Ann. For. Sci. 61 (2004) 125–128
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DOI: 10.1051/forest:2004003



**Note**

# Influence of exogenous L-proline on embryogenic cultures of larch (*Larix leptoeuropaea* Dengler), sitka spruce (*Picea sitchensis* (Bong.) Carr.) and oak (*Quercus robur* L.) subjected to cold and salt stress

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(Received 8 July 2002; accepted 6 March 2003)

**Abstract –** The effect of exogenous L-proline (1 mM, 10 mM, and 100 mM) on embryogenic cultures of larch, sitka spruce and oak subjected to environmental stresses was examined. Low temperature (4 °C) completely inhibited growth of the cultures and this was partially alleviated by the addition of proline. Our studies show that not only can cultures survive low temperatures, but are capable of active growth while the cold stress is still being applied. Growth was inversely related to [NaCl] with complete inhibition at 200 mM. Proline stimulated growth at all concentrations tested permitting growth with 200 mM NaCl even at low (1 mM) proline concentrations. Release of internal cellular potassium was inversely related to freezing temperature and this release was reduced by exogenous proline. These results for cultures of forest species are consistent with findings previously reported for deciduous herbaceous angiosperms and suggest that proline may have a role in protection of forest species from environmental stresses.

**trees / chilling / environmental stress / tolerance**

**Résumé – Effet de la L-proline apportée de manière exogène à des cultures embryogènes, de mélèze hybride (*Larix leptoeuropaea* Dengler), d'épicéa de sitka (*Picea sitchensis* Bong.) et de chêne (*Quercus robur* L.), soumises à des stress au froid et salin.** Des cultures embryogènes de mélèze hybride, d'épicéa de sitka et de chêne ont été soumises à différentes conditions de culture et leur croissance étudiée en fonction de l'ajout de L-proline au milieu de culture (1 mM, 10 mM, et 100 mM). Si des températures basses (4 °C) inhibent totalement la croissance des cultures, celle-ci redevient partiellement normale en présence de proline. Nos résultats montrent que les cultures non seulement survivent à de basses températures mais sont aussi capables de croître activement au cours de la durée d'application du froid. De même, la croissance est inversement corrélée à la concentration en sel avec sa complète inhibition en présence de 200 mM de NaCl. L'addition de L-proline au milieu de culture (quelles que soient les concentrations testées) stimule la croissance des cultures, même en présence de 200 mM de NaCl. La libération de potassium intracellulaire est inversement corrélée à la température de congélation, libération qui est réduite en présence de proline exogène. Ces résultats, obtenus pour des cultures d'espèces forestières, sont en accord avec ceux précédemment rapportés pour des espèces herbacées. Ils suggèrent le rôle potentiel de la proline dans la protection de ces espèces forestières soumises à des stress abiotiques.

**arbre / froid / stress abiotique / tolérance**

## 1. INTRODUCTION

The amino acid proline is thought to play an important role as an osmoregulatory solute in plants exposed to high levels of salt or drought [5, 8, 10]. The accumulation of proline is also associated with plant responses to chilling [4, 6, 23]. Plants often overproduce proline in response to these abiotic stresses. For example, tobacco cells adapted to NaCl accumulate proline to 80-fold higher levels, and this is accounted for by increased synthesis [17]. Possible roles suggested for proline are: osmoregulation, protection of cellular membranes and enzymes and conservation of energy and amino groups for post stress growth [2].

A number of studies on deciduous angiosperms have shown the effect of exogenous proline on cold tolerance of species such as *Solanum* [22] and maize [6] and also on osmotolerance [18]. However, there have been no studies on the effects of exogenous proline on forest species in vitro culture. It has been shown that the growth and physiological condition of oak, when grown in culture, was affected by NaCl, even at low concentration [1]. There have been no published studies on the effects of NaCl on cell cultures of gymnosperms. In the present work the influence of exogenous L-proline on embryogenic cultures of larch (*Larix leptoeuropaea* Dengler), sitka spruce (*Picea sitchensis* (Bong.) Carr.) and oak (*Quercus robur* L.) subjected to cold and salt stress was examined.

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## 2. MATERIALS AND METHODS

### 2.1. Plant material

#### *2.1.1. Larch* (Larix leptoeuropaea *Dengler*)

Embryogenic cultures (ECs) were induced from immature zygotic embryos of the hybrid larch [11]. One cell line (69.18) was maintained in suspension culture in MSG medium supplemented with 1.46 g $L^{-1}$ glutamine, 9 µM 2,4-D and 2.25 µM benzyladenine.

#### *2.1.2. Sitka spruce* (Picea sitchensis *(Bong.) Carr.)*

Sitka spruce ECs were raised from immature embryos of sitka spruce clones and one cell line (574F) was maintained in sitka spruce embryo initiation medium [9].

#### *2.1.3. Oak* (Quercus robur *L.*)

A single embryogenic cell line of oak was initiated from an immature zygotic embryo of pedunculate oak. Embryogenic cell suspension cultures were maintained on Murashige and Skoog medium (MS) [13] supplemented with 0.1 g $L^{-1}$ inositol, 0.2 g $L^{-1}$ glutamine, 10 µM benzyladenine and 30 g $L^{-1}$ sucrose.

### 2.2. Growth conditions

For each species, 50 mL suspension cultures were maintained in the above mentioned multiplication medium in sterile 250 mL Erlenmeyer flasks on an orbital shaker at 110 rpm and subcultured every 10 days. Except where otherwise stated, all cultures were maintained at 24 °C under a 16 h photoperiod with a light intensity of 30 µmoles/$m^2$/s.

### 2.3. Experimental conditions

Proline (Sigma-Aldrich) was filter sterilised into medium at the time of subculture to give a final concentration of 0, 1, 10 or 100 mM. Experiments were always carried out in triplicate for larch, sitka spruce and oak.

To study the effect of cold treatment on growth, embryogenic cultures were incubated at 24 °C and 4 °C.

To study the effect of salt stress on growth, embryogenic cultures were incubated at 24 °C. NaCl (Sigma-Aldrich) was added to the multiplication medium at the time of subculture to give a final concentration of 0, 50, 100, 150 or 200 mM.

For studies on both cold and salt stresses, cultures were maintained for 14 days. Every 2 days, the flasks were briefly removed from the orbital shaker, the cells allowed to settle for 10 min and the volume of settled cells (SCV) recorded [7]. From these measurements, the specific growth rate of the cultures was determined.

We used potassium leakage from the cells to study the effect of low and freezing temperatures [14]. Proline was added to 5 day old embryogenic cultures and after 48 h the cells were harvested by sieving on a 200 µm nylon mesh. They were then washed three times with 50 mL aliquots of distilled water by resuspension and filtration. Samples (500 mg) were cooled to (24, 0, –5, –10, –20 and –30 °C) and potassium release into distilled water measured by atomic absorption spectrometry. $K_I$ (initial potassium) was measured. The samples were then autoclaved for 5 min at 121 °C and potassium levels were re-measured ($K_F$). Percent $K^+$ release was calculated as:

$$\% K^+ \text{ Release} = (K_I / K_F) \times 100.$$

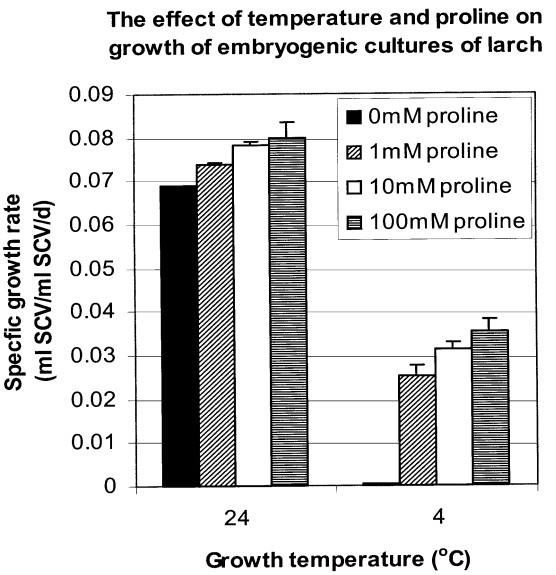


**Figure 1.** Influence of proline on the specific growth rate of larch (*Larix leptoeuropaea* Dengler) embryogenic cultures at 4 °C and 24 °C. Mean values ± the standard error of the mean (SEM) are shown.

### 2.4. Proline assay

Embryogenic cultures were incubated in maintenance medium supplemented with 0, 1, 10 or 100 mM proline for 2 days. They were then washed three times with aliquots of distilled water (50 mL) by vacuum filtration on 200 µm nylon mesh and resuspension. 200 mg samples of cells were analysed in triplicate. The free proline concentration in the cells was determined using a modified ninhydrin method [3].

### 2.5. Statistical analysis

Factorial analysis of variance was carried out using SPSS for Windows (version 8). Tukeys' HSD test was used for Post-Hoc testing.

## 3. RESULTS

### 3.1. Influence of proline in relation to temperature

Figure 1 shows the growth rate of larch embryogenic cultures grown at 24 °C and 4 °C. Cultures grown at 4 °C without exogenous proline turned brown within seven days indicating necrosis. Cultures grown at 4 °C with exogenous proline did not turn brown and morphologically resembled those grown at 24 °C. Culture at 4 °C, with no proline supplementation, completely inhibited growth of all three species. Factorial ANOVA showed that there was a very highly significant effect of proline and temperature, and of the interaction between them ($p < 0.0005$), with a very highly significant effect of proline at each temperature. Similar results were seen for sitka spruce and oak (results not shown).

### 3.2. Influence of proline in relation to concentrations of NaCl

Figure 2 shows the growth rate of larch embryogenic cultures grown in varying concentrations of NaCl. There was a

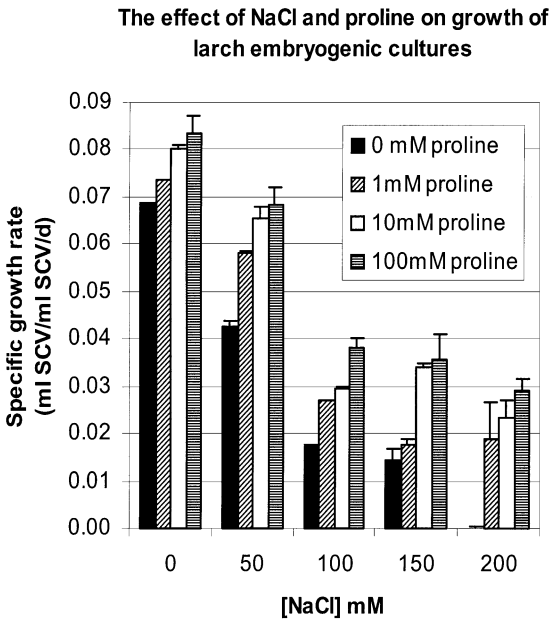


**Figure 2.** Influence of proline on the growth rate of larch (*Larix leptoeuropaea* Dengler) embryogenic cultures in varying concentrations of NaCl. Mean values ± SEM are shown.

very highly significant effect of increasing NaCl concentration on the growth of embryogenic cultures ($p < 0.0005$) with NaCl causing a progressive decline in the growth rate and with complete inhibition of growth at 200 mM NaCl in the absence of proline. Proline significantly improved cell growth at every concentration of NaCl ($p < 0.0005$). Similar results were seen for sitka spruce and oak (results not shown).

### 3.3. Potassium leakage from embryogenic cultures subjected to below freezing temperatures

Figure 3 shows the effects of proline on $K^+$ release for larch embryogenic cultures subjected to low and freezing temperatures. Potassium leakage was inversely proportional to temperature. Addition of proline reduced the amount of potassium released at every temperature, and the protective effect was related to the concentration of exogenous proline. Similar results are seen for sitka spruce and oak (results not shown).

### 3.4. Measurement of intracellular proline concentration in embryogenic cultures subjected to below freezing temperatures

The concentration of proline in embryogenic cultures of larch, sitka spruce and oak is shown in Table I. With no proline addition, intracellular proline levels are correspondingly low. When proline is added, intracellular proline levels recorded correspond approximately to the amount of proline added to each culture.

## 4. DISCUSSION

Exogenous proline has been shown to have a positive effect on recovery from cold stress in cultures of maize and potato [6, 23]. Our studies show that not only can cultures survive low temperatures, but moreover that they are capable of active growth while the cold stress is still being applied.

Salt can dramatically reduce plant growth. The addition of NaCl caused a progressive decline in elongation of shoots of *Hordeum vulgare* L. cv. Maris Mink (cultured Barley) as well as a decrease in tissue fresh weight [12]. Addition of proline at 10 mM was reported to reduce the inhibition of growth caused by the addition of NaCl with no additional protection at higher concentrations. We have shown very similar results of salt (NaCl) on the growth of larch, sitka spruce and oak cultures and similar effects of exogenous proline.

Frost-hardy species produce cryoprotectants such as proline, which reduce damage by freezing-induced desiccation. Positive correlations have been found between leaf proline content and frost tolerance in a wide range of species [15, 20]. Ion leakage is used as an indicator of freezing injury in plants [14]. It can be seen that exogenous proline reduces $K^+$ leakage from larch, sitka spruce and oak.

**Table I.** Intracellular proline concentration 48 h after addition of exogenous proline. Mean values ± SEM are shown.

| Proline added to cultures (mM) | µMoles proline / g fresh weight | | |
|---|---|---|---|
| | Larch | Sitka spruce | Oak |
| 0 | 0.0424 ± 0.006 | 0.0402 ± 0.023 | 0.0283 ± 0.009 |
| 1 | 1.708 ± 0.029 | 1.621 ± 0.036 | 0.834 ± 0.017 |
| 10 | 9.829 ± 0.002 | 9.632 ± 0.021 | 7.8107 ± 0.014 |
| 100 | 77.938 ± 0.012 | 76.572 ± 0.065 | 51.816 ± 0.091 |

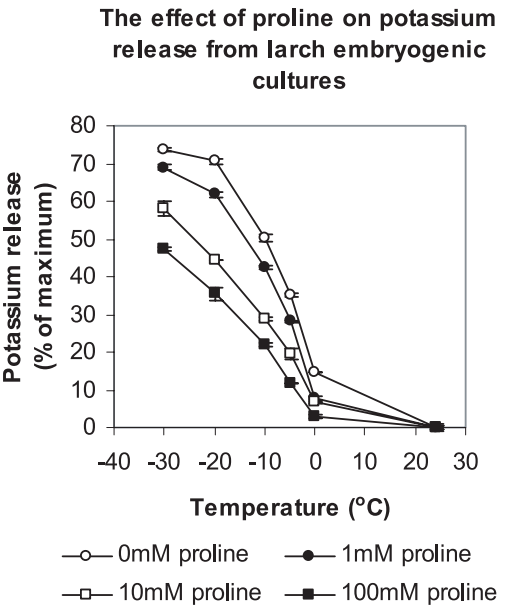


**Figure 3.** Influence of proline on potassium leakage from larch (*Larix leptoeuropaea* Dengler) embryogenic cultures at below freezing temperatures. Mean values ± SEM are shown.

Thus exogenous proline protected the cells from the effects of the salt, cold and freezing stresses applied and in a similar manner to that of herbaceous, deciduous angiosperms. This raises the possibility that forest species may also be protected from environmental stresses by manipulation of the accumulation of endogenous proline. Recent studies show that introduction of a gene for the rate-limiting enzyme in proline biosynthesis has produced improved environmental stress resistance in herbaceous dicots [16] and monocots [19]. The introduction of this gene into embryogenic cultures of forest species may therefore be a potent mechanism for introduction of stress tolerance into forest species, and their subsequent mass propagation [21].

**Acknowledgments:** We would like to thank Dr. David Thompson of Coillte Research Laboratory, Newtownmountkennedy, Co. Wicklow, Ireland for donation of sitka spruce and oak cultures.

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