Cantos, M., Cuerva, J., Zárate, R. and Troncoso, A. (1998), *Seed Sci. & Technol.*, **26**, 193–198

Embryo rescue and development of *Juniperus oxycedrus* subsp. *oxycedrus* and *macrocarpa*

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(Accepted July 1997)

Summary

Germination of intact seeds, seeds without testa and isolated embryos of two subspecies of *Juniperus oxycedrus* (*oxycedrus* and *macrocarpa*) was compared both *in vitro* and under greenhouse conditions (for intact and seeds without testa). Intact seeds did not germinate in the greenhouse or *in vitro*. Seeds without testa did not germinate under greenhouse conditions, although these showed a low response *in vitro* (12%) on 1/3 strength of Murashige and Skoog medium with 3% sucrose with and without 0.5 g l^{-1} of GA_3. However, isolated embryos *in vitro* reached germination levels of about 50% on the same media, providing a promising method for improving germination and propagation of *Juniperus oxycedrus*. Acclimatization of plantlets in soil under greenhouse conditions was very successful. The survival rate was 80% and the average plant height after one year was 20 cm.

Introduction

The genus *Juniperus* includes about 50 species of trees and shrubs adapted to very different environments (Scortichini, 1986). In Spanish environments, there are species such as *J. communis* L., *J. oxycedrus* L. (subsp. *oxycedrus*), *J. phoenicea* L., (subsp. *phoenicea*) and *J. thurifera* L. growing in continental and river bank conditions. In the high mountains, species such as *J. communis* (subsp. *alpina*) and *J. sabina* L. (Aparicio and Silvestre, 1987), and on the coasts (cliffs and dunes) *J. navicularis* Gand., *J. oxycedrus* L. (subsp. *macrocarpa*), *J. phoenicea* and *J. turbinata* Guss. (Castroviejo, Lainz, López González, Montserrat, Muñoz Garmendia, Paiva and Villar, 1986) are found.

Juniperus has an important ecological value in preventing erosion by soil formation and protection (Hernández and Clemente, 1994), together with some commercial applications *e.g.* hard wood (Ilahi, 1986), galbulus (berry like seed cone), medicinal uses (Zaman, Khan and Khan, 1968). Nevertheless, in Spain *Juniperus oxycedrus* is an endangered species because of the use of its habitat for new estates, uncontrolled tree cutting, fires, diseases (Morelet, 1982; Rutherford, Epton and Benton, 1989), slow rate of propagation (Pardos and Lázaro, 1983) and slow growth (Ceballos and Ruíz de la Torre, 1979).

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In this article we present a novel method to improve germination and propagation of *J. oxycedrus*, using seeds without testa in which isolated embryos are cultured *in vitro*.

Materials and methods

Plant material

The galbulus of *J. oxycedrus* subsp. *oxycedrus*, were collected from plants growing in the Viar river gorge in Cazalla de la Sierra (Sevilla, southern Spain) and those of *J. oxycedrus* subsp. *macrocarpa* on the coast of Cabo Roche in Conil de la Frontera (Cádiz, southern Spain). In both cases, the sampling was carried out in November 1992 when the seeds were mature. The galbulus were opened and the seeds removed and counted. The seeds were then washed with xylol 98% (Panreac, Barcelona, Spain) to remove any remaining resin and water, and then stored at room temperature until used.

Preparation of seeds and isolation of embryos

The seeds were pressed with a carpenter's clamp, so that the testa broke easily, showing the endosperm which contained the embryo (seeds without testa = SWT). Depending on the morphology of the SWT, these were classified as empty, deformed or normal. The normal seeds without testa were selected and the embryos removed. These were held with a pair of forceps and a longitudinal cut performed in the endosperm with a scalpel, not affecting the embryo. The cut was then opened and the embryo extracted with the sharp end of the scalpel.

Pre-conditioning and sterilization treatments and in vitro germination

Intact seeds received the following treatment prior to inoculation on the culture media.

T_1: 100 seeds were immersed in a commercial sodium hypochlorite solution (12.5 g l^{-1} of active chlorine) stirred for 60 min, and then rinsed with sterile distilled water.

T_2: 100 seeds were immersed in ethanol 96° for 15 min and then in a commercial sodium hypochlorite solution (35.5 g l^{-1} of active chlorine) stirred for 20 min, and finally rinsed with sterile distilled water.

T_3: 100 seeds were immersed in ethanol 96° for 15 min, followed by immersion in a mixture of commercial sodium hypochlorite solution (35.5 g l^{-1} of active chlorine) and potassium nitrate (2 g l^{-1}) (Merck, Darmstad, Germany) for 20 min, and then rinsed with sterile distilled water.

T_4: 100 seeds were soaked in sulphuric acid, a solution of 96% purity (Merck), for 15 min and then immersed in a 10% NaOH solution (Merck) for 20 seconds , and finally rinsed with sterile distilled water.

T_5: 100 seeds were treated in a solution of 25% NaOH (Merck) for 15 min then solution of 10% of sulphuric acid (Merck) for 20 seconds, and then rinsed with sterile distilled water.

T_6: 100 seeds were held with a pair of pincers and passed quickly over a flame several times. This procedure was performed to simulate the improvement of *J. oxycedrus* germination after fire damage. The seeds were then inoculated onto the nutrient media described below.

The SWT were treated with a solution of commercial sodium hypochlorite (35 g l^{-1} of active chlorine) for 5 min for surface sterilization. Finally, these were immersed in sterile distilled water for 24 h at room temperature to promote swelling and facilitate the extraction of the embryo.

For *in vitro* culture, 1/3 strength of Murashige and Skoog (1962) medium, (MS/3) with 3% sucrose and 0.8% agar (Merck) at a pH 5.7, adjusted before autoclaving, was routinely used. A second culture medium with the same composition but supplemented with 0.5 g l^{-1} of GA_3 ($MS/3+GA_3$) was also used to inoculate SWT and isolated embryos of the two subspecies studied. The culture media (8 ml per tube) were contained in glass tubes (150 × 25 mm) closed with a plastic cap and incubated at 25 ± 2 °C and a 16 h photoperiod with a light intensity of 30 μE $m^{-2}s^{-1}$.

Greenhouse germination

The method of Catalán Bachiller (1991) was followed for preliminary preparation of the seeds. Two hundred intact seeds per subspecies were sown 0.5–1 cm in depth in soil which was taken from the plant habitat and mixed with peat (4:1) (Grünland, ASB, Ludwigsburg, Germany), contained in PVC trays (48×33×7cm). For the experiments with SWT, these were sown as described previously but using 300 ml pots. In both cases, the culture conditions were 25±2 °C, with a photoperiod of 16h, with regular watering to keep the soil moist.

Adaptation to greenhouse conditions

In vitro germinated plants derived from isolated embryos with 3 cm long stems and 2 cm long roots, were transplanted individually to a mixture of sandy soil and peat (4:1) contained in PVC pots (330 ml). The substrate was watered to saturation and then the plant and pot covered with a translucent plastic bag, to which water was previously sprayed to provide a moist environment, to prevent the plantlet desiccation. These were then placed in a growth chamber at 25±2 °C, 16 h photoperiod with a light intensity of 111 μE $m^{-2}s^{-1}$. After 10 days, the plastic bag was cut (3 cm) in one of the corners and this was repeated for the remaining corner until achieving total contact with the environment, then the bag was removed. Later, the plants were transferred into the greenhouse where their growth was monitored.

Table 1. Percentage of the number of seeds per galbulus of *J. oxycedrus* subsp. *oxycedrus* and *macrocarpa* (Average of 2000 galbulus).

Subspecies	Galbulus with 1 seed (%)	Galbulus with 2 seeds (%)	Galbulus with 3 seeds (%)	Galbulus with 4 seeds (%)
oxycedrus	4.0	24.0	70.0	2.0
macrocarpa	4.0	23.0	70.0	3.0

Table 2. Physical appearance of *J. oxycedrus* subsp. *oxycedrus* and *macrocarpa* mature seeds.

Subspecies	Total no. of seeds	Empty	Deformed	Normal
oxycedrus	3289	327 (10%)	1705 (52%)	1257 (38%)
macrocarpa	2700	130 (5%)	1110 (41%)	1460 (54%)

Results and discussion

Table 1 shows the proportion of galbulus bearing 1, 2, 3 or 4 seeds for each subspecies of *J. oxycedrus*. It can be seen that there was no difference between subspecies with most of the galbulus containing 3 seeds. The physical appearance of *J. oxycedrus* SWT (seeds without testa) is presented in Table 2. It is observed that a high percentage of SWT (empty + deformed) appear unable to germinate, 62% for subsp. *oxycedrus* and 46% for subsp. *macrocarpa*. This seems to be a partial explanation of the low germination of *Juniperus* seeds.

From the different experiments carried out with intact seeds, both in the greenhouse and *in vitro* conditions, germination was not achieved, despite the different surface treatments employed. Indeed, none of the pre-treatments used (T_1–T_6) with intact seeds cultured *in vitro* induced any response in germination. Similarly, when SWT were cultured under greenhouse conditions, germination did not take place. These results would confirm the frequent low germination rate of *Juniperus* seeds reported by other authors (*e.g.* Ilahi, 1986). Furthermore, the absence of germination in intact seeds also indicated the possible existence of factors hindering this process.

Seeds without testa (SWT) of *J. oxycedrus* and *J. macrocarpa* cultured on MS/3 medium did show a low percentage germination (*ca.* 12% mean value) (Table 3). After an incubation period of 45 days, the subsp. *oxycedrus* reached a maximum of 17% with an average germination rate of 10%. Similarly, the subsp. *macrocarpa* reached 20% and 14.6% respectively but this was not significantly different. On the other hand, the addition of GA_3 to the nutrient medium did not result in a significant higher germination rate (*ca.* 9.2%), and did not break the dormancy of the SWT as it was expected. These results indicate that *in vitro* conditions and the removal of the testa have favoured germination.

Conversely, *in vitro* culture of isolated embryos did increase the percentage germination with a maximum above 50% for both subspecies. The average germination rate in the four experiments was 47% for *oxycedrus* and 41% for *macrocarpa* (Table 4). A

Table 3. *In vitro* germination SWT (seeds without testa) of *J. oxycedrus* subsp. *oxycedrus* and *macrocarpa* after 45 days incubation on MS/3 medium (30 seeds per experiment with 5 replications).

Subspecies	Total no. of seeds	Contaminated	Inactive	Germinated
oxycedrus	150	32	103	15 (10.0%)
macrocarpa	150	20	109	22 (14.6%)

Table 4. *In vitro* germination of isolated embryos of *J. oxycedrus* subsp. *oxycedrus* and *macrocarpa* after 45 days incubation on MS/3 medium (average of 4 experiments).

Subspecies	Total no. of embryos	Contaminated (%)	Inactive (%)	Germinated (%)
oxycedrus	100	4	49	47
macrocarpa	100	12	47	41

similar germination rate was obtained with MS/3+GA_3 medium (data not shown), which once again shows that the use of GA_3 did not improve dormancy breaking as observed with SWT. Isolated embryo germination rate was significantly higher (t-test 14.72, $p<0.01$) than that obtained with SWT (Table 3). It appears that the presence of the hard cover and/or the endosperm in the seed did not favour germination, possibly due to the presence of inhibitors, similar to the results reported by LePage-Degivry (1969) with several species of *Gymnospermae*. This author attributed seed dormancy to the presence of a compound identical to ABA. Similarly, Zhiri, Jaziri, Homes, Vanhaelen, and Shimomura (1994) postulated that a lack of germination of seeds of *Taxus* species was due to the presence of the same compound as indicated by LePage-Degivry (1969). Therefore, the low germination potential of *J. oxycedrus* appears to be a coat-imposed dormancy mechanism and not inherent to the embryo, since isolated embryos cultured on either medium exhibited greatly increased germination, which is also consistent with Bewley and Black (1985).

Using the method indicated above, plants derived from isolated embryos were gradually adapted to the environment. Acclimatization in soil of *in vitro* germinated plants of *J. oxycedrus* subsp. *oxycedrus* and *macrocarpa* was successful. The survival rate was respectively 83 and 75% for the subsp. *oxycedrus* and for *macrocarpa*. The average height reached by these plants under greenhouse conditions was 20 cm after the first growth year.

The techniques and strategies shown in this investigation provide a promising method for overcoming the low germination rates of *Juniperus* species and for improving their propagation.

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