In vitro cultivation of *Ruscus aculeatus* L. and *Ruscus hypoglossum* L. (*Liliaceae*)

Teodora A. Ivanova

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Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences;
23 Acad. G. Bonchev Str., Sofia 1000, Bulgaria; e-mail: tai@bio.bas.bg;

PhD supervisor: Assoc. Prof. Tatyana Stoeva

Summary

Ruscus aculeatus L. and *R. hypoglossum* (*Liliaceae*) are small evergreen shrubs used both in gardening and as cut foliage. The rhizomes of *R. aculeatus* are widely collected as a drug source of steroid saponins with antiinflammatory, venotonic and antihaemoroidal activity. Both species are considered conservationally important in several countries due to the fact that cut foliage and rhizomes have been collected predominantly from the wild. In Bulgaria a regulated regime of gathering was prescribed for *R. aculeatus* and *R. hypoglossum*. The natural slow reproduction cycle and specific culture requirements hinder wide field cultivation. Recently, *in vitro* cultivation of both species has been performed for the production of planting material and conservation purposes mainly with material of Spanish, Portuguese and Romanian origin. However many technological aspects remained unsolved and the micropropagation protocols contained unequivocal and unclear parameters.

The aim of this thesis was to initiate *in vitro* cultures of *R. aculeatus* and *R. hypoglossum* from donor material of Bulgarian wild origin and to assess the influence of different culture factors on *in vitro* propagation and conservation: explant type, plant growth regulators and their concentration, culture type and sugar content. The characterization of the clonal variability and effect of culture conditions on plant genetic integrity was conducted by means of flow cytometry (DNA content and genome stability) and PAGE isozyme analyses of peroxidase (POD), asterase (EST), and acid phosphatase (ACP). Determination of biosynthetic potential and localization of saponins in *R. aculeatus* cultures was carried out using phytochemical HPLC analysis of ruscogenins (ruscogenin, neoruscogenin, ruscin and desglucoruscin).

Twelve clones of *R. aculeatus* from 4 origins and 3 clones of *R. hypoglossum* from 2 origins were initiated and protocols for micropropagation were developed. Culture initiation from seeds was limited by the deep morpho-physiological dormancy, low germination rates (*R. aculeatus* 15% and *R. hypoglossum* 35%), and prolonged 6-12 months lag-phase. Vegetative explants were unsuitable due to the high contamination or lack of development. Shoots were obtained through direct regeneration on *in vitro* rhizome explants of *R. aculeatus* on MS media supplemented with BAP (1 mg/l) and NAA (0.5 mg/l). *Ruscus hypoglossum* cultures regenerated shoots through callus, initiated on media with 0.5 mg/l thidiazuron (TDZ). This caused

somaclonal variation of shoots and cladodes – crispate shoots, monstrous cladodes and random inflorescence development.

Up to 70 shoots per initial *R. aculeatus* explant were obtained in the propagation stage using combination of pre-cultivation in liquid culture and subcultivation on agar media with NAA and BAP. Propagation rate increased 4 times for 3 years on media with BAP. Paclobutrazol was most efficient in agar cultures resulting in 5.88 shoots per explant. Sucrose concentration of 15 g/l was found to be most suitable for propagation on agar media without growth regulators.

Although it was not useful for propagation, two-phase cultivation of *R. aculeatus* proved to be effective for *in vitro* conservation at ambient conditions, ensuring 16-month effortless storage and fast recultivation on agar media without regulators or low concentrations of TDZ and KIN.

Micropropagation of *R. hypoglossum* was successful only on media with TDZ reaching highest multiplication rate of 5 shoot per explant on agar media with TDZ (0.5 mg/l) and NAA (1 mg/l). Consecutive cultivation on media without growth regulators and 60 g/l sucrose provided 8 shoots per explant.

Development of rooted shoots in the propagation stage allowed direct planting of clusters *ex vitro*. Survival of *R. aculeatus* plantlets was about 90% and reached 100% in regenerants, raised on paclobutrazol containing media. *Ruscus hypoglossum* plantlets, with their brittle and delicate cladodes, were more sensitive to *ex vitro* conditions and about 50% survived after one month of adaptation.

Cytometric assessment of both species proved that statistically significant changes in DNA content of the regenerants were not always related to the observed morphological variations and vice versa. No significant changes in DNA content and genome instabilities were detected in the obtained clones. Isozyme analyses showed clonally related induction and loss of isoforms that could be assigned to culture conditions, stage of differentiation or intraspecific variation.

Phytochemical screening of the *R. aculeatus in vitro* cultures demonstrated that all cultures produced saponins and the content was higher in the underground parts of the directly regenerated plants. Neoruscogenin dominated in the roots and ruscin in the rhizomes. Comparison of the clones showed differential synthetic potential. Clone Vel133 originated from Strandzha mountain showed highest contents of neoruscogenin (1.12 mg/g DW) and desglucoruscin (1.66 mg/g DW) in the underground parts.