by treatment with 70% ethanol (30 sec.) and 5% sodium hypochlorite (15 min), and then rinsed three times with sterile water (3, 7).

Bacterial Strains

Agrobacterium rhizogenes strains 15834, R1000, 2628 and 9365 were used for inoculation of cotyledons. The strains were kindly provided by S. Çetiner and M. Tör. These strains were grown on NB (Nutrient Broth) medium at 28˚C for 2 days (8).

Induction of Hairy roots via A. rhizogenes Infection

Surface-sterilised cotyledons were wounded and infected with *A. rhizogenes* strains 15834, 2628, R1000 and 9365. The inoculated cotyledons were co-cultivated with *A. rhizogenes* strains for 2 days at 25˚C with a 16 h photoperiod (9). The experiment was designed to be completely randomized with four replicates. Forty explants were used for each population. After co-cultivation, explants were transferred to semi-solid MS (Murashige and Skoog) medium solidified with 0.8% agar, and contained 3% sucrose (10), plus 0.4 g/l Augmentin to kill the bacteria (pH: 5.7) at a density of 10 explants per plate (9 cm petri dish), and cultured at 25˚C, with a 16 h photoperiod. Frequency of hairy root formation for each treatment was scored 30 days after co-cultivation. Individual roots emerged from the wound sites were excised and subcultured onto the same medium. Forty days after co-cultivation, hairy roots were weighed out and transferred to 50 ml of MS liquid medium (pH= 5.7) containing 3% sucrose, and shaken in an orbital shaker at 120 rev/min at 25˚C in the dark (9). The roots were then subcultured onto the same medium every 4 weeks. After 4 months in liquid culture, hairy roots from each explant were weighed out and the mean weight for each treatment was calculated.

Statistical Analyses

Analysis of variance (ANOVA) was used to determine the significance of variance. According to the results, the Duncan test was applied to determine the groups.

Determination of Anthraquinone Contents

Hairy roots were dried in the dark at 60˚C for 2 days. Dried roots were powdered by mortar and pestle, and 50 mg of this fine powder was then soaked in 50 ml of distilled water for 16 h. This suspension was heated in water bath at 70˚C for 1 h. After the suspension was cooled, 50 ml of 50% methanol (MeOH) was added and then filtrated. The clear solution was measured by spectrophotometer (Shimadzu UV-160A) at a wavelength of 450 nm and compared with a standard solution containing 1mg/100ml alizarin and 1 mg/100ml purpurin with the absorption-maximum 450 nm (11).

Results and Discussion

In this research, genetically modified roots of *R. tinctorum* were produced in order to determine the virulent strains and their effects on the content of anthraquinones.

Induction of Hairy roots

Hairy roots were initiated on the cut surface of the cotyledon explants from four populations 10-12 days after co-cultivation with strain 15834 (Figure 1). Hairy root formation occured at the highest frequency (75%) in Konya, followed by Izmir (60%), Usak (34%) and Bademagaci (33%) via *A. rhizogenes* strain 15834. The strain R1000 induced hairy roots at a frequency of 50% and 15% in Konya and Izmir populations, respectively (Table 1). The untreated control cotyledon explants failed to elicit any hairy root response or substantial amounts of callus formation in any of the experiments.

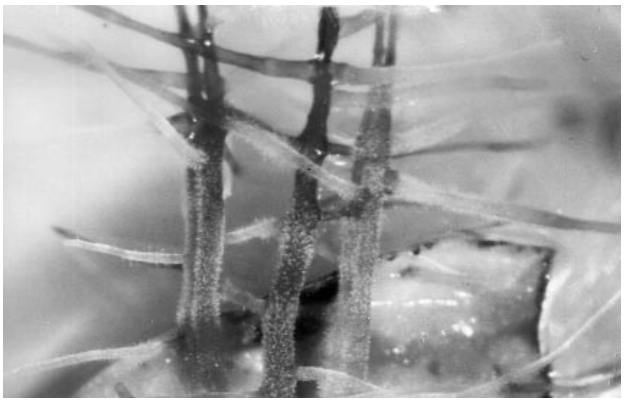

Figure 1. Hairy roots emerged from wound sites of cotyledon explants of *R. tinctorum* (Konya population) via strain 15834.

Table 1. Frequency of hairy root formation on different *R. tinctorum* populations via *A. rhizogenes* strains (%)

	A. rhizogenes strains			
Populations	15834	R1000	9365	2628
Konya	75	50	18	23
Izmir	60	15	17	-
Usak	34	-	-	-
Bademagaci	33	-	15	-

Frequency of hairy root formation=Percentage of explants that produced hairy roots

Significant variations were found among locations and *A. rhizogenes* strains at a level of $p<0.01$. The interaction between locations and *A. rhizogenes* strains was also significant at a level of $p<0.01$.

The Konya population produced the highest amount of hairy roots, followed by the Izmir, Bademagaci and Usak populations (Table 2). Differences between populations proved to be statistically significant. Strain 15834 resulted in the highest amount of hairy root formation in all populations. Strain 9365 was beneficial but not as effective as strain 15834. In contrast to these two strains, strain 2628 did not induce hairy roots in any of the populations used in this study. Thus, this strain seems to be totally avirulent to *R. tinctorum.* Similar results, were also reported by Sato et al. (9, 12) who obtained hairy roots from cotyledonary explants of *R. tinctorum* with strain 15834.

Strain R1000 induced hairy roots in Konya and Izmir populations, while strain 9365 induced hairy roots in Konya, Izmir and Bademagaci populations within 20 days of co-cultivation.

Cotyledonary explants from the Bademagaci population co-cultivated with strain 15834 produced the highest amount of hairy root formation, followed by explants from the Izmir population co-cultivated with strain 9365 and the Konya population co-cultivated with strain 15834 (Table 2). Bademagaci x strain 15834 was statistically significant. Izmir x strain 9365 and Konya x 15834 were also significant, but had lower mean values.

Table 2. Hairy root weights (mg) in locations, *A. rhizogenes* strains and locations x *A. rhizogenes* strains (40 days after co-cultivation)

Locations	Mean	Locations x *A. rhizogenes*	Mean
Konya	0.04793a	BA-15834	0.07420a
Izmir	0.04542b	I-9365	0.06083b
Bademagacı	0.04198c	K-15834	0.06067b
Usak	0.01084d	BA-9365	0.05173c
		I-15834	0.04910d
LSD value= 0.0007222		K-9365	0.04227e
A. rhizogenes strains	Mean	K-R1000	0.04087f
15834	0.05412a	U-15834	0.03253g
9365	0.03871b	I-R1000	0.02633h
R1000	0.01680c	BA-R1000	0.00000ı
		U-9365	0.00000ı
		U-R1000	0.00000ı
LSD value= 0.0007222		LSD value= 0.0007222	

BA; Bademagaci, I; Izmir, K; Konya, U; Usak

Mean followed by the same letters are not significantly different from each other at the 1% level as determined by LSD.

In terms of hairy root induction, different populations exhibited varying degrees of responses. All strains except 2628 induced hairy roots in Konya and Izmir populations. Hairy roots were induced by strains 15834 and 9365 in the Antalya population and only strain 15834 in the Uşak population. These results show that genotype is an important factor for hairy root induction via *A. rhizogenes*. These results support the findings of Dobigny et al. (8), who demonstrated that hairy roots of two cultivars of potato were produced after inoculation with the strains 2659 and 2659 GUS. In contrast, few *rhizogenic* responses or no response at all were obtained with the strains 15834 and 8196 GUS.

In the present study, the Konya population was found to be the most amenable for induction of hairy roots, and strain 15834 was found to be the most virulent strain towards *R. tinctorum* in the present conditions.

Growth of Hairy Roots in Liquid Culture

Fresh weights of the hairy roots after 4 months in liquid culture were significantly higher than their initial weights (Tables 2 and 3).

Table 3. Hairy root weights (mg) in locations, *A. rhizogenes* strains and locations x *A. rhizogenes* (after 4 months in liquid culture)

Locations	Mean	Locationsx*A.rhizogenes*	Mean
Konya	0.7249a	K-15834	1.5160a
Izmir	0.4923b	I-9365	0.8135b
Bademagacı	0.2345c	I-15834	0.6373bc
Usak	0.1588c	BA-15834	0.5474c
		U-15834	0.4765c
LSD value= 0.2167		K-R1000	0.4558c
A. rhizogenes strains	Mean	K-9365	0.2031d
15834	0.7942a	BA-9365	0.1560d
9365	0.2932b	I-R1000	0.0260d
R1000	0.1205b	BA-R1000	0.0000d
		U-9365	0.0000d
		U-R1000	0.0000d
LSD value= 0.2167		LSD value= 0.2167	

BA; Bademagaci, I; Izmir, K; Konya, U; Usak

Mean followed by the same letters are not significantly different from each other at the 1% level as determined by LSD.

As shown in Table 3, the fresh weight of hairy roots in the Konya population induced by strain 15834 increased by approximately 25 times. The corresponding increase for the Izmir population induced by strain 9365 was 13 times. Similarly, the fresh weights for the Konya population induced by strain R1000 increased by approximately 10 times; inducement by strain 15834 increased the fresh weight of the hairy roots by more than 10 times in Izmir and Usak populations and by approximately 7 times in the Bademagaci population.

However, in the Izmir x strain R1000 treatment, the amount of hairy roots was not increased by shaking in liquid culture.

As a result, inoculation with strain 15834 was found to be an effective means of inducing hairy root formation on *Rubia tinctorum* collected from different regions of Turkey. Previous studies have also revealed that the induction frequency of hairy root formation in madder cotyledonary explants was the highest with *A. rhizogenes* strain 15834 (8, 9, 12). Strain 9365 was not as effective as strain 15834 in this study.

Determination of Anthraquinone Content of Hairy Roots and Plant Roots

The anthraquinone contents of both hairy roots grown in vitro, and field grown control plant roots were compared. In all populations, particularly, in Antalya and Usak populations, the in-vitro anthraquinone contents of hairy roots were found in all cases to be lower than those measured in field grown plant roots. (Table 4)

The anthraquinone content of the Usak population was 3.22% in the plant roots, while it was 1.13% in hairy roots. In the Izmir population, the anthraquinone content of plant roots and of hairy roots grown in vitro was 1.29% and 1.05%, respectively. These values for the Konya population were 1.05% and 0.95%, respectively.

Recently, the differences between secondary metabolites produced by mother plants and the corresponding in vitro cultures have attracted the attention of many researchers (3, 6, 12). Sato et al. (12) reported that hairy roots of *R. tinctorum* produced anthraquinone pigments, but always at lower levels than in field grown plants. This was attributed to the influence of phytohormones and sucrose concentrations in the culture medium. They found that anthraquinone production was maximal in phytohormone-free medium with 12% sucrose (9). In the present study, only 3% sucrose in culture medium was tested, resulting in lower anthraquinone production.

The anthraquinone contents of different *R. tinctorum* populations collected from different regions of Turkey were found to be different (Table 4). In field condition, the Usak population produced the highest anthraquinone content (3.22%), while the Konya population produced the lowest percentage of anthraquinone (1.11%).

In-vitro root formation in *R. tinctorum* is important since it may enable early testing of anthraquinone contents of various plants in breeding programs.

Table 4. Comparison of anthraquinone contents between the field grown plants and in-vitro hairy roots.

Locations	In-vitro (%)	Field (%)
Konya	0.95	1.11
Izmir	1.05	1.29
Bademagaci	0.72	2.25
Usak	1.13	3.22

Acknowledgements

This research was financially supported by TUBITAK.

We are grateful to Dr. Simone Siebenborn for photometric analyses and Prof. Dr. Selim Çetiner and Assoc. Prof. Dr. Mahmut Tör for kindly providing us with *A. Rhizogenes* strains 9365 and 15834.

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