Scientia Horticulturae 230 (2018) 78–85

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Research Paper

Biological active compounds from *Limonium insigne* and alternative methods for its micropropagation

Ana Ortuño[a], Licinio Díaz[a,*], Irene Pérez[a], Francisco Sánchez[b], José Antonio Del Río[a]

[a] Departament of Plant Biology (Plant Physiology Unit), Faculty of de Biology, University of Murcia, E-30100 Murcia, Spain
[b] Centre of Germoplasma Bank, Counseling of Water, Agriculture and Environment of Region of Murcia. La Alberca, E-30150 Murcia, Spain

ARTICLE INFO

Keywords:
Antioxidant activity
Apigenin 7-*O*-glucoside
Flavonoids
Luteolin-7-*O*-glucoside
Naringenin

ABSTRACT

The flavonoid composition of the *Limonium insigne* plants was analyzed by high-performance liquid chromatography, and mass spectroscopy (MS/MS and TOF). The results revealed the presence of luteolin-7-*O*-glucoside and apigenin-7-*O*-glucoside, as the major flavone glycosides, together with the aglycon flavanone naringenin. The maximum levels of these flavonoids in *Limonium insigne* plants were detected in inflorescence stems (148, 173 and 40 mg/100 g DW, respectively). Followed by leaves (129, 140 and 43 mg/100 g DW, respectively) and roots (29, 31 and 11 mg/100 g DW, respectively).

The *in vitro* multiplication of *Limonium insigne* from axillary buds and by direct caulogenesis from leaf explants were evaluated. Of the hormonal treatments assayed (2 μM 6-benzylaminopurine; 5 μM kinetin; 2 μM 6-benzylaminopurine + 5 μM kinetin), the best response in the case of development from buds was obtained with 2 μM 6-benzylaminopurine + 5 μM kinetin, since a 57%, 82% and 196% increase in the number of leaves per explant with respect to the control was observed after 20, 40 and 60 days of culture, respectively. Leaf size was also greater, with a 64% increase in the longitudinal axis over the control values after 60 days. As regards caulogenesis, the Kinetin + ANA combination (5 μM + 0.5 μM) was the most successful for shoot formation (90%). For shoot rooting the best results were obtained with 10^{-6} M IBA. In the case of flavonoid content in micropropagated plants, it is very similar to the mother plants, being much lower *in vitro* plants.

The possibility of using these two methods for the micropropagation of this rare and endangered plant speciesand as a source of flavonoids of pharmaceutical interest is discussed.

1. Introduction

The genus *Limonium* Miller (Plumbaginaceae) includes herbaceous species, most of them native to the Mediterranean region and Canary Islands (Wilfret et al., 1973). They have a high ornamental value as cut flowers for both fresh and dry-flower arrangements (Stewart and Coring, 1970; Harazy et al., 1985; Martín and Pérez, 1995).

This is a complex genus, characterised by high intraspecific variabity as a result of, among other reasons, their extraordinary capacity for hybridisation or retrohybridisation. An enormous number of hybrid taxa have been described and it is sometimes difficult to identify samplesand differentiate between them because of this high level of hybridisation.

This genus comprises more than 400 species, fourteen of which grow in the province of Murcia (SE Spain). Most of these are perennial, except some annual species which grow in a reduced area, some of which, in turn, are considered rare and endangered. Such is the case with the object of this study, *Limonium insigne*.

Many higher plants are major sources of natural products used as pharmaceuticals, agrochemicals, flavour and fragrance ingredients, food additives and pesticides (Balandrin and Klocke, 1988). The search for new plant-derived chemicals should thus be a priority in current and future efforts towards sustainable conservation and rational utilization of biodiversity. Nowadays, there is a great interest in the study of new sources of natural antioxidants, as oxygen reactive species are the major cause of material degradation in food and in particullar free radicals, are involved in the a variety of pathological conditions such as cancer, cardiovascular disease, arteriosclerosis and neurodegenerative diseases (Halliwell et al., 1992; Markesbery and Carney, 1999). Even aging may be considered as the result of deleterious free radicals reactions which occur throughtout cells and tissues (Maxweell, 1995). In this way, although rare, there are some contributions that highlight some *Limonium* species to express biosynthetic pathways typical of the secondary metabolism of plants, having described the presence of some phenolic compounds (Kandil et al., 2000; Chaung et al., 2003; Zhang and Zou, 2004; Ye and Huang, 2006; Inocenti, 2010). Flavonoids are a group of

* Corresponding author.
E-mail address: l.diazexposito@um.es (L. Díaz).

https://doi.org/10.1016/j.scienta.2017.11.022
Received 15 June 2017; Received in revised form 8 November 2017; Accepted 10 November 2017
Available online 20 November 2017
0304-4238/

polyphenolic compounds of great commercial interest for food and pharmacological industries, due to known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes, and biological activity to treat many diseases (Benavente-García et al., 1997, 2007).

On the other hand, las techniques for the *in vitro* propagation of the genus *Limonium* Mill. are of great interest since (i) such techniques, from the conservation point of view, provide individuals of good genetic quality from selected wild samples, enabling the restitution of natural populations and diminishing the threat represented by further hybridisation with other species of the same genus, in this way preserving and regenerating the germoplasm; (ii) given the ornamental value of the species of this genus, obtaining propagules as a "phytogenetic resource" for agricultural and gardening purposes and increasing our knowledge of propagation methods will permit their use both for growing ornamental plants and in genetic improvement programmes; and (iii) a way to increase the multiplication of these plants for production of possible bioactive compounds of interest for differents industries: biomedical, pharmaceutical or food companies.

To date, there have been few contributions to this line of research and the studies that do exist tend to be based on the culture of inflorescence explants (Amo-Marco and Ibañez, 1998), or on the isolation of inner buds located at the base of the rosulated plants, with the consequent problem of depending on the flowering season to obtain flowering stems in the first case, and the unavoidable destruction of the mother plants in the second.

The objetive of this work was to investigate new sources of plant materials to obtain natural biological active compounds. For this, it has been conducted a study for the identification and quantification of main flavonic compounds present in plants of this specie, as well as it has been analyzed their antioxidative properties, and carry on the optimization of *Limonium insigne* micropropagation, based on the direct induction of caulogenesis from leaf explants or the culture of axillary buds from *Limonium insigne* rosettes obtained from *in vitro* plants, to ensure the availability of plant material with a view to its possible use for the production of secondary metabolites of industrial interest.

2. Material and methods

2.1. Extraction, measurement and identification of flavonoids

Six-month old *Limonium insigne* non-micropropagated mother plants, micropropagated plants, and *in vitro* plants were used in the different assays. Non-micropropagated whole mother plants, and leaves, roots and inflorescence stems of the different above described plant materials were collected, and divided into different lots, in each case. The plant material of each lot was dried at 50 °C immediately after collection to constant weight. The dried leaves were ground and shaken with dimethylsulphoxide (DMSO) (Castillo et al., 1992) for 2 h in a proportion of 200 mg of dry weight/ml. The resulting extracts were filtered through a 0.45 μm nylon membrane before analysis in a Jasco liquid Chromatography system equipped with a Jasco quaternary pump (model PU-2089 Plus), a Jasco photodiode array detector (model MD-2010 Plus) and a Jasco autosampler (model AS-2055 Plus). The stationary phase was a LiChroCARTR C_{18} (Agilent, USA) analytical column with an average particle size of 5 μm (250 × 4 mm i.d.) at 30 °C. The flavonoids were separated using a binary gradient of water: methanol: acetonitrile: acetic acid (15:2:2:1) as solvent (A) and acetonitrile as solvent (B). The initial solvent composition consisted of 100% (A) for 40 min; then, the solvent composition was changed in a linear gradient to 20% (A) over 30 min. Between 70 and 80 min, the composition was maintained and then the solvent composition was changed in a linear gradient to 100% (A). Eluent flow was 1 ml/min. Changes in absorbance were recorded in the UV/Vis diode array detector at 280 nm for the flavanone and 340 nm for the flavones.

For the identification of main phenolic compounds by HPLC-ESI–MS/MS, a mass ion trap spectrometer model VL was used, equipped with a ESI interphase, coupled to a HPLC Agilent 1100A. A 5 μm (250 × 4 mm i.d.) C_{18}Kromasil 100 (Tecnokroma) column was used for the separation, which was performed by means of a similar elution gradient to that described above to quantify flavonoids. The column was maintained at 30 °C. ESI mass spectra were acquired in both positive and negative ion modes by scanning over the 50–1000 mass range. The ESI parameters were: source voltage 3.5 kV, dry temperature 350 °C, nebullizer 60 psi, and dry gas 9 l/min.

For mass exact spectrometry analyses (HPLC-APCI-TOF), a mass spectrometer with detector of time of flight (TOF) (MS-TOF 6220, Agilent) was used. It has a mass range of 50–3000 *m/z*, ionization by a APCI-electrospray source in both positive and negative mode and with a resolution of 16000, allowing analyze masses with a less error of 2 ppm, coupled to the same HPLC described above.

The quantities of these secondary compounds were determined from the area given by the integrator using the response factor of the corresponding standards.

2.2. Antioxidant activity measurement

For this assays, non-micropropagated whole mother plants of *L. insigne* were used. The plant material was dried at 50 °C immediately after collection to constant weight. For the extraction, 10 g of powdered dry plant material were mixed with 100 ml ethanol and stirred at 500 rpm for 24 h at 20 °C. After stirring and filtering under vacuum, the filtrate was evaporated in vacuo to dryness, a brown residue (0.45 g) was obtained.

The antioxidant activity was evaluated by the ability as free radical scavenger of crude extracts and pure compounds, according to procedure previously described (Murray et al., 2004). For this, a spectrophotometric assay was used as follows: 200 μl of a ethanolic solution of the test compounds or crude extracts were added to 3.2 ml of a 0.004% DPPH (1,1-diphenyl-2-picrylhydrazyl) solution in ethanol. Six concentrations, ranging from 1 to 100 μM, were prepared for each sample and analyzed in triplicate. 3.2 ml of ethanol plus 200 μl of each compound solution were used for blank solutions. 3.2 ml of 0.004% DPPH solution plus 200 μl of ethanol were used for negative control. The mixture was incubated in the room temperature for 30 min. Absorbance was recorded at 517 nm using an UNICAM UV–vis spectrometer UV2 (Unicam Limited, UK), and the percentage of DPPH reduction was calculated taking into account the absorbance of the blank solutions and the negative control. Quercetin was used as reference compound under the same experimental conditions.

2.3. In vitro culture of Limonium insigne seeds to generate seedlings

Limonium insigne seeds were supplied by the Banco de Germoplasma de la Región de Murcia (La Alberca, Murcia, Spain) and placed in a muslin cloth for sterilisation with a solution of 5% NaClO for 5 min. The chemical sterilant was then decanted in a laminar flow chamber and the seeds were washed thoroughly with sterile distilled water three times before sowing on Murashige and Skoog (1962) culture medium supplemented with 3% sucrose and 0.8% agar at pH 5.8. All media were then sterilised in an autoclave at 1 atmand121 °C for 20 min.

Cultures were maintained in a growth chamber at 25 °C with a 16-h light photoperiod, provided by fluorescent tubes (Sylvania, Gro-Lux, F36W/Gro, 1000 lx).

The *Limonium insigne* seedlings thus obtained were used as starting material to obtain explants (axillary buds from the rosettesor leaf sections) to establish the corresponding cultures. Since the seedlings were sterile, the corresponding explants can be used directlywith no additional sterilizing procedures that may cause damage to the tissues and affect regeneration.