SUPPLEMENTARY MATERIAL

Micropropagation of *Salvia wagneriana* Polak and hairy root cultures with rosmarinic acid production

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Abstract

Salvia wagneriana Polak is a tropical species native in Central America, well adapted to grow in the Mediterranean basin for garden decoration. Micropropagation has been assessed from axillary shoots of adult plants using a Murashige and Skoog basal medium, with the addition of 1.33 μM 6-benzylaminopurine for shoot proliferation; the subsequent rooting phase occurred in plant growth regulator-free medium. The plants were successfully acclimatized with high survival frequency. Hairy roots were induced after co-cultivation of leaf lamina and petiole fragments with *Agrobacterium rhizogenes* and confirmed by PCR. The establishment and proliferation of the selected HRD3 line was obtained in hormone-free liquid medium and the production of rosmarinic acid was evaluated after elicitation. The analysis of rosmarinic acid was performed by LC-ESI-DAD-MS in the hydro-alcoholic extracts. The addition of casein hydrolysate increased the rosmarinic acid production, whereas no enrichment was observed after the elicitation with jasmonic acid.

Keywords*: Salvia wagneriana* Polak, , *Agrobacterium rhizogenes*, elicitation, casein hydrolysate, LC-ESI-DAD-MS, rosmarinic acid,

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3. Experimental

3.1. - Abbreviations

BA = 6-benzylaminopurine; CRN = carnosol; CA = carnosic acids; HR = hairy roots; MSO = Murashige and Skoog hormone-free medium; MSCX = MSO + cefotaxime 100 mgL^{-1}; UV = ultraviolet detector; LC-DAD-ESI-MS = Liquid Chromatography-Diode Array Detector-Electron Spray Ionization-Mass Spectrometry; SRM = Selective Reaction Monitoring; RA= rosmarinic acid

3.2. - *In vitro* propagation and *ex vitro* establishment

Plants of *S. wagneriana* Polak were grown at Hanbury Botanical Garden Collection (Ventimiglia, Italy). Apical and axillary buds collected from adult plants, were sterilized with 70% ethanol (v/v) for 30 s, then with sodium hypochlorite solution (1% free chlorine) for 15 min and finally five times rinsed in sterile, distilled water. They were transferred for 4 weeks to hormone-free Murashige and Skoog medium (MS, 1962) containing 30 g/L of sucrose, 7 g/L technical agar and pH 5.7 set before sterilization. 500 ml glass vessels with transparent caps were used containing 160 ml of medium. The media were sterilised in autoclave at 1 atm and 120°C for 20 min. All *in vitro* experiments were carried out in a growth chamber at 24 ± 1°C, with a 16 h photoperiod at 30 μmol/m^2 s light intensity. Half of the shoots were then transferred onto MS base medium (including vitamins) supplemented with BA (1.33 μM) and half onto hormone-free medium as control. The experiment was carried out with 5 glass vessels per treatment containing 8 explants each. Multiplication rate, height of shoots and presence of roots were recorded after 40 days. Rooted plants were transferred to the greenhouse for acclimatization in plastic plateau with a peat-perlite (1:1 v:v) substrate at 80% relative humidity (R.H.) supplied by mist system (10 s every 30 min). They were grown in natural light with 50% relative shade. After a period of 20 days, they were kept at 60% R.H. for a further 20 days.

3.3. - Hairy root development and characterization of hairy roots

Transformation was performed with *A. rhizogenes* wild-type strains ATCC 15834 (American Type Culture Collection) or 1855 NCPPB (National Collection of Plant Pathogenic Bacteria). Bacterial suspensions were grown overnight on appropriate YMB medium medium (Hooykaas et al. 1977). Leaf fragments (30-40 mm^2) and petioles (20-30 mm) of micropropagated shoots of *S. wagneriana* were soaked for 20 min in the bacterial suspensions (A_{600} = 0.1). Control explants were soaked in sterile distilled water for the same time. Infected and control fragments were placed on hormone-free MS medium for 3 days and then transferred to MS medium added with cefotaxime (100 mg/L - MSCX).
Hairy root formation was monitored and recorded as percentage of fragments developed after 30 d and 60 d from co-cultivation. Several putative HR lines were recognised by their characteristic phenotype, rapid branching, fast growth rate and development of white hairs after the growth on hormone-free medium; such roots were excised from parental tissue and sub-cultured at 5 week intervals on MSCX medium under the environmental conditions described above.
Putative HRs were confirmed by PCR analysis. The total genomic DNA was extracted and purified from 11 putative HR lines (100 mg) and from petioles of micropropagated plants (100 mg, negative control) using a mini-DNeasy Plant Kit (Quiagen). DNA was also extracted from *A. rhizogenes* strains 15834 and 1855 (Klimyuk et al. 1993).

DNA amplification was performed in a programmable Thermal PTC-100TM controller (MJ Research, Inc, USA). Each PCR reaction was performed in a final volume of 50 μl with 200 μM dNTPs, 0.3 μM of each primer, 1x PCR buffer, 1 unit of Taq DNA polymerase, 2.5 mM $MgCl_2$, and 80 ng of DNA template. PCR conditions for *rol*C gene amplification band of 514 bp (Scorza et al. 1994) were as follows: an initial denaturation at 94°C for 5 min was followed by 30 cycles at 94°C for 60 s denaturation, 65°C for 60 s annealing, 72°C for 2 min elongation and a final elongation step of 10 min at 72°C. The absence of bacterial contamination of plant tissue was confirmed after PCR

amplification of 326 bp fragment of the *vir*C1 gene (Vaira et al. 1995). Amplification products were resolved by electrophoresis on 2% agarose gel in TE buffer, and stained with ethidium bromide (figure S1).

3.4. - Optimization of HR culture medium and elicitation

The HR line D3 (HRD3) was chosen among the *rol*C gene positives-ones from ATCC 15834 transformation for the fast and efficient growth and was sub-cultured in MSCX liquid medium in glass vessels (1:3, biomass: liquid medium). The cultures were kept in the dark at 24 ± 1°C in continuous agitation at 60 rpm.

The plant material was subsequently cultured and multiplied in hormone-free liquid MS medium. In order to maintain the active growth, subcultures were made every 28 days. For the determination of the growth curve, eighteen round glass vessels containing 2 g HR in 160 mL liquid medium were prepared and maintained in agitation at 60 rpm. Fresh and dry weights were recorded at 0, 8, 14, 21, 28 and 35 d, each result based on 3 replicates. The experiment was repeated twice.

Twenty eight day-old HR batch cultures were treated for 24 h with jasmonic acid (JA, 3.3 or 6.6 mg/L) or casein hydrolysate (CH, 200 or 400 mg/L) and kept in dark 24 ± 1°C in continuous agitation at 60 rpm, then transferred to the usual hormone-free liquid MS medium. After 1 week the biomass was filtered in a laminar air-flow cabinet then stored at -80 °C until it was used for phytochemical analysis.

3.5. - Extraction of Rosmarinic acid and LC-DAD-ESI-MS analysis

An aliquot of *in vitro* 28 day-old HRD3 biomass (0.5 g fresh weight) was extracted by maceration in an appropriate volume of EtOH-water (7:3), while the corresponding liquid culture media were purified by Solid Phase Extraction (1g, SPE-RP-18, MeOH), then the solvent was removed in a rotary evaporator at 42°C. Each extract sample was dissolved in methanol, filtered (0.45 µm PTFE) and analysed by LC-DAD-ESI-MS.

The LC-DAD-ESI-MS apparatus consists of Surveyor ThermoFinnigan liquid-chromatograph pump equipped with a Surveyor Autosampler, a ThermoFinnigan Photodiode Array Detector and an ion trap LCQ Advantage mass spectrometer. The analyses were carried out by an analytical RP-18 column (Synergy Fusion, 250 x 4 mm i.d., 3 µm, Merck) using a linear gradient with water, 0.1% formic acid (solvent A) and acetonitrile (solvent B) from 10:90 v/v (B/A) to 75:15 (15 min), at a flow rate of 0.2 ml/min (injection volume 20 µl, triplicate). The qualitative screening was carried out for the target compound carnosol (CRN), carnosic acid (CA) and rosmarinic acid (RA), while the quantitative LC-ESI-MS method was performed only for RA (negative ion mode) as CRN and CA were not found with our procedure. The LC-chromatograms were registered for quali-quantitative analyses by UV at 328 nm, as well as MS detection (full scan m/z 100 to 600 amu, Selected Reaction Monitoring mode -SRM). Table 1S summarizes the performance parameters of LC-DAD-MS method used for the screening of the three selected phenolic compounds (RA, CA, and CRN) both in the hydro-alcoholic extracts and in liquid culture media. The RA content was determined by an external calibration method (0.1-25 µg/ml, MeOH solution, Figure 2S).

3.6. - Statistical analysis

All the data were processed using COSTAT, which enabled ANOVA. Means were compared using the Student Newman Keuls test ($p<0.5$). Percentages were transformed in angular values prior to analysis.

3.7. - Chemicals

All the chemicals acetonitrile, methanol and formic acid were purchased from J.T.Baker® (Phillipsburg,New Jersey,USA), and were used for LC-DAD-MS analysis. MS medium, technical agar, sucrose, BA, cetofaxime, carnosol,

carnosic acid and rosmarinic acid were purchased from Sigma-Aldrich, Saint Louis, Mo, USA. Carnosol (CRN, 10 mg,), carnosic acid (CA, 10 mg) and rosmarinic acid (RA, 10 mg) were used as reference materials (HPLC purity grade 97-98%), each analyte was dissolved in methanol.

References

Hooykaas, PJ, Klapwijk, PM, Nuti, MP, Schilperoort, RA and Rorsch, A. 1977. Transfer of the *Agrobacterium tumefaciens* Ti plasmid to avirulent agrobacteria and to explanta. *Journal of General Microbiology,* 98**:** 477–484.

Klimyuk, VI, Carroli, BJ, Thomas, CM, and Jones JAG. 1993. Alkali treatment for rapid preparation of plant material for reliable PCR analysis. *The Plant Journal* 3(3): 493-494

Murashige, T, and Skoog, K. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum,* 15: 473-597.

Scorza, R, Zimmerman, TW, Cordis, JM, Footen, KJ, and Ravelonandro, M. 1994. Horticultural characteristics of transgenic tobacco expressing the rolC gene from *A. rhizogenes.* *Journal of the American Society for Horticultural Science* , 119(5): 1091-1098.

Vaira, AM, Semeria, L, Crespi, S, Lisa, V, Allavena, A, and Accotto, GP. 1995. Resistance to tospoviruses in *Nicotiana benthamiana* transformed with the N gene of Tomato Spotted Wilt Virus: correlation between transgene expression and protection in primary trasformants. *Molecular Plant-Microbe Interactions***,** 8(1): 66-73.

Table S1. Effect of BA on *in vitro* cultures of *S. wagneriana.* Values are means of n= 40, with statistical differences P=0.05, SNK test.

Culture medium	Multiplication rate (No shoots / explant)	Shoot height (cm)	Rooting (%)
MS	1.39 a	7.42 b	94.04 a
MS + BA 1.33 µM	3.32 b	5.34 a	87.37 a

Table S2. LC-DAD-ESI-MS screening of the target phenolic compounds (RA rosmarinic acid, CRN carnosol, CA carnosic acid).

Compound	UV (λ nm)	MW	Base peak (m/z)	Collision energy (%)	MSn (m/z) (collisional fragment ions)	Collision energy (%)	MSn (m/z) (collisional fragment ions)	Adducts
RA	288 sh, 328	360	$[M-H]^-$ 358.9	26	161.2	38	133.1	
CRN	215 sh, 288	330	$[M-H]^-$ 329.1	26	285.2			658.8 $[M-Hx2+23]^-$
CA	215sh, 288	332	$[M-H]^-$ 331.1	30	287.2	50	244.2, 271.3	685.1 $[M-Hx2+23]^-$

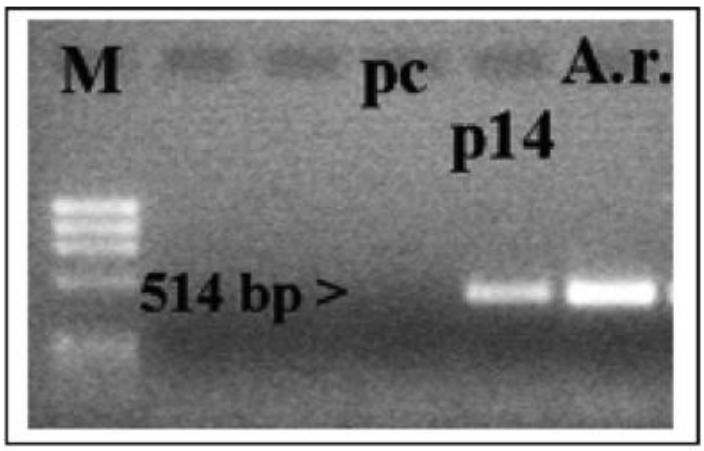

Figure S1

Figure S1. Production of hairy roots from *S. wagneriana* petioles. A) Agarose gel electrophoresis of PCR products of roots transformed by *A. rhizogenes* ATCC 15834. Extract-N-Amp Plant PCR Kit was performed with *rol*C primers. M= ΦX/HaeIII marker; pc= *S. wagneriana* not-transformed petioles (negative control); p14= *S. wagneriana* putative transformed root line (called 14, positive transformed HR) A.r = *A. rhizogenes* ATCC 15834 sample (positive control). Arrows show amplified fragments of *rol*C (514bp) gene.

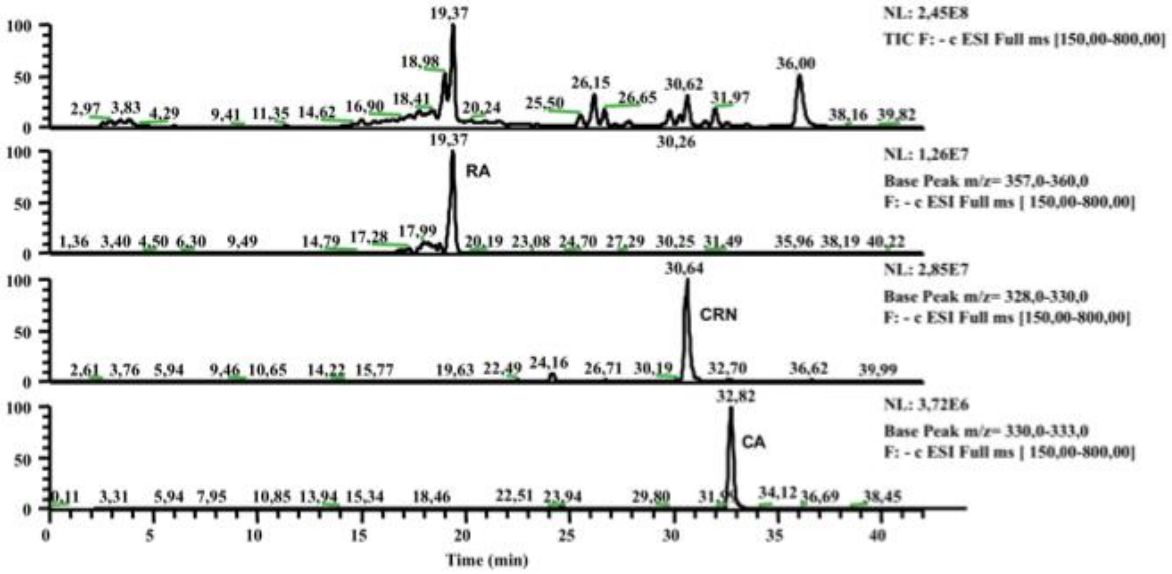

Figure S2

Figure S2. LC-ESI-MS chromatogram of the spiked hydroalcoholic extract of *S. wagneriana* HRD3 line. RA= rosmarinic acid, CRN= carnosic acid; CA= carnosic acid. Artificial standard solution of markers (100 µg/ml).