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ORIGINAL RESEARCH PAPER

Two-phase temporary immersion system for *Agrobacterium rhizogenes* genetic transformation of sage (*Salvia tomentosa* Mill.)

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Abstract Hairy root cultures of *Salvia tomentosa* were initiated by transformation with *Agrobacterium rhizogenes*. To prevent necrosis in the explants and to protect young hairy roots, Amberlite XAD-4 resin, in combination with a temporary immersion cultivation system, was applied. HPLC analyzes showed that the resin adsorbed more than 93% of the released phenolic acids and 100% of the released flavonoids. The decreased content of the released phenolics significantly reduced their destructive effects on the plant tissues, prevented, and speeded up the appearance of hairy roots.

Keywords *Agrobacterium rhizogenes* · Amberlite XAD4 · Necrosis · Phenolics · Sage · *Salvia tomentosa* Mill.

Introduction

Balsamic sage (*Salvia tomentosa* Mill.) is widely used either for tea preparation or as flavoring agent in perfumery and cosmetics. The aerial parts of mature plant accumulate essential oils and produce various compounds with strong antimicrobial and antioxidant activities (Tepe et al. 2005). In Bulgaria this plant is under threat and spreads in very limited populations. Since 2007 its collection has been prohibited for commercial purposes. Plant in vitro systems are promising technologies for obtaining biologically active substances and also for basic research of molecular biology and biochemistry of plant cells. Secondary metabolites are defined as products of differentiation and for this reason, research has been done on in vitro cultures of hairy roots derived from differentiated cells (Georgiev et al. 2007; Veena and Taylor 2007). However, there are no reports for hairy root cultures initiation of by *Salvia tomentosa* Mill. Probably the main reason is due to the presence of highly active molecules in the tissues, inhibiting the growth of bacteria and/or new formed hairy roots.

In the present work we describe an effective protocol for hairy roots obtaining of *Salvia tomentosa* Mill. mature plants using two-phase temporary immersion system for *Agrobacterium rhizogenes* transformation.

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Materials and methods

Plant material and explants sterilization

Young leaves of naturally growing mature *Salvia tomentosa* Mill. plants were harvested in an

experimental field of the Institute of Botany, Bulgarian Academy of Sciences, near Sofia, Bulgaria. They were sterilized by 70% (v/v) ethanol for 20 s followed by treatment with either calcium hypochlorite [4, 6 and 8% (w/v)] or Domestos [10, 20 and 30% (v/v)] for 3, 6 or 9 min.

Transformation techniques

Classical transformation approach: For hairy roots, suspensions of *Agrobacterium rhizogenes* ATCC 15834 were used with OD_{600} 0.5, 0.75 or 1. The methods of direct infection and co-cultivation were applied (Georgiev et al. 2007). 72 h after transformation, explants were transferred to solid MS media + 100, 200, or 400 mg Cefotaxime/l to kill the bacteria. Half of the explants were cultivated in darkness, and the remainder with illumination (16 h light and 8 h dark).

Submerged two-phase system for Agrobacterium rhizogenes transformation: Co-cultivation with *A. rhizogenes* ATCC 15834 (OD_{600} = 0.75) was used. After 72 h, the explants were transferred into flasks with 100 ml liquid MS media + 200 mg Cefotaxime/l and 1 g Amberlite XAD-4 resin, held in a net. Cultivation was at 26°C with shaking (110 rpm) and illumination (16 h light, 8 h dark).

Two-phase temporary immersion system for Agrobacterium rhizogenes transformation: Co-cultivation with *A. rhizogenes* ATCC 15834 (OD_{600} = 0.75) was used. After 72 h, the explants were transferred into a RITA apparatus (CIRAD Ltd., France) with 200 ml MS medium + 200 mg Cefotaxime/l and 2 g Amberlite XAD-4 resin in a net. Cultivation was at 26°C, with illumination (16 h light, 8 h dark), at immersion frequencies of 15 min flooding and 4, 8, and 12 h stand-by periods, respectively. The inlet air during flooding periods was AT 60 l/h for each RITA apparatus.

Amberlite XAD-4 activation

The activation was carried out according to the manufacturer's instructions. The activated resin was washed with double distillated water to neutral pH. The packs were sterilized in liquid MS medium at 121°C for 30 min before using in the experiments.

Extraction of phenolics

From the culture liquid: culture media were evaporated to dryness at 60°C, and the residue was dissolved in 10 ml methanol, cooled at −20°C for 3 h and the supernatant was passed through a 0.22 μm filter.

From Amberlite XAD-4 resin: the resin was extracted with 200 ml methanol for 2 h on a shaker in triplicate. The extracts were evaporated to dryness and the residue was dissolved in 10 ml methanol, filtered through 0.22 μm filter.

Analysis

Total phenolics: Total phenolics were assayed by the Folin–Ciocalteu method with gallic acid as standard (Georgiev et al. 2010).

HPLC: Samples were analysed for phenolic acids and flavonoids using Supelco Discovery HS C18 column (25 cm × 4.6 mm, 5 μm) at 25°C. Details given in Table 1.

PCR analysis

Integration of the Ri T-DNA into the plant genome was confirmed by PCR. Genomic DNA from isolated bacteria-free hairy root lines, the intact plant leaves, as well as from *A. rhizogenes* ATCC 15834 was extracted using InnuPREP Plant DNA kit (Analytik-Jena, Germany) and quantified by NanoDrop 2000C spectrophotometer (Thermo-scientific, USA). PCR was performed in 25 μl with 80 ng gDNA, 2 μM of each *rol*A gene-specific primer : forward 5′-AGA ATG GAA TTA GCC GGA CTA-3′ and reverse 5′-GTA TTA ATC CCG TAG GTT TGT TT-3′, 200 μM of each nucleotide, 1% reaction buffer (10 mM Tris/HCl, pH 8.3; 50 mM KCl, 1.5 mM $MgCl_2$) and 0.5 U Taq DNA polymerase per reaction-Ready-to go PCR Analysis Beads kit (GE Healthcare, USA), in Quanta Biotech PCR system QB-24 thermal cycler. PCR was carried out using an initial denaturation at 94°C for 5 min followed by 35 cycles each of 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C with a final extension of 72°C for 10 min. PCR products were separated on a 1.5% agarose gel electrophoresis in 0.6% TBE buffer [0.09 M TrisBoric acid and 2 mM EDTA (pH 8.0)] at

Table 1 HPLC analyses of phenolic acids and flavonoids

Setup	Phenolic acids							Flavonoids						
Time (min)	1	30	45	50	60	65	70	1	10	15	16	30	35	40
Solvent A (%)	95	65	30	20	0	95	95	70	50	50	48	20	70	70
Solvent B (%)	5	35	70	80	100	5	5	30	50	50	52	80	30	30
Flow rate (ml/min)	0.8	0.4	0.4	1.2	1.2	0.8	0.8	1.0	1.0	1.0	0.8	0.8	1.0	1.0

HPLC system–Waters, Milford, MA, USA [binary pump (Waters 11525), UV–VIS detector (Waters 2487) and Breeze 3.30 SPA software]. Solvents: for phenolic acids determination–2.0% (v/v) acetic acid (A) and 0.5% (v/v) acetic acid: acetonitrile (1:1, v/v) (B); for flavonoids determination–2.0% (v/v) acetic acid (A) and methanol (B). Detection at: 280 nm (for phenolic acids) and 380 nm (for flavonoids)

90 V, visualized by staining with ethidium bromide (10 mg/ml) and documented using a gel documentation system (BioDoc-it, UVP, USA). PCR products were determined by comparison to external size standard (50–2000 bp), USB Corporation, USA.

Results and discussion

Sterilization of the plant tissues prior to transformation with *Agrobacterium* was most effective with 6% (w/v) $Ca(ClO)_2$ for 6 min. More than 90% of leaves remained viable and were free of contaminants.

The effective removal of the *Agrobacterium* and the best viability of explants were achieved at cultivation with illumination on MS medium + 200 mg Cefotaxime/l. However, leakages of brown sap from the wounding points were observed and at the end of the third week plant tissues became dark brown (Fig. 1a, b). Under these conditions, root tips were observed at the infection points. Obviously, the *A. rhizogenes* ATCC 15834 had clearly transformed *S. tormenotsa* explants but the formed young hairy roots were killed by the release of necrotic tissue compounds (Fig. 1c). Most of these compounds possess significant phytotoxic activity (Reigosa and Pazos-Malvido 2007) or have strong allelopathic effects by inhibiting the plant root growth (Makoi and Ndakidemi 2007).

To overcome this problem, a two-phase cultivation system was applied. Such systems were widely used for target product recovery in various plant in vitro biotechnologies (Park et al. 2011). The application of this technique allowed us to remove the phenolics from the cultivation medium, to prevent the explants and the formed young hairy roots from their destructive effects. Recently, the polymeric resin Amberlite XAD-4 was successfully applied as adsorbent of the released toxic phenolics in the selection procedure for regeneration of transgenic *Aloe vera* plants (Velcheva et al. 2010). Explants cultivated with this resin remained fresh, without necrosis, and 50% of them formed hairy roots at the end of forth week (Fig. 1d). The analyses of the secreted phenolics showed that 73% of them were adsorbed from the resin. However, in spite of the effective explants protection and the successful hairy root initiation, this approach had a serious disadvantage, because developed hairy roots were easily broken to small pieces by the mechanical interactions of the explants and resin nets.

For solving the problem, a temporary immersion cultivation system was applied. These cultivation systems were developed mainly for the needs of plants micropropagation (Debnath 2009) but, recently, they found application in transgenic plant regeneration (Hanhineva and Karenlampi 2007) and secondary metabolite production (Pavlov and Bley 2006; Ivanov et al. 2011). Their suitable design allowed an effective separation of the resin nets from the explants. Further, it was established that the best cultivation regime was at 15 min flooding and 12 h stand-by periods. Under these conditions, 100% of the explants intensively formed fast growing hairy roots at the end of the second week. The explants remained green and transformed roots were white, branched and possessed intensive growth (Fig. 1e). The positive effect on the hairy roots formation was due to the depletion of the phenolic compounds. HPLC analyses (Table 2) showed that explants of *S. tomentosa* secreted high concentrations of phenolic acids and flavonoids, as most of them were adsorbed from the resin. These results are important because phenolic acids above 1000 μg/l have strong growth inhibitory effects on plants roots and seedlings (Einhellig et al. 1985;

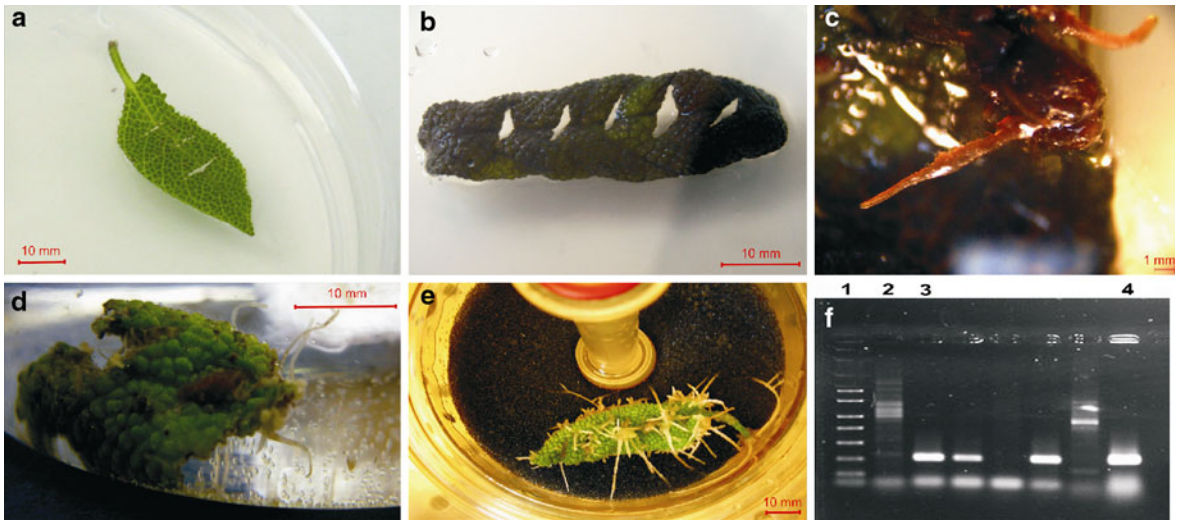

Fig. 1 Explants of *Salvia tomentosa* Mill. plants, infected with *Agrobacterium rhizogenes* ATTC 15834: one day after infection (**a**), three weeks after infection (**b**); Dead hairy roots on necrotic leaves, cultivated for three weeks on solid MS medium (**c**). Explants with hairy roots, cultivated in flasks with Amberlite XAD-4 for four weeks (**d**); Two weeks old explants and hairy roots, cultivated in two-phase temporary immersion systems at 15 min flooding and 12 h stand-by periods (**e**); PCR amplification of *rol*A gene (**f**): DNA marker (*1*), the extracted DNA from the leaf of the intact plant *Salvia tomentosa* Mill. (*2*), the extracted DNA from the hairy roots of the transformed *Salvia tomentosa* Mill. (*3*), the amplified DNA of *rol*A gene from *Agrobacterium rhizogenes* ATTC 15834 plasmid (*4*)

Table 2 Phenolic compounds determined in two-phase temporary immersion system during *Agrobacterium rhizogenes* transformation of *Salvia tomentosa* Mill. explants

Compound	Culture medium, μg/RITA[a]	Adsorbed on Amberlite XAD-4, μg/RITA[a]	Degree of adsorption, %
Phenolic acids			
Gallic acid	51 ± 17	39 ± 6	39
Protocatechuic acid	9 ± 2	188 ± 29	95
Salicylic acid	35 ± 14	446 ± 137	93
Chlorogenic acid	24 ± 5	331 ± 115	93
Vanillic acid	9 ± 2	332 ± 121	97
Caffeic acid	22 ± 5	131 ± 36	86
Syringic acid	3	167 ± 69	98
p-Coumaric acid	4 ± 1	44 ± 18	92
Sinapic acid	4 ± 2	521 ± 136	99
Ferulic acid	6 ± 3	232 ± 62	98
Cinnamic acid	5 ± 3	27 ± 7	86
Flavonoids			
Myricetin	0	47 ± 10	100
Hesperidin	0	21 ± 3	100
Quercetin	0	25 ± 6	100
Luteolin	0	4	100
Kaempferol	0	11 ± 2	100

The presented values are means ± SD

[a] Amount in one RITA apparatus

Machrafi et al. 2006; Makoi and Ndakidemi 2007; Reigosa and Pazos-Malvido 2007). Moreover, some compounds, such as salicylic acid, vanillic acid, caffeic acid, p-coumaric acid and ferulic acid, have root growth inhibitory effect depending of their concentration (Mandal et al. 2010). 85% of these

compounds were removed from the medium. Clorogenic acid, having strong prooxidant nature (Sakihama et al. 2002), was also removed (93%) from the medium. In contrast, only 39% of gallic acid, which acts primary as antioxidant (Mandal et al. 2010), was absorbed from the resin.

Flavonoids were completely adsorbed from the Amberlite XAD-4 resin (Table 2). Removing such active allelopathic compounds, with strong toxic and growth inhibitory actions, as myricetin, hesperidin and quercetin (Einhellig et al. 1985; Inderjit and Dakshini 1991; Makoi and Ndakidemi 2007), from the cultivation medium, provided optimal conditions for developing young hairy roots. Absent of flavonoids in the culture medium probably was the main reason for shorter period of hairy roots appearance (only two weeks after transformation in comparison with the four weeks at the submerged cultivation in flasks).

The successful transformation of obtained hairy roots was proved by PCR amplification of *rol*A gene (Fig. 1f). The confirmed transfer of the *rol*A gene into the transformed *S. tomentosa* hairy root line was clear proof for its successful genetic transformation (Rahimi et al. 2008).

In conclusion, for the first time we reported successful *Agrobacterium rhizogenes* transformation of plant tissues based on combined application of an adsorbent resin and a temporary immersion cultivation system. This approach is overcoming the negative effects of the secreted phenolic compounds with antimicrobial and phytotoxic activities. The developed protocol is essentially suitable for transformation of plants biosynthesizing huge amounts of phenolic compounds. Following the described procedure, 111 hairy root lines of *Salvia tomentosa* Mill. were obtained. After six months of cultivation (subcultured every 25 days), 35% of them (38 lines) showed stable growth and morphological characteristics. Further these hairy roots will be evaluated for bioactive secondary metabolites production.

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