Rapid assessments of cold hardiness and quality deterioration during storage of bare root conifer transplants

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Abstract

Chlorophyll fluorescence and trace gas analysis techniques were evaluated with the aim of providing nursery and establishment managers a rapid prediction of dormancy status and seedling quality deterioration during storage. Dark-adapted maximal fluorescence (Fv/Fm) assessments of excised shoots following freezing treatments accurately predicted cold tolerance as determined using the temperature at which 50% of seedlings are damaged (LT_{50}). The Fv/Fm of excised shoots was correlated with visual assessments of needle damage and survival following extended storage of seedlings at different temperatures. We suggest that these Fv/Fm-based methods provide a more rapid and robust indicator of seedling quality during period of extended storage when compared to other physiological measures, such as root electrolyte leakage (REL) and the build-up of volatile compounds such as ethanol and ammonia. This modified fluorescence-based estimate of LT_{50} ($F.LT_{50}$) can be conducted within 3 days, compared to 14 days using the conventional visual assessment LT_{50}. The $F.LT_{50}$ fluorescence based technique is now been routinely used to assess hot lift and cold storage suitability of Sitka spruce seedlings in Coillte nurseries.

Keywords: S*eedling quality, hardiness acclimation, deterioration in storage*.

Introduction

The use of freshly lifted and cold stored bare-root seedlings to establish a forest is a common silvicultural practice in both Ireland and the U.K. Nursery production factors or management decisions which influence the likelihood of successful seedling establishment include dormancy status of the material before lifting and the amount of seedling quality deterioration during cold storage or on site before planting (McKay 1992 and 1993, Mason 1994, O'Reilly et al. 1999).

Lifting and cold storage of insufficiently hardened trees may lead to reduced vitality, frost damage and desiccation. Although lifting and planting windows have been well established for most commercially grown species, these guidelines should be interpreted carefully (O'Reilly and Keane 2002) because the recommendations reflect average conditions that prevail over relatively large geographical locations and dormancy status or stress resistance levels vary considerably from year to year

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or from nursery to nursery (Ritchie 1984). The routine identification of lifting and cold storage windows requires rapid characterisation of hardening acclimation using plant physiological or phenological indicators. The most common testing procedure for conifers involves the visual assessment of shoot damage after artificial freezing to estimate temperature at which only half the seedlings (LT_{50}) would recover. The method has been shown to be consistently related to hardening and de-hardening acclimation processes and field performance after different lift dates in many species (Colombo et al. 1989, O'Reilly et al. 1999, 2000). However, this method is of limited practical value because of the long period required for visual damage symptoms to develop (up to 14 days depending on extent of freeze damage).

Following dispatch, seedlings are often left on site for extended periods before planting. This has been suggested to be one of the reasons for establishment failure in Ireland (O'Reilly and Keane 2002). This warrants the development of an operational test to assess seedling quality on site if quality deterioration is suspected. Whilst numerous tests have been developed to assess seedling quality during storage, few are rapid and simple enough to routinely use under operational conditions. Physiological assessments of seedling survival potential following periods of extended storage have been determined by assessing root membrane integrity through measurement of root electrolyte leakage (e.g. McKay 1992). This method is best suited for use under laboratory conditions and results are difficult to interpret in some cases (O'Reilly et al. 2000).

The use of two techniques for rapid analysis of seedling quality under operational conditions is investigated in this study:

a) Chlorophyll fluorescence, a proxy field measurement of photosynthesis., is a rapid, non-destructive method for assessing seedling survival potential of numerous forest seedling species in response to stress (Perks et al. 2000, Black et al. 2005, 2008). Perks et al. (2004) demonstrated a rapid method for freeze tolerance using chlorophyll fluorescence of shoots ($F.LT_{50}$) following artificial freezing. This can potentially reduce the time to derive results to 3 days, when compared with up to 14 days for the more traditional visual assessment LT_{50} technique. We report on the further development of a quick, cheap, simplified $F.LT_{50}$ method, initially developed by Perks et al. 2004) for routinely assessing dormancy status of conifer crops under operational conditions over six seasons.

b) The use of a method to rapidly analyse volatile compounds in the airspace of storage bags to detect fermentation activity or ammonia build up, which is indicative of plant decomposition during storage, is reported. These techniques, together with simple chlorophyll fluorescence assessments, were developed with the aim of providing a cheap rapid test of seedling quality under nursery or field conditions.

Materials and methods

Description of nurseries

Bare root transplants were grown at Ballintemple nursery, Co. Carlow (52° 44' N, 06° 42' W, 100 m elevation) and Killygordon nursery Co. Donegal, Ireland (55° 15' N, 07° 35' W, 80 m elevation). The Ballintemple nursery soil is a brown earth with a pH of 5.7, 8–12% organic matter content and sand, silt and clay fractions of 66, 19 and 15%, respectively. The soils in Killygordon are also represented by a brown earth with a similar pH, 7-9% organic matter and a sand, silt and clay content of 69, 21, and 10%, respectively. Plants received monthly additions of nitrogen at c. 150 kg N ha^{-1} from April to July, with top dressings in July of P, K and Mg in the final year to achieve a final top height of 60 cm (c. 50 kg P, 100 kg K and 30 kg Mg ha^{-1}).

On each sampling date, seedlings of Sitka spruce (*Picea sitchensis* (Bong.) Carr.), Norway spruce (*Picea abies* (L.) H.Karst), lodgepole pine (*Pinus contorta* Dougl. ssp. *contorta*), Scots pine (*Pinus sylvestris* L.) and Douglas fir (Pseudotsuga menziesii (Mirb.) Franco) were lifted and graded as per normal forest nursery operational practice. Graded seedlings were placed in polyurethane coextruded bags (see different experiment's sample numbers below) and labelled prior to dispatch to University College Dublin (UCD).

Nursery climatic conditions

The air temperature at 2 m above the crops located at both nurseries was monitored using temperature loggers (Tingtag, Gemini data loggers, U.K.) to derive a measure of accumulated day degrees above 5 °C for May to December from 2006 to 2011 (O'Reilly et al. 2000). Data for Killygordon was only collected for the period 2006 to 2008. The number of cumulative chilling hours below 0 °C from May to December was also calculated as an indicator of ground frost frequency (Keane and Sheridan 2004).

Experiment 1: Evaluation and calibration of physiological indicators of seedling dormancy

Transplants from Ballintemple and Killygordon nurseries were lifted at 1 to 4 week intervals from September to April for the 2006/7 and 2007/8 seasons and dispatched to UCD for freeze tolerance and root electrolyte leakage (REL) assessments. On each sampling occasion, all physiological assessments were performed using eight seedlings per bag, replicated three times (three bags per sampling interval).

Standard cold hardiness tests (LT_{50})

To assess the accuracy and predictability of the newly developed fluorescence-based cold hardiness test ($F.LT_{50}$), a standard cold hardiness (LT_{50}) method, as described by (O'Reilly et al. 2000), was used as a comparison. This was conducted only on the Sitka

spruce Washington provenance plants. The sampling frequency of the standard LT_{50} test was reduced to once a month. First-order lateral shoots (10-15 cm long), representing the current year's growth, were excised from seedlings (n = 10) and placed in empty spectrophotometer curvette styrofoam holder trays (10 × 10 rows, CEL1060, Spark laboratory suppliers, Dublin) prior to freezing. Sets of trays were then subjected to a series of target freeze temperatures -4, -8, -12, -20 and -30 °C (10 shoots × 5 temperatures × 2 nurseries × 3 reps per sample point). The programmable freezer was set to cool from ambient room temperature (c. 20 °C) to the desired temperature at a rate of 6 °C h^{-1}. The freezer temperature was maintained at the target temperature for 3 h and then warmed at a rate of 10 °C h^{-1} to 10 °C. The series of freeze temperatures were selected to bracket the range causing 50% damage, as subsequently assessed by visual examination of needles. On removal from the freezer, the cut base of excised shoots were submerged in tap water by filling the styrofoam trays and placed in a controlled environment chamber (Vindon Scientific Ltd., UK) at 20 °C day/night, 16 h photoperiod, and an irradiance of 300 μmol m^{-2} s^{-1}. Visual assessments of needle damage were made after 14 days incubation in a controlled environment (Cannell et al. 1990). Scores were based on the degree of browning of the current year's needles, in increments of 10%. The temperature damaging 50% of the needles (LT_{50}) was estimated using the GLM procedure of SAS v 9.1. (after O'Reilly et al. 2000). The results of previous research has shown the validity of this approach for determining lifting windows for Sitka spruce and other conifer species (O'Reilly et al. 1999, 2000).

Fluorescence-based cold hardiness (F.LT_{50}) assay

Modifications to the original quick freeze tolerance method described by Perks et al. (2004) included:

a) The use of domestic chest freezers instead of programmable freezers, thereby reducing setup costs, but re-producing a freeze profile similar to that obtained using programmable freezers.

b) A reduction in the number of target freeze temperatures required to determine LT_{50}. Domestic chest freezers were set up to maintain a constant temperature of -4, -12 and -30 °C. The series of target temperatures were reduced to simplify the method, reduce labour costs and turn-around time, which is an important pre-requisite for the establishment of a routine operational assay. These specific temperatures where selected to bracket the range causing 50% damage over the hardening phase for the establishment of hot lifting and lifting for cold store windows using the thresholds described by O'Reilly et al. (2000, 2002).

c) Control of cooling and heating rates in domestic freezers.

A series of tests were conducted to manually control the cooling and heating rates

similar to those obtained with the programmable freezer. This was achieved by placing the shoots (in Styrofoam holder trays, as described above) in 50 mm thick Styrofoam boxes with internal dimensions of 19 (l), 21 (b) and 26 cm (h) (provided by Sigma Chemicals, U.K.) prior to the freezing treatment. The temperature inside the Styrofoam boxes was monitored using temperature loggers. A separate box of shoots was used for each target temperature. The cooling and heating rates within the freezing/melting point range were reduced by adding 50 ml of tap water to each Styrofoam box before freezing. A cooling rate of <6 °C h^{-1} was achieved, which reduced the formation of large ice crystals in tissues (Figure 1). All boxes were placed in the freezer destined for freezing to -4 °C overnight; this freezer had been switched off and allowed to warm-up to ambient temperature. A timer switched the freezer on at 4.30 am, which cooled the samples to c. -4 °C by 9 am. At this time, one box was left at -4 °C for 4 h while the other two were transferred to the -12 or -30 °C freezer for 5 and 7 h, respectively (Figure 1). Once the target temperature was maintained for c. 3 h, the -12 or -30 °C treatment boxes were transferred back to the -4 °C freezer for 1 h, removed from the freezer and allowed to warm up at ambient conditions in the lab. (c. 20 °C). Trays containing the shoots were removed from the boxes, filled with tap water and incubated in the controlled environment chamber at 20 °C for a minimum of 4 h in the light before fluorescence assessments were performed.

Estimation of $F.LT_{50}$

Dark-adapted maximal fluorescence (Fv/Fm) was determined within 24 h of the freeze

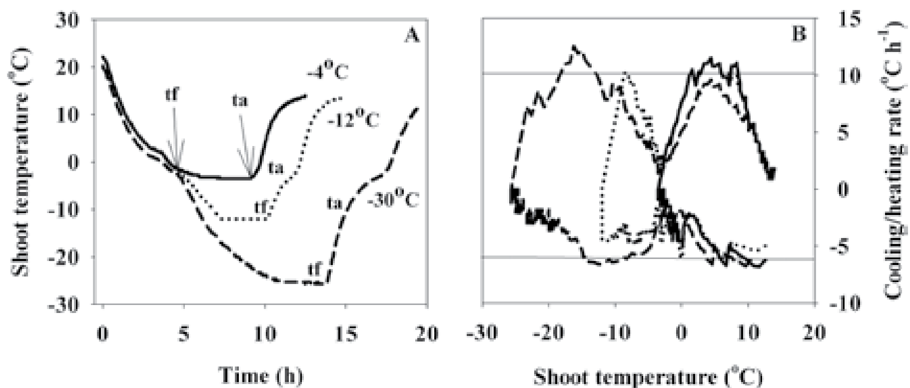

Figure 1: *The final freeze profiles (A) and cooling/warming rates as a function of shoot temperature (B) for different target temperatures (-4 (solid line), -12 (dotted line) and -30 °C (short dashed line), using the modified cold hardiness methodology. The symbol* ***tf*** *indicates when the boxes for the -12 and -30 °C target temperatures were transferred from the -4 °C freezer to respective freezers and returned after 5 to 7 hrs. The symbol* ***ta*** *indicates were boxes were removed from the -4 °C freezer and left at room temperature. The solid grid lines in Figure 1 B indicate the desired maximum cooling and heating rates during the freezing protocol.*

treatment using a modulated fluorimeter (Hansatech PEA2, Hansatech Instruments Ltd, Kings Lynn, UK) as described by Perks et al. (2004). An empirical equation was used to predict cold tolerance using dark-adapted (Fv/Fm) fluorescence measurements (Perks et al., 2004). In order to allow for the operationalisation of the Fv/Fm method, Perks et al. modified the original equation published by Fisker et al (1995). This was done by assuming that the Fv/Fm at which LT_{50} occurs (i.e. $F.LT_{50}$) at a threshold fluorescence value of approximately 0.43-0.59, depending on species (Eq. (1), Table 2), as evidenced from experimental data (see Figure 2). The $F.LT_{50}$ was determined using the following formula (Perks et al. 2004):

$$F.LT_{50} = \frac{F50 - F2}{\beta} + T2 \tag{1}$$

The Fv/Fm value at which 50% (F50) of the shoots died was determined using regression analysis (Figure 2). F2 is the mean Fv/Fm value for shoots (n = 10) subjected to the lowest freeze temperatures (usually -12 or -30 °C depending on extent of cold hardiness), T2 is the freeze temperature (-12 or -30 °C).

$$\beta = \frac{F1 - F2}{T1 - T2} \tag{2}$$

where F1 is the mean Fv/Fm value for shoots (n = 10) subjected to a sub-lethal temperature and T1 is the sub-lethal temperature (-4 or -12 °C).

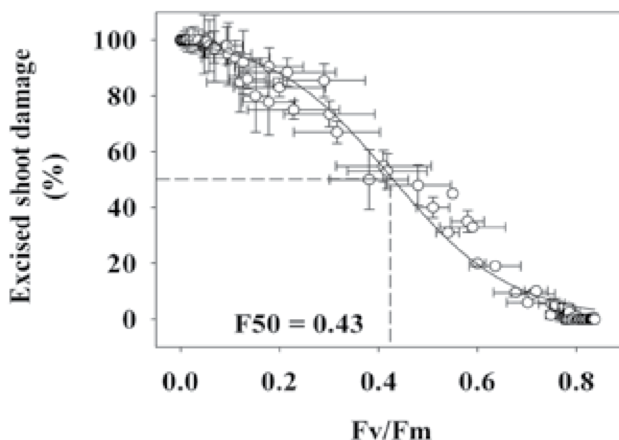

Figure 2: *Relationships between mean fluorescence (Fv/Fm) and visual assessments of needle browning from grouped means (n = 30) over all sample dates lift dates and freeze temperatures from both the Ballintemple and Killygordon nurseries over the 2006/7 season (using paired mean data).*

Table 1: *Species and provenances sampled for F.LT50 assays over the period 2006 to 2011. The threshold fluorescence at which 50% of samples do not survive (F50) value used in Eq. 1 was obtained from the overall regression of grouped data for each provenance of species in the form of a 3 parameter sigmoidal function.*

Species	Provenance	Seed source	F50
Sitka spruce	Washington	SW-IERATH-A140	0.43
Sitka spruce	QCI	SQ-DKFP622-E12	0.45
Sitka spruce	Oregon	SR-ORO53-W72	0.47
Lodgepole pine	Northern	LN-UKNT15-J60	0.49
Lodgepole pine	Southern	LS-IEGY-J27	0.52
Norway spruce	Lund	NS-DKLUN-J65	0.59
Scots pine	Scottish	SP-IEWX-F57	0.53
Douglas fir	Washington	DF-WA030-H79	0.50

Root electrolyte leakage (REL)

Measurements of REL were determined on excised fine roots (<2 mm diam., fresh mass 300–500 mg) of three plant replicate bags (10 seedlings per bag) for each lift date. The relative conductivity method of Wilner (1955) was used to determine REL, following the modifications of McKay (1992).

Experiment 2: Seasonal patterns for dormancy acclimation and dehardening for six conifer species

Following calibration of the quick F.LT$_{50}$ methodology, additional assessments were performed using six different conifer crops from 2006 to 2011 (See Table 1). For these assessments, all of the crops were grown at Ballintemple nursery under the same conditions as those described under the seedling material section. Sampling was limited to this nursery for the time series due to logistical reasons and because of the limited numbers of species produced at the Killygordon nursery. The assessments were made on three provenances of Sitka Spruce, QCI, Washington and Oregon material. Two provenances of lodgepole pine were also tested over the same period. Characterisation of the threshold fluorescence vales was determined for each species (Table 1). On each sampling occasion, six to eight seedlings were used from each of three replications (bags).

Experiment 3: Indicators of quality deterioration in the field

The potential impact of plant quality status on seedling performance was assessed using Sitka spruce (2+1) of Washington provenance (seed lot SW-IERATH-A140) samples at Ballintemple nursery. Cold stored seedlings (72 bags of 50 seedlings each), lifted at Ballintemple nursery, were graded, packed in polyurethane coextruded bags

and cold stored (at 1 to 2 °C) in January 2005, were dispatched to UCD in March 2006 for the storage deterioration experiment. Tap water (2 l) was poured over seedlings in half of the bags to simulate the lifting and packaging of wet seedlings. Each bag was sealed in a second polyurethane coextruded bag before dispatch to UCD to minimise the likelihood of puncturing and loss of air from the inside of the bag during transport and handling.

Bags set up in a factorial design with soaked (wet) and non-soaked (dry) bags, incubated at 5, 10 and 20 °C in controlled environment chambers (Vindon Scientific Ltd., UK) for a total of six treatment combinations (2 bag moisture × 3 temperatures). The inside temperature of the bags was recorded using temperature loggers (Tingtag, Gemini data loggers, U.K.). Three bags were sampled at 0, 1, 5, 10, 14, 21, 25 and 32 days after initial storage for REL, Fv/Fm, visual needle damage assessments, seedling survival and quantification of volatile compounds.

Seedling survival

Sampled seedlings from the experiment were planted into 2 l pots containing a 3:1 (v/v) mixture of peat compost and perlite and placed in a controlled environment chamber (Vindon Scientific Ltd., UK) at 20/16 °C day/night, 16 h photoperiod, and PPFD of 300 mmol m^{-2} s^{-1}. Pots were regularly saturated with tap water and survival was recorded after 3 weeks. Plant condition was assessed using a plant health index based on needle browning, for the whole plant (value range: 100% = brown, presumed dead to 0% = completely green, presumed healthy).

Volatile compounds

A replicate set of treated bags from the experiment (described above) were sampled at the same time intervals for volatile ethanol and ammonia assessments using the Gastec detector tube system (Gastec Corporation, Ayase City, Japan). Ethanol is a product of fermentation resulting from the breakdown of sugars and has been suggested to be indicative of deterioration of seedling tissues during storage (Steve Colombo, pers. comm. 2005). The ethanol concentration of air in the bag head space was measured using Gastec 112L tubes with a detection limit of 5 ppm over a range of 50 to 200 ppm in air and an accuracy tolerance of 25% using the Gastec multi-stroke gas sampling pump (Model GV 100S, Gastec) as described by the manufacturer. Ammonia, a breakdown product of plant proteins during decomposition, was also measured using Gastec 3L tubes with a detection limit of 0.2 ppm over a range of 0.5 to 78 ppm. Air in sealed bags was sampled by puncturing the coextruded bag with the tip of the sample tube and extracting 100 to 200 cm^3 air using the GASTEC pump, depending on type and concentration of the volatile compound (see 112L and 3L product information charts www.Gastec.co.jp). Holes in the bags created by the insertion of tubes were sealed with insulation tape after each sample was taken.

Statistical analyses and data presentation
Percentage and index scores for REL, needle damage and plant survival values were transformed to arc sine, square root values prior to analyses. Concentration values for volatile compounds were log transformed to normalise the data before ANOVA analysis.

The effects of soaking, incubation time, incubation temperature and their interactions with mortality, REL, volatile organic compounds and Fv/Fm during storage was analysed using Fisher's PSLD method of analysis of variance (ANOVA) in SAS v.9.1 (SAS Institute Inc., Cary, NC). Significance levels quoted are at $p < 0.05$. The relationship between the physiological measures and seedling survival was evaluated using the regression procedure in GLM of SAS v.9.1 (SAS Institute Inc., Cary, NC).

Non-linear regression analysis of Fv/Fm and seedling condition was performed on un-transformed data using a three parameter sigmoidal function using SigmaPlot (v 8.0, SSPS Inc. USA).

Analysis of REL and $F.LT_{50}$ values of seedlings lifted from Ballintemple and Killygordon nurseries for the 2006/7 season was compared using Fisher's PSLD method of ANOVA in SAS. Comparison and regression analysis of conventional LT_{50} and $F.LT_{50}$ mean values were conducted using polynomial regression and residual analysis (SigmaPlot v. 8.0, SSPS Inc. USA).

Results

Experiment 1

A. Validation and calibration of the rapid $F.LT_{50}$ technique

An important calibration of the rapid $F.LT_{50}$ method was the determination of the Fv/Fm value at which 50% (F50) of the shoots died for the parameterisation of equation 2 (see Materials and methods). A F50 value of 0.43 for the Washington provenance of Sitka spruce was obtained by regression analysis of needle damage scores following freezing and chlorophyll fluorescence assessments of shoots taken from seedlings lifted at Ballintemple and Killygordon over the 2006/7 season (Figure 2). The relationship between shoot damage and Fv/Fm across different freeze temperatures did not vary when values for shoots from Killygordon or Ballintemple were compared.

Estimates of cold hardiness using the rapid (3 days) Fv/Fm-based assessment of needle damage following freezing in domestic freezers ($F.LT_{50}$) compared well with conventional visual needle damage assessments (LT_{50}), performed 14 days after freezing in the programmable freezer (Figure 3). The partial coefficients (i.e. the slope and y-intersects) of the linear relationship between LT_{50} and $F.LT_{50}$ estimates did not vary when values for seedlings taken from Killygordon and Ballintemple were

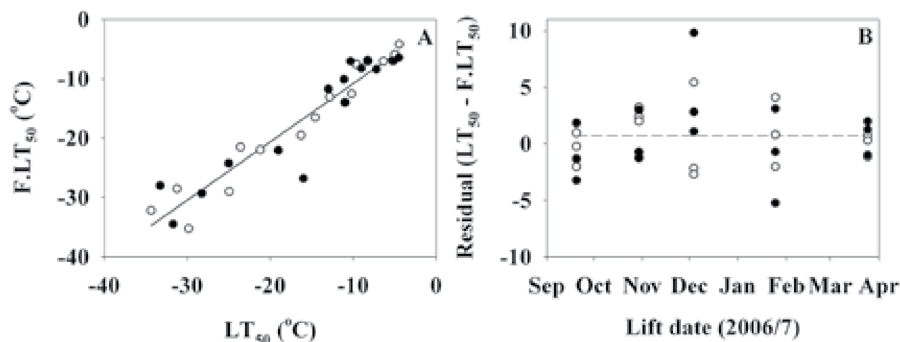

Figure 3: *A linear regression showing the comparison of conventional visual assessments (LT50) and instantaneous Fm/Fm estimates (F.LT50) of cold hardiness (A) and comparison of residuals as a function of lift date (B) for seedlings lifted from Ballintemple (○) and Killygordon (●) nurseries during the 2006/7 season. The solid regression line (A) indicates a significant relationship (r2 = 0.91, p < 0.001, slope = 1.03, SEE = 3.1). The broken regression line (B) for the residuals over time was not significant (r2 = 0.002, p = 0.87).*

analysed separately and compared. The F-values (156 and 169) and standard error of estimate (3.5 and 3.3%) for data derived from the two nurseries were also similar. Therefore, data from both nurseries were pooled for combined regression analysis (Figure 3). Regressions and residual analysis suggested that F.LT_{50} was neither under- nor over-estimated, when compared to conventional LT_{50} assessments, in different nurseries and across different lift dates.

B. Comparison of physiological indicators of hardiness acclimation

REL values of lifted seedlings showed significantly different trends over the 2006/7 season at each nursery (Figure 4A). REL for seedlings lifted at Ballintemple nursery was below the suggested hot lift threshold value of 25% for the entire 2006/7 season. In contrast, REL values of seedlings from Killygordon were generally above 25% until the last week of December 2006 (Figure 4A). REL values of seedlings from both nurseries did increase slightly prior to the onset of bud break in early May, but these values were not significantly different to those in measured in late March 2006 (Figure 4).

Comparison of REL compared to F.LT_{50} results in 2006, suggested contrasting hardiness acclimation and lift dates for crops from the different nurseries. These F.LT_{50} results suggest that both the Ballintemple and Killygordon crops were suitably acclimated for lifting and cold storage in late November 2006. In contrast, REL results suggest that the crop from Ballintemple was suitable for cold storage from late September, compared to late December for the Killygordon crop.

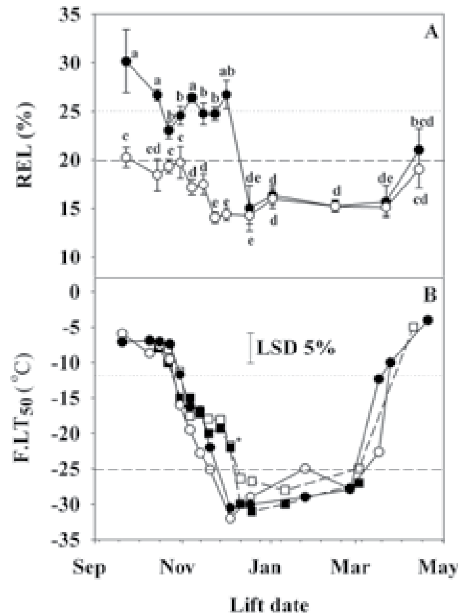

Figure 4: *Cold hardiness development of Sitka spruce at time of lifting from Ballintemple (clear symbols) and Killygordon (black symbols) nurseries for the 2006/7 (circle symbols) and 2007/8 (square symbols) season as determined using REL (2006/7 only, panel A) and F.LT50 assessments (B). All symbols represent a mean and standard deviation (n = 30). REL symbols with different letters are significantly different at p ≤ 0.05. The grid lines across the y axis indicate the suggested % REL and LT50 threshold values for hot lift (dotted lines) and cold storage (broken lines) (after O'Reilly et al 2000, 2002). For panel B, individual error bars could not be displayed because this impeded on the clarity of the presented data. Therefore, LSD error was used to represent the mean error of all the measurements. The asterisk indicates (*) a different lift date for cold storage in the 2006/7 and 2007/8 seasons.*

Estimates of cold hardiness acclimation and de-acclimation, based on $F.LT_{50}$, were similar at both nurseries (Figure 4B). However, the data do show that both crops exhibited different hardening acclimation trends in 2007/8, compared to 2006/7, particularly at the onset of suitability for cold storage (i.e. $F.LT_{50}$ <-25°C). Crops lifted in 2006 were sufficiently acclimated for cold storage by early November, compared to mid-December in 2007. In early December, seedlings were c. 10°C less hardy in 2007 than in 2006 (Figure 4B). The later hardiness acclimation response in 2007, compared to 2006 was associated with the lower accumulated day degree temperature, and particularly the lower cumulative chilling hours experienced in 2007 (Table 2). The number of air frost hours, as indicated by the cumulative chilling hours <0 °C was c. three-fold lower in 2007, compared to 2006.

Table 2: *Inter-annual variations in temperature at nursery sites.*

Year	Nursery	Cumulated day degrees > 5 °C	Cumulated chilling hours < 0 °C
2006	Ballintemple	1,753	31
	Killygordon	1,503	27
2007	Ballintemple	1,552	12
	Killygordon	1,421	9
2008	Ballintemple	1,652	25
	Killygordon	nd	nd
2009	Ballintemple	1,633	56
	Killygordon	nd	nd
2010	Ballintemple	1,489	987
	Killygordon	nd	nd

Experiment 2:

Inter-annual and species variations in dormancy status.

Figure 5 shows the seasonal and inter-annual variation of the different species over six successive seasons for Sitka spruce (SS) and five seasons for the other conifer species (2007 to 2011). For some years, species could not be sampled because crops were lifted for sale at Ballintemple nursery. There were noticeable differences in species dormancy induction responses. Generally, Norway spruce (NS) and Douglas fir hardened off earlier than pine (lodgepole (LP) or Scots pine (SP) species and Sitka spruce (i.e. NS >DF >LP >SP >SS for hot lift). The onset of hardiness sufficient for cold storage is, however slightly different (i.e. NS > SP > LP > SS > DF).

Estimates of cold hardiness acclimation and de-acclimation, based on F.LT50, showed different seasonal trends for lifted crops (Figure 5). The most notable differences occurred in the Sitka spruce crops in 2006/7 and 2010/11. The data show that the Sitka spruce crops exhibited different hardening acclimation trends in 2006/7, compared to 2010/11, particularly at the onset of suitability for cold storage (i.e. LT50 <-25 °C). Crops lifted in 2006 were sufficiently acclimated for cold storage by late December, compared to early November in 2010. In early December, seedlings were c. 10 °C less hardy in 2006 than in 2010 (Figure 5). Ireland experienced the coldest winter in 40 years in 2010, characterised by early onsets of frost and extended period of snow from early December to January. The later hardiness acclimation response in 2006, compared to 2010, was associated with the lower accumulated day degree temperature, and particularly the lower cumulative chilling hours experienced in 2010 (Table 2).

The pattern of cold hardiness acclimation and de-acclimation differed among provenances (Figure 6). Generally, the northern provenances tended to harden off sooner than the more southern provenances. For example, the Oregon (SR) Sitka spruce provenance hardened off about a month later than the QCI provenance.

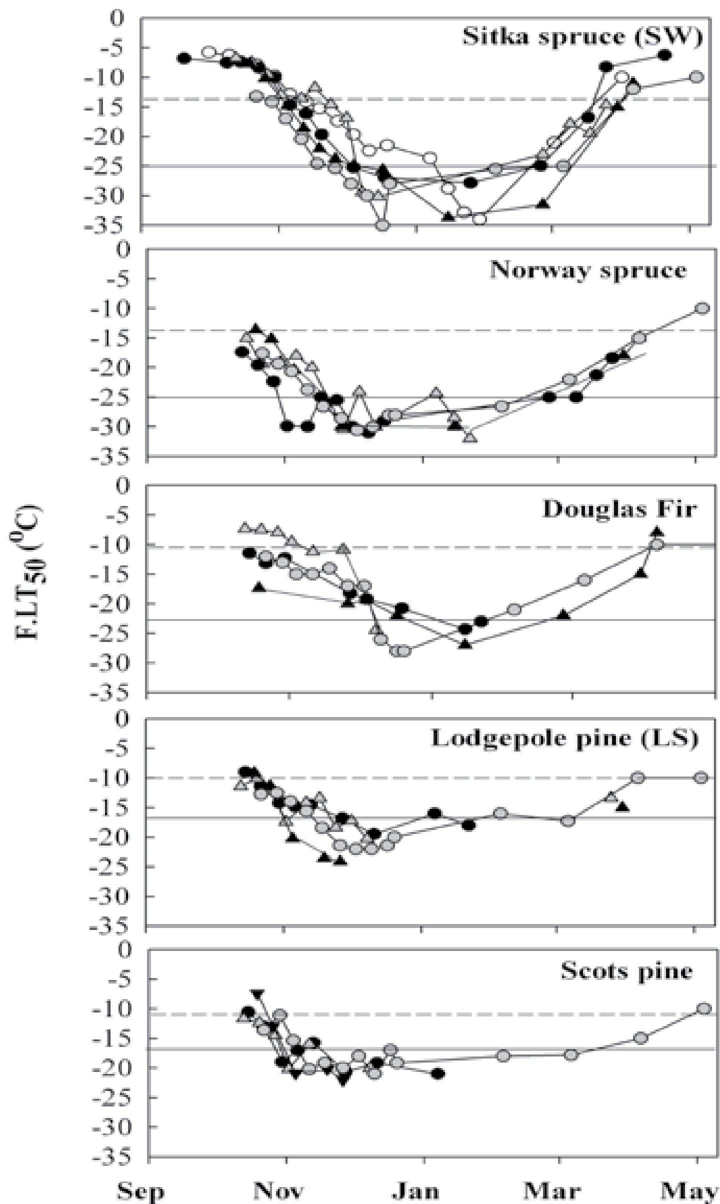

Figure 5: *Seasonal and inter-annual variations in hardiness levels for different species and provenances from 2006 to 2011. Each symbol is a mean value and the vertical bar at top of each graph is the overall error mean square. Symbols for different seasons are 2006/7 (○), 2007/8 (●), 2008/9 (▲) 2009/10 (▲) and 2010/11 (●). Dashed line presents the hot lift threshold and the solid line is the cold store threshold applied at commercial nurseries.*

Experiment 3:

Comparisons of quick indicators of seedling quality during storage

Bare root seedling survival and needle damage was significantly influenced by ambient storage temperature, the presence of water in bags and the duration of incubation following cold storage (Figure 7 and Table 3). Seedlings stored in wet bags and at higher temperatures (10 and 20 °C) deteriorated at a faster rate than those stored in dry bags and those stored at 5 °C (Figure 7). The Fv/Fm was also significantly influenced by ambient storage temperature, the presence of water in bags and the duration of incubation (Figure 8 and Table 3).

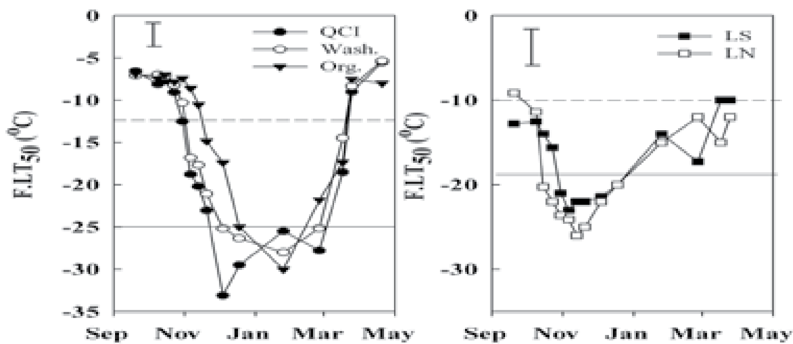

Figure 6: *Seasonal and inter-annual variations in dormancy status for different provenances of Sitka spruce; Washington (SW), Queen Charlotte Island (QCI) and Oregon (SR), Northern (LN) and Southern provenances of Lodgepole pine. All symbols represent a mean value for all years tested (2006-2011). The overall standard error (SE) for all dates is shown, but to improve the clarity of presentation, individual SEs are not shown. Dashed line presents the hot lift threshold and the solid line is the cold store threshold applied at commercial nurseries.*

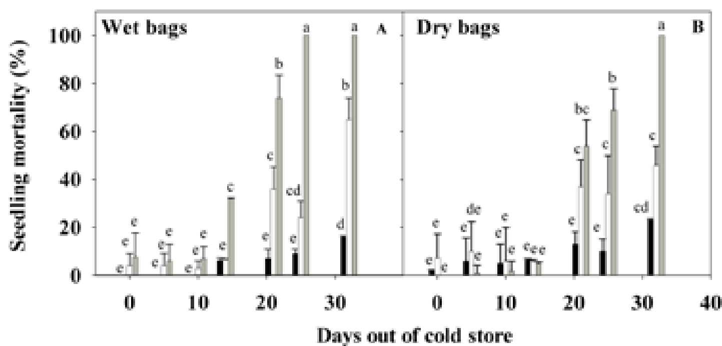

Figure 7: *The effect of bag moisture (dry or wet bags), storage temperature (5 °C (■), 10 °C (□) and 20 °C (■)) and storage duration on seedling mortality following removal from cold storage. Histograms and error bars represent a mean and standard deviation of 3 replicate sample bags (10 seedling samples per bag). Histograms with different alphabetical letters are significantly different at p ≤ 0.05.*

Table 3: *Sources of variation of different physiological parameters, expressed as F-ratios, for the three different storage temperatures, bag treatments (wet and dry) and storage durations.*

Variable	**Treatment**			**Interactions**			
	Storage duration (D)	**Incubation temperature (T)**	**Wet/Dry treatment (W)**	**D × T**	**D × W**	**T × W**	**D × T × W**
Mortality	36***	25**	4*	8**	5*	3*	6*
Needle damage	78***	34**	9**	14**	7**	5*	12*
REL	2	5*	<1	2	<1	<1	<1
Fv/Fm	49***	29**	5*	16**	5*	10*	7*
Ethanol	3*	4*	<1	<1	<1	<1	<1
Ammonia	<1	<1	<1	<1	<1	<1	<1

F-ratio with an asterisk was significant at * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Chlorophyll fluorescence appeared to be the best predictor of mortality and seedling deterioration during storage after removal from cold storage. There was a significant correlation ($p \leq 0.001$) between seedling mortality and estimates of maximum, dark-adapted, quantum efficiency (Fv/Fm) of shoots (Figures 7 and 8). In contrast, seedling mortality was not significantly correlated ($p > 0.1$) with REL values or volatile compound concentrations in the airspace of stored bags (Figure 7 and 8). The pattern of REL response varied with storage treatment. REL for those stored at the low temperature (5 °C) stayed about the same or decreased during storage. For seedlings stored at higher temperatures, REL increased slowly or decreased during the early storage period, but values increased towards the end of the storage period. REL increased more quickly in seedlings stored under wet conditions. Volatile ethanol concentrations in the stored bag air space were influenced by storage duration and temperature (Table 3), but concentrations varied and were erratic over the duration of the experiment. Ammonia was detected in stored bags on two occasions only.

Discussion

Quick detection of seedling quality deterioration in warm storage

We demonstrate that assessments of maximal potential quantum efficiency (Fv/Fm) can provide a robust indicator of seedling quality following storage (see Figure 9). The equipment is portable, easy to use and numerous measurements can be taken within a 30 min following an initial dark adaptation period. A major limitation regarding the use of this technology in the past was the prohibitive cost. However, low cost portable fluorimeters are now available for c. €2,000 depending on manufacturer and functionality. Fv/Fm values below 0.6 are generally associated with about 20 to 25%

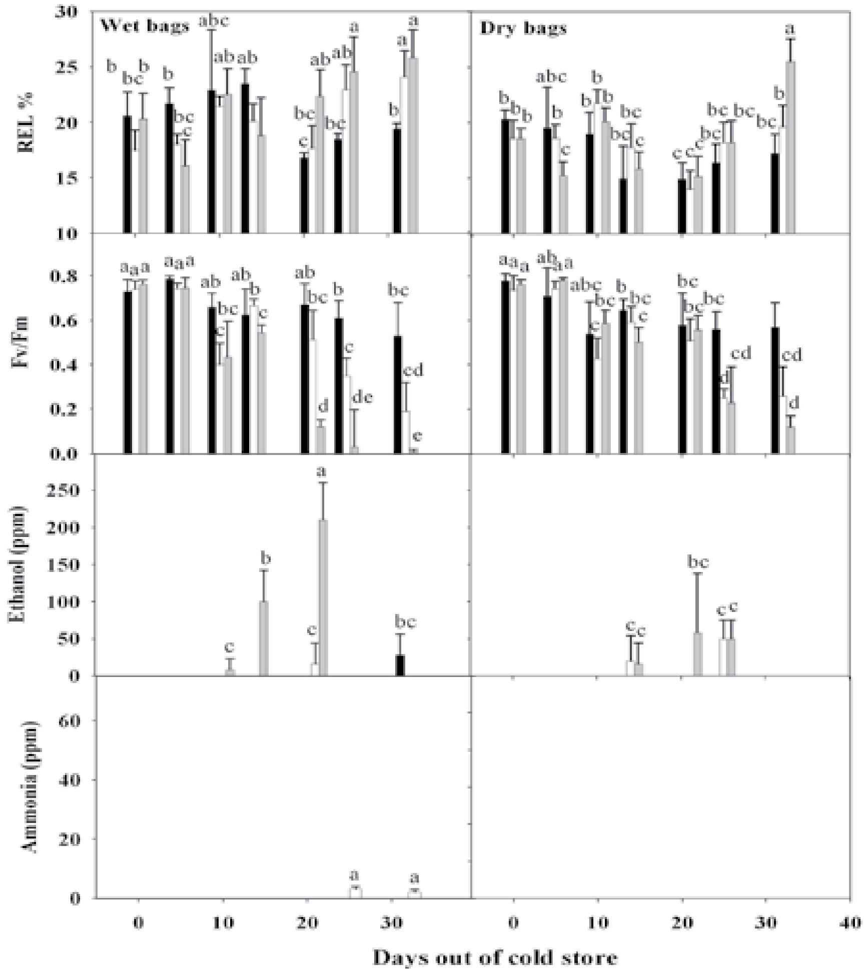

Figure 8: *The effect of bag moisture (dry or wet bags), storage temperature (5 °C (■), 10 °C (□) and 20 °C (■)) and storage duration on root electrolyte leakage (% REL), shoot maximum potential quantum yield (Fv/Fm), volatile ethanol and ammonia concentrations following removal from cold storage. Histograms and error bars represent a mean and standard deviation (n=10 per rep) for REL and Fv/Fm determinations). Means with the same letters are not significantly different at $p \leq 0.05$.*

mortality in bare root Sitka spruce stock that has grown under close to ideal climatic conditions after planting. This threshold is consistent with other recommended Fv/Fm thresholds for seedling survival of conifer species (Perks et al. 2001, Black et al. 2005; Colombo 2005). However, the relationship between Fv/Fm and mortality can

vary considerably depending on climatic conditions following out-planting (Black et al. 2005) and seedling morphological condition such as shoot height or shoot to root ratio (O'Reilly and Keane, 2002).

The reliance on a single measure of seedling quality may not be advisable (Puttonen 1996), so the REL technique and a volatile compound accumulation were also assessed. Contrary to expectation, there was no relationship between seedling survival and REL in response to warm storage stress. Similar results have been reported for Douglas fir (Harper and O'Reilly, 2000) and oak (Carbral and O'Reilly 2005). The observed REL trends for warm stored seedlings following removal from cold storage followed a tri-phasic pattern (see Figures 4 and 8); 1) an initial increase due to deterioration of membrane function and higher metabolic activity after removal from cold store (Mc Kay, 1992; Harper and O'Reilly, 2000); 2) followed by a decline in REL after 10 days possibly due to the exhaustion or redistribution of electrolytes in the plant (as suggested by Carbral and O'Reilly, 2005) and 3), a subsequent loss of membrane permeability/ integrity or root cell lyses and release of metabolites and ions required cell maintenance after prolonged storage resulting in high REL values after extended storage (20 to 30 days). Black et al. (2008) suggest the use of a combined quality index using both physiological (e.g. Fv/Fm and REL) and morphological parameters (e.g. shoot to root ratio and sturdiness) when assessing stored seedling suitability for out planting. These authors have demonstrated in field trials that combined quality indices correlate better with field survival following out planting than estimates based on a single parameter.

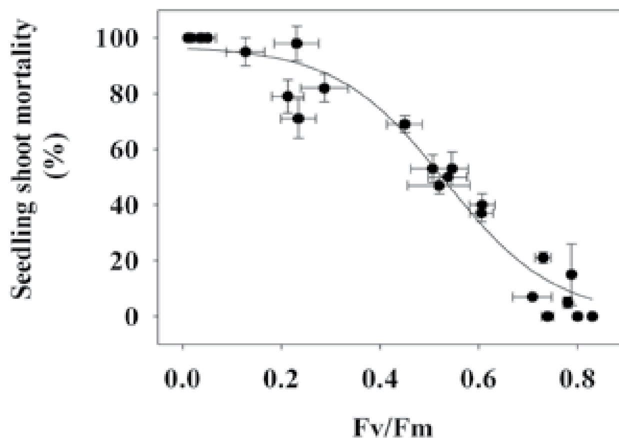

Figure 9: *The relationships between mean fluorescence (Fv/Fm) and mortality of Sitka spruce seedlings, grouped (n = 30) over all sample dates during the storage experiment (experiment 3). Symbols correspond with data as shown in Figures 1 and 2 (r^2 = 0.96, SEE = 7.9). All partial coefficients and the overall regression coefficient were significant at p ≤0.001).*

This paper also reports on the development of an on-site test for seedling deterioration in co-extruded bags following dispatch to site. Although it was possible to detect ethanol and ammonia in the bag air space, these trends were also not consistent with seedling mortality, underlying the weakness of this technique. We suggest this technique does, however, offer potential if the following technical difficulties can be overcome. Firstly, loss of air from the bag head space during handling or puncturing of bags results in the loss of volatile stress compounds. Secondly, compounds such as ethanol can be further metabolised to other end-products, perhaps explaining the responses shown in Figure 8. Finally, the detection limit of the Gastec tubes may be too low for the detection of ammonia. A possible solution to these technical problems, and an avenue worth investigation, may involve the use of Gastec Dosi-tubes (Gastec Corporation, Ayase City, Japan), which can be placed in bags at packaging to detect low levels of stress type compounds over long time periods (2 to 3 days).

Rapid establishment of lifting windows

There was good agreement between the modified freeze tolerance methodology ($F.LT_{50}$) and traditional visual LT_{50} technique. Seasonal hardening acclimation trends observed for Sitka spruce using the rapid $F.LT_{50}$ technique over the 2006/7 and 2007/8 seasons (Figure 4B), were similar to those reported for Sitka spruce (Washington and Oregon origins) from Ballintemple nursery for the periods 1992 to 1995 (O'Reilly et al. 2000) and for QCI lifted in the U.K. (Cannell et al. 1990), using conventional LT_{50} and other physiological methods.

The methodology described in this paper can be implemented in operational nurseries to provide a rapid assessment of hardiness acclimation for establishment of suitability for lifting operations. Routine testing of Sitka spruce and other conifer species has been implemented at Ballintemple nursery since October 2007. The advantage of this technique over other assessments of hardiness acclimation is that results are available within 3 days, compared to 14 days for the root growth potential or conventional visual assessments of cold hardiness. Furthermore, the method is cheap since it involves use of domestic freezers to generate freeze profiles.

Whilst there are other alternatives to the $F.LT_{50}$ approach for estimating dormancy status under operational conditions, few are robust or rapid enough to be routinely used in an operational context. Recent developments in cDNA based techniques (for review see Howe et al. 2003, Thomashow 1999) have resulted in the launch of a commercial service (e.g. Nsure; www.afsg.wur.nl) that can provide and independent measure of cold tolerance within 2 days of sample receipt, but it might take another 1-2 days for the samples to reach the laboratory after dispatch from nursery. Cheaper and rapid microscopic assessment of mitotic index (Grob and Owens 1994, O'Reilly

2000) and REL (McKay 1993, O'Reilly et al. 2000), have been used to determine safe lifting "windows" but trends were not entirely consistent with other physiological parameters tested in this and other studies (O'Reilly et al. 2000). A possible alternative involves freeze tolerance assessments using REL measurements of thawed shoots (Colombo, 2005). The advantage of this technique is that it could be applied to deciduous species. The downside is that the assay may take 12 to 24 hours longer to perform when compared to the Fv/Fm-based assessment.

A tool for investigating ecophysiological control of hardiness acclimation?

Our data support the view that variations in the physiology of hardiness acclimation from season to season are dependent on climatic factors such as cumulative day degrees >5 °C and cumulative chilling hours <0 °C. These findings are consistent with the findings of O'Reilly et al. (2000), Greer (1983) and Burr et al. (1989). The early stages of hardiness acclimation are thought to be strongly influenced by warm temperatures and photoperiod, while the later stages are believed to be more heavily dependent on low temperatures (Greer 1983; Burr et al. 1989). Other factors (e.g. nutrient and water availability) and the interactions of all factors may also play a role in the hardiness process (Colombo et al. 2001). Different species may display a similar pattern of hardiness acclimation most years, probably because the main drivers (photoperiod and temperature) closely track each other most years. However, some species may respond more strongly to photoperiod than other cues, but differences may be manifested in certain years only. In studies conducted in Ireland for example (O'Reilly et al. 1999, 2000), both Douglas and Sitka spruce sampled from the same nursery showed similar patterns of hardiness development over several years, but differed during a mild autumn. Both species hardened off later during the mild year than in other years, but Douglas fir seedlings did not harden beyond -15 °C for about 5 weeks from late October until early December, which was about the time that chilling temperatures began to accumulate. In contrast, Sitka spruce continued to harden off during this 5-week period. This observation that Douglas fir hardens off initially primarily in response to photoperiod is consistent with previous findings (Burr et al. 1989). Some of the species response difference described in this study may reflect similar ecophysiological response differences. This presents a challenge for nursery operations, especially given that "atypical" patterns of hardening may become more common as a result of climate change. Therefore, more research is needed to elucidate the relationship between these factors and hardiness development, especially the role of chilling and warm temperatures. To this end, the rapid freeze tolerance test, as described in this study, may be useful for the routine characterisation of physiological acclimation in response to different climates.

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