

Screening for efficient cold hardening in a breeding population of *Salix* using near infrared reflectance spectroscopy

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Abstract – The inheritance of cold hardening components – the timing of onset and the inherent rate – was studied in *Salix* spp. This was achieved by characterising the F₂ population of a cross between an early-and-rapidly hardening clone and a late-and-slowly hardening clone. The cold hardiness of stems was estimated using the infrared reflectance spectra of dried and homogenised samples. This method was first calibrated against the freeze test method. The timing of growth cessation was used to determine the onset of cold hardening. In the F₂ progeny, traits were partly recombined as indicated by the occurrence of clones with early-and-slowly hardening characteristics. The frequency distributions of clones also indicated that the timing of onset and the inherent rate of hardening were independently inherited traits. None of the clones exhibited the desirable late-and-rapidly hardening characteristics, combining a long growing period with effective cold hardening. This is not surprising since few F₂ clones exhibited late hardening.

cold hardiness / growth cessation / near infrared spectroscopy / *Salix* / tree breeding

Résumé – Criblage d'une population de *Salix* pour une acclimatation au froid optimale au moyen de la spectroscopie de réflectance en proche infrarouge. La transmission des facteurs d'acclimatation au froid – le délai d'action et le taux d'acclimatation intrinsèque – a été étudiée chez *Salix* spp. Ceci a été effectué par caractérisation d'une population F₂ issue d'un croisement entre un clone à acclimatation précoce et rapide et un clone à acclimatation tardive et lente. L'acclimatation au froid des tiges a été estimée à partir du spectre de réflectance infrarouge d'échantillons séchés et homogénéisés. Cette méthode a tout d'abord été calibrée par comparaison avec la méthode de test par congélation. Le minutage de l'arrêt de la croissance a servi à déterminer le délai d'acclimatation. Dans la génération F₂, les caractères étaient partiellement recombinants, comme indiqué par l'occurrence des clones présentant une acclimatation précoce et lente. Les distributions de fréquence des clones ont montré également que le délai d'action et le taux d'acclimatation intrinsèque étaient transmis de façon indépendante. Aucun clone n'a présenté les caractéristiques recherchées, associant une longue période de croissance avec une acclimatation au froid optimale. Ceci n'est pas une surprise étant donné que peu de clones F₂ montraient d'acclimatation tardive.

acclimatation au froid / arrêt de croissance / spectroscopie du proche-infrarouge / *Salix* / amélioration génétique des arbres

1. INTRODUCTION

In Europe, fast-growing willows (*Salix* spp.) are increasingly being used for biomass production [15]. In northern areas such production is limited by injuries inflicted as a result of autumn frost [9]. In *Salix*, as with most other woody species, cold hardening does not begin until growth has stopped in response to the reduction in day length [17]. The small variations in cold hardiness that plants display while actively growing seem to be phenotypic, rather than genetic in origin [13]. Northern ecotypes of *Salix* cease to grow earlier – at longer day lengths – than the southern ones [12], as do other woody species [10]. This linkage between growth and hardening cycles imposes restrictions on the extent to which either of these factors can be improved in isolation [19, 22]. However, we have previously

presented evidence that cold hardiness is also affected by differences in the inherent rate of cold hardening [13, 14]. Some northern and continental ecotypes have the ability to undergo cold hardening up to three times as quickly as typical southern and maritime clones. This suggests that autumnal cold hardiness can be improved without adversely affecting growth by selecting for the combination of late commencement and a rapid rate of hardening. None of the clones tested so far, however, have displayed this combination. Breeding could create the desired combination, provided that the underlying traits are independently inherited.

Levels of cold hardiness can be evaluated practically by field trials. However, such trials cannot discriminate between the two factors contributing to hardening, namely the inherent rate and the timing of onset. This is because genotypes that begin

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to harden late in the autumn will experience lower hardening temperatures than those starting early. Hardening is a temperature dependent process, with a temperature optimum at around 10 °C at the start, but at lower temperatures during later stages [14]. A few studies using other woody plants suggest that there is variation in the inherent rate [1, 7], but none of them have been carried out under controlled conditions. In potato, however, genetic variation in the rate of hardening has been confirmed [25].

Under controlled hardening regimes, levels of cold hardness can be evaluated by means of freeze tests. Such tests, however, have the disadvantage of being laborious and destructive. Alternatively, cold hardness can be evaluated indirectly by means of underlying physiological and genetic factors. Characterising quantitative trait loci (QTLs) could supply the desired information in the future [3], but currently detailed quantitative data are not available [4, 6]. Quantitative data can be obtained using electrical impedance spectroscopy during the early hardening phase [20]. Recently, promising results have been obtained using reflectance spectroscopy in the visible and near infrared ranges. Most of the genetic and phenotypic variation shown by conifer seedlings [23], and *Salix* [14] could be evaluated using this technique. Its value is that it detects the chemical changes associated with cold hardening, for instance in pigmentation and water and sugar content [14]. Similarly, this technique is useful for the analysis of wood properties [2], characteristics that also have a strong chemical basis.

The objectives of the present study were twofold: to study the inheritance of hardening components by characterising the F₂ progeny of a cross between a late-and-slowly hardening clone and an early-and-rapidly hardening one; and to test the value of the spectral technique for diagnosing cold hardness in a breeding situation.

2. MATERIALS AND METHODS

2.1. Plant material

A cross between the female clone 'Jorunn' (*Salix viminalis* L., cultivar of Svalöf Weibull AB, Sweden) and the male clone 'SW901290' (*S. dasyclados* Wimm., collected wild from Kirov, Russia) was used to produce F₂ progeny [21]. Of these, 78 were used in the present study. Replicate plants of the F₂, F₁ and P clones were raised from 10 cm cuttings. They were grown for the first three weeks in 0.1 dm³ pots and thereafter in 2.6 dm³ pots, using the substrates and nutrient solutions described in [17]. During the day, growth room temperatures varied from 20 to 30 °C and at night from 15 to 20 °C. The irradiance varied from 300 to 600 μmol·m⁻²·s⁻¹ (19 h photoperiod), measured at the top of the plants using a quantum sensor (Li-189; Li-Cor, Lincoln, Neb., USA). After seven weeks of growth, when plants had reached a height of 1–2 m, they were divided into three sets and subjected to various regimes to induce hardening (see below).

2.2. Hardening regimes

The timing of growth cessation, which marks the onset of cold hardening [17], was determined using a set of three replicate plants of each clone, and subjecting them to a natural day length reduction. From the middle of August they were kept in a greenhouse room shielded from

any extraneous artificial light. During the day, except the twilight hours, additional light was provided by lamps to maintain an irradiance of approximately 300 μmol·m⁻²·s⁻¹ at the top of the plants. Temperatures varied from 20 to 25 °C during the day, and from 15 to 20 °C at night. Plants of similar height were placed adjacent to each other so as to minimize shading. All the remaining plants were treated to drastic day length reduction (from 19 to 6 h), known to trigger growth cessation [13]. Thus, the rate of cold hardening could be determined from a single measurement at a later stage. Three growth rooms were used with day/night/overall temperatures averaging 8.8/7.5/8.5 °C, 10.3/4.1/8.8 °C, and 9.4/7.1/8.8 °C, measured using thermocouples (0.05 mm) logged at 5 s intervals (CR10, Campbell Scientific, Logan, Utah, USA). The irradiance at the top of plants varied from 250 to 350 μmol·m⁻²·s⁻¹ in all the growth rooms. The plants treated to the abrupt day length reduction represented two sets. The first set – the calibration set – comprised ten replicates each of 15 representative clones, four of which were the parental and grandparental clones. These were used for assessing cold hardness and reflectance spectra, in order to calibrate the multivariate model for predicting cold hardness from spectral data. The second set – the prediction set – comprised four replicates of each of the clones. The cold hardness of these plants was assessed, using the model, from their spectral data. The replicates from the prediction set were evenly distributed between the three growth rooms whereas all replicates of a particular clone from the calibration set were kept together, so there were five clones in each growth room. After 10 weeks of cold hardening, the plants from the prediction set were harvested: the 34–38 cm stem segment below the stem tip was cut off and stored at –25 °C for subsequent spectral analysis (see below). After 8–12 weeks of cold hardening, the clones from the calibration set were assessed, individually, for cold hardness by means of the freeze tests described below.

2.3. Freeze tests

Freeze tests were performed on the calibration set using the protocol described in [13], except that plants were assessed individually. The 20–54 cm section below the stem tip was cut into fifteen 2 cm segments, leaving the central 4 cm segment for subsequent spectral analysis. Prior to analysis, this segment was stored at –25 °C. The smaller segments were separated into fourteen test tubes, to form a temperature series with controls (two segments were used for the control). Segments from two replicate plants were placed in the same series of tubes, with one replicate marked for later identification. All ten replicate plants were prepared this way, resulting in five series of tubes. The thirteen test temperatures were set apart by 1.4 °C starting from a temperature within the range of –6 to –12 °C depending on the expected cold hardness of the clone as determined by the length of hardening treatment and genetic factors. The cooling rate was 3 °C·h⁻¹. In order to initialise ice nucleation, cooling was temporarily halted at –2 °C and small amounts of ice added to the tubes. After a test temperature had been reached, the tubes were transferred to Dewar flasks, which had been pre-cooled to the same temperature. This allowed slow thawing when they were subsequently transferred to 5 °C. After thawing and subsequent recovery in darkness at 10 °C for 13 d, freezing damage was determined by measuring the ratio of variable to maximal chlorophyll fluorescence, a method that was validated before by comparison with scorings of tissue browning and regrowth capacity [17]. Data for individual plants were fitted to a sigmoid function by regression analysis (SPSS software, Chicago, USA), to determine the inflection point corresponding to 50% injury (LT50), as detailed in [13]. For fifteen out of the 150 plants tested no LT50 value could be obtained. For three of these this was because water was not added to the samples during recovery, so they became desiccated. For the remaining twelve, the data were too variable to allow regression analysis.

2.4. Reflectance spectroscopy

The stem segments to be used for spectral analyses were freeze-dried and ground to a powder, using a ball mill. Samples were handled and analysed in a random order. Reflectance spectra were recorded within the interval 1100 to 2500 nm (2 nm intervals; Model 6500, FOSS NIR Systems, Silver Spring, USA), using the accompanying software. Each sample was pressed into a sample holder, 2 mm deep and 5 mm wide, using a metal plate attached to a spring to produce a standard force. One spectrum per sample was recorded. The reflectance (R) values were converted into absorbance (A) values using the formula $A = \log(1/R)$. In order to identify outliers among data, all spectra were combined in a principal component analysis plot, produced using the SIMCA 8.0 package (Umetrics, Umeå, Sweden). Nine outliers were identified. However, when re-analysed, these samples were suitable for inclusion, suggesting that they were initially handled incorrectly. In further analysis of the calibration set, one sample was identified as an outlier in the X versus Y correlation space (see below). After excluding this sample, and the 15 samples for which no LT50 could be obtained (see above), 134 out of the 150 samples could be used for modelling.

In order to predict LT50 values from the spectral data, a partial least squares projection to latent structures (PLS) model was created using the SIMCA 8.0 package. Only a brief description is provided here, but a full review of the method can be found in [16]. A data matrix, X , formed by measurements of p variables (absorbance spectra) from n samples, can be projected down onto an A -dimensional subspace to obtain a good approximation of matrix X , as well as a good correlation with matrix Y (LT50), on the basis of the least-squares criterion. The statistically significant number of dimensions, A , for the projection is determined by the model's ability to predict the Y -matrix for deleted samples (the method of cross validation). Using this method, one-quarter of the samples are deleted and a model is developed for the remaining three-quarters; this model is then applied to the deleted samples to predict their Y -matrix. This procedure is repeated to obtain predictions for all samples. The method of orthogonal signal correction (OSC), described by Wold et al. [26], was used to remove irrelevant systematic data, mainly the result of light scattering. In short, the method involves the removal of spectral X data that is unrelated to Y . The data from the samples of the prediction set were then imported and the same OSC algorithm as for the calibration set was applied. This allowed prediction of their LT50 values from their spectral data.

2.5. Growth cessation and growth rate

The timing of growth cessation was assessed for plants subjected to natural day length reduction. The length of the shoot apex was assessed daily. The length of the growing leaf whose width was closest to 3 mm was also measured. Cessation of growth was determined by comparing this measurement with the length of the shoot apex. Growth was deemed to have ceased when the apex length was reduced to half that of the leaf. The rate of shoot growth was determined, for the prediction set, by dividing the height of the plants when growth ceased by the time since planting.

3. RESULTS

A multivariate model was developed to predict LT50 values for *Salix* stems from the reflectance spectra of dried and milled samples. Reflectance was determined for wavelengths ranging from 1100 to 2500 nm. A subset of 15 clones was used to calibrate the model. The model was able to predict 73% of the variation in LT50 values for the calibration set during the early stage of cold hardening studied here (Fig. 1). The model com-

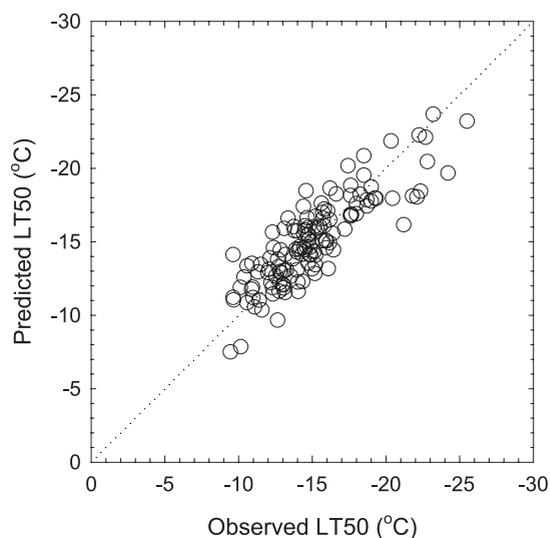


Figure 1. Predicted versus observed LT50 values for the 134 plants in the calibration set. Predictions were based on multivariate modelling using the absorbance spectra (1100–2500 nm) as X variables and the LT50 value assessed from freeze tests as the Y variable. The dotted line represents the 1:1 relationship.

prised only two dimensions, A , further demonstrating its credibility and value in practical work. Although the model was validated for LT50 values from -10 to -25 °C, it may have underestimated the true level of cold hardiness at the lower end of this range (Fig. 1). However, only a few of the plants reached this end: plants that had been hardening for 12 weeks instead of the standard period of 10 weeks.

The model was then used to predict LT50 values for the prediction set, comprising all clones of the breeding population. The standard error of determination for individual plants was <0.7 °C throughout (data not shown). Figure 2a shows the relationship, for all clones, between the predicted LT50 value and the timing of the onset of growth cessation and hence the start of cold hardening. All clones are known to start cold hardening when treated to drastic day length reductions and they are known to be equally cold sensitive while actively growing [13]. A single measurement of LT50, therefore, after 10 weeks in this case, provides a measure of the rate of cold hardening. The range of variation across clones with respect to the timing and the inherent rate of hardening was extensive, with the onset beginning between 240 and 285 days from the beginning of the year, and LT50 values after 10 weeks ranging from -10 to -20 °C. Figures 2b and c show the frequency distributions for clones with respect to both characters. Clearly, the male grandparent (P^*), which originated from continental Russia, displayed an earlier start and a more rapid rate of cold hardening than the female grandparent (P^{**}), which originated from a temperate climate. The range of values exhibited by the F_2 clones extended the range displayed by the grandparents with respect to both characters. Both F_1 parents exhibited intermediate values, although these were shifted towards those of the P^* grandparent. With respect to LT50 values, the frequency distribution of clones apparently had the characteristics of a

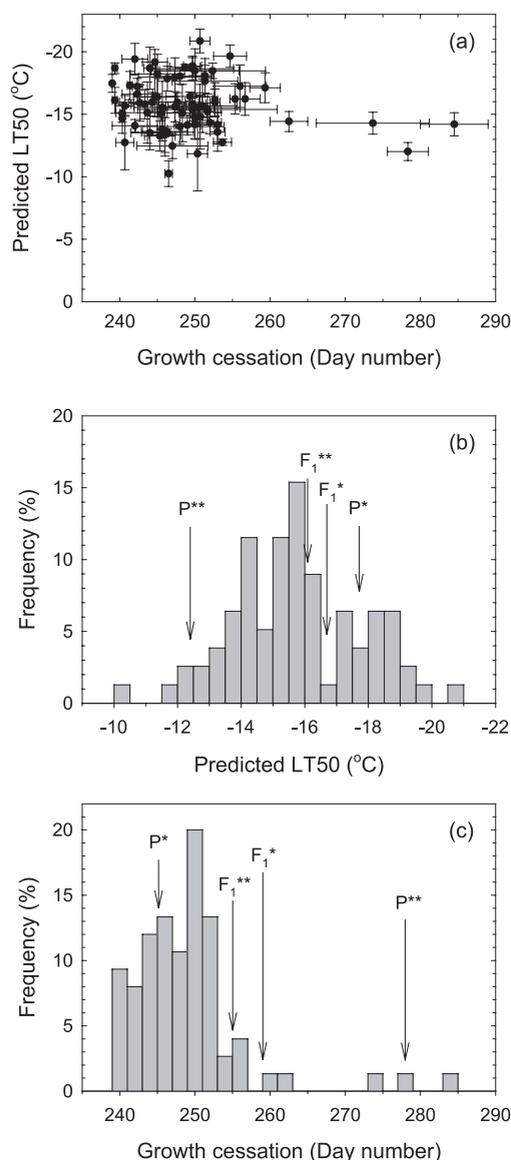


Figure 2. Cold hardening characteristics of the 78 F₂ clones, the two F₁ parents and the two P grandparents: the LT50 value, predicted from spectral measurements, after 10 weeks of cold hardening induced by a drastic reduction in day length, and, during natural day length reduction, the number of days from the beginning of the year to the cessation of growth and the onset of cold hardening. (a) LT50 values versus number of days to growth cessation; (b) the frequency distribution of clones with respect to LT50 values; (c) the frequency distribution of clones with respect to number of days to growth cessation. The male (*) and female (**) clones of parents and grandparents are indicated. In (a), mean \pm SE values are shown for four (LT50) and three (growth cessation) replicates.

normal distribution, with its centre shifted slightly towards the P* grandparent (Fig. 2b). By contrast, the frequency distribution for the timing of onset of cold hardening was heavily skewed towards the P* grandparent (Fig. 2c). A Shapiro-Wilk normality test confirmed that only the former distribution was normal ($P = 0.23$ for LT50 and $P = 0.00$ for growth cessation).

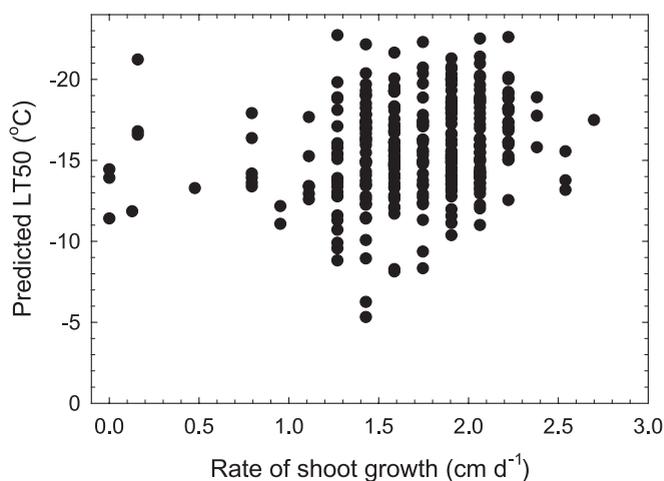


Figure 3. The relationship between LT50, predicted from absorbance spectra after 10 weeks of cold hardening, and the rate of shoot growth prior to cold hardening. The data represent individual plants of the 78 F₂ clones, the two F₁ parents and the two P grandparents.

The different hardening characteristics of the grandparents were partly recombined in the F₂ offspring: among the F₂ clones exhibiting early onset of hardening, like the P* grandparent (at around day 245), there were several with a slow hardening rate, like that of the P** grandparent (Fig. 2a). Although the rapid hardening of the P* grandparent was not recombined with the late onset of hardening of the P** grandparent, this may be coincidental and reflect the low numbers of late hardening clones.

There was a wide variation in growth rate between the clones, but this was unrelated to the variation in the inherent rate of cold hardening (Fig. 3).

4. DISCUSSION

4.1. Assessing cold hardiness using reflectance spectroscopy

The levels of cold hardiness of *Salix* stems could be assessed from the reflectance spectra of dried and milled samples: 73% of the variation in the calibration set could be predicted (Fig. 1). In a previous study, however, we were able to predict as much as 96% of the variation in cold hardiness by analysing intact tissues [14]. It, therefore, seems that intact tissues provide better estimates than homogenised and dried ones, despite the fact that the former are optically more heterogeneous. However, the intact tissues also provided more hardening-related information: in the near infrared range, they revealed the decreasing relative water content with advancing cold hardening, and, in the visible range, they revealed the decreasing chlorophyll content and increasing anthocyanin content [14]. Neither water nor pigment could be assessed using dried samples. The pigments were degraded by the drying process.

Cold hardening was monitored during its relatively early stages. Beyond an LT50 value of about -20 °C, however, the spectral method may underestimate the true level of cold hardiness

(Fig. 1). Other techniques for estimating cold hardiness from chemical changes also seem to provide underestimates beyond this point [5, 18]. This may be because the hardening process undergoes a shift at about this point from mainly broad chemical changes to mainly qualitative changes, such as the formation of the glass state [11]. This complication does not seriously limit the value of the spectral technique because the early hardening stage is more critical than the later stages. In *Salix* plantations, autumn frosts cause more severe injuries than winter frosts, and even frost-sensitive *Salix* clones have sufficient capacity for cold hardening in relation to prevailing winter temperature minima [9]. Similarly, a greater genetic variation in cold hardiness in the autumn than in the winter has been observed in sessile oak [8] and Douglas fir [1].

In conclusion, the spectral method for determining cold hardiness is best applied to intact tissues undergoing initial cold hardening, and by utilising information in both the visible and near infrared spectral ranges.

4.2. Inheritance of cold hardening traits

Previously we have demonstrated the existence of a large genetic variation in *Salix* with respect to both the timing of onset and the rate of cold hardening [13]. Clones from colder climates exhibit an earlier start and a higher rate than those from milder climates. None of the clones examined showed the desirable combination of a late start and a rapid rate. One of the objectives of the present investigation was to look for such a combination in a cross between an early-and-rapidly hardening clone and a late-and-slowly hardening one. Traits were partially recombined in the F₂ offspring, with several of the clones showing the early-and-slowly hardening combination (Fig. 2a). Although none of the clones showed the desirable late-and-rapid hardening recombination, this may be the result of the low numbers of late-hardening F₂ clones overall. The distribution of F₂ clones is asymmetrical with respect to the timing of onset, with the majority of clones showing the early-hardening character. This is probably the result of a difference in the ploidity of the grandparents: after completion of the study we learned that the early-hardening grandparent is hexaploid, whereas the late-hardening one is diploid (Rönnerberg-Wästljung, personal communication). The greater impact of the hexaploid grandparent on the progeny, however, was restricted to the timing of onset of hardening. In contrast, the rate of cold hardening was normally distributed with the centre of the distribution only slightly shifted towards the hexaploid grandparent (Fig. 2b). This may suggest that the rate of hardening is controlled by a larger number of genes, thus cancelling out the difference in ploidity. In general, cold hardening is a quantitative trait involving many genes with small additive effects [24], but genetic studies in the past have not distinguished between the different hardening components. Another possible explanation for the rate of hardening being normally distributed but not the timing of onset may be a higher degree of heterozygosity of genes controlling the rate.

In conclusion, the results presented here suggest that the inherent rate and the timing of onset of cold hardening are independently inherited traits in *Salix*. This, in turn, suggests that it should be possible to improve cold hardiness without adversely affecting growth, by selecting for a high rate and a

late onset. The discovery that hardening rate and growth rate are unrelated traits (Fig. 3) lends further support to this possibility. Wide-scale screening will, clearly, be required to find desirable genotypes, but the spectral analysis method should make this possible.

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