Comparative Study of Differentiation Levels and Valepotriate Content of in Vitro Cultures and Regenerated and Wild Plants of *Valeriana edulis* ssp. *procera*

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Valepotriate content levels in samples of in vitro cultures of *Valeriana edulis* ssp. *procera* were compared with those of roots and rhizomes of wild plants in the reproductive stage. Rhizomes and roots of regenerated and wild plants showed a similar valepotriate content. The data obtained support the hypothesis that valepotriate production in *V. edulis* spp. *procera* is closely related to rhizome and root differentiation. The large-scale propagation of this endangered plant may offer an attractive alternative for its production for medicinal purposes.

Plants of the genus *Valeriana* (Valerianaceae) have sedative, antispasmodic, and relaxing properties which have been ascribed to the presence of iridoid esters called valepotriates,[1,2] as well as isovalerenic acid[3] and borneol derivatives,[4] which are predominately produced in the roots and rhizomes of the intact plants. As an alternative to obtain these active compounds of great pharmaceutical interest, in vitro production has been evaluated, mostly in differentiated callus cultures and various tissues from some Valerianaceae such as *V. officinalis*, *V. wallichi*, *Centranthus ruber*, and *C. macrosiphon*.[5,6]

Valeriana edulis ssp. *procera* (Kunth) Meyer known as "Valeriana Mexicana" is a commercially important species. It is indigenous to Mexico[7] and is an endangered medicinal plant used as a sedative and hypnotic remedy in traditional medicine.[8] Phytochemical analysis has shown that this plant contains high concentrations of valepotriates, from which valtrate (**1**) is perhaps the most important active compound of this group.[9] A recent report demonstrated the hypnotic effect and safety of acute treatment of a standarized hydro alcoholic extract of *V. edulis* ssp. *procera* on patients suffering from insomnia.[10] In view of the pharmacological relevance of this species, Castillo et al. have reported a regeneration process for this plant species by organogenesis and somatic embryogenesis via a morphogenetic callus.[11] In this paper, we describe the correlation between the degree of differentiation and the valepotriate content in in vitro and in regenerated plants and the comparison of the valepotriate level between the wild and the in vitro regenerated plants of *V. edulis* ssp. *procera*.

Friable creamy calli of *V. edulis* ssp. *procera* with some relatively compact zones were developed from 6 to 8 weeks of in vitro culture (Figure 1A), originated from leaf explants of a typical dicotyledon leaf (Figure 2A). Histological analysis showed that these calli consist of small cells located both in the central region and in the surface of the callus (Figure 2B). Groups of cells approximately isodiametric with relatively large nuclei and cells having large vacuoles, thin walls, and some intercellular spaces were observed (Figure 2C).

In a previous study we reported the effect of growth regulators in the induction of the morphogenetic callus and plant regeneration of *V. edulis* ssp. *procera*.[11] In this work, the presence of the morphogenetic callus was confirmed after 12 weeks of culture (Figure 1B), as well as the regeneration of complete plants (Figure 1C).

HPLC quantification of valtrate (**1**), isovaltrate (**2**), dihydrovaltrate (**3**), dihydroisovaltrate (**4**), and valerenic acid (**5**) was performed. Valepotriate levels from 6, 8, 10, and 12 week old calli and from shoots and rhizomes of plantlets and regenerated plants were compared with the aerial parts and rhizomes of wild plants in the reproductive stage. None of the nonmorphogenetic and morphogenetic calli, shoots, and roots of plantlets and shoots of regenerated plants contained either valepotriates or valerenic acid. Rhizomes and roots of regenerated plants produced the highest valepotriate level, namely, 0.19% (dry weight) of valtrates and 0.21% (dry weight) of dihydrovaltrates. This concentration was similar to that found in the rhizomes and roots of wild plants in the reproductive stage (0.29% dry weight). Valerenic acid (**5**) was not detected in any tissue tested from both wild and regenerated plants. These results agree with those described by Shimmer and Roeder, who did not find valerenic acid (**5**) in this species.[12] On the other hand, it has been reported that roots and rhizomes of wild plants of *V. mexicana* contain 2.9% of valtrate/isovaltrate and 2.5% of dihydrovaltrate;[13] along the same lines, roots and rhizomes of *V. officinalis* contain a 1.1−1.4% (dry weight) of valepotriates,[14] and subterranean

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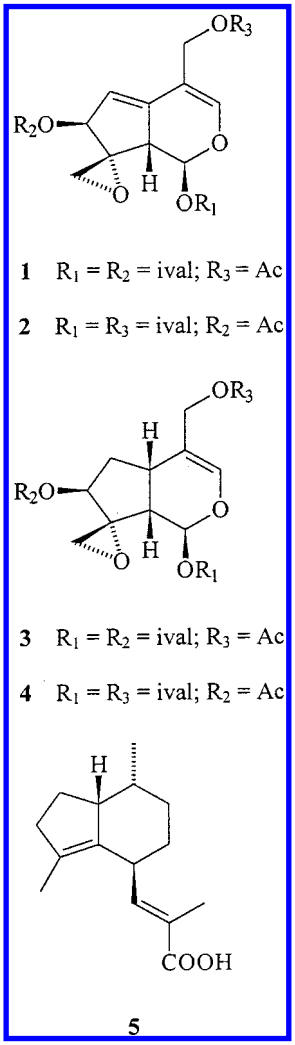
parts of *V. wallichii* contain 0.09−1.30% of valepotriates on a dry weight basis.[15]



These results are in agreement with the hypothesis that the failure to produce valepotriates in morphogenetic calli and plantlets is linked to the level of differentiation and that it is an essential prerequisite for the expression of genes associated with the biosynthetic pathway of secondary metabolism. Therefore, the organogenetic process, which results in the formation of roots and rhizomes in the plant, is indispensable for the production of quantifiable levels of valepotriates. This interpretation is in accordance with the results obtained by Violon et al.,[4] who established a close correlation between valepotriate content and the level of differentiation in various tissues of Valerianaceae, and with the study of Becker and co-workers, who did not find valepotriates in callus cultures of *V. officinalis*.[5,16]

These results indicate that the plant regeneration process did not affect the production of valepotriate in mature plants. The large-scale propagation of this endangered plant may offer an attractive alternative for its production for medicinal purposes.

Experimental Section

Plant Material. The plants and seeds of *V. edulis* ssp. *procera* were collected from a wild population in the village of San Felipe Neri, Tlalnepantla, Morelos, México, in June 1997. Samples were deposited in the Herbarium of the Biological Research Center (MORE) of the University of Morelos State (voucher No. 5591). Seeds were in vitro germinated aseptically.

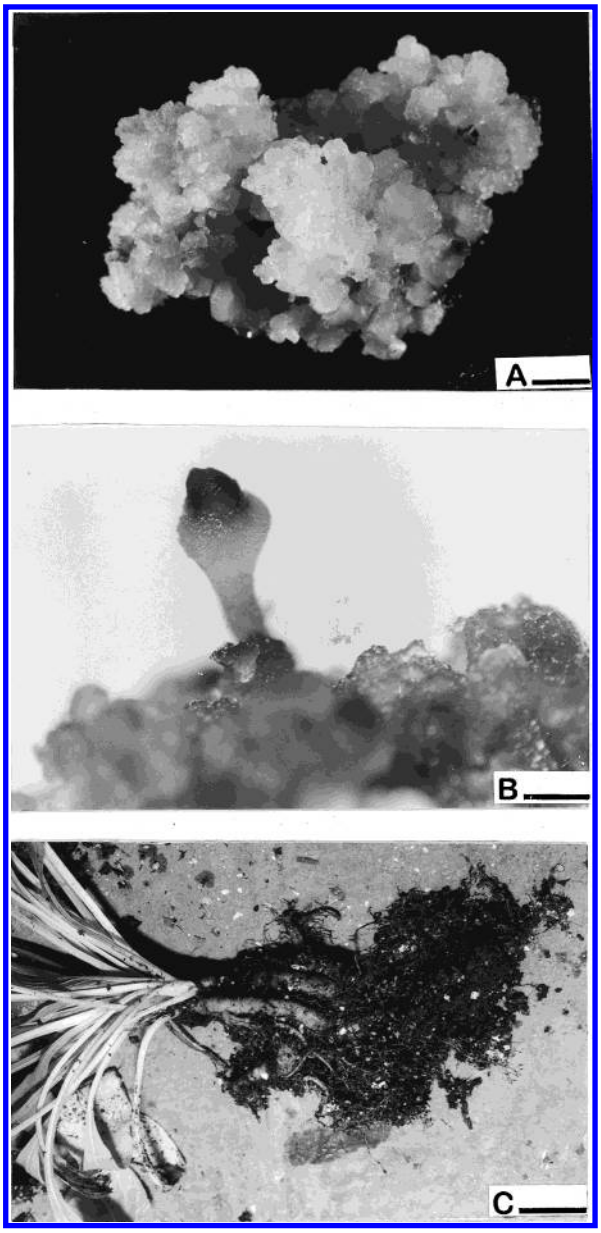


Figure 1. Morphological aspects of the callus induction and regenerated plants of *Valeriana edulis* ssp. *procera*. (A) Appearance of friable callus after 8 weeks of in vitro culture (bar = 10 mm). (B) Morphogenetic callus showing shoots at 12 weeks (bar = 2.5 mm). (C) Regenerated plant showing a rhizome well developed after 6 months in a greenhouse (bar = 10 cm).

Seedling leaves were used as explants according to the procedure of Castillo et al.[11]

Establishment and Maintenance of Cultures. Explants were cultured in semisolid MS (Murashige and Skoog) medium, supplemented with Gamborg vitamins (B5), 3.8% g/L of phytagel, 30 g/L of saccharose, and 2.0 mg/L of 2,4-dichlorophenoxyacetic acid in combination with 0.15 mg/L of kinetin. The pH was adjusted to 5.6 ± 0.1 before autoclaving for 20 min at 120 °C. Samples were subcultured every 2 weeks. The regeneration of plants was based on the procedure described by Castillo et al.[11]

Determination of Differentiation Level. Levels of differentiation were determined macroscopically and microscopically. Fresh material was observed on a stereoscopic microscope (20× magnification). Following this, calli of 6, 8, 10, and 12 weeks of age were fixed for 24 h in collidine buffer (pH 7.2) containing 3% glutaraldehyde and 1.5% paraformaldehyde. Dehydration was performed in ethanol. Tissues were embedded in JB4 resin, sectioned (0.8−1 μm) with a ultramicrotone, or embedded in paraplast and sequentially sectioned (8 μm).

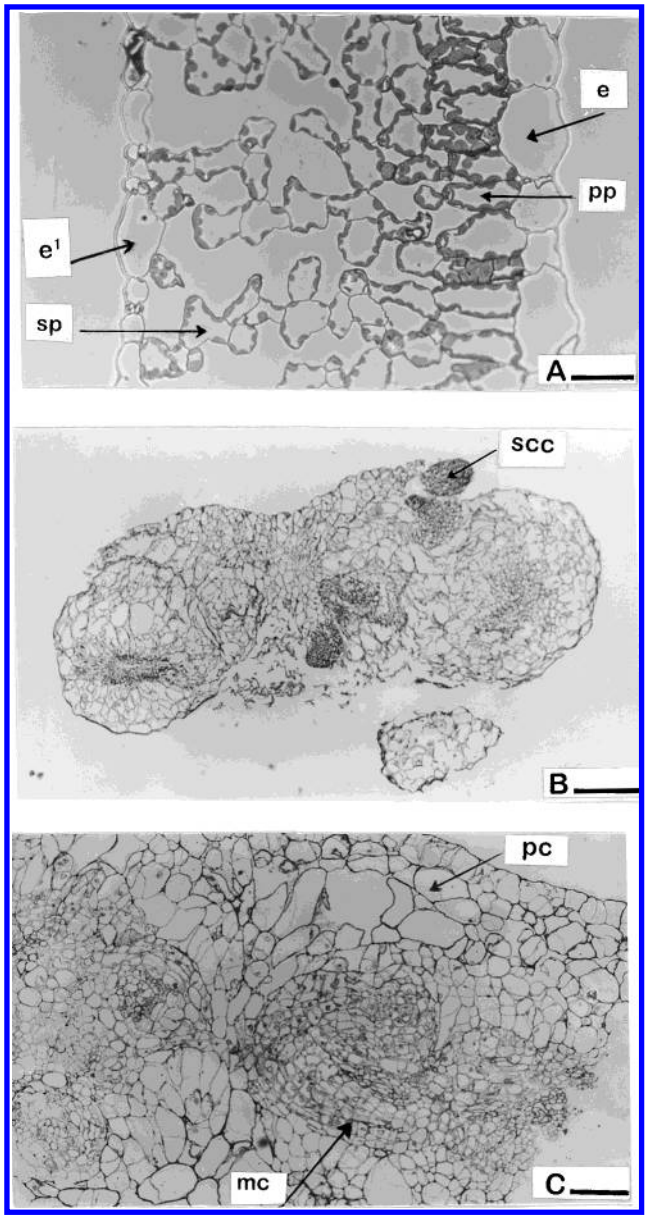


Figure 2. Histological characterization of leaf explant and callus. (A) Transversal section of a *Valeriana edulis* ssp. *procera* seedling leaf (e = adaxial epidermis, e^1 = abaxial epidermis, pp = palisade parenchyma, sp = spongy parenchyma, bar = 50 μm). (B) General aspect of callus after 8 weeks of culture showing clusters of small cells (scc, bar = 250 μm). (C) Callus after 10 weeks showing meristematic (mc) and parenchymatic (pc) cells (bar = 100 μm).

All samples were mounted on glass slides and stained with 1% toluidine blue. Observations and pictures were achieved under a Carl Zeiss standard with a TMAX film optical microscope.

Extraction of Plant Material. Calli were harvested from 6, 8, 10, and 12 week old cultures; shoots, roots, and rhizomes were obtained from 6 month old regenerated plants. The fresh and dry weights after lyophilization were individually determined. The powdered samples were extracted with (3 × 15 mL) ethanol and filtered. The filtrate was evaporated at room temperature under vacuum, and the residue was dissolved in 2 mL of acetonitrile and filtered through a filtration sample kit (Waters). Liquid media cultures were filtered, freeze-dried, and extracted using the same procedure. Root and rhizome tissues of regenerated and wild plants of *V. edulis* ssp. *procera* were also lyophilized and extracted according to the above protocol.

HPLC Analysis. Valepotriates for HPLC analysis were obtained by fractionation of 10 g of the hydro alcoholic (7:3) extract of wild plants of *V. edulis* ssp. *procera* on a silica gel cartridge (Sep-pak vac Silica, Waters), previously equilibrated with *n*-hexane and attached to a 6 mL syringe. Valtrates (54.4 mg) were eluted with *n*-hexane−EtOAc (9:1), and dihydrovaltrates (51.0 mg) were obtained with *n*-hexane−EtOAc (7:3). Valerenic acid (**5**) was kindly supplied by Farmasa-Schwabe (Morelos, México).

Extracts were analyzed on a Waters Delta Prep 4000 modular HPLC system, consisting of an U 6K injector, a 600E pump system controller (Millenium 2110 software), and a photodiode array detector. The analyses were carried out on a μ Porasil C_{18} (5 μm) column (125 × 3.9 mm i.d.); the mobile phase flow-rate was 2.4 mL/min. The initial solvent was 35% acetonitrile, 65% water acidified to pH 2.5 with H_3PO_4 (35:65), with a linear gradient to acetonitrile−water (49:51) over 38 min. Calibration curves were constructed separately for valtrates, dihydrovaltrates, and valerenic acid using solutions at the following dilutions: 10, 20, 40, and 80 μg/mL in acetonitrile. Valtrates were detected at 254 nm, dihydrovaltrates at 207 nm, and valerenic acid at 224 nm; a volume of 20 μL was injected. The identities of each peak were confirmed by co-injection of purified samples of valtrate (**1**), isovaltrate (**2**), dihydrovaltrate (**3**), dihydroisovaltrate (**4**), and valerenic acid (**5**). The calibration curves were based on the peak areas of the HPLC chromatograms. The experiments were performed in five replicates. Values were expressed in terms of percent on the basis of the dry weight in grams.

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