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SUCROSE-RELATED EFFECTS ON SHOOT DEVELOPMENT AND DOUBLE-PHASE CULTIVATION OF *RUSCUS ACULEATUS* L.

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Summary: *Ruscus aculeatus* is an economically important plant species with remarkable shade and drought tolerance. Its slow growth habit and specific growing requirements limit its frequent field cultivation. Furthermore, micropropagation of *R. aculeatus* is greatly influenced by determined shoot growth and choice of the source material (origin and explant type). This paper presents our experiments to stimulate the propagation and growth of *R. aculeatus* shoots by modification of the carbon source (sucrose) and cultivation in a double-phase system. The propagation rate and shoot development were assessed and genome-size stability of the regenerants was verified by flow cytometry. The excess of sucrose in the media prevented shoot proliferation and development. The lowest concentrations were more suitable for shoot induction and even for root growth, contrasting with the general view that the carbon source stimulates the growth in rhizomatous species. The obtained shoots performed a normal morpho-physiological habitus mostly with non-branched stems with typical cladodes. The unaffected genome size status of the regenerants was also confirmed. Double-phase cultivation was effective only in a medium without growth regulators which supported better shoot growth. Addition of paclobutrazol was beneficial both in agar and double-phase cultures producing 5.8 and 3.5 shoots per explant, respectively. However, the propagation rates were not higher than those on agar. The obtained results give useful insights in the micropropagation of *R. aculeatus* that could reduce production and conservation costs.

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Keywords: Paclobutrazol; retardant; cytometry; liquid culture; micropropagation.

Abbreviations: BAP – 6-Benzylaminopurine; NAA – α-naphtaleneacetic acid; DMRT – Duncan's multiple range test.

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INTRODUCTION

Ruscus aculeatus L. (Butcher's broom) is a small rhizomatous semi-shrub valued for its ornamental foliage and suitable for landscaping and floral arrangements. Moreover, it is used as a medicinal plant due to the steroidal saponins (ruscogenins) in its rhizomes and roots (Blumenthal et al., 2000). Plants have typical slow growth and development that limit their cultivation, hence over-collection in wild populations is quite common (Coskun et al., 2006). The species is listed in European Red List of Vascular Plants, in the 'Habitats' Directive 92/43/EEC. According to the Biodiversity Act in Bulgarian its collection is under regulation.

Different aspects of *in vitro* cultivation of *R. aculeatus* have been investigated previously showing some irregularities in propagation related to a recalcitrance, slow development and unclear effects of growth regulators (Balica et al., 2005; Moyano et al., 2006; Banciu and Aiftimie-Păunescu, 2012). A liquid culture was tested with limited success as explant development stopped and propagation rate declined (Ivanova et al., 2008). The latter was related to the detrimental effect of the fully-submerged type of cultivation. Double-phase cultivation was proposed as an approach for a long-term storage of *R. aculeatus*, ensuring easy conservation for over a year (Ivanova et al., 2011).

The aim of the study was to assess the possibility to stimulate propagation and shoot development in *R. aculeatus* by variation in sucrose concentration and by double-phase cultivation. Evaluation of the genome fidelity was performed by flow cytometry.

MATERIALS AND METHODS

Seeds were collected in the framework of the Millennium Seed Bank Partnership collection missions. Voucher specimens were deposited in the Herbarium of the Institute of Biodiversity and Ecosystem Research (SOM), Bulgarian Academy of Sciences. Shoot cultures were initiated from rhizome segments of seedlings as previously described by Ivanova et al. (2008).

Cultures were maintained continuously on MS medium (Murashige and Skoog, 1962). All media were adjusted to pH 5.75 prior to sterilization and autoclaved at 121°C/1atm for 20 min. The cultures were maintained in VitroVent® containers (Duchefa, NL) with 125 ml medium, at a 16/8 h photoperiod and 23±1°C.

The effect of sucrose was tested at four concentrations ranging from 15 to 90 $g.l^{-}$ added to MS nutrient media without any growth regulators and solidified with 0.8 $mg.l^{-1}$ Plantagar (Duchefa, NL).

The double-phase cultivation was tested on rhizome explants fixed on water-agar base and liquid media (20ml) added not covering entire explants. NAA, BAP or paclobutrazol were added to the liquid media at concentrations of 1 and 2 $mg.l^{-1}$. Shoot cultures were transferred onto fresh media every 8 weeks and every shoot which was at least 0.5 mm high was detached and grown separately on the same medium. The propagation rate, rooting and shoot growth were recorded at subcultivation. Every treatment was performed in four repetitions with 20 explants each.

DNA content of the cultures was measured by flow cytometry with propidium iodide staining, using Partec CyFlowR SL and following the protocol

provided by Partec (Germany). *Pisum sativum* (2C=8.84 pg*)* was used as an internal standard. Native *R. aculeatus* plant was used as a reference control. Fresh cladode samples were collected and genome size was measured in three replicates, each run thrice to 5000 counts.

Data were processed statistically by single factor ANOVA followed by Duncan's multiple range test.

RESULTS

Effect of sucrose on shoot development

Shoot development in all treatments started at the end of the first month. The most suitable sucrose concentration for propagation of *R. aculeatus* was 15 g.l^{-1}. The propagation rate decreased with the increase of sucrose in the media (Table 1). Still in the range of 15-60 g.l^{-1} sucrose most of the explants (78-89%) produced new shoots. The shoots were from 1.5 to 3 cm in size, unbranched, with 2 to 4 normally developed cladodes. The largest shoots were produced on 15 g.l^{-1} sucrose. Higher amounts of the carbon source (30 and 60 g.l^{-1} sucrose) did not result in improved growth and the size of the regenerated shoots was even smaller than the one achieved on 15 g.l^{-1} sucrose (Fig. 1). The highest sucrose concentration

Table 1. Effect of sucrose concentration on shoot development of *R. aculeatus* cultures.

Sucrose [g.l^{-1}]	Average explants with regeneration [%]	Average shoots/ explant	Average rooted shoots [%]	Average shoot height [cm]
15	89.30^{a}	1.86^{a}	7.5^{b}	2.92^{a}
30	77.50^{a}	1.15^{bc}	12.0^{b}	1.97^{a}
60	88.75^{a}	1.45^{b}	37.3^{a}	2.48^{a}
90	54.30^{b}	0.89^{c}	11.5^{b}	1.57^{a}

Values followed by the same letters are not significantly different at p=0.05 (DMRT).

Figure 1. Habitus of *R. aculeatus* shoots on different sucrose concentrations. Left to right 15, 30, 60, 90 g.l^{-1}.

(90 g.l^{-1}) decreased the propagation rate and caused necrosis in the explants. The overall culture state worsened with time and shoots produced in this treatment were least developed. Rooting was recorded in 37% of the shoots on 60 g.l^{-1} and less than 15% in the rest four treatments.

Double-phase cultivation

The addition of the growth regulators paclobutrazol and BAP was beneficial for shoot induction both in agar and double phase cultures and more effective than NAA treatments and the control. However, propagation rates in all treatments with growth regulators on agar culture were superior to those in the double-phase system (Fig. 2). Only in the control, the double-phase system ensured better shoot regeneration than on agar. Among all tested regulators paclobutrazol was most efficient both in agar and double-phase cultures producing 5.8 and 3.5 shoots per explant, respectively. The positive effect of PAC was associated with a considerable stem reduction (Fig.

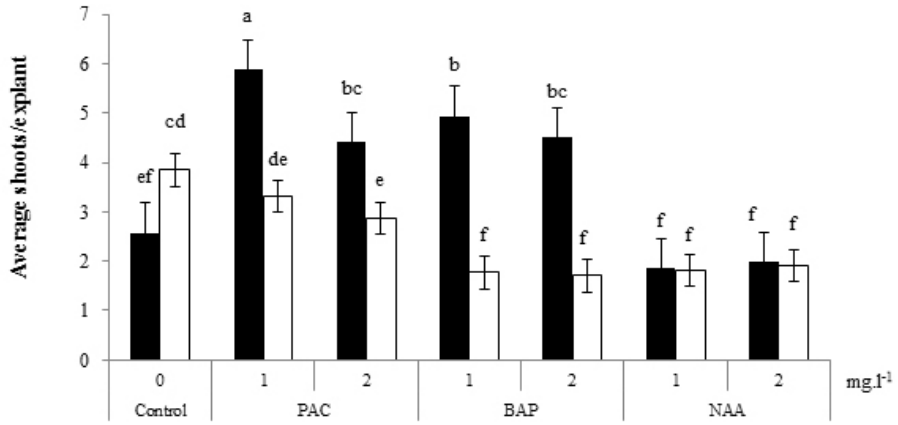

Figure 2. Propagation rate in agar and double-phase cultures of *R. aculeatus*. ■ Agar culture; □ Double-phase culture. Values (±SD) followed by the same letter are not significantly different at p=0.05 (DMRT).

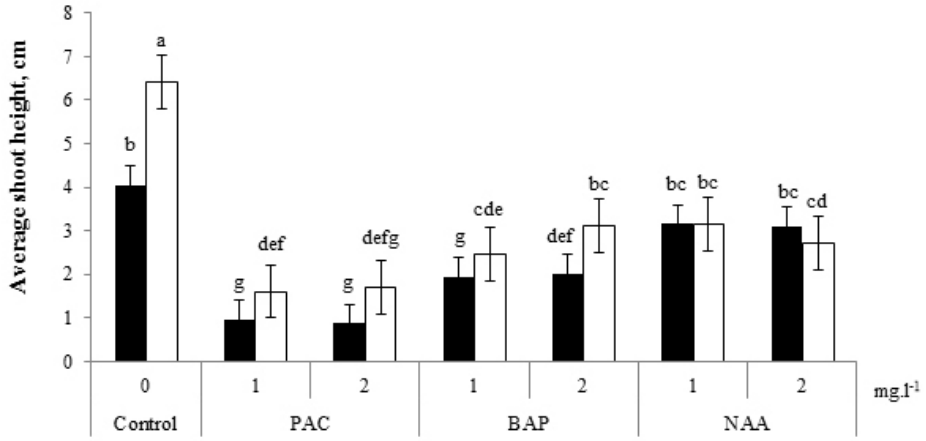

Figure 3. Shoot growth in agar and double-phase cultures of *R. aculeatus*. ■ Agar culture; □ Double-phase culture. Values (±SD) followed by the same letter are not significantly different at p=0.05 (DMRT).

3). Nevertheless, both morphology and size of the cladodes were not affected. The positive effect of BAP on shoot production was visible and considerably improved compared to previous results obtained for *R. aculeatus* (Ivanova et al., 2008). Regardless of the culture type and concentration, NAA was less effective for shoot production. However, NAA was beneficial for production of bigger shoots compared to PAC and BAP. Shoots obtained in double-phase cultures were higher than the ones on agar in almost all treatments.

Root induction was strong in agar cultures treated with NAA, poor on PAC and was almost absent on BAP-supplemented media (Fig. 4). NAA at 1 mg.l^{-1} was most effective inducing up to 7 roots per explant. In double-phase

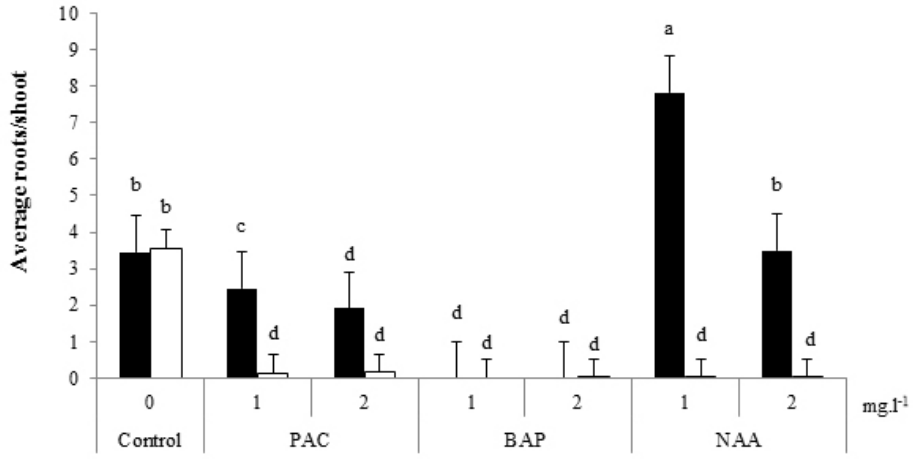

Figure 4. Rooting in agar and double-phase cultures of *R. aculeatus*. ■ Agar culture; □ Double-phase culture. Values (±SD) followed by the same letter are not significantly different at p=0.05 (DMRT).

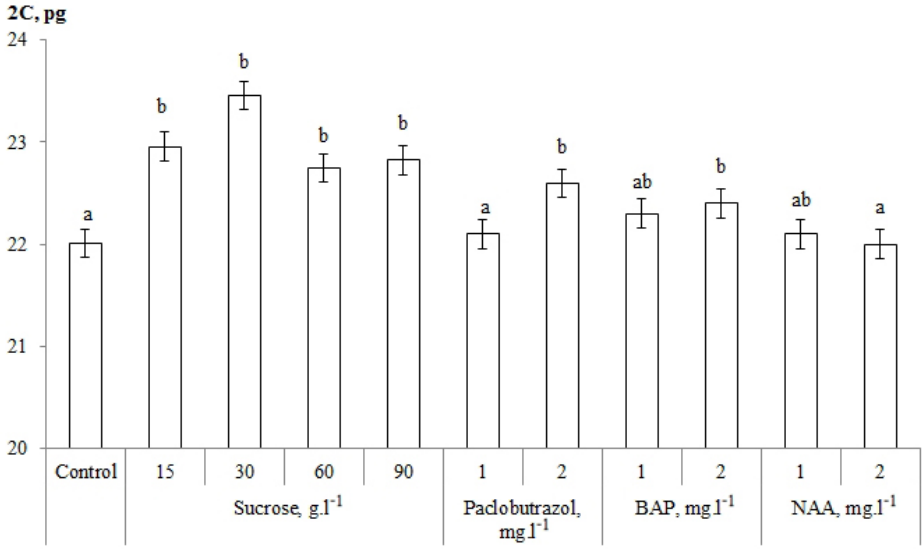

Figure 5. DNA content of *in vitro* regenerants of *R. aculeatus*. Values (±SD) followed by the same letter are not significantly different at p=0.05 (DMRT).

cultures noticeable rooting was observed only in the control where results were similar to those on agar. Poor rooting in double-phase cultures did not allow direct transfer of the obtained shoots for *ex vitro* establishment.

The assessment of the genome stability did not show distinctive deviations (Figs. 5, 6). All treatments resulted in higher DNA content compared to the control. The increase in sucrose level as well as addition of growth regulators were not related to significant changes in the genome size.

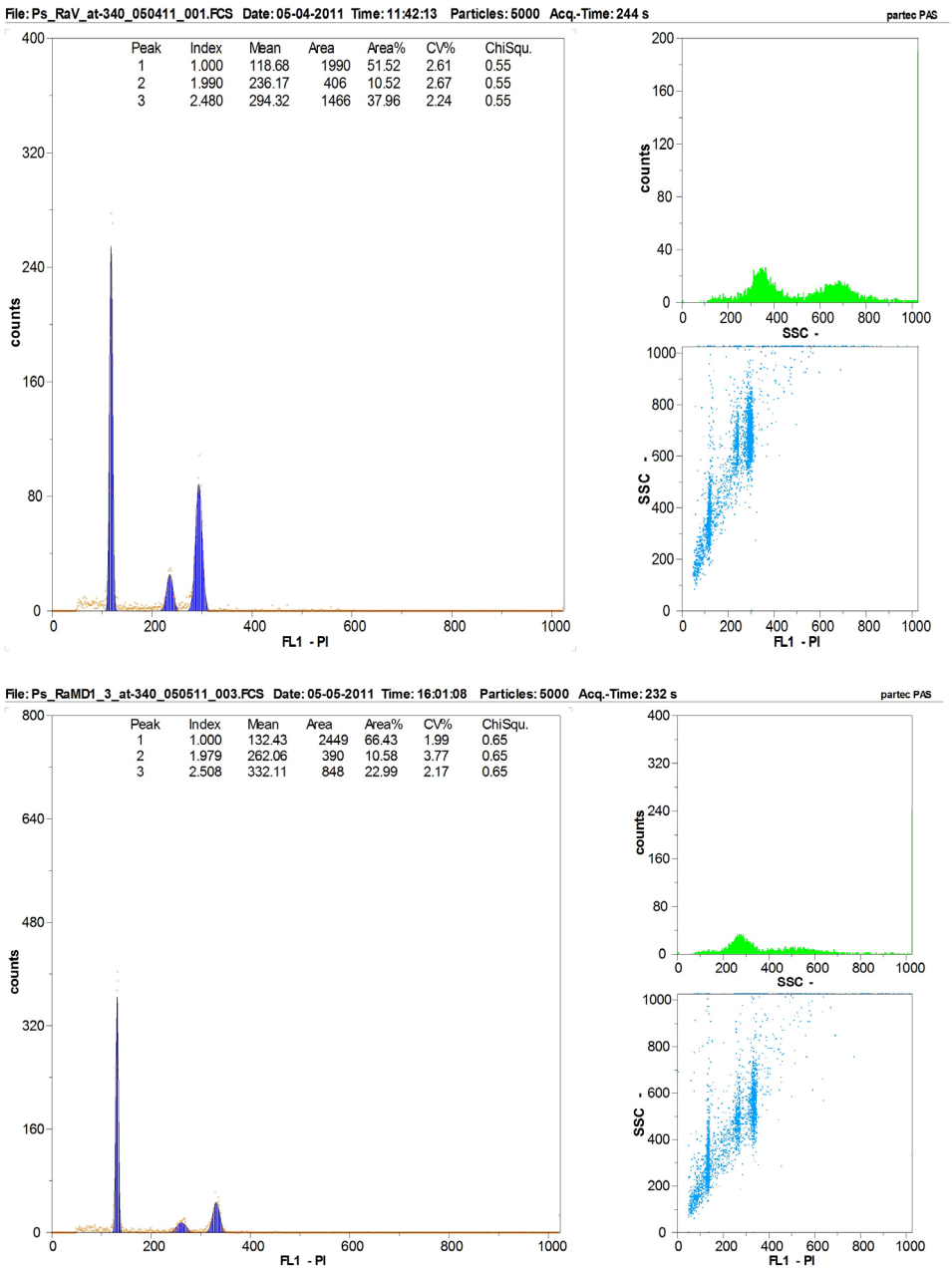

Figure 6. Histograms of cytometric analysis in native and *in vitro R. aculeatus* plants. Up: native plant; Down: *in vitro* plant.

DISCUSSION

The sucrose-related effects observed in *R. aculeatus* similar to those in *R. hypoglossum* showed considerable lower carbon source demands compared to other rhizomatous species where increased carbon concentrations stimulated growth and development (Ivanova et al., 2013). The optimal concentration of sucrose for shoot production typically varied between 60 and 80 $g.l^{-1}$ depending on the species (Islam et al., 2004; Archana et al., 2013). Higher concentrations could induce more but smaller shoots or their further establishment is inferior compared to shoots grown on less sugar-supplemented media (Ebrahim, 2004; Archana et al., 2013). In *Asparagus* higher levels of sucrose were important mainly as a nutrition source for the roots (Conner and Falloon, 1993). In *R. aculeatus* 15 $g.l^{-1}$ sucrose in the medium was most favorable in the multiplication phase ensuring maximum proliferation and relatively low rooting. The increase of sugar alone was not enough for significant stimulation of rooting and results were considerably lower than those obtained on agar media with NAA and BAP (Ivanova et al., 2008).

The addition of liquid media on the surface of the agar was found to promote culture growth providing both support and beneficiary effect of the liquid phase (Maene and Debergh, 1985; Han et al., 2004). However, shoot production in double-phase systems could be rather variable depending on the plant species and applied conditions. Prasad and Dutta Gupta (2006) reported improved shoot multiplication in *Gladiolus* in double-phase cultures on BAP and NAA. However, prolonged incubation in liquid media was reported to cause a culture decline and low bud proliferation in *R. aculeatus* and *R. hipophyllum* cultures (Ivanova et al., 2008). Our results showed that adding paclobutrazol to the double-phase culture could be used effectively to achieve the highest propagation rate in *R. aculeatus* reported so far. Several authors reported paclobutrazol as a promotive regulator of shoot proliferation (Ziv, 1989; Steinitz and Lilien Kipnis, 1989). On the other hand, like other triazole-type compounds, paclobutrazol causes a reduction of shoot length by shortening the internodes without changing the developmental patterns or being phytotoxic (Rademacher, 2000). Thus its application should be cautious and not for prolonged periods of time.

The assessed DNA content of the regenerants was compatible with previously reported data for *R. aculeatus* (Carvalho et al., 1998; Vesely et al., 2012). Slightly elevated values could be either caused by the *in vitro* cultivation or result from natural variation in the species.

In conclusion, the presented results imply that micropropagation of *R. aculeatus* could be performed with less supplementation of carbon source and using paclobutrazol in agar or double-phase cultures to reduce production and conservation costs.

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