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Medium-term conservation of redwood (*Sequoia sempervirens* (D. Don.) Endl.) in vitro shoot cultures and encapsulated buds

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ABSTRACT

This study evaluated the survival and recovery of non-encapsulated and encapsulated shoots of *Sequoia sempervirens* after storage at 4 °C in the dark for up to 15 months on four different culture media. Survival and regrowth of encapsulated shoots declined within 3 months, regardless of the storage medium composition. By contrast, no significant decrease in survival and regrowth was noted with non-encapsulated shoots after 12 months of storage on Quoirin and Lepoivre medium supplemented, or not, with 1 mg l^{-1} benzyladenine. Regrowth dropped to 60–61% after 15 months of storage on the same media. Medium-term conservation of *S. sempervirens* germplasm is therefore possible using in vitro storage of non-encapsulated shoot cultures.

1. Introduction

Sequoia sempervirens (D. Don) Endl., also called 'redwood' due to the reddish brown colour of its heart-wood (Arnaud et al., 1993), is an evergreen conifer tree, and a sole living species of the genus *Sequoia* in the Taxodiaceae family (Stokey, 1981). Redwood trees are natively distributed along the Pacific coastal areas of USA from southwest Oregon to northern California (Sawyer et al., 2000). It has also been introduced to some other countries, including Russia, France, Great Britain and Turkey, and domesticated successfully (Donnet, 1984; Gerçek, 2002; Liu et al., 2006).

Redwood is highly valuable, not only for ornamental purposes but also for reforestation, timber and plywood production due to its resistance to pathogen attacks and diseases, tolerance to adverse climatic conditions, rapid growth and high wood quality (Arnaud et al., 1993; Sul and Korban, 2005). However, its low rooting capacity, shoot dormancy, low seed germinability (about 10% only, Boe, 1974) and low seedling viability (Donnet, 1984; Bourgkard and Favre, 1989; Liu et al., 2006) contribute to its loss in nature and render the use this species for silviculture purposes impossible. Uncontrolled urbanization and increasing demand for its lumber also contribute to such loss. Redwood trees have thus received since 2006 a 'vulnerable conservation status' by the International Union for Conservation of Nature (IUCN, http://www.iucnredlist.org/details/34051/0).

At present, *S. sempervirens* genetic resources are conserved in the form of whole trees, in situ in national parks in the USA and ex situ in botanic gardens worldwide. Even though redwood seeds display orthodox storage behaviour (Hong et al., 1996), seed storage is not an effective option for this species as seed production is often erratic and seed germinability very low (Donnet, 1984; Bourgkard and Favre, 1989).

In vitro techniques have been widely used for multiplication and conservation of species whose propagation and storage by classical techniques is problematic (Engelmann, 1997; Ozudogru et al., 2010), such as *S. sempervirens*. Various micropropagation protocols have been established for redwood (Korban and Sul, 2006); however, no protocol has been published for medium or long-term storage of this species.

For short- and medium-term storage, the aim is to reduce growth and to increase the intervals between subcultures. For most species, growth reduction is achieved by modifying the environmental conditions and/or the culture medium. The most widely

Abbreviations: BA, N^6-benzyladenine; HCl, hydrochloric acid; MS, Murashige and Skoog medium; NaCl, sodium hypochlorite; QL, Quoirin and Lepoivre medium.

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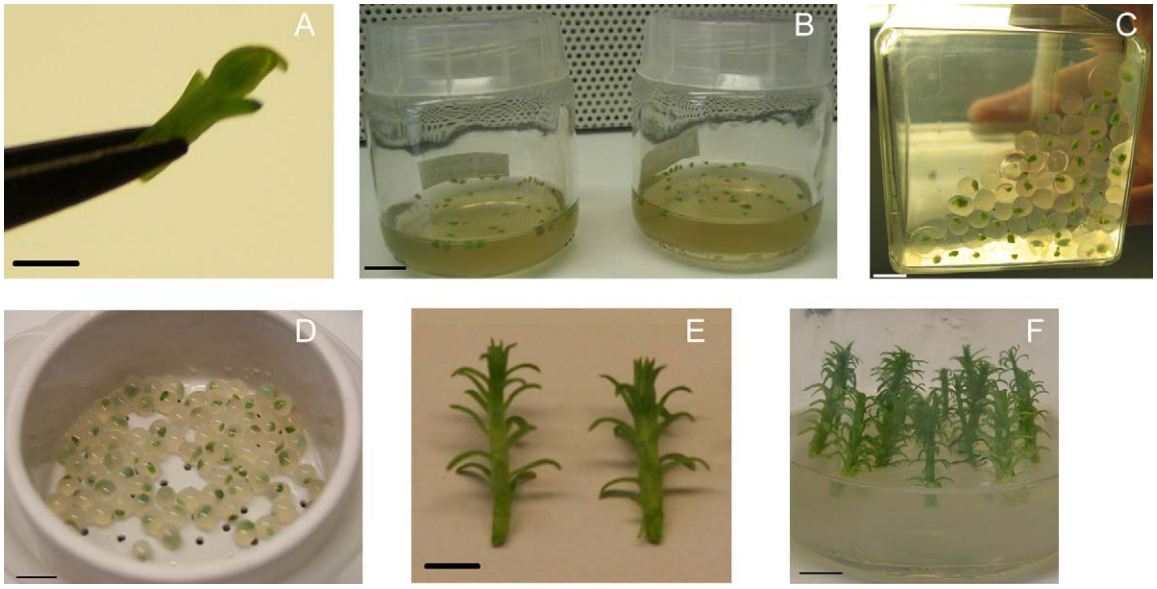

Fig. 1. Medium-term conservation of *Sequoia sempervirens* encapsulated buds and shoot cultures. (A) Buds, used for synthetic seed preparation (bar, 1 cm), (B) suspension of buds in 3% Na-alginate solution (bar, 1.25 cm), (C) polymerization in $CaCl_2$ solution (bar, 1 cm), (D) capsules, collected on a sterile sieve (bar, 1 cm), (E and F) single shoots, used for cold storage (bars, 1 cm).

applied technique is temperature reduction, which can be combined with a decrease in light intensity or culture in the dark (Engelmann, 1997; Ozudogru et al., 2010).

Another promising technique is based on the production and storage of synthetic seeds. The concept of synthetic seed has been established by Murashige (1977). A synthetic seed or artificial seed is referred to as artificially encapsulated somatic embryo, shoot bud or any other meristematic tissue that can be used as functionally mimic seed for sowing and possesses the ability to convert into a plant under in vitro or ex vitro conditions and that can retain this potential even after storage (Redenbaugh, 1993; Ara et al., 2000). In vitro cultures of various species including fruit trees (Rai et al., 2009; De Carlo et al., 2009; Lisek and Orlikowska, 2004) and ornamental plants (Preece and West, 2009; Swaroopa et al., 2007; Duong et al., 2005) have been successfully stored for various durations in the form of synthetic seeds consisting of encapsulated shoots.

The present study aimed at exploring the possibility of using in vitro slow growth storage for medium-term conservation of *S. sempervirens* germplasm. In short, in vitro shoot cultures and synthetic seeds consisting of encapsulated apical and basal buds of *S. sempervirens* were maintained at 4 °C in the dark on different media and returned to standard culture conditions after various storage periods to evaluate their survival and recovery. To our knowledge, this is the first attempt to conserve *Sequoia* germplasm by means of biotechnological approaches, which are complementary to conventional in situ and ex situ conservation methods.

2. Materials and methods

2.1. Plant material

In vitro shoot cultures of *S. sempervirens* (D. Don.) Endl. were initiated from an old elite tree, growing in a public garden of Florence (Italy), and had been maintained in vitro for over 5 years by periodic 4-week subcultures.

2.2. Standard culture conditions

Cultures were maintained by periodic transfers (4-week intervals) of 1–1.5 cm long apical shoots on semi-solid Murashige and Skoog medium (MS, Murashige and Skoog, 1962), supplemented with 1 mg l^{-1} N^6-benzyladenine (BA), 30 g l^{-1} sucrose and gelled with 7 g l^{-1} agar (MS1, proliferation medium). MS basal salts, vitamins and amino acids were supplemented from Sigma®. The pH was adjusted to 5.8 with 1 N NaOH or HCl before addition of agar and media were autoclaved for 20 min at 121 °C. Cultures were incubated at 23 ± 2 °C, under a 16 h light/8 h dark photoperiod, with a light intensity of 36.3 μmol m^{-2} s^{-1} provided by cool daylight fluorescent lamps.

2.3. Slow growth storage of synthetic seeds and in vitro shoot cultures

Apical and basal buds were excised from in vitro shoot cultures which had been cold-hardened for 4 weeks at 4 °C. After excision, they were precultured on MS medium supplemented with 0.5 M sucrose for 24 h under standard culture conditions (23 ± 2 °C, 16 h light/8 h dark photoperiod). For synthetic seed preparation, buds (~0.5 cm in length, Fig. 1A) were suspended in a 3% sodium alginate (200 cps) solution (Fig. 1B) and dropped in a 100 mM $CaCl_2$ solution, each drop containing one explant (Lambardi et al., 2006). The beads were kept for 25 min at room temperature in the $CaCl_2$ solution to ensure complete polymerization of the calcium alginate (Fig. 1C), collected on a sterile sieve and washed with sterile distilled water (Fig. 1D). They were transferred to Petri dishes (⌀90 mm, 20–23 beads/Petri dish) containing 30 ml of one of the following media (all supplemented with 30 g l^{-1} sucrose and gelled with 7 g l^{-1} agar): (i) MS medium, hormone-free (MS0); (ii) MS medium, supplemented with 1 mg l^{-1} BA (MS1); (iii) Quoirin and Lepoivre medium (QL, Quoirin and Lepoivre, 1977), hormone-free (QL0); and (iv) QL medium, supplemented with 1 mg l^{-1} BA (QL1), which were placed under cold storage.

For preparation of shoot cultures, multiple shoots, obtained under standard culture conditions, were separated and single shoots (~2 cm in length, Fig. 1E) were transferred to glass jars (⌀60 mm, 90 mm height, 20 shoots/jar) containing 35 ml of the same four media (Fig. 1F). They were maintained under standard culture conditions for 14 days, then transferred to cold storage.

Synthetic seeds and shoot cultures were maintained at 4 °C in the dark (cold storage) for up to 15 months. Every 3 months, sam-