DEVELOPMENT OF PROTOCOLS FOR THE CRYOPRESERVATION OF ZYGOTIC EMBRYOS OF *PINUS RADIATA* AND SUBSEQUENT PLANT REGENERATION

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ABSTRACT

Whole zygotic embryos of *Pinus radiata* D.Don were evaluated for survival after storage in liquid nitrogen (–196°C). Zygotic embryos were cryoprotected with selected levels of sorbitol and dimethylsulphoxide (DMSO) and were then frozen, employing an isopropanol-filled cryovial container and –80°C freezer for 1.5 hours prior to immersion in liquid nitrogen. After removal from liquid nitrogen the condition of embryos was assessed and then cotyledons were excised for shoot induction. Up to 61% of cotyledons cryopreserved in a cryoprotectant formula of 0.4 M sorbitol and 15% DMSO regenerated shoots. A sample of the regenerated shoots was successfully rooted. Cryopreserved cotyledons from 84% of the genotypes were able to regenerate shoots upon return to *in vitro* culture as compared to 92% of the unfrozen controls.

Keywords: cryopreservation; zygotic embryo; *Pinus radiata*.

INTRODUCTION

Control-pollinated seed from top-ranked *Pinus radiata* parents is expensive to produce and relatively scarce. Tissue culture via organogenesis is one of the techniques available for amplifying this seed. Techniques for organogenesis of *P. radiata* have been developed at the New Zealand Forest Research Institute (Reilly & Washer 1977; Aitken *et al.* 1981; Horgan

& Aitken 1981; Smith *et al.* 1982) and have been commercialised by Fletcher Challenge Forests to produce 2–3 million plantlets per year via this technique (Gleed 1993).

To improve gains achieved from the progeny of controlled pollinations, selections may be made of outstanding individuals which can then be propagated and tested as clones. Mass propagation of proven outstanding individuals is the essence of clonal forestry. Clones from within top families have demonstrated marked improvements in performance when compared with the family averages (Johnson 1988). Genetic uniformity is an additional benefit of clonal forestry.

However, propagules generated clonally from mature *P. radiata* (more than 8 years old) have a slower initial growth rate than those from younger material (Menzies *et al.* 1989). Conversely, many characteristics of commercial interest, such as stem form, disease resistance, and wood density, can be identified only when trees are 8 to 12 years old. Thus, at the age elite trees are identified they are mature and clonal propagules exhibit reduced growth rate. A storage protocol which preserved juvenile growth characteristics in representative propagules while field tests of the same propagules took place would allow the forest grower to achieve better selection. Once elite trees were identified, amplification of stored material would provide identical planting stock with juvenile growth rates.

Options for storage of juvenile material include cool storage of *in vitro* grown shoots (Aitken-Christie & Singh 1987) and maintenance of stool beds in the nursery (Menzies *et al.* 1985). Cryopreservation, based on storage in liquid nitrogen, offers several advantages over other nursery and low-temperature storage options. It requires minimal space and equipment and only occasional maintenance. It can be run without electricity and the absence of subculturing minimises the opportunity for bacterial and fungal contamination and mislabelling (Nairn 1992). Growth abnormalities sometimes experienced with low-temperature storage are also minimised (Son *et al.* 1991).

The present investigation was undertaken to devise a protocol for the cryopreservation of zygotic embryos of *P. radiata,* as cotyledons from embryos have been used successfully in adventitious shoot regeneration systems (Reilly & Washer 1977; Horgan & Aitken 1981; Aitken-Christie *et al.* 1988).

MATERIALS AND METHODS

Seed used for these experiments was from a bulk seedlot (GF16) from Proseed in Rotorua. Seeds were surface-sterilised in a 50% Chlorodux (calcium hypochlorite 5% v/v) plus surfactant (0.1 ml silwet L-77, a wetting agent) solution for 20 minutes, followed by overnight rinsing under running water. This facilitates imbibition of seeds. Following imbibition, seeds were re-sterilised in 6% H_2O_2 solution plus 0.1 ml silwet for 10 minutes, then rinsed three times in sterile water. Zygotic embryos were dissected from surface-sterilised seeds and placed on modified Quoirin and Lepoivre medium (LP) (Quoirin & Lepoivre 1977). The modification was derived by K.Horgan and J.Aitken-Christie on the basis of analysis of tissue-cultured shoots and normal levels of elements expected in *P. radiata* foliage from the nursery and field (Aitken-Christie *et al.* 1988).

Preconditioning and Preculture Treatments

Two separate experiments were performed, using different concentrations of sorbitol and dimethylsulphoxide (DMSO). In Experiment 1 (Table 1), dissected embryos (300) were

TABLE 1–Chemical composition of preconditioning and preculture media

Preconditioning medium M sorbitol	Preculture medium M sorbitol +	% DMSO	Replications (5 embryos per rep.) Experiment 1	Experiment 2
0.0*	0.000	0	5	5
0.0	0.000	10		5
0.0	0.000	15		5
0.0	0.000	20		5
0.0	0.000	25		5
0.0	0.000	30		5
0.1	0.090	10		5
0.1	0.085	15		5
0.1	0.080	20		5
0.1	0.075	25		5
0.1	0.070	30		5
0.2	0.180	10		5
0.2	0.170	15		5
0.2	0.160	20		5
0.2	0.150	25		5
0.2	0.140	30		5
0.3	0.300	0	5	
0.3	0.285	5	5	
0.3	0.270	10	5	5
0.3	0.255	15	5	5
0.3	0.240	20		5
0.3	0.225	25		5
0.3	0.210	30		5
0.4	0.400	0	5	
0.4	0.380	5	5	
0.4	0.360	10	5	5
0.4	0.340	15	5	5
0.4	0.320	20		5
0.4	0.300	25		5
0.4	0.280	30		5
0.5	0.500	0	5	
0.5	0.475	5	5	
0.5	0.450	10	5	
0.5	0.425	15	5	

* Control embryos placed directly on to $^{1}/_{2}$LP5 medium after dissection

placed in cryo-vials containing a preconditioning medium of 1 ml liquid LP medium plus 0.3, 0.4, or 0.5 M sorbitol. There were five embryos per cryo vial and 20 vials for each sorbitol concentration (100 embryos per preconditioning treatment). Vials were placed in the dark at 4°C and incubated for 5 days. After incubation, 0.5 ml liquid was removed from each vial and 0.5 ml DMSO solution was added to give 0, 5, 10, and 15% preculture treatment concentrations. In Experiment 2 (Table 1), the same protocols were followed except that sorbitol concentrations were 0, 0.1, 0.2, 0.3, and 0.4 M. The DMSO solutions were added to give 10, 15, 20, 25, and 30% preculture treatment concentrations. The addition of DMSO diluted the sorbitol concentrations and these changes are detailed alongside the preconditioning and preculture treatments (Table 1). For each experiment a further 25 embryos were

dissected and placed directly on to half strength LP medium containing 5 mg benzylaminopurine/l ($^1/_2$LP5), and were cultured in a light incubator as a non-pretreated, non-cryopreserved control.

Dissected embryos were left to equilibrate with DMSO solutions for 10 minutes at 0°C in a "Mr Frosty" container (Nalgene) which holds 18 cryo-vials surrounded by isopropyl alcohol. The container was then placed in a freezer at –80°C. The isopropyl alcohol in conjunction with the freezer gave a steady cooling rate of approximately 1°C/min. After 75 minutes in the freezer, vials were removed from the "Mr Frosty" and plunged into liquid nitrogen.

Regrowth—Test of Viability

After 24 hours of storage, vials were thawed in a 45°C water bath for 2 minutes. Contents of the vials were poured on to nybolt cloth (30 μm Scapa Filtrations) resting on several pieces of absorbent paper-towel and were left to stand for 5 minutes, allowing cryoprotectant solutions to be absorbed. During thawing it was noted that in some treatments cotyledons had spontaneously abscissed where they joined the hypocotyl. A record was made of numbers of embryos with cotyledon retention.

Embryos were transferred to $^1/_2$LP5 medium and were placed in the light incubator under shade cloth (PPF 30 μmol/m^2/s), 16 hours light at 24°C and 8 hours dark at 18°C. After 14 days, embryos were assessed for viability by colour. Green tissue was used as an indicator of tissue most likely to form meristematic tissue, then shoots, following further culture (a score was also made of white embryos (dead) and partially coloured (piebald) embryos but these data are not presented). After the above assessments, all cotyledons which had not already abscissed were severed from embryos and placed in contact with $^1/_2$LP5 medium.

After 26 days on $^1/_2$LP5 medium, cotyledons were transferred to LP medium. Shoot formation was recorded 10 weeks after thawing. In Experiment 2, a record was also made of the genotypes which formed shoots. A sample of shoots grown from the cryopreserved cotyledons was taken through to the rooting stage using techniques described by Horgan & Aitken (1981) and Horgan & Holland (1989).

Performance of the non-frozen controls is noted below each Table of results.

Data Analyses

Embryo and cotyledon responses to the cryoprotectant treatments were tested by ANOVA (Sokal & Rohlf 1981). Transformation by $\sqrt{X}$ was required to normalise the data on cotyledon shoot formation in Experiment 1.

RESULTS

Viability of Recovered Tissue

Green embryos

In Experiment 1 the concentrations of both DMSO ($p<0.001$) and sorbitol ($p<0.0006$) had a significant effect on numbers of surviving embryos as measured by their turning green 14 days after thawing (Table 2). However, the interaction between DMSO and sorbitol was

TABLE 2–Green embryos (%) per treatment in Experiment 1

DMSO (% in solution)	Sorbitol (molar concentration)			Mean
	0.3	0.4	0.5	
0	36	4	0	13.3
5	20	12	8	13.3
10	52	40	28	40.0
15	64	36	32	44.0
Mean	43.0	20.3	17.0	

Non-cryopreserved control had 100% green embryos

not significant. The greatest percentage of green embryos was usually obtained in the treatments with the highest DMSO percentage combined with lowest sorbitol concentration. Absence of DMSO (0% DMSO) in the 0.4 and 0.5 M sorbitol preculture solutions gave much lower percentages of green embryos than the solutions containing 10 and 15% DMSO, although these results were not significantly different. In Experiment 2 (Table 3), DMSO ($p<0.0008$), sorbitol ($p<0.0001$), and the interaction ($p<0.0001$) of the two cryoprotectants had significant effects on the number of green embryos although there were no clear patterns relating to increased doses of either chemical. Sorbitol at 0.3 M gave the highest average yield of green embryos, but the 0.4 M treatment yielded the highest values at low (10%, 15%) levels of DMSO.

TABLE 3–Green embryos (%) per treatment in Experiment 2

DMSO (% in solution)	Sorbitol (molar concentration)					Mean
	0	0.1	0.2	0.3	0.4	
10	12	28	20	40	60	32.0
15	32	48	12	72	76	48.0
20	40	15	8	68	4	27.0
25	28	4	0	48	16	19.2
30	28	20	52	48	4	30.4
Mean	28.0	23.0	18.4	55.2	32.0	

Non-cryopreserved control had 92% green embryos

Cotyledon retention

DMSO had a significant effect ($p<0.0001$) on retention of cotyledons by embryos in Experiment 1 (Table 4). Spontaneous cotyledon abscission was an unexpected and unwanted result. It was more prevalent in the lower-concentration DMSO treatments and may indicate a poorer penetration of cryoprotectants. However, neither the effects of sorbitol nor the interaction between DMSO and sorbitol were significant. Cotyledon retention was much greater in treatments with 10% and 15% DMSO (Table 4). In Experiment 2, neither DMSO, sorbitol concentration, nor the interaction between sorbitol and DMSO had a significant effect on cotyledon retention (Table 5).

Shoot Formation

Numbers of cotyledons that formed shoots 10 weeks after thawing in Experiment 1 showed a significant response to DMSO ($p<0.0001$) at 10% and 15% (Table 6). Effects of

TABLE 4–Embryos (%) with cotyledon retention in Experiment 1

DMSO (% in solution)	Sorbitol (molar concentration)			Mean
	0.3	0.4	0.5	
0	4	0	4	2.7
5	24	8	8	13.3
10	60	40	56	52.0
15	64	80	68	70.7
Mean	38.0	32.0	34.0	

Non-cryopreserved control, 100% of embryos retained cotyledons

TABLE 5–Embryos (%) with cotyledon retention in Experiment 2

DMSO (% in solution)	Sorbitol (molar concentration)					Mean
	0	0.1	0.2	0.3	0.4	
10	84	68	90	76	72	78.0
15	84	100	80	88	92	88.0
20	96	100	96	88	88	93.6
25	84	100	84	84	88	88.0
30	88	80	88	100	96	90.4
Mean	87.2	89.6	87.6	87.2	87.2	

Non-cryopreserved control, 100% of embryos retained cotyledons

TABLE 6–Cotyledons (%) that formed shoots 10 weeks after thawing in Experiment 1

DMSO (% in solution)	Sorbitol (molar concentration)			Mean
	0.3	0.4	0.5	
0	1 (165)*	1 (176)	1 (142)	1.0 (161)
5	0 (158)	1 (165)	3 (136)	1.3 (153)
10	18 (165)	20 (150)	16 (160)	18.0 (158)
15	46 (172)	20 (176)	15 (155)	27.0 (168)
Mean	16.3 (165)	10.5 (167)	8.8 (148)	

Non-cryopreserved control, 80% of cotyledons had formed shoots at 10 weeks with a total of 182 cotyledons in treatment.
* Numbers in brackets = total number of cotyledons in each treatment

sorbitol and the interaction of sorbitol and DMSO were not significant. The highest level of DMSO (15%) in combination with the lowest concentration of sorbitol (preconditioning sorbitol 0.3 M, preculture sorbitol 0.255 M) produced the greatest shoot-formation response from cotyledons (46%) (Table 6). Concentrations of DMSO lower than 10% were clearly deleterious.

Higher concentrations of DMSO and lower concentrations of sorbitol were tested in Experiment 2. The percentages of cotyledons that formed shoots 10 weeks after thawing showed significant responses to different levels of DMSO ($p<0.0001$) and sorbitol ($p<0.0001$) although there was no clear pattern related to dosage. The interactive effect of sorbitol and DMSO was also significant ($p<0.0001$) (Table 7). The best shoot formation (61%) occurred

TABLE 7–Cotyledons (%) that formed shoots 10 weeks after thawing in Experiment 2

DMSO (% in solution)	Sorbitol (molar concentration)					Mean
	0	0.1	0.2	0.3	0.4	
10	12	12	11	24	22	16.2
15	8	39	8	48	61	32.8
20	24	9	8	48	2	18.2
25	14	1	1	29	15	12.0
30	18	14	33	32	11	21.6
Mean	15.2	15.0	12.2	36.2	22.2	

Non-cryopreserved control, 75% of cotyledons formed shoots after 10 weeks

with DMSO at 15% in combination with 0.4 M sorbitol (preconditioning sorbitol 0.4 M, preculture 0.34 M). This response compared favourably with that of the controls in which 75% of the unfrozen cotyledons produced shoots. Higher concentrations of DMSO (25% and 30%) in combination with lower concentrations of sorbitol (0.1 or 0.2 M) may not have been advantageous, but this was not statistically significant.

Genotype Differentiation

The high percentage of cotyledon retention (Table 5) observed in Experiment 2 allowed genotype performance to be evaluated as the cotyledons from the five embryos in each replication had not "mixed" within the cryopreservation vial (Table 8). The highest percentage of genotypes (84%: 21 of the 25 embryos formed shoots) occurred with a combination of 0.4 M sorbitol (preculture 0.34 M) and 15% DMSO (Table 8), compared with the non-frozen controls where 92% (23 of 25 embryos) of the genotypes tested formed shoots.

TABLE 8–Percentage of genotypes that formed shoots 10 weeks after thawing in Experiment 2

DMSO (% in solution)	Sorbitol (molar concentration)					Mean
	0	0.1	0.2	0.3	0.4	
10	20	21	16	44	40	28.2
15	20	60	8	76	84	49.6
20	36	12	16	76	8	29.6
25	32	4	4	48	28	23.2
30	28	29	60	56	16	37.8
Mean	27.2	25.2	20.8	60.0	35.2	

Non-cryopreserved control, 92% of genotypes had formed shoots after 10 weeks

Rooting Trial

The sample of shoots grown from cryopreserved cotyledons and taken through to rooting performed normally, rooting within 6 weeks. Foliage appeared normal, with the presence of primary needles and then secondary needles as expected (Fig. 1).

FIG. 1–Shoots grown from cryopreserved cotyledons of *Pinus radiata*

DISCUSSION

Successful cryopreservation of excised non-germinated cotyledons of white spruce (*Picea glauca* Moench Voss) was reported by Toivonen & Kartha (1989). Their best recovery (35%) occurred with 5% DMSO and 0.4 M sorbitol (compared with 61% of *Pinus radiata* cotyledons in 15% DMSO and 0.4 M sorbitol reported here). Other forest tree species (*Quercus petraea* (Mattuschka) Liebl., *Fagus sylvatica* L., and *Aesculus hippocastanum* L.) have had recovery of 90–100% of zygotic embryos frozen with a cryoprotectant mixture of 10% DMSO and 5% sucrose (Jorgensen 1990). Survival was assayed by callus formation or further growth.

For the practical application of this research, cotyledons will be frozen after being severed from the embryo, facilitating subsequent removal of individual cotyledons from cryopreservation. These experiments were initiated to test a range of DMSO and sorbitol concentrations for effectiveness as there were reports published on toxicity and interactive effects in cell cultures and cotyledons of *Picea glauca* (Kartha *et al.* 1988; Toivanen & Kartha 1989). Interactive effects of sorbitol and DMSO were significant when shoot formation was considered in Experiment 2, although this was not apparent in the earlier observations of cotyledon abscission and embryo greening in *Pinus radiata*.

The results presented here show a high representation of genotypes after cryopreservation. It is critical that any protocols developed do not become a selection process,

but embryogenic cell cultures of both *Abies nordmanniana* (Steven) Spach and *Picea abies* (L.) Karsten have shown genotypic variability in relation to cryotolerance (Nørgaard *et al.* 1993 a, b).

Protocols developed for the cryopreservation of axillary and apical meristems are preferred as these offer less risk of unwanted somaclonal variation than meristems which arise adventitiously (Scowcroft 1985; Berlyn *et al.* 1986). But, axillary and apical meristems are more difficult to cryopreserve than adventitious meristems. This probably relates to the larger and physiologically more heterogeneous cell population that meristems provide (Chen & Kartha 1988).

The work reported here is significant in that it is the only description of the successful cryopreservation of organised *Pinus radiata* tissue and is the first step towards developing a successful cryopreservation protocol for axillary meristems.

CONCLUSIONS

Cotyledons from cryopreserved zygotic embryos of *P. radiata* formed meristematic tissue that subsequently gave rise to shoots. Combinations of 0.4 M sorbitol as a pre-conditioning treatment followed by preculture with 15% DMSO and 0.34 M sorbitol tended to have more shoot production than lower DMSO or higher sorbitol concentrations. Concentrations of DMSO above 5% appeared to prevent cotyledon abscission, where this was a problem.

The high percentage of genotypes which gave rise to shoots after cryopreservation (84% *v.* 92% of the non-frozen control) was encouraging.

These results indicate that, using the protocols developed, cotyledon tissue with juvenile characteristics can be both successfully amplified and cryopreserved, thereby allowing multiplication of selected juvenile clones after clonal field testing.

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REFERENCES

AITKEN, J.; HORGAN, K.J.; THORPE, T.A. 1981: Influence of explant selection on the shoot forming capacity of juvenile tissues of *Pinus radiata*. *Canadian Journal of Forest Research 11*: 112–117.

AITKEN-CHRISTIE, J.; SINGH, A.P. 1987: Cold storage of tissue cultures. Pp. 285–304 *in* Bonga, J.M.; Durzan, D.J. (Ed.) "Cell and Tissue Culture in Forestry", Volume 2. Martinus Nijhoff Publishers, Dordrecht.

AITKEN-CHRISTIE, J.; SINGH, A.P.; DAVIES, H. 1988: Multiplication of meristematic tissue: A new tissue culture system for radiata pine. Pp.413–432 *in* Hanover, J.W.; Keathley, D.E. (Ed.) "Genetic Manipulation of Woody Plants". Plenum Publishing Corporation.

BERLYN, G.P.; BECK, P.C.; RENFROE, M.H. 1986: Tissue culture and the propagation and genetic improvement of conifers: Problems and possibilities. *Tree Physiology 1*: 227–240.

CHEN, T.H.H.; KARTHA, K.K. 1988: Cryopreservation of plant cells and organs. Pp. 217–240 *in* Valentine, F.A. (Ed.) "Forest and Crop Biotechnology, Progress and Prospects". Springer-Verlag, New York, Berlin, Heidelberg, London, Paris, Tokyo.

GLEED, J.A. 1993: Development of plantings and stockings of radiata pine. Pp.149–157 *in* Ahuja, M.R.; Libby, W.J. (Ed.) "Clonal Forestry II, Conservation and Application". Springer-Verlag, Berlin.

HORGAN, K.; AITKEN, J. 1981: Reliable plantlet formation from embryos and seedling shoot tips of radiata pine. *Physiologia Plantarum 53*: 170–175.

HORGAN, K.; HOLLAND, L. 1989: Rooting micropropagated shoots from mature radiata pine. *Canadian Journal of Forest Research 19*: 1309–1315.

JOHNSON, G.R. 1988: A look to the future: Clonal forestry. Pp.79–84 *in* Menzies, M.I.; Aimers, J.P.; Whitehouse, L.J. (Ed.) "Proceedings of Workshop on Growing Radiata Pine from Cuttings, Rotorua, May 1986". *Ministry of Forestry, New Zealand Forest Research Institute, FRI Bulletin No.135.*

JORGENSEN, J. 1990: Conservation of valuable gene resources by cryopreservation in some forest tree species. *Journal of Plant Physiology 136*: 373–376.

KARTHA, K.K.; FOWKE, L.C.; LEUNG, N.L.; CASWELL, K.L.; HAKMAN, I. 1988: Induction of somatic embryos and plantlets from cryopreserved cell cultures of white spruce (*Picea glauca*). *Plant Physiology 132*: 529–539.

MENZIES, M.I.; KLOMP, B.K.; HOLDEN, D.G. 1989: Optimal physiological age of propagules for use in clonal forestry. Pp.142–146 *in* Miller, J.T. (Ed.) "FRI/NZFP Forest Ltd Clonal Forestry Workshop". *Ministry of Forestry, New Zealand Forest Research Institute, FRI Bulletin No.160.*

MENZIES, M.I.; FAULDS, T.; DIBLEY, M.; AITKEN-CHRISTIE, J. 1985: Vegetative propagation of radiata pine in New Zealand. Pp. 167–199 *in* South, D.B. (Ed) "Proceedings of the International Symposium on Nursery Management Practices for the Southern Pines", 4–9 August. IUFRO/Alabama Agricultural Experiment Station, Auburn University, Montgomery, Alabama, USA.

NAIRN, B.J. 1992: Commercial micropropagation of radiata pine. Pp. 383–394 *in* Ahuja, M.R. (Ed) "Micropropagation of Woody Plants". Kluwer, Netherlands. *Forestry Sciences No.41.*

NØRGAARD, J.V.; BALDURSSON, S.; KROGSTRUP, P. 1993a: Genotypic differences in the ability of embryogenic *Abies nordmanniana* cultures to survive cryopreservation. *Silvae Genetica 42*: 93–97.

NØRGAARD, J.V.; DURAN, V.; JOHNSON, Ø.; KROGSTRUP, P.; BLADERSON, S.; ARNOLD, VON S. 1993b: Variation in cryotolerance of embryogenic *Picea abies* cell lines and the association to genetic, morphological and physiological factors. *Canadian Journal of Forest Research 23(12)*: 2560–2567.

QUOIRIN, M.; LEPOIVRE, P. 1977: Etudes des milieux adaptés aux cultures *in vitro* de *Prunus*. *Acta Horticulturae 78*: 437–442.

REILLY, K.; WASHER, J. 1977: Vegetative propagation of radiata pine by tissue culture: Plantlet formation from embryo tissue. *New Zealand Journal of Forestry Science 7*: 199–206.

SCOWCROFT, W.R. 1985: Somaclonal variation: The myth of clonal uniformity. Pp. 217–245 *in* Hohn, B.; Dennis, E.S. (Ed.) "Plant Gene Research". Springer Verlag.

SMITH, D.R.; HORGAN, K.J.; AITKEN-CHRISTIE, J. 1982: Pp.723–724 *in* Fujiwara, A. (Ed.) "Micropropagation of *Pinus radiata* for Afforestation". Proceedings of the 5th International Congress on Plant Tissue and Cell Culture, Tokyo, Japan.

SOKAL, R.R.; ROHLF, F.J. 1981: Assumptions of analysis of variance. Pp. 400–453 *in* "Biometry, The Principles and Practice of Statistics in Biological Research", second ed. W.W.Freeman and Co., New York.

SON, S.H.; CHUN, Y.W.; HALL, R.B. 1991: Cold storage of *in vitro* cultures of hybrid poplar shoots (*Populus alba* L. × *P. grandidentata* Michx.). *Plant Cell, Tissue and Organ Culture 27*: 161–168.

TOIVONEN, P.M.A.; KARTHA, K.K. 1989: Cryopreservation of cotyledons of non-germinated white spruce (*Picea glauca* (Moench) Voss) embryos and subsequent plant regeneration. *Journal of Plant Physiology 134*: 766–788.