ISOENZYME MARKERS FOR ASSESSMENT OF *IN VITRO* PLANTS FROM *RUSCUS ACULEATUS* L.

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ABSTRACT

PURPOSE: *Ruscus aculeatus* L. is an economically important ornamental and medicinal plant gathered mainly from the wild. Micropropagation protocols have been suggested for rapid production of clonal material and *ex situ* conservation of *Ruscus* species to avoid further over-exploitation of its natural resources. Variances in propagation rate and plant habitus were reported previously as important traits of the specific clones. Isoenzyme markers are proposed for assessment of the possible somaclonal variability induced during the *in vitro* propagation and the efficiency of the micropropagation procedure. **METHODS:** The isoenzyme profiles of three enzyme systems (peroxidases, esterases and acid phosphatase) of seven *in vitro* clones and native control were compared using discontinuous PAGE system. **RESULTS:** Induction of additional isoforms was frequent in the *in vitro* plants and clone-specific variations were detected. Acid phosphatase profiles were most polymorphic compared to less variable esterase and peroxidase ones. Isoenzyme profiles were not particularly correlated with the geographical origin of the accessions. **CONCLUSIONS:** The less polymorphic peroxidase and esterase systems could be used as markers for somaclonal changes by *in vitro* cultivation of *R. aculeatus* and selection of prospective clones.

Key words: PAGE electrophoresis, micropropagation, butcher's broom

INTRODUCTION

Ruscus aculeatus L. is naturally occurring in the Mediterranean area (incl. North Africa), East Europe, Central Hungary, Caucasus and Crimea (1). The species is widely known medicinal and ornamental plant gathered mainly from the wild. Hence its natural resources are diminishing. The species is included in 'Habitats' Directive 92/43/EEC (Annex Vb) and the European Plant Red List. In Bulgaria the collection of *R. aculeatus* from its natural accessions is regulated by the national Biodiversity Act (2007) and the Medicinal Plants Act (2000). *In vitro* cultivation of *R. aculeatus* has been investigated previously and shows some irregularities in the propagation due to recalcitrance, slow development and unclear effect of growth regulators (2). Clonal origin was pointed as an important factor for the distinctiveness in the development pattern,

propagation rate and ruscogenin biosynthetic capacity of the *in vitro* clones (3).

Analysis of the isoenzyme profiles of seven *R. aculeatus in vitro* clones is presented in order to assess the variability in directly regenerated shoots and to test the possibility for the isoenzymes to be used as markers in assessment of the micropropagation effectiveness.

MATERIAL AND METHODS

Plant material and culture conditions

Seeds were collected from wild populations in Northwest (S) and Southeast (V, MD) Bulgaria as part of the Millennium Seed Bank Partnership collection missions. Voucher specimens were deposited in the Herbarium of Institute of Biodiversity and Ecosystem Research (SOM). Shoot cultures were initiated from rhizome segments of seedlings by previously described technique (4). Cultures were maintained continuously on MS media with 1 mg/L 6-benzylaminopurine, 0.5 mg/L α -naphthaleneacetic acid, 0.8 mg/L Plantagar (all supplied by Duchefa, Netherlands) and 30 g mg/L commercial sugar. All media were

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adjusted to pH 5.75 prior to sterilization and autoclaved at 121 °C/1 atm for 20 minutes. The cultures were maintained in VitroVent® containers (with 125 mL medium, at 16/8 h photoperiod and 23±1 °C. Cultures were transferred onto fresh media every 8 weeks and every shoot with minimal height of 0.5 mm was detached and grown separately on the same medium. Propagation rate, shoot growth and appearance were recorded for at least 18 months. Data were processed statistically by Duncan's multiple range test (SPSS Inc.).

Samples for the isoenzyme analysis were collected from fully-developed shoot clusters.

Protein extraction and isoenzyme profiles of anodal isoforms of esterase (EST), peroxidase (POD) and acid phosphatase (ACP) using discontinuous PAGE system were done according to Angelov (2003) (6).

RESULTS

Propagation rates of the clones differed significantly being about twice as high in clones S52 and MD1 compared to others from same geographical origin (**Figure 1**). Shoot morphology in all clones was normal and abnormalities were uncommon. However some differences in phylloclade size and form were observed (**Figure 2**).

Figure 1. Propagation rate of *Ruscus aculeatus* *in vitro* clones.

Values (±SD) followed by same letters are not significantly different at $p=0.05$ (Duncan's multiple range test).

All *in vitro* clones had one common POD isoform with the native control and additional one or two slower ones (**Figure 3**). Most clones had one or two POD isoforms. Three or four isoforms were detected in two of the clones from the Southeast Bulgaria – V0 and V133 and one from the Northwest – S52.

Most of the *in vitro* clones exhibited at least one of the EST isoforms typical for the native control (**Figure 4**). Only clone S28B has exactly same profile as the control. Clone

V133 expressed none of the native isoforms. Induction of new slower isoform 12 was detected by clones S33B and V0.

ACP profiles appeared most polymorphic with induction of many new isoforms in the *in vitro* plants (**Figure 5**). Most of the induced isoforms were slow; some were unique for the specific clone. Largest number of newly induced isoforms was found in clone S33B. The slowest isoforms 10 and 12 were unique for the same clone.

Figure 2. Shoot morphology of *R. aculeatus* *in vitro* clones
 Clones S33B and S12 – Northwest Bulgarian origin; clones MD1 and V133 – Southeast Bulgarian origin

Figure 3. Schematic peroxidase isoenzyme profiles of *R. aculeatus* plants.

Figure 4. Schematic esterase isoenzyme profiles of *R. aculeatus* plants.

Figure 5. Schematic acid phosphatase isoenzyme profiles of *R. aculeatus* plants.

DISCUSSION

The distinctive difference in propagation rate and phylloclade morphology of the studied *R. aculeatus* plants imply that the observed variation in the isoenzyme profiles could not be attributed only to the cultivation conditions. Previously reported *in vitro* cultivation of *R. aculeatus* from Romania did not showed effect on POD patterns (7). However their data showed at least 3 or 4 isoforms both in native and *in vitro* plants. Common POD isoform 52 was typical for *R. hypoglossum* as well (8). Our results suggest that isoenzyme profiles of the *R. aculeatus* plants are clone-specific and could not be particularly correlated with the geographical origin of the accessions. Previously reported highly ruscogenin yielding clones S12 and V133 (3) exhibited same or less polymorphic EST patterns. On the other hand POD pattern of the V133 was most polymorphic. The dissociation of the POD and EST isoforms and induction of new forms was related to somaclonal variation in *in vitro* plants in other species as well (9-11). All isoform of the acid phosphatase were very intensive unlike previous report previously (7) and highly polymorphic regardless of the clone origin. In conclusion, obtained results demonstrate that less polymorphic POD and EST systems could be used as markers for somaclonal changes by *in vitro* cultivation of *R. aculeatus* and selection of prospective clones.

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