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BRIEF COMMUNICATION

In vitro micropropagation of *Ruscus aculeatus*

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Abstract

We have developed three protocols for the rapid micropropagation of *Ruscus aculeatus*. The primary explants utilised were immature embryos, aerial buds excised from rhizomes and shoot buds regenerated from organogenic calli. In order to increase the plant regeneration from the primary explants, we used organogenic calli from cladode, stem and rhizome segments. We tested more than 20 culture media for callus induction and shoot regeneration and the best results were obtained when rhizome segments were cultured on Murashige and Skoog medium supplemented with 0.5 mg dm^{-3} 2,4-dichlorophenoxyacetic acid and 1 mg dm^{-3} kinetin.

Additional key words: 2,4-dichlorophenoxyacetic acid, embryo cultures, kinetin, naphtalenacetic acid, organogenic callus.

Ruscus aculeatus L. (butcher's broom) belonging to the *Liliaceae* family, is a small evergreen shrub widespread in western, southern and south-central Europe (Tutin *et al.* 1972, Pignatti 1982). It has tough, erect, and striated stems forming numerous short branches and very rigid leaves that are actually extensions of the stem (cladodes) and terminate in a single sharp spine. The reproductive success was very low, mainly due to the lack of pollen transport for this species (Martinez-Pallé *et al*. 2000), which could become extinct in the near future. Most *in vitro* research has focused on other *Ruscus* species such as *R. racemosus*, a plant of some interest in floriculture (Curir *et al*. 1986). Although there are many reports of micropropagation protocols for ligneous species, such as the medicinal trees *Crataeva nurvala* (Walia *et al*. 2003) and *Ginkgo biloba* (Tommasi and Scaramuzzi 2004), and for monocotyledonous plants (Ket *et al*. 2004) there are no reports on the successful micropropagation of *R. aculeatus*, partly because of the difficulties in establishing primary explants. In this paper, we describe three new protocols for rapid micropropagation of *R. aculeatus* utilising different sources of primary explants: immature embryos, rhizome buds and organogenic calli.

Ruscus aculeatus L. fruits are bright red and about 1.3 cm long. The berries were collected in Sant Cugat del Vallés (Spain) at the end of October when the fruits are mature. The fruits containing 1 - 4 seeds were sterilised by conventional protocols (Halámková *et al.* 2004). The embryos were isolated in aseptic conditions and cultured on Murashige and Skoog (1962; MS) medium without plant growth regulators. Between 80 - 100 embryos were incubated in Petri dishes with 25 cm^3 of hormone-free MS medium under a 16-h photoperiod irradiance of 28 - 36 μmol m^{-2} s^{-1} and at a temperature of 25 ºC. After 2 - 3 weeks embryos germinated (90 %) showing a root of 1 - 2 cm, and one month later the aerial part was visible (Fig. 1*A*). When the developing plantlets achieved height of about 5 cm, they were transferred to a mixture of sand and peat and were cultured in a greenhouse at 25 ºC. Approximately 60 % of the plants transferred to *ex vitro* conditions were acclimatized correctly (Fig. 2).

In order to develop *Ruscus* micropropagation independent of seasonal variations, and taking into account the high frequency of contamination when underground organs of plants are cultured *in vitro*, *Ruscus* rhizomes were sterilised by immersion in 70 % ethanol for 30 s, then in $HgCl_2$ (1 %) for 15 min and

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Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; NAA - naphtalenacetic acid; Kin - kinetin; PGR - plant growth regulator; MS - Murashige and Skoog (1962).
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NaClO (3 %) for 30 min, and finally the rhizomes were rinsed 5 times with sterile H_2O. In these conditions the percentage of initially contaminated explants was lower than 30 %, whereas using conventional sterilization systems the percentage of contaminated explants was greater than 70 %. Between 20 - 40 excised aerial buds from rhizomes were incubated in MS hormone free medium, in the conditions mentioned above for embryo cultures. Buds developed one month later were subcultured in MS medium supplemented with naphtalenacetic acid (NAA; 2 mg dm^{-3}) to obtain roots; 68 % of transferred shoots rooted in this medium. When the roots of plantlets were developed, we transferred them to *ex vitro* conditions for acclimatization the plantlets in a greenhouse, and 58 % of plants survived in these conditions. The rhizomes of *Ruscus* present a high number of aerial buds and consequently they constitute an excellent source of vegetative explants for micropropagation. The period after the introduction of the excised buds into *in vitro* culture until the plants were acclimatized did not last more than 4 months. Fig. 1*B* shows the different steps of plant regeneration.

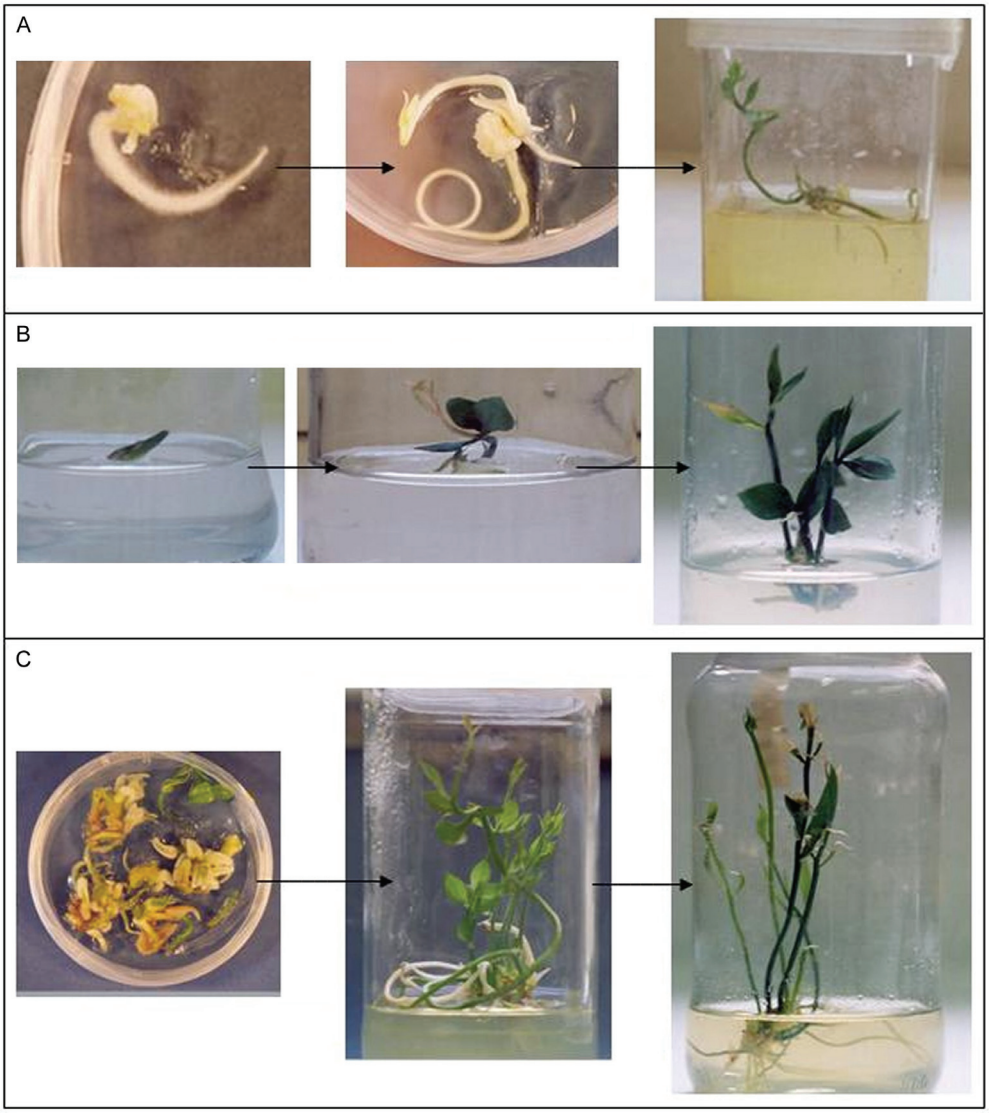

Fig. 1. *Ruscus aculeatus* plant regeneration from embryos (*A*), excised rhizome buds (*B*) and organogenic calli (*C*).

With the aim of increasing the number of shoots from primary explants we developed organogenic calli from *R. aculeatus*. We tested segments of stem, cladode and rhizome as primary sources of explants. For each culture condition, between 30 - 40 explants were assayed. Aerial parts of plants were sterilised as described previously. The different types of explants were inoculated in MS medium with plant growth regulators (Table 1). Following previous assays, we used 2,4-dichlorophenoxyacetic acid (2,4-D) instead of NAA for a better induction of calli (Zapata *et al.* 2004) and to avoid a reduction in the frequency of shoot organoganesis (Çöçü *et al*. 2004). In all of the media tested segments of stems and cladodes only developed small pieces of callus,

whereas segments of rhizomes developed abundant callus formation (Fig. 1*C*). Depending on the hormonal concentration (0.5 mg dm^{-3} 2,4-D; 0.1 and 1 mg dm^{-3} kinetin), *Ruscus* calli regenerated aerial buds, but no correlation between the auxin/cytokinin ratio and the capacity of callus cultures for regenerating shoots was established. Our results also show that high 2,4-D concentrations (more than 1 mg dm^{-3}) inhibit callus growth and organogenesis, so it is necessary to add a cytokinin into the culture medium to obtain high levels of proliferating cells and, consequently, callus biomass or aerial buds. Similar results were obtained by Raimondi *et al.* (2001) working with *Asparagus officinalis*, a plant species with a similar pattern to *Ruscus* when cultured *in vitro*. When the concentration of 2,4-D was increased the callus cultures developed roots but symptoms of cellular necrosis appeared in the callus pieces. The shoots excised from the organogenic calli were rooted in MS medium with 2 mg dm^{-3} of NAA (Fig. 1*C*).

Table 1. The effect of 2,4-D and Kin on percentage of rhizome discs that developed calli after 6 weeks of culture. PGR were added to basic MS medium in different concentrations. * - organogenic callus.

[mg dm^{-3}]	2,4-D [0.5]	2,4-D [1.0]	2,4-D [2.0]	2,4-D [2.5]
Kin [0]	33 %	28 %	10 %	-
Kin [0.1]	40 %*	63 %	39 %	10 %
Kin [1.0]	80 %*	75 %	67 %	25 %

Fig. 2. *Ruscus aculeatus* plant derived from embryo, cultured in a greenhouse, 4 weeks after transplantation.

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