The use of *in vitro* culture
to improve the propagation
of *Rhododendron ponticum*
subsp. *baeticum* (Boiss. & Reuter)

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Abstract: *Rhododendron ponticum* subsp. *baeticum* is endemic in the southern region of the Iberian Peninsula. The relict populations of this species are vulnerable, due mainly to difficult conditions for the establishment of seedlings, resulting in a virtual lack of sexual recruitment. In order to preserve the surviving populations, *in vitro* culture methods have been applied for both the sexual and the agamic propagation of the species. The *in vitro* germination of seeds was high when conducted with Anderson's medium without plant growth regulators. The self-rooted seedlings obtained were easily transplanted to outside conditions. The presence of growth regulators in the medium interfered with the development of the seedlings, causing heavy callus formation. The *in vitro* growth of explants took place readily in Anderson's medium plus 0.072 mg L⁻¹ of BA and 0.036 mg L⁻¹ of NAA although the explants did not form roots. Rooting was achieved by the basal dipping of the explants in hydroalcoholic solutions of 500 mg L⁻¹ IAA during the outside transplanting process. Therefore, the combination of *in vitro* grown explants together with *ex vitro* rooting, results in a good method for the agamic propagation of *Rhododendron ponticum* subsp. *baeticum*.

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Abbreviations

2iP - 6-(gamma,gamma-Dimethylallylamino) purine

BA- 6-benzylaminopurine

IAA - Indole-3-acetic acid

NAA - naphthaleneacetic acid

PVC - Polyvinyl chloride

1 Introduction

Rhododendron ponticum L (Ericaceae) is an evergreen shrub of up to 8 meters. It was widely distributed throughout Europe during the Tertiary period while at present it has a reduced, discontinuous distribution, including the Iberian Peninsula, the Black Sea coast and Lebanon [1–3]. Two subspecies have been recognized: *R. ponticum* L. subsp. *ponticum* in the Black Sea region and Lebanon, and *R. ponticum* L. subsp. *baeticum* (Boissier & Reuter) Handel-Mazzetti, paleoendemic in southern Spain and Portugal.

The Spanish populations are restricted to the Natural Park of “Los Alcornocales”, situated in the northern side of Strait of Gibraltar (Figure 1), where populations of *R. ponticum* subsp. *baeticum* survive mainly on riverbanks running through steep terrain, with shadow-rainy environment, or in the mountains with frequent fogs. These populations, although locally abundant, are formed mainly by adult plants showing low or no effective recruitment, and a lack of young plants [4]. The populations persist probably because it has been observed that lower branches of adult plants produce new ramets by layering.

Fig. 1 Location of the Natural Park of “Los Alcornocales” where Spanish populations of *Rhododendron ponticum* subsp. *baeticum* occur.

Relict populations are successful because the lower branches of adult plants have been observed to produce new ramets by layering. Occasionally, young seedlings have been observed, but generally they do not survive. This fact together with losses of plants from clearings, fires, *etc.*, causes the *R. ponticum* subsp. *baeticum* to be an endangered plant in southern Spain that requires protection [5].

In contrast, this species was introduced -most likely from southern Spain- to serve as a garden plant in the British Isles [6], where is now considered an invasive species and a major threat to the natural ecosystems there [7].

Great differences in the seed germination rate have been detected among different *Rhododendron* species. Sakharova [8] reported very high percentages of germination for *R. catawbiense* (92%) or *R. camtschaticum* (78%), and very low for *R. hirsutum* (3%) or *R. vaseyi* (2%).

In general, propagation of *Rhododendron* is difficult [9], but the commercial value of some of these plants encouraged a number of authors to try *in vitro* multiplication [10–16].

Up to date, little work has been done on the propagation of *R. ponticum* subsp. *baeticum*. Recently, Almeida *et al.* [17] have reported a micropropagation method for this species starting from plants growing in the field. Nevertheless, since a major problem for the survival of this species is the lack of recruitment, it would be advisable to use seeds as starting material in order to introduce biodiversity in the relict populations still existing. Thus, the aim of this work is to develop a method for the propagation of *R. ponticum* subsp. *baeticum* employing seeds, which will be useful for conservation programs. To achieve this, *in vitro* culture techniques is applied.

2 Experimental procedures

Mature fruits were harvested from plants of *R. ponticum* subsp. *baeticum* growing in the Natural Park, “Los Alcornocales”. In the laboratory, seeds were extracted and stored at 4 °C.

2.1 Germination in substratum.

After 20 months of cold storage, 1650 seeds were sown in trays with peat : sand (3 : 1) substratum (with a pH of 5.5), and placed in a growth chamber at 23 ± 1 °C, $30 \mu\text{Em}^{-2}\text{s}^{-1}$ of light intensity and 16 h photoperiod. The substratum was kept moist by irrigation with water at pH 5.2.

2.2 Germination in Petri dishes.

After 72 days of cold storage, 400 seeds were placed in Petri dishes (50 per dish) on wet filter paper. Half of the dishes were covered with aluminium foil to carry out germination in darkness. All the Petri dishes were placed in a culture chamber as before.

2.3 *In vitro* germination.

After 72 days of cold storage, 2000 seeds were disinfected by immersion in 90% ethanol for 5 seconds and then in sodium hypochloride (3.5% active chlorine) for 15 minutes, followed by several rinses with sterilized water. After this, half of the seeds were placed individually in test tubes (150 × 25 mm) with Anderson's medium [11] plus 20 g L⁻¹ of sucrose, 0.4 mg L⁻¹ of thiamine, and 100 mg L⁻¹ of inositol, without using plant growth regulators. The other half was sown the same medium plus Anderson's plant growth regulators (15 mg L⁻¹ of 2iP and 4 mg L⁻¹ of IAA). In both cases, the medium was adjusted to pH 4.7. All the tubes were covered with a plastic cap, sealed with parafilm, and placed in a culture chamber as before.

After 90 days of culture, half of the seedlings grown with plant growth regulators were subcultured in the same medium without plant growth regulators in the same culture conditions indicated above, while the other half were recultured in the same conditions but with the Anderson's growth regulators.

2.4 *In vitro* micropropagation.

From the seedlings obtained after *in vitro* germination, 260 uninodal, 0.5 cm length explants were prepared and individually placed in similar test tubes with the medium described above plus 0.072 mg L⁻¹ of BA and 0.036 mg L⁻¹ of NAA. Culture conditions were as mentioned before. After 30 days of culture, 30 explants were transferred to fresh medium, supplemented respectively with 4, 8 or 12 mg L⁻¹ of IAA, added by microfiltration.

2.5 Rooting.

After 52 days of culturing, a short basal section was cut to 90 explants. Then the explants were immersed by the injury for 10-15 seconds in a hydroalcoholic solution of 500 mg L⁻¹ of IAA, and established in 300 ml pvc pots with peat : perlite (3 : 1) as substratum (with a pH of 5.5). The pots were covered with a transparent plastic bag according to Cantos *et al.* [18], and placed in a culture chamber at 25±1 °C, 111 μE m⁻² s⁻¹ of light intensity and 16 h photoperiod.

3 Results and discussion

Only 42 seeds (2.5%) of *R. ponticum* subsp. *baeticum* germinated in the substratum after 90 days. No more germination was observed during the following 100 days of the experiment. Such a low germination rate was similar to that described for *R. hirsutum* or *R. vaseyi*, considered some of the most difficult to germinate species of *Rhododendron* [8].

Table 1 shows the rate of seedling germination considering 42 seedlings as 100% of total germination. The greatest appearance of seedlings was observed between the 15th day and the 60th day.

Days from sowing	% Germination	
	Partial	Accumulated
7	10	10
15	22	32
30	20	52
50	16	68
60	17	85
75	11	96
90	4	100

Table 1 Relative percentage of *R. ponticum* subsp. *baeticum* seed germination in peat:sand (3 : 1) substratum, through the first 90 days from sowing considering the total germination reached (2.5%) as 100%.

The germinating seeds presented an epigynous nascency with a quick growth of the epicotyl. Afterwards, the seedlings showed normal development, but after a few days many of them died. Similar results were reported by F. Besnier-Romero [19]. Also, Mejías *et al.* [4] observed virtually no germination when seeds were buried *in situ*.

If this behaviour could be assumed for field conditions, then this could be one of the reasons for the lack of sexual recruitment in wild populations of *Rhododendron ponticum* subsp. *baeticum*. Mejías and Arroyo [20] indicated that seeds of *Rhododendron ponticum* subsp. *baeticum* need special ecological conditions to germinate and grow.

When seeds were germinated in Petri dishes in the dark, the germination rate was also very low, only 3% (Table 2), but when culture was carried out under light, germination rate increased up to 46% after 90 days, although 30% of germination was recorded by day 10. Again, these results are very similar to those reported by Mejías *et al.* [4]. Consequently, this method significantly increased the percentage of germination and decreased the time needed for it. Nevertheless, the lack of nutrients and room, severely limited the development of the seedlings which were not suitable for outside transplanting and could not be maintained for long inside the Petri dishes.

Days from sowing	% Germination			
	Light		Dark	
	Partial	Accumulated	Partial	Accumulated
10	30	30	0	0
20	10	40	1	1
33	4	44	1	2
90	2	46	1	3

Table 2 Percentage of *R. ponticum* subsp. *baeticum* seed germination in Petri dishes through the first 90 days from sowing (influence of light).

In vitro seed germination -with or without growth regulators- was very quick and effective, reaching over 80% of germination in the first 15 days and over 90% 15 days later. However, the development of the seedlings was strongly dependent on the application of growth regulators (Table 3). Thus, without growth regulators, the seedlings grew very well, and after 90 days of culture, they reached an average height of 4 centimeters with 10 leaves as well as secondary roots (Figure 2). When there were growth regulators in the medium, the seedlings only reached 1 centimeter and 4 leaves with a poor radicular system and abundant callus tissue formation. Consequently, the seedlings raised in medium without plant growth regulators were suitable for transplanting to outside conditions while those grown with growth regulators were not.

Development of cultures	Growth regulators	
	With	Without
Callus (%)	67b	0a
Shoot length (mm)	10a	40a
Nº of leaves per shoot	4a	10b

Means in rows with the same letter do not differ at $P \leq 0.05$ (Student's t test).

Table 3 *In vitro* seed germination of *R. ponticum* subsp. *baeticum* on Anderson's medium with (15 mg L^{-1} of 2iP plus 4 mg L^{-1} of IAA) or without growth regulators in the media, after 90 days of culture. Callus (%) indicates percentage of seedlings that formed callus at the end of the culture time.

Fig. 2 Seedling of *Rhododendron ponticum* subsp. *baeticum* germinated *in vitro* in Anderson's medium without plant growth regulators, after 90 days of culture.

When the seedlings were transferred to outside conditions according to Cantos *et al.* [18], over 80% of them survived and grew (Figure 3).

Fig. 3 Plant of *Rhododendron ponticum* subsp. *baeticum* obtained by *in vitro* seed germination after transferred to pot.

To check the effect of the growth regulators, half the seedlings germinated with growth regulators were transferred to medium without them while the other half were recultured on medium with growth regulators. The first half of the seedlings grew quickly to reach 3 centimeters in height and formed new leaves and roots, good enough condition for outside transplanting. The other half of seedlings only formed callus tissue that covered the seedling. After a few days, many small shoots with leaves but without roots began to appear on the surface of the callus (Figure 4). When these small shoots were transplanted to growth regulator-free medium, normal development resumed and healthy shoots were formed. These new shoots raised from the callus tissue can be used to improve the multiplication rate.

Fig. 4 Seedling of *Rhododendron ponticum* subsp. *baeticum* germinated in media with plant growth regulators and recultured in media with 12 mg L^{-1} 2iP plus 4 mg L^{-1} IAA. Small shoots can be observed appearing on the surface of the callus.

Thus, *in vitro* germination of *Rhododendron ponticum* subsp. *baeticum* seeds in modified Anderson's medium without growth regulators is an effective tool for obtaining of new seedlings of this species that could be used for recovery of endangered populations.

In vitro culture of explants of *Rhododendron ponticum* subsp. *baeticum* was easy with low concentrations of growth regulators (0.072 mg L^{-1} of BA and 0.036 mg L^{-1} of NAA). The explants developed a healthy aerial part but no roots were produced. After 120 days of culture, the explants had grown up to 20 mm and formed an average of 20 new buds and 4 lateral shoots (Table 4). Almeida *et al.* [17] reported higher proliferation rates using zeatin as growth regulator although our results show that BA could be used also at low concentrations and longer periods of culture. Nevertheless, it should be noted that our cultures were not clones as in [17] and thus variability is greater. Additions of different concentrations of IAA (4, 8 and 12 mg L^{-1}) to the medium improved the development of the explants, particularly with 8 mg L^{-1} , but did not result in rooting (Table 5). Due to the high variability found, no significant differences could be established among the IAA concentrations added.

Days	Shoot Length (mm)	Nº of buds per shoot	Nº of shoots
0	5.0 a		
15	6.0 a	7.4 a	1.5 a
30	6.5 a	8.0 a	1.5 a
45	7.5 a	9.5 a	1.6 a
60	8.0 a	10.5 a	1.9 ab
75	10.0 ab	12.5 ab	1.9 ab
90	12.5 b	13.0 ab	2.3 ab
105	15.0 bc	16.5 bc	2.5 b
120	20.0 c	25.0 c	4.0 b

Means in columns with the same letter do not differ at $P \leq 0.05$ (Student's *t* test).

Table 4 *In vitro* development of *R. ponticum* subsp. *baeticum* explants on Anderson's medium with 0.072 mg L^{-1} of BA and 0.036 mg L^{-1} of NAA, along the experiment.

IAA (mg L^{-1})	Shoot Length (mm)	Nº of buds per shoot	Nº of lateral shoots
0	20.0 ± 10.3	24.0 ± 8.9	3.7 ± 1.3
4	24.5 ± 19.4	24.0 ± 18.6	2.9 ± 2.4
8	25.5 ± 12.2	38.5 ± 21.8	4.9 ± 2.6
12	15.0 ± 8.6	19.0 ± 9.7	3.2 ± 1.6

Mean $\pm$ SD.

Table 5 Effect of the addition of different concentrations of IAA to the medium (Anderson's plus 0.072 mg L^{-1} of BA and 0.036 mg L^{-1} of NAA). Results after 115 days of culture.

Rooting was obtained by basal dipping of the shoots in hydroalcoholic solutions of IAA (500 mg L⁻¹) before transplanting to pots. With this method, 67% of the shoots rooted, resulting in the formation of whole plants that could be afterwards transferred to 2 L containers to continue growth. Almeida *et al.* [17] obtained a 100% of rooting with this method. The difference could be due to the more concentrated solution of auxin or maybe to a better response to the auxins used by those authors (NAA or IBA).

Our results show that *in vitro* germination of *R. ponticum* subsp. *baeticum* seeds is easy and effective, allowing high rates of germination in only 30 days from sowing. Without growth regulators in the medium, the seeds produce self-rooted seedlings, well developed and ready to be transplanted to ex-vitro conditions with high survival rates. Since the seeds of *R. ponticum* subsp. *baeticum* barely germinate in the nature as reported previously [4, 20], *in vitro* germination would provide an important tool to overcome this problem, and thus raise seedlings suitable for reintroduction in their natural habitat.

Furthermore, the use of seeds as starting material, rather than propagating new plants from pre-existing individual plants, is a more advantageous strategy for the species conservation, since the former method preserves the genetic diversity of the populations.

In conclusion, *in vitro* culture methods are a powerful tool for both sexual and agamic propagation of *Rhododendron ponticum* subsp. *baeticum*, and useful for the preservation and recovery of this endangered subspecies.

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