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Endogenous isoflavone methylation correlates with the *in vitro* rooting phases of *Spartium junceum* L. (*Leguminosae*)

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ABSTRACT

Spartium junceum L. (*Leguminosae*) is a perennial shrub, native to the Mediterranean region in southern Europe, widespread in all the Italian regions and, as a leguminous species, it has a high isoflavone content. An *in vitro* culture protocol was developed for this species starting from stem nodal sections of *in vivo* plants, and isoflavone components of the *in vitro* cultured tissues were studied by means of High Performance Liquid Chromatography (HPLC) analytical techniques. Two main isoflavones were detected in the *S. junceum* tissues during the *in vitro* propagation phases: Genistein (4′,5,7-Trihydroxyisoflavone), already reported in this species, and its methylated form 4′,5,7-Trimethoxyisoflavone, detected for the first time in this plant species (0.750 ± 0.02 mg g^{-1} dry tissue). The presence of both of these compounds in *S. junceum* tissues was consistently detected during the *in vitro* multiplication phase. The absence of the methylated form within plant tissues in the early phases of the *in vitro* adventitious root formation was correlated with its negative effect displayed on root induction and initiation phases, while its presence in the final "root manifestation" phase influenced positively the rooting process. The unmethylated form, although detectable in tissues in the precocious rooting phases, was no longer present in the final rooting phase. Its effect on rooting, however, proved always to be beneficial.

Introduction

Spartium junceum L., known as Spanish Broom or Weaver's Broom, is an economically important leguminous shrub, evergreen, perennial, 2–4 m tall, native to the Mediterranean region in southern Europe, southwest Asia and northwest Africa (Oggiano et al., 1997). In addition to its use in perfumery (Miraldi et al., 2004), *S. junceum* is utilized in Europe for cordage, in badlands recovery and land restoration (Preti and Giadrossich, 2009). It is a species characterized by an abundant production of secondary metabolites, such as alkaloids, saponins, simple phenols and flavonoids (Yeşilada et al., 2000; Proestos et al., 2006). These molecules have been studied from a pharmacological point of view (Cerchiara et al., 2012) but they have not been associated in this species to any particular physiological state of the plant. Phenolics, however, in addition to their strictly metabolic roles, may be related to plant specific physiological phases, such as flowering, rooting, juvenility, senescence, fruit production, response to biotic and abiotic stresses (Ozyigit, 2008) during which they may display quali-quantitative variations (Fernandez-Lorenzo et al., 1999). Adventitious root formation in microcuttings, a fundamental step of the *in vitro* propagation of plant species, may fall within the above-mentioned physiological states. Rhizogenesis is indeed a very complex process in which, in addition to other physico-chemical factors (Da Rocha Correa et al., 2012), endogenous flavonoids play important roles (De Klerk et al., 2011). In this context, flavonoid quali-quantitative changes could therefore be expected during *in vitro* rooting of *S. junceum* microcuttings. During preliminary investigations (unpublished data),

Abbreviations: BA, 6-benzylaminopurine; BSA, bovine serum albumin; CEM, Channel Electron Multiplier; CPE, crude protein extract; DTT, Dithiothreitol; DMSO, Dimethyl sulfoxide; EDTA, Ethylenediaminetetraacetic acid; E.I., Ebullition Interval; MCE, methanolic crude extract; HPLC, High Performance Liquid Chromatography; mAU, Absorbance Unit; MeOH, Methanol; MS, Murashige and Skoog; R_t, retention time; SAM, S-adenosyl-methionine; TMIF, 4′,5,7-Trimethoxyisoflavone.

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we observed important isoflavone variations in *S. junceum* L. tissues occurring when *in vitro*-cultured plants were induced to root. Isoflavones, however, have rarely been associated with plant rooting events and this class of flavonoids has been mainly studied in relation to symbiotic partnership establishment (Larose et al., 2002) and as plant defensive molecules active against various pathogens (Treutter, 2005).

The lack of information concerning a possible involvement of isoflavones in plant rooting processes prompted us to carry out the present work, with the aim of finding a possible correlation between the previously observed isoflavone changes in *S. junceum* *in vitro* cultured tissues and the *in vitro* rooting process.

Materials and methods

Plant material

Stem and leafy branches 10 months old from *Spartium junceum* L., *Leguminosae*, used for the *in vitro* culture experiments, were collected from a selected genotype grown in an open air cultivation near Sanremo (IM, Italy). A plant specimen is conserved at the Botanical Garden of University of Turin, Italy (Voucher nr. 4.04, Prof. Silvano Scannerini, Vegetal Biology Dept., University of Turin).

Tissue culture and rooting stages

Softwood branches were used as a starting material for tissue culture. Branch pieces, cut in nodal sections 4 cm long, 1 cm diameter and with a pair of axillary buds, were sterilized in a 1.5% free chlorine solution for 10 min, rinsed three times with sterile distilled water, then aseptically inoculated into MS basal medium (Murashige and Skoog, 1962) without growth regulators and left on this medium for one month to induce their adaptation to the *in vitro* culture conditions. Explants were then transferred onto a multiplication medium, consisting of MS macro and micro elements and vitamins, 0.5 mg L^{-1} BA supplemented with 100 mg L^{-1} inositol, 30 g L^{-1} sucrose, 8.8 g L^{-1} agar. All media were brought to pH 5.8 before sterilization by autoclaving at 121 °C at 1 atm for 20 min.

Each subculture had a duration of one month and the culture conditions were 20 °C temperature and 16 h photoperiod. Illumination was provided by Philips 84 white fluorescent lamps, with an irradiance of 22 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR). This phase was considered as the multiplication phase.

Explants to be rooted (microcuttings 4 cm tall) were transferred onto a RM rooting medium composed as the above mentioned multiplication medium, but without hormones. In particular, no rooting hormones were applied to evaluate the tissue response under natural physiological conditions and in the absence of exogenous stimuli. The rooting stages, based on observations on free hand obtained histological sections, were conventionally divided as follows, according to Uribe et al. (2008): (1) phase of induction (0–7 days of culture), neoformation of parenchymatous cells/vascular elements; (2) phase of initiation (8–18 days of culture), differentiation of meristematic areas within callus tissues; (3) phase of manifestation (19–40 days of culture), well developed primary and secondary roots. The *in vitro* explants were considered rooted when they showed at least three adventitious roots, each with a length of 2 cm.

Phenol extraction and column chromatography procedures

20 g fresh tissues from *in vitro* plants were harvested at different times during both explant multiplication and each of the three rooting phases described above. Tissues were extracted with MeOH:H_2O (1:1) in a soxhlet apparatus two times for 1 h. Extracts were filtered through paper filters (Whatman 2V), put in a separation funnel, brought to pH 5.0 by HCOOH addition and then partitioned in layers with Petroleum ether (E.I., 40–60 °C); the upper phase, containing pigments, was discarded while the hydro-alcoholic fraction, collected and neutralized with Na_2CO_3, was evaporated to dryness through a rotary evaporator (Buchi, Rotovapor L – 200); the residue was then re-dissolved in MeOH and stored as methanolic crude extract (MCE) at −20 °C until needed.

Column chromatography separation and purification of the investigated compounds were performed according to a previously published protocol (Ferracini et al., 2010).

Several mg of an uncommon isoflavone constituent of *S. junceum*, not commercially available, were obtained from plant extract after repeated column chromatography over silica gel 100 C_8 reverse phase column (40–63 μm particle size, Fluka, Germany) with a linear gradient eluting profile according to a previous protocol (Ferracini et al., 2010). After purification and identification, this compound was dried under nitrogen reflux, stored as powder and used as a pure reference molecule in the further analyses. All solvents and reagents from various suppliers were of the highest purity. Water was HPLC grade.

Isoflavone methyl transferase activity evaluation

The entire procedure was performed at 4 °C. Ten g of fresh *in vitro* tissues, from multiplication, induction, initialization and elongation phases, respectively, were homogenized with a Turrax T 65 homogenizer (IKA Werke, Staufe, Germany), at 9.500 rpm, in 0.2 M Tris HCl buffer, pH 7.5, containing 14 mM mercaptoethanol, 5 mM EDTA and 5 g L^{-1} insoluble Polyvinylpolypirrolidone (Polyclar). The homogenates were centrifuged for 10 min at 8000 × *g* and the pellet discarded, while to the supernatant $(NH_4)_2{\cdot}SO_4$ 80% of saturation point was added. The precipitate was separated by centrifugation as above, re-dissolved in 0.2 M Tris HCl buffer, pH 7.5, and desalted overnight in a collodion bag against the same buffer, to yield the crude protein extract (CPE).

Two isoflavone substrates were assayed: Genistein (4′,5,7-Trihydroxyisoflavone) and Daidzin (Daidzein-7-O-glucoside), standard pure samples obtained from Sigma (USA). They were dissolved in 12 mM DMSO while 36 mM SAM was prepared in the same solvent.

10 mL CPE (with a total protein cncn. of 8 mg mL^{-1}), was incubated with 0.5 mL of each substrate and 0.5 mL SAM for 1 h at 28 °C. The reaction was stopped by the addition of 20 mL MeOH and the precipitate was removed by centrifugation at 8000 × *g* for 2 min; the supernatant was analyzed both through HPLC and column chromatography as described below for a first identification of possible new methylated products. The purified molecules obtained from the enzymatic reaction were submitted to MS analyses for their final identification. Data concerning the isoflavone methyl transferase activity of CPE were obtained according to the protocol of Curir et al. (2003).

Isoflavone demethylase activity evaluation

To cover a wide range of possible plant demethylases (Hagel and Facchini, 2010), two kinds of experiments were set, according to the procedure of Berim et al. (2012). Protein extract was submitted to $(NH_4)_2{\cdot}SO_4$ 80% of saturation point fractionation, as above mentioned. Briefly, in both types of experiments 25 μM substrate were added to 0.5 mL CPE (with a total protein content of 10 mg mL^{-1}) in a total volume of 2.0 mL 100 mmolar Tris HCl buffer solution, pH 7.5, and 1 mmolar DTT. Then, 2 mM NADPH and 1 mM dithiocarbamate were added in the first series of experiments, while in a second series of trials 10 mM 2-oxoglutarate, 10 mM sodium ascorbate and 250 μM $FeSO_4$ were added. Reactions were allowed to develop for