



## MICROPROPAGATION OF *RHODIOLA KIRILOWII* PLANTS USING ENCAPSULATED AXILLARY BUDS AND CALLUS

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This study assessed regrowth of micropropagated *Rhodiola Kirilowii* buds after encapsulation of axillary buds and differentiating callus in calcium alginate hydrogel and low-temperature preservation. This method of micropropagation was applied to obtain enough plant material for studies on chemical compounds of *Rhodiola Kirilowii*, a plant difficult to obtain from its natural environment. Axillary buds and differentiating callus were encapsulated in calcium alginate and stored at 4°C for 1 to 15 weeks and then transferred to hormone-free MS medium. The best results were obtained after six weeks of preservation: 100% of the encapsulated explants survived and developed into shoots and plantlets after subculture on basal MS medium. Longer storage of encapsulated axillary shoot buds decreased their regrowth, but the duration of storage of encapsulated differentiating callus had no significant influence on its survival.

**Key words:** *Rhodiola Kirilowii*, axillary buds, differentiating callus, encapsulation, cold storage, plant regeneration.

### INTRODUCTION

*Rhodiola Kirilowii* Rgl. ex Maxim (= *Sedum Kirilowii* Rgl. in Rgl. et Tiling; *Sedum elongatum* Kar. et Kir.; Hongjintian), of the family Crassulaceae, is found in North Asia and China. Of the many species of the genus *Rhodiola* listed in INDEX KEVENSIS (Jackson, 1960), only two, *R. rosea* and *R. quadrifida*, grow in Europe (Tutin et al., 1964). According to Ohba (2003), *Rhodiola Kirilowii* (Fig. 1a) is a typical mountain plant that grows at sites difficult to access, at 2000–5000 m a.s.l.

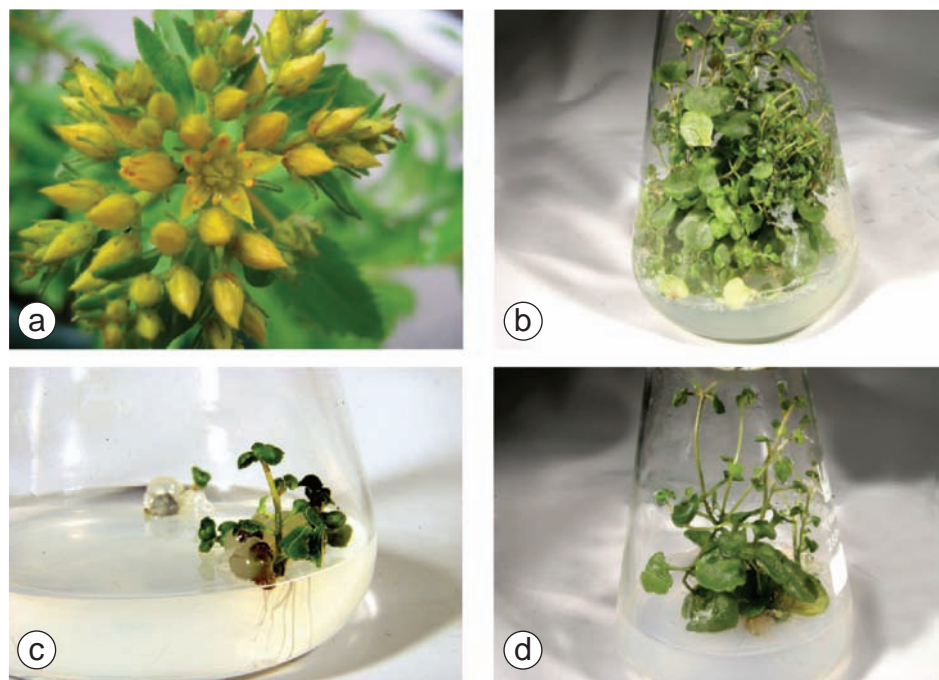
There are published reports in the Chinese language on studies of *R. Kirilowii* by Chinese researchers. Such chemical compounds as salidroside, tyrosol, daucosterol, lotaustralin, sucrose (Peng et al., 1994) and beta-sitosterol (Kang et al., 1992) have been isolated from petroleum extracts of the plant. Oral administration of *R. Kirilowii* was shown to protect inhabitants of mountain regions from cardiopulmonary disorders caused by high altitude (Zhang et al., 1989). *R. Kirilowii* was also found to reduce damage to rat viscera caused by a hypoxic high-altitude environment (Zhang et al., 1990).

The best-known *Rhodiola* species is *R. rosea*, which is used medicinally as an adaptogen (Brown et al., 2002) in Russia, Sweden (Rosen Root), Germany (Lentaya) and Poland (Antystress Forma, Cellu Vita). These products are standardized for salidroside or rosavin content.

In our experiment we adapted our own method (Furmanowa et al., 1991) and also methods cited in this article to obtain plants from encapsulated axillary buds or differentiating callus of *Rhodiola Kirilowii*. Encapsulation of meristematic tissue and storage at 4°C decelerates its development, which is of practical significance. This method of micropropagation has been used for some medicinal plants to facilitate storage and transport. Micropropagation via encapsulation of different propagules can be used to achieve a higher micropropagation rate and accelerate plant development in a temperate climate. We

**Abbreviations:** MS – Murashige and Skoog (1962) medium; MS/2 – Murashige and Skoog medium with half amounts of all components; BA – benzyl adenine; IAA – indole-3-acetic acid; SA – sodium alginate; CA – calcium chloride; NAA – alpha-naphthaleneacetic acid; AC – adenine chloride.

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**Fig. 1.** *Rhodiola Kirilowii* culture. (a) Flowering plant growing in the Botanical Garden, (b) Plantlets established from existing meristems growing on MS/2 medium, (c) Plantlet developed from capsule containing axillary bud after 6 weeks of storage at 4°C on MS/2 medium and 2 weeks at 24°C on MS medium, (d) The same plant growing on MS medium for 6 weeks at 24°C.

searched for the easiest and cheapest procedure for encapsulation, using media without growth regulators, bearing in mind that *R. Kirilowii* is a very hardy plant that grows in a harsh environment. We introduced this technique for *R. Kirilowii* propagation because of its inaccessibility. In light of the findings from Chinese studies, *R. Kirilowii* is of potential use as a medicinal plant in European countries.

Synthetic seed technology is gaining increasing attention as an important procedure for cost-effective micropropagation of different plant species. The artificial cover protects the embryoid or meristematic tissues against mechanical damage, drying or dehydration during storage and transportation. Capsules with sterile material can be exchanged easily between laboratories. The encapsulation process is very useful when normal seeds cannot be used for propagation, when plants are affected by various diseases, or when the plants do not form seeds. The shoots and plantlets developed from synthetic seeds are used for further micropropagation of plants. Efficient micropropagation of *Rhodiola Kirilowii* is an attractive option since it is so difficult to obtain otherwise.

There have been many studies on encapsulation of different plant material. Somatic embryos were used as a reproducible system for *Carum carvi* (Furmanowa et al., 1991), sugarcane (Nieves et al., 2003) and *Pau-*

*lownia elongata* (Ipekci et al., 2003). Less attention has been given to encapsulation of nonembryogenic vegetative propagules such as nodal segments, axillary buds, shoot tips, hairy roots or callus, but there are more and more reports on successful encapsulation of various parts of plants: apical and axial shoot buds of *Valeriana wallichii* (Mathur et al., 1989), buds of *Zingiber officinale* (Sharma et al., 1994), hairy roots of *Azadirachta indica* (Repunte et al., 1995), shoots isolated from *Ananas comosus* (Soneji et al., 2002), shoot buds of *Rubus chamaemorus* (Thiem, 2002), shoot buds of *Catalpa ovata* (Wysokińska et al., 2002) and apical buds of *Malus pulmila* Mill (Micheli et al., 2002).

There are also reports of coating calcium alginate beads with various substances such as paraffin (Repunte et al., 1995; Wysokińska et al., 2002) to prevent damage to explants.

Patel et al. (2000) studied a novel encapsulation technique in which plant material was suspended in a solution containing carboxymethylcellulose and calcium chloride and then dropped into alginate sodium solution (hollow beads). This procedure should protect the explants like a natural seed.

Encapsulation of axillary buds and callus has not been attempted in *Rhodiola* species before. Somatic embryos of *Rhodiola Kirilowii* form very rarely, so we could not use them for our experiment.

## MATERIALS AND METHODS

### PLANT MATERIAL USED FOR ENCAPSULATION

In vitro culture of *R. Kirilowii* was started from seeds received from the Botanical Garden of the Research Institute of Medicinal Plants in Poznań, where the plants had been cultivated for four years in soil. Seeds were washed in running tap water and submerged in 96% ethanol for 15 sec. Afterwards the seeds were sterilized for 15 min in a solution of sodium hypochlorite (diluted 1:1) and then rinsed four times in sterile distilled water. Sterilized seeds used for the initiation of plantlet culture were put on modified Murashige and Skoog (1962) solid medium with all components reduced by half (MS/2).

Axillary shoot buds ( $n = 42$ ) excised from developed shoots of plantlets (Fig. 1b) were taken for encapsulation. Another pool of plant material used for the same process consisted of 45 pieces of differentiating callus developed from excised hypocotyl segments or parts of shoots growing in vitro on Murashige and Skoog (MS) medium with 2 mg l<sup>-1</sup> benzylaminopurine (BA) and 2 mg l<sup>-1</sup> indole-3-acetic acid (IAA).

### ENCAPSULATION

#### Encapsulation of axillary buds

Encapsulation was accomplished by mixing the described tissues in 5% sodium alginate (SA) and then dropping them individually into stirred 50 mM calcium chloride (CA) solution according to the method in Mathur et al. (1989) and Furmanowa et al. (1991). Each bead contained one explant. A calcium alginate coating was formed around the tissues after 30 min incubation in calcium chloride. After hardening, the capsules were rinsed in distilled water to remove calcium chloride residues. Capsules obtained by this procedure were stored at 4°C as generally described in the literature. After six, eleven and fifteen weeks of preservation on MS/2 solid medium, 14 capsules were transferred to solid MS medium without growth regulators and cultured in a growth chamber at 24°C under a 12 h photoperiod to develop shoots and plantlets with new lateral shoots.

#### Encapsulation of differentiating callus

Fifteen small pieces of callus (2–3 mm) were mixed successively in each of the following solutions:

- a) 5% sodium alginate
- b) 4% sodium alginate with MS medium
- c) 4% sodium alginate with MS medium containing 2 mg l<sup>-1</sup> benzyl adenine (BA), 2 mg l<sup>-1</sup> naphthalene-1-acetic acid (NAA) and 1 mg l<sup>-1</sup> adenine chloride (AC).

Then they were dropped individually into a 50 mM solution of calcium chloride.

In preliminary studies we tested five types of storage:

- 1) Petri dishes
- 2) Petri dishes with dry blotting paper
- 3) Petri dishes with moist blotting paper
- 4) MS medium with BA (2 mg l<sup>-1</sup>), NAA (2 mg l<sup>-1</sup>) and AC (1 mg l<sup>-1</sup>)
- 5) 0.7% agar.

We found no differences in shoot development from encapsulated buds between the tested storage media (data not shown), and decided to store the beads with callus for 1 to 6 weeks at 4°C on Petri dishes in the further experiments. After preservation, the beads were put on solid MS basal medium and cultured in a growth chamber at 24°C under a 12 h photoperiod. All media, solutions and equipment were autoclaved at 121°C for 15 min before use. All procedures were carried out in sterile conditions.

## RESULTS AND DISCUSSION

Axillary buds and differentiating callus were successfully encapsulated in calcium alginate beads. The capsules containing axillary shoot buds and differentiating callus developed into shoots and plantlets after transfer to solid MS basal medium. Each experiment was done twice. The results were similar. Details are shown in Table 1 for axillary buds and in Table 2 for callus.

We found that capsules of *R. Kirilowii* could be stored at low temperature for different periods of time. Axillary buds and differentiating callus survived and showed very good tolerance to the encapsulation procedure. After cold preservation for 1 to 6, 11 or 15 weeks the encapsulated plant material developed into shoots and next into plantlets that did not differ in morphology from plants cultivated without encapsulation. Longer preservation at 4°C prolonged shoot development from capsules at 24°C (1 week at 4°C: 5 days of shoot development at 24°C; 15 weeks at 4°C: 12 days of shoot development at 24°C).

Axillary buds of *Rhodiola Kirilowii* survived and showed 100% viability after six weeks of low-temperature preservation. As shown in Table 1, longer storage decreased the regrowth of encapsulated buds. Similarly, Danso and Ford-Lloyd (2003) found cassava plant regrowth considerably affected by the duration of low-temperature storage. In *Dalbergia sissoo* Roxb., Chang and Singh (2004) achieved only 72% conversion of encapsulated nodal segments into plantlets stored at 4°C for 30 days.

The capsules that survived in our experiment developed into shoots after ten days of culture at 24°C irrespective of the duration of preservation at 4°C (6, 11, 15 weeks).

In capsules formed from differentiating callus, the percentage of developed shoots after 1 to 6 weeks of

TABLE 1. Development of *Rhodiola Kirilowii* axillary buds encapsulated in calcium alginate hydro gel

Plant material used for encapsulation	Method of encapsulation	Time of preservation at 4°C (weeks)	% of developed axillary buds	Time of development in 24°C (days)
	5% SA			
Axillary shoot buds	+	6	100	10–12
of plantlets developed in vitro	+	11	83	10–12
	+	15	33	10–12

TABLE 2. Development of *Rhodiola Kirilowii* differentiating callus encapsulated in calcium alginate hydro gel

Plant material used for encapsulation	Methods of encapsulation			Time of preservation at 4°C (weeks)	% of developed shoots from encapsulated callus	Time of development in 24°C (days)
	5% SA	4% SA + MS	4% SA + MS + BA, NAA, AC			
Differentiating callus of hypocotyl origin	+	+	+	1	100	5
	+	+	+	2	100	7–8
	+	+	+	3	99	8–10
	+	+	+	4	95	8–10
	+	+	+	5	95	8–10
	+	+	+	6	95	8–10

storage was between 95% and 100% (Tab. 2). Time of storage had no significant influence on the survival of encapsulated callus. Supplementation of sodium alginate with MS medium containing growth regulators had no effect on the viability of encapsulated callus or on subsequent shoot development.

In the experiment with axillary bud encapsulation we abandoned supplementation with nutrients in order to reduce the cost of the procedure, and to avoid reduction of viscosity and reduction of the gel's ability to form solid capsules, reported by Piccioni and Standardi (1995). These authors also suggested that supplying nutrients during the encapsulation process did not benefit plant regrowth. Other authors have stated that adding growth regulators can significantly enhance plant regrowth (Danso and Ford-Lloyd, 2003).

Our results are comparable with those obtained for synthetic seeds of *Carum carvi* (Furmanowa et al. 1991). For both species, the duration of coating calcium alginate around the axillary buds and callus was 30 min, and the optimal preservation period was 6 weeks.

Meristematic tissue of *Rhodiola Kirilowii* proved capable of maintaining the genotype during these biotechnological procedures, and could be stored at 4°C in calcium alginate capsules for at least 6 weeks and then successfully transferred to media and cultured in vitro and in vivo, yielding enough plant material for further chemical studies on *Rhodiola Kirilowii*. The method we used is of practical value. *Rhodiola Kirilowii* grows in mountain regions and is hard to obtain in its natural

environment, hence encapsulation technology is of critical importance for faster propagation of this plant in a temperate climate. From one plantlet growing in vitro for three months, about 20 buds could be obtained and used for encapsulation. One plantlet developed from one bead containing a single bud after 10 days of cultivation at 24°C (Fig. 1c), and then produced many axillary shoots (Fig. 1d). The encapsulation procedure can be successfully used for cost-effective clonal plant micropropagation of *Rhodiola Kirilowii*.

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