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SHORT COMMUNICATION

Cold storage of initial plant material affects positively somatic embryogenesis in *Pinus radiata*

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Abstract Traditionally, many efforts have been carried out in order to improve the success of somatic embryogenesis process in conifers, but little attention has been paid to the influence of the plant material storage conditions in the subsequent phases of the somatic embryogenesis process. In this work our objective was to study the feasibility of storing *Pinus radiata* plant material at 4 °C for long periods in order to make easier the initiation with high amount of cell lines. The effect of cold storage on the different stages of somatic embryogenesis process has been evaluated. Storage periods of 1–3 months enhanced initiation rates and the number of somatic embryos obtained in the embryogenic cell lines. These results demonstrate the beneficial effect of cold storage and open the possibility of considering a cold preconditioning of plant material as a good alternative to improve the somatic embryogenesis process in conifers.

Keywords Conifer · In vitro · Micropropagation · Radiata pine · Somatic embryo

Introduction

Pinus radiata, a species native to California, is well known as an exotic conifer species deployed in forest plantations, predominantly in New Zealand, Australia, Chile and Spain. Due to its high productivity, it is a highly valued resource for construction timber, furniture, heating, pulp and paper (Charity et al. 2005).

As reviewed thoroughly by Lelu-Walter et al. (2013), in order to meet the world's future wood demand, the use of vegetative propagation in forestry is the fastest, the most flexible

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and effective way to produce enough genetically improved material. In the past, the only way to propagate in vitro juvenile or adult material from radiata pine was via organogenesis. This technology has been used until recently (Hargreaves et al. 2005; Montalbán et al. 2011a, 2013) but nowadays, somatic embryogenesis (SE) is being used in many breeding programs.

Since the first reports on SE from radiata pine (Smith 1997), improvements have been made at different stages of the SE process, such as initiation (Hargreaves et al. 2009; Montalbán et al. 2012) or maturation (Montalbán et al. 2010, 2011b). But still, a drawback of this technique is that it can only be accomplished using immature zygotic embryos as initial explants. This type of material implies that the resulting clones have to be tested in the field to check their ex vitro performance, as well as a narrow competence window for SE initiation (MacKay et al. 2006). The first problem is solved by cryopreserving the embryogenic cell lines (ECLs) while field testing is carried out; then, after ex vitro testing has shown which clones are the best, the ECLs can be defrosted and repropagated (Park et al. 1998). To try to overcome the second problem, the narrow competence window for SE, the cones are usually stored at 4 °C for 1 (Salajová and Salaj 2005) to 4 weeks (Yildirim et al. 2006) while processing them; as a consequence, a high amount of human resources is needed to introduce the amount of initial explants demanded for initiation stage in a short period of time. For this reason, it is necessary to develop systems able to increase the efficiency of SE process.

Cold storage has been used to promote SE in several angiosperm species (Janeiro et al. 1995; Aslam et al. 2011). But in *Pinus* genus there are only few reports on the influence of cold storage on SE initiation. Häggman et al. (1999) tried to improve *P. sylvestris* initiation rates through cold preconditioning, while Park (2002) aimed to expand the availability of plant material from *P. strobus* to somehow overcome the narrow competence window.

This study examined the hypothesis of whether it is possible to cold store SE plant material for extended periods of time without a detrimental effect on initiation, proliferation, maturation and plantlet quality.

Materials and methods

Plant material

One-year-old green female cones of *Pinus radiata* D. Don were collected from two open-pollinated trees (12 and 14) in a seed orchard established by NEIKER-TECNALIA in Deba-Spain. All cones were collected in June in 2012, when the average stage of the zygotic embryo was between two and four (Montalbán et al. 2012). Intact cones were sprayed with 70 % (v/v) ethanol, wrapped in filter paper and stored at 4 °C inside expanded polystyrene boxes on a layer of silica gel. The cones were sampled the day of collection [0 days (Fig. 1a)], after 2 weeks, 1, 2, 3 and 4 months (Fig. 1b) at 4 °C. At each sampling date, immature seeds were dissected and sterilized following Montalbán et al. 2012. Seed coats were removed and whole megagametophytes were excised out aseptically and placed horizontally onto EDM initiation medium (Walter et al. 2005) supplemented with 3 g L^{-1} Gelrite®.

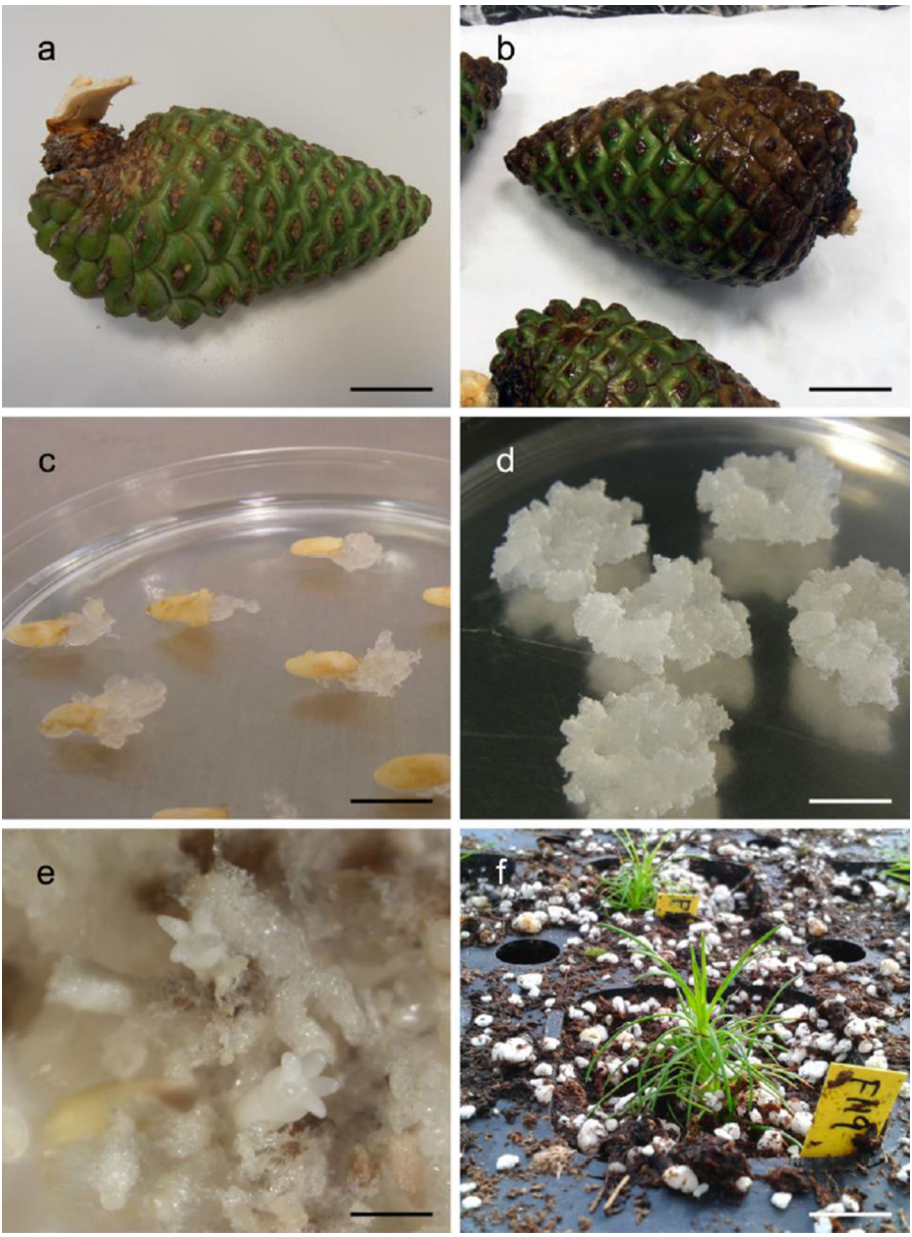

Fig. 1 **a** One year-old cones from *Pinus radiata* the day of collection, bar length 50 mm; **b** One year-old cones from *P. radiata* after 4 months at 4 °C, bar length 60 mm; **c** Initiation of embryogenic tissue in *P. radiata* megagamethophytes cultured on EDM medium, bar length 15 mm; **d** proliferation of embryogenic tissue of *P. radiata* cultured on EDM medium, bar length 9 mm; **e** Somatic embryos obtained from an embryogenic cell line from a cone stored at 4 °C for 2 months, bar length 3 mm; **f** Somatic plantlet growing in the greenhouse obtained from a cone stored at 4 °C for 1 month, bar length 16 mm

Initiation and proliferation

Eight megagametophytes per Petri dish and a total of ten Petri dishes per mother tree and cold storage period were cultured, a total of 960 megagametophytes.

After 4–8 weeks, proliferating embryogenic tissue (ET) (3–5 mm in diameter) was separated from the megagametophytes. ET was subcultured every 2 weeks; the maintenance medium had the EDM composition, but a higher concentration of Gelrite® (4.5 g L^{-1}). After 6–8 subcultures, proliferating ECLs were subjected to maturation.

Maturation and conversion

Maturation was carried out following the protocol described by Montalbán et al. (2010). Three to five ECLs per mother tree and cold storage period were chosen for maturation experiments, giving a total of 50 ECLs subjected to maturation. Four replicates per ECL were kept in darkness at 21 ± 1 °C.

After 15 weeks, 40 mature somatic embryos (se) per ECL were selected and isolated from ET. Germination and acclimatization were carried out following Montalbán et al. 2010.

Data collection and statistical analysis

Eight weeks after each sampling date, the number of proliferating ETs per Petri dish was recorded and initiation percentages per mother tree and cold storage period were calculated. After four subculture periods, actively growing ETs were recorded as ECLs, and the percentage of initiated lines that had proliferated successfully was calculated.

To evaluate the effect of mother tree and cold storage period, a logistic model and its corresponding analysis of deviance was carried out. To assess multiple comparisons among the different levels of mother trees and cold storage period, estimable linear functions of model coefficients were computed (McCulloch and Searle 2001) and p values were conveniently adjusted, following Benjamini-Yekutieli method (Benjamini and Yekutieli 2001).

After 15 weeks on maturation medium, the ECLs subjected to maturation that had produced mature se were recorded and the number of mature se per gram was registered. A logistic model and its corresponding analysis of deviance were carried out to evaluate the effect of cold storage period on the proportion of ECLs that had produced se.

Among the ECLs that had produced se, a non-parametric test was performed (Kruskal–Wallis test) to assess the effect of the cold storage period on the number of se per gram of ET. Multiple comparisons are based on rank differences (Conover 1999).

After 16 weeks on germination medium the overall germinated se related to the total number of se introduced (conversion, %) was recorded.

Results

Statistical analysis of initiation percentages showed a significant effect of the cold storage period (p value <0.001), the mother tree (p value = 0.007) and an interaction between the cold storage period and the mother tree (p value <0.001) (Table 1).

The significant interaction between cold storage and mother tree is displayed in Fig. 2. The highest initiation percentages were obtained after 1–2 months (in mother 14 and 12,

Table 1 Analysis of deviance of the logistic regression for proportion of initiation, proliferation, maturation of *Pinus radiata* embryogenic tissue after different storage periods at 4 °C of the green cones. The initial explants were megagametophytes containing immature zygotic embryos

Source	Initiation			Proliferation			Maturation		
	df	χ^2 test	*p* value	*df*	χ^2 test	*p* value	*df*	χ^2 test	*p* value
Mother tree (MT)	1	7.352	0.007	1	2.940	0.086	1	0.900	0.343
Cold storage period (CSP)	5	93.952	<0.001	5	7.964	0.158	5	5.694	0.337
MT × CSP	5	22.698	<0.001	5	2.164	0.826	4	2.758	0.599

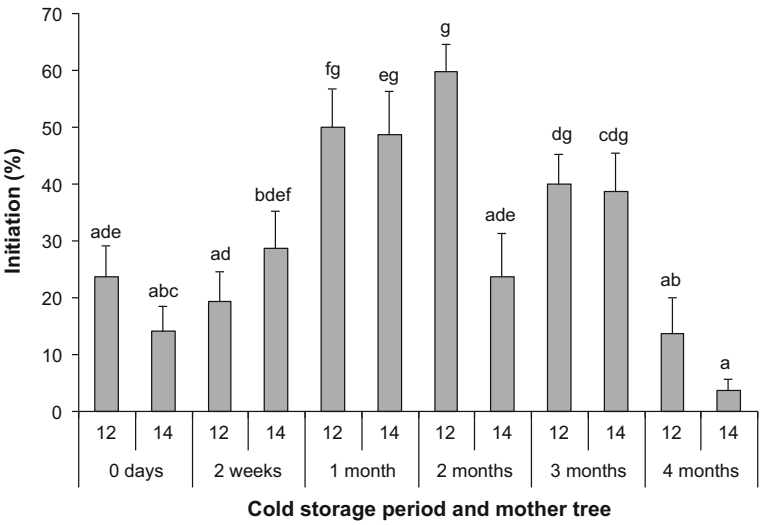

Fig. 2 Embryogenic tissue initiation (%) in *Pinus radiata* somatic embryogenesis process after different periods of storage at 4 °C in two mother trees (12 and 14), M ± S.E. Same letters are not statistically different at significance level $\alpha = 0.05$

respectively) of cold storage. The cones from mother tree 12 stored for 1–2 months produced significantly higher initiation percentages than the cones not stored at 4 °C. When cold storage period lasted 4 months, the lowest initiation percentages were obtained, these percentages were significantly lower than those obtained after 1, 2 or 3 months of cold storage. Nevertheless initiation percentages after 4 months were not statistically different from those of the controls (no cold storage, Fig. 1c).

The highest number of ECLs was achieved when the cones were stored for a month, after a storage period of 2 and 3 months a similar number of ECLs was obtained (42 and 41, respectively), while shorter storage periods (0 days or 2 weeks) led to a lower number of ECLs (Fig. 3).

Considering the percentage of proliferating ECLs (Fig. 1d) on the total embryogenic lines initiated, the effects of the cold storage period (p value = 0.158), the mother tree (0.086), or an interaction between both factors (p value = 0.826) were not statistically significant ($\alpha = 0.05$) (Table 1). The overall proliferation percentage was 74 % and the

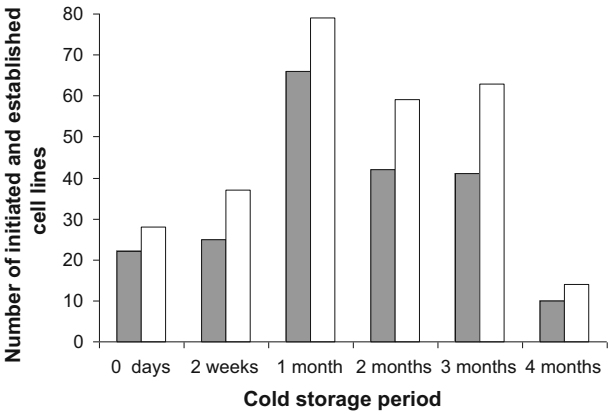

Fig. 3 Total number of embryogenic cell lines proliferating (*grey*), and total embryogenic lines initiated (*white*), in *Pinus radiata* somatic embryogenesis process after different periods of storage at 4 °C of the green cones. The initial explants were megagametophytes containing immature zygotic embryos

values for the different storage periods assayed ranged from 67 (3 months of cold storage) to 84 % (1 month of cold storage).

Regarding the percentage of ECLs producing se (Fig. 1e), the effects of the cold storage period (p value = 0.337), the mother tree (0.343), or an interaction between both factors (p value = 0.599) were not statistically significant ($\alpha = 0.05$) (Table 1). The lowest percentage of ECLs producing se was obtained in those ECLs from no cold storage (75 %) and the highest percentages of ECLs producing se were obtained after 1 and 3 months of cold storage (100 %).

Taking into account only those ECLs producing se, the number of se per gram of ET was significantly affected by the cold storage time of the cones (Kruskal–Wallis p value <0.001). The ECLs from cones cold stored for 1–4 months showed a significantly higher number of se (ranging from 302 to 405 embryos g^{-1} ET) than those stored for 0 days or 2 weeks (112 and 89 embryos g^{-1} ET, respectively) (Fig. 4).

The conversion rate of se was 70 % and the somatic seedlings were successfully acclimatized in the greenhouse (Fig. 1f).

Discussion

We found that cold storage of cones for 1–2 months increased SE initiation rates. On the contrary, a strong decline in *P. strobus* initiation frequencies was observed as the cold storage time increased (Park, 2002). In this sense, Häggman et al. (1999) reported that cold treatment had no effect on initiation frequencies in *P. sylvestris*. In our experiments, although slight differences were observed between mother trees, we can conclude that the best results for initiation were obtained after 1–3 months of cold storage of cones. Some authors have pointed out the importance of applying a short cold stress to initial explants as a necessary step to induce SE, particularly when this material is in a differentiated state (Krul 1993; Bonga 1996). Using megagametophytes as initial explant, it is possible to achieve SE initiation without a cold preconditioning period, but in agreement with

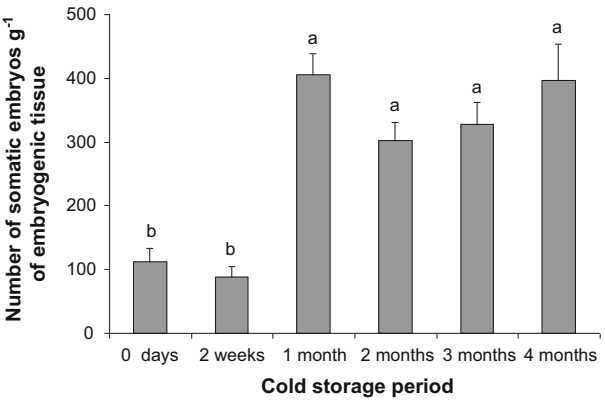

Fig. 4 Number of mature somatic embryos per gram of embryogenic tissue in *Pinus radiata* SE process after different 4 °C storage periods of the green cones, M ± S.E. The initial explants were megagametophytes containing immature zygotic embryos. Same letters are not statistically different at significance level $\alpha = 0.05$

Tomaszewski et al. (1994) in *Dactylis glomerata* or Luo et al. (2003) in *Astragalus adsurgens*, initiation rates were enhanced by this kind of treatment.

In our study, proliferation of ET was not significantly affected by mother tree or cold storage period tested. This result is encouraging as high initiation rates did not drop in the next phase of the process. It would be interesting for a further study to asses the effect of a cold treatment at proliferation stage, since there is a study in *Catharanthus roseus* showing that the effect of a cold treatment at proliferation stage is beneficial for the subsequent phases of the process (Aslam et al. 2011).

The percentage of ECLs that gave se was high, ranging from 75 to 100 % and was not significantly affected by cold storage period. However, the ECLs from plant material stored for 1 month or more at 4 °C produced a significantly higher number of se per gram of tissue. This beneficial effect of a cold preconditioning in the number of se obtained has been observed in other angiosperms species (Krul 1993). Some authors have reported higher conversion rates to plantlets when ETs or se were maintained at low temperatures (Corredoira et al. 2003). Thus, it seems cold storage or cold culture periods enhance later stages of SE by means of promoting the maturity of somatic embryos.

As reviewed by Neilson et al. (2010), it is clear that temperature stress cause distinct molecular responses in plant tissues; low temperature stress response is characterized by significant effects on chloroplast components, reactive oxygen species detoxification, and energy production. In other study, Kvaalen and Johnsen (2007) showed the existence of a mechanism in *Picea abies* that operates during embryo development and adjusts the timing of bud set in accordance with the temperature conditions in which the mother tree lives. As reviewed by Achrem et al. (2012) low temperature stress induces temporary and stable (epigenetic) changes in several species. The presence of "stress memory" keeps plants prepared for upcoming stresses. In keeping with these reports, it would be interesting for a future research to analyse different temperature stress tolerance of the plantlets obtained. To summarize, we report that it is possible to storage plant material of *P. radiata* for over 1 month and that this cold storage period increases SE initiation rates and the production of se. Moreover, cold treatment does not affect proliferation rates or maturation percentages. These findings are important from a practical point of view and should be proven in other

Pinus species because on one hand, offer the possibility of processing plant material for a longer period of time making easier initiation process and permitting introduction of a higher amount of initial explants, and on the other, increase success rates in initiation and maturation phases of SE.

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