portance for survival of the ovules during winter period and subsequent fertilization of the egg cell in the second developmental season. It is evident therefore that this feature of pollinated ovules reflects very sensitively the hybridological status of the species under investigation and that it may be utilized effectively in the evolutionary study of the hard pines as recommended earlier by McWilliam (1959).

A close relationship between P. nigra and P. thunbergii at the biochemical level has been demonstrated in our comparative study concerning the serological properties of the pollen grains (Kormutak and Lanakova, 1988) and the restriction profiles of the chloroplast DNA (Kormutak et al., in press). All the findings obtained so far correlate positively with the established compatibility of P. nigra x P. thunbergii combination. Prus-Glowacki et al. (1985) in their serotaxonomical investigation of pines revealed the presence of seed antigens common for both the above species. The authors share opinion that except for the continuing speciation of pines and considerable intraspecific variability, it is irregular hybridization which complicates the systematics of the genus Pinus. The last mentioned aspect is fully applicable to the group Larici as well. Of no less strange than compatible hybridological status of geographically distinct species of P. nigra x P. thunbergii and P. nigra x P. tabulaeformis is also a strong reproductive isolation of the species combinations of P. mugo x P. nigra (Kormutak and Lanakova, 1988), P. nigra x P. sylvestris and reciprocal (Vidakovici and Jurkovic-Bevilacqua, 1970; Vidakovici and Borzan, 1973) as well as a negligible crossbility between P. sylvestris and P. mugo (Dobrinov and Jagdunov, 1971; Nester-Sarquella et al., 1988) the evolution of all of which was to a large degree bound to common territory of Europe.

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Clonal Variation in Heat Tolerance and Heat Shock Protein Expression in Black Spruce

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Summary

One-year-old Picea mariana rooted cuttings representing 9 families and a total of 24 clones were used to assess variability in heat tolerance and, for 2 clones, differences in heat shock protein (HSP) synthesis. Different clones of the same family exposed to a temperature of 47 °C for 30 minutes varied widely in damage, indicating a large component of single tree variability in the factors contributing to heat tolerance. The predominance of clones from family 347 possessing above-average heat tolerance suggested that tolerance is, in part, also heritable within families. Clone 86—109 was both more heat tolerant and possessed higher constitutive levels of HSP compared to clone 347—36. Levels of HSP synthesis offer promise as a means of molecular selection for tree breeding and clonal propagation. Rapid screening for heat tolerance of large numbers of individuals is possible by means of direct hot water immersion of shoots.

Key words: Picea, stress tolerance, rooted cutting, heat shock.

Introduction

For black spruce (Picea mariana Mill. B. S. P.), a boreal temperate zone species with a vast range spanning the

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northern section of North America from the Pacific to the Atlantic Oceans, heat tolerance is not commonly considered an important trait for tree improvement. However, high temperatures can cause damage during nursery stock production, stock handling, and after planting. During nursery stock production, damaging high temperatures can occur to germinants and seedling foliage in greenhouses (Colombo, 1980) and after transplanting in nursery beds (Korstanje and Fetherolf, 1921). The heat tolerance of foliage is also of importance during nursery stock handling and shipping, particularly when stock is packaged in bags or boxes when direct exposure of the foliage to high temperatures can occur (Kauppi, 1984). High soil surface temperatures after planting can be a significant factor causing injury or mortality in trees up to age 10 years, even in northerly latitudes (Baker, 1929; Helgerson, 1990; Smith, 1951; Vaartaja, 1949). In addition, if predictions concerning global environmental warming are accurate, the ability of native trees to tolerate high temperatures, thermotolerance, may be an important attribute for tree improvement purposes, as has been the case for various agricultural species (Maxin et al., 1985; Reynolds and Ewing, 1989; Wang and Nguyen, 1989).

Tree improvement of black spruce is facilitated by the fact that shoots from juvenile individuals of this species can be propagated relatively easily using rooted cuttings. This has led to the implementation of operational clonal propagation programs for black spruce, including the Moonbeam Clonal Forestry Centre located near Kapuskasing in northern Ontario, Canada. Clonal propagation offers many potential advantages in comparison to seed-based propagation, chief among which are the ability to propagate large numbers of genetically superior individuals in a relatively short period of time for direct transmission of desirable traits into tree planting operations (Lamb and Rauter, 1984). However, in order to derive this advantage it is necessary to identify traits conferring genetic superiority while individuals are still young enough to possess high juvenile rooting ability. The early identification of individuals possessing genetically controlled traits is also important in tree improvement programs based on family selection. One means of such early testing is the assessment of seedling stress tolerance. This paper presents a technique for screening large numbers of individuals for heat tolerance, which can be used both in clonal screening and selective tree breeding programs.

Exposure of black spruce to high, non-damaging temperatures increases subsequent thermotolerance (Koppenaal et al., 1990; Colclough et al., 1992). The induction of thermotolerance is dependant on temperature and duration of exposure, whereby seedlings exposed to 38 °C for 3 hours are able to survive subsequent high-temperature stresses which would otherwise be lethal. The development of thermotolerance is characterized by increased synthesis of Heat Shock Proteins (HSPs) (Colclough et al., 1992). During exposure to high temperatures the synthesis of several heat shock proteins is induced and/or enhanced, and HSPs have been implicated in the development of thermotolerance in black spruce (Colclough et al., 1992).

The purpose of this investigation is to (1) establish the sensitivity of black spruce clones to heat stress and (2) to assess the relationship between clonal differences in heat tolerance and heat shock protein synthesis.

**Materials and Methods**

Heat tolerance of black spruce rooted cutting shoots was evaluated using plant material originating from seedlings produced from controlled pollination between plus trees. On September 7, 1989, rooted cuttings from 9 families representing 24 clones were exposed to a temperature of 47 °C for 30 minutes to test heat tolerance levels. Fourteen clones came from two full-sib families, 9 from family 347 and 5 from family 348. Clones from families 347 and 348 were selected on the basis of the results of field screening trials (unpublished) conducted by the Northern Region of the Ontario Ministry of Natural Resources near the towns of Cochrane, Hearst and Kapuskasing: clones with the best and poorest survival were selected for screening based on the hypothesis that field survival rates were at least partly due to post-planting heat stress. An additional 10 clones from 7 families other than families 347 and 348 were tested to compare inter-family differences in heat tolerance. Sixteen rooted cuttings per clone were tested. A list of all clones tested is shown in figure 1.

Heat tolerance was tested between 12:30 pm and 3:30 pm. High temperature exposure was accomplished by inverting the trays of transplanted rooted cuttings and plunging the shoots to within approximately 1 cm of the container surface in a 47 °C ± 0.1 °C circulating hot water bath for 30 minutes. Exposure for 30 minutes to 47 °C was chosen on the basis of other investigations (Colombo and Timms, 1992), which indicated this to be an exposure level sensitive to differences in heat tolerance. In order to ensure that no injury resulted from immersion in water apart from the

![Table: Clone Number]

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<thead>
<tr>
<th>Clone Number</th>
<th>200-60</th>
<th>293-4</th>
<th>294-19</th>
<th>295-4</th>
<th>296-1</th>
<th>303-22</th>
<th>309-20</th>
<th>347-18</th>
<th>349-10</th>
<th>359-47</th>
<th>360-70</th>
</tr>
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<tr>
<td>Heat Tolerance Rating</td>
<td>0-10</td>
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<td>25-40</td>
<td>41-56</td>
<td>57-64</td>
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*Figure 1. — Clonal variation in heat tolerance. Black spruce rooted cuttings were exposed to 47°C for 30 minutes as described in Materials and Methods. Damage to the terminal 5 cm of each rooted cutting was rated according to a qualitative scale 4 weeks after heat exposure, where: 0 = entire terminal 5 cm of shoot dead; 1 = stem alive, needles all killed, no buds growing; 2 = stem alive, needles all killed, buds growing on the lower half of the terminal 5 cm of stem; 3 = buds growing, damage restricted to the apical meristem and the needles immediately surrounding it; and, 4 = no damage to the terminal 5 cm of the shoot.*
effects of elevated temperature, black spruce shoots were immersed in room temperature water for up to 3 hours; this immersion caused no damage (Colombo and Timmer, 1992). Shirley (1956) similarly found that 5 hours immersion of conifer seedling shoots in room temperature water caused no damage.

Following removal from the water bath the trays of rooted cuttings were placed in a greenhouse for four weeks to allow the full expression of damage symptoms to take place. At the end of four weeks, damage to the terminal 5 cm of each rooted cutting was rated according to a qualitative scale, where: 0 = entire terminal 5 cm of shoot dead; 1 = stem alive, needles all killed, no buds growing; 2 = stem alive, needles all killed, buds growing on the lower half of the terminal 5 cm of stem; 3 = buds growing, damage restricted to the apical meristem and the needles immediately surrounding it; and, 4 = no damage to the terminal 5 cm of the shoot. A clonal heat tolerance rating was calculated by summing the code values for the 16 rooted cuttings per clone, with clonal heat tolerance being greater the higher the value. This non-destructive assessment of heat tolerance was used to preserve the surviving rooted cuttings for use in other trials.

A second test of clonal heat tolerance was conducted in June, 1990, using clones 86—109 and 347—36, in order to compare heat tolerance to the ability of these clones to produce heat shock proteins (HSPs). These clones were selected on the basis of their wide variation in heat tolerance in a prior investigation (Colombo, unpublished). In this test, 15 rooted cuttings per clone were immersed in 47 °C water for 75 minutes. This longer duration of exposure than was used in the first test was necessary, as plants in June had initiated buds, which is known to increase heat tolerance (Koppensaal and Colombo, 1988), whereas those tested the previous September had not initiated buds. No difference in interpretation due to rooted cuttings being bud-initiated is expected, as species differences in heat toleration do not usually interact with season (Bannister, 1970; Bannister and Smith, 1983). Damage in the June test was expressed as the proportional dry weight of damaged to non-damaged shoot, four weeks after heat exposure.

For heat shock protein extraction and immunological detection of heat shock proteins, 25 rooted cuttings each of clones 86—109 and 347—36 were removed from the growing medium, the roots rinsed with tap water and placed in aerated plastic containers with 500 ml of complete nutrient solution (20—20—20) and incubated overnight under normal growth conditions. The following day, rooted cuttings were exposed to 126 °C or 48 °C air temperature in a growth chamber for 1 hour (relative humidity 70±5% to 80±5% and light intensity 220 μmol m⁻² sec⁻¹ to 240 μmol m⁻² sec⁻¹), and soluble and membrane-bound proteins were extracted as described before (Colclough et al., 1992). This temperature-duration combination was chosen based on previous investigations of heat shock protein expression (Colclough et al., 1992). Nuclear membrane proteins were collected by centrifugation of homogenate at 200 x g for 15 minutes. Proteins were separated electrophoretically using SDS-PAGE on 10% discontinuous linear acrylamide gels and thereafter transferred onto nitrocellulose by electro-blotting under constant voltage (30V) for 21 hours. Immunological detection of HSPs was done using a mouse monoclonal antibody specific for the constitutive HSP70 and inducible HSP72 from Hela cells (StressGen Biotech. Corp., Sidney, B. C., Canada).

Results and Discussion

Wide differences in heat tolerance occurred between black spruce clones exposed to high temperature (Fig. 1). Three clones (347—18, 347—110 and 347—131) had clonal heat tolerance ratings of 60 or greater, indicating virtually no damage. In contrast, three clones (347—36 and 348—39) had heat tolerance ratings below 10, indicating severe damage or mortality of almost all rooted cuttings tested.

Clones belonging to family 347 tended to be more heat tolerant than clones from family 348 (Fig. 1). This may indicate that genetic differences at the family level play a role in determining heat tolerance. Since variation in heat tolerance between clones from the same family was also great (eg. clones 347—110 and 347—36), it suggests that both inter- and intra-family factors play important roles in determining heat tolerance. Heat tolerance and metabolism under elevated temperatures have been found to be heritable traits in a wide range of plant species (Larson, 1988; Marsh et al., 1988; Martineau et al., 1976; Neales, 1968; Reynolds and Ewing, 1988; Wang and Nguyen, 1989).

One of the principal difficulties in the development of clonal forestry programs for large scale reforestation is the identification of superior clones. The results of the clonal screening of heat tolerances described in this trial offers one means of selecting clones. High temperature is an environmental constraint upon plant development for which a clonal selection program may prove an effective means of improving stress tolerance. Similar screening protocols to those described here could easily be adapted to the selection of parents for controlled pollination to increase the heat tolerance of offspring in a tree breeding program. In this trial, it appears that family variability in heat tolerance of black spruce exists, which would enable a breeding program based on this trait to yield improvements in heat tolerance of the offspring. This screening protocol can also be applied to individuals from general collection seed sources. This has been done

![Figure 2](image-url)
(Colombo, unpublished) using trays of general seed source collection container-grown seedlings in which the shoots were immersed in hot water, as was done in this trial for clonal stock. Individuals selected using this approach can then either be propagated clonally or used in conventional tree improvement programs. This assumes that a high level of heat tolerance and HSP expression is a superior attribute, with no unforeseen negative side effects. Field testing in support of this assumption is necessary to safely apply this protocol in a tree improvement program.

In order to assess the possible role of HSPs in the tolerance of heat stress, two clones found in an earlier trial to differ in levels of heat tolerance (Colombo, unpublished) were evaluated to compare the relative levels of heat tolerance and HSP expression. As shown in figure 2, clone 86—109 was significantly more heat tolerant than clone 347—36. These same clones were exposed to heat shock conditions (sudden exposure to 48 °C), and the constitutive and inducible levels of HSP72/73 were studied by immunological detection using a monoclonal antibody for the HSP72/73 from human HeLa cells (Colclough et al., 1992). We have found that this antibody specifically recognized HSP72/73 from black spruce seedlings, and that the enhanced synthesis of HSP72/73 was correlated with heat-tolerance (Colclough et al., 1992).
Both heat-resistant and heat-sensitive black spruce clones displayed constitutive synthesis of HSP27 at 26 °C (Fig. 3). HSP27 was observed in soluble, nuclear, mitochondrial, and microsomal protein fractions. In the soluble protein fractions of both clones, the HSP27 antibody not only recognized two polyepitides of molecular masses 72 kD and 73 kD, but also recognized two polyepitides of molecular masses 42 kD and 23 kD.

The expression of the heat-sensitive clone 347—36 to 48 °C resulted in a decrease of soluble HSP27 and an increase in nuclear, mitochondrial and microsomal membrane-bound HSP27. In comparison, exposure of heat-resistant clone 86—108 to 48 °C resulted in a decrease of HSP27 in membrane-bound protein fractions, while soluble HSP27 was significantly increased (Figure 3).

Despite their designation as heat shock proteins, most black spruce HSPs are expressed constitutively without the need for high temperature exposure (Colclough et al., 1997), thus suggesting a role for these proteins in the normal physiological activity of black spruce cells. Proteins of the HSP27 family have been shown to participate in the stabilization of protein substrates that are in the process of maturation and in posttranslational protein assembly and translocation (Chirico et al., 1988; Deshaies et al., 1988).

Recently, Beckmann et al. (1990) suggested that most newly synthesized proteins in the cell interact transiently with cytosolic HSP27. Through such an interaction, newly synthesized proteins are maintained in a stable conformation until translation is completed. The new protein may then begin to fold into its final conformation with a concomitant release of HSP27. Moreover, the HSP27 group of proteins have autoregulatory capabilities (Tilly et al., 1982) and a positive correlation between the accumulation of HSP27 and repression of its synthesis has been observed (D’Immenico et al., 1982; Colclough et al., 1982). The concentration of free versus substrate-bound HSP27 is tightly regulated; thus, when the equilibrium is shifted toward substrate-bound forms, the reduction of free HSP27 will induce HSP27 synthesis (Beckmann et al., 1990). In the current trial, heat-shock induced a marked increase in cytosolic HSP27 in a heat-tolerant black spruce clone. In a heat-sensitive clone, cytosolic HSP27 decreased, although membrane-bound HSP27 increased in mitochondrial, microsomal and nuclear fractions. These results suggest that although newly synthesized proteins were able to complete their insertion in the membranes, they were unable to release the substrate-bound HSP27. Moreover, our results also suggest that the regulatory mechanisms of HSP synthesis were affected in heat-sensitive clones, since the increase in the bound form of HSP27 failed to induce HSP27 synthesis.

Clonal differences in HSP synthesis and heat tolerance suggest that these traits are under genetic control in black spruce. Genetic variation in the level of HSP synthesis has been reported in higher plants. In sorghum, genetic differences in high temperature sensitivity are correlated with variations in the capacity for HSP synthesis (Ougham and Stoddard, 1986). In wheat, a correlation was identified between the synthesis of specific low molecular weight heat shock proteins and the degree of thermotolerance expressed following exposure to high temperatures (Krishnan et al., 1989). In the case of black spruce in the present study, clonal differences in heat tolerance and HSP synthesis may similarly reflect genetic control over these traits.

In conclusion, large differences in clonal heat tolerance suggest the existence of both inter- and intra-family control of the physiological attributes controlling the ability to tolerate high temperatures. Relative levels of heat tolerance were associated with the constitutive levels of heat shock proteins in two clones, and with the ability to produce HSPs in response to high temperature exposure. Using the protocols described here heat tolerance can be tested rapidly and for large numbers of individuals in tree improvement programs.

Acknowledgements

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References


Provenance Trial in Eucalyptus grandis and its Implication to Forestry Programmes

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Summary
A provenance trial involving 17 exotic provenances from Australia and one local (Valparai) seed source of Eucalyptus grandis was undertaken to identify suitable provenances for a general plantation programme. The study revealed the presence of significant variation among the provenances. The provenances Paluma, Ravenshoe, Gadgara and Herberton were found to be the best. It is suggested that the local provenance of E. grandis which has till now been used for plantation forestry in the region should be replaced with the fast-growing provenance, Paluma. This may also be used as the population for the genetic improvement of E. grandis, if undertaken.

Key words: Eucalyptus grandis, provenance, variability, correlation.

Introduction
Selection of the best provenances of the best species for a given site or region is necessary for achieving maximum productivity both in plantation forestry and with agroforestry systems. The concept of provenance testing has been well established and demonstrated through nationally or internationally conducted co-ordinated provenance trials. Eucalyptus grandis, an exotic species, is being introduced to India for its high value for fuel, pulp, paper and timber and is the most sought-after species for paper production. It is also resistant to "pink disease" and grows well at high altitude in Tamil Nadu and Kerala (Siyam Sundar, 1979). Information on the amount of provenance variation in growth rate and on selection of superior provenance of this species for commercial planting is meagre. A trial was therefore established so that suitable provenances of Eucalyptus grandis could be recommended for general and agroforestry plantation programmes.

Materials and Methods
The experimental materials consisted of 17 Australian provenances of Eucalyptus grandis received from CSIRO, Australia and one local seed source (Table 1). The provenance trial was planted in 1981 at Valparai which is a hill station, and at 1666 metres a.m.s.l. The provenances were planted in a randomized block design with four replications. Sixteen plants were planted in each plot at an spacing of 2 metres x 2 metres. Height and girth were measured annually. At the final assessment at age 9 years, height, clear bole height, girth and diameter at breast height (dbh) and basal area were measured. Growth parameters at the age of 7 and 9 years form the basis of this paper. The data were subjected to analysis of variance using plot means and correlation coefficients between characters were calculated at the plot mean level.

Results
Significant differences among the provenances were shown in the analysis of variance for all the characters under study (Table 2).

Provenance means for different characters are presented in Table 3. All the provenances except S. of Tyalgum, northern N. S. W., Port Stephens and Eungella were superior to the Valparai local seed source for height which ranged from 17.8 m (Port Stephens) to 25.5 m (Paluma) at the age of 7 years. However, at the age of 9 all the Australian provenances were growing better than the Valparai local source. Of the 17 provenances, 10 showed 25% better growth in height than control. Mean height ranged from 26.2 m (Valparai local) to 44.0 m (Paluma). The correlation between heights at the ages of 7 and 9 years was positive and significant (r = 0.7188). Clear bole height measured at the age of 9 years was higher in all Australian provenances than the Valparai local and ranged from 10.3 m (Valparai local) to 26.1 m (Paluma).

12 and 7 provenances respectively had higher girth at the age of 7 and 9 years than the Valparai local. While Paluma showed the highest girth (82.0 cm), the Port Stephens provenance recorded the lowest girth (46.2 cm). The correlation between the girths at 7 and 9 years of age was positive and significant (r = 0.9008).

Mean values for dbh ranged from 13.5 cm (Port Stephens) to 23.6 cm (Paluma). 11 provenances had better diameter growth rate than the local plantation.

11 out of 17 provenances recorded higher mean basal area than the Valparai local and the values ranged from 143.9 cm² (Port Stephens) to 446.3 cm² (Paluma). 6 provenances showed 50% increased basal area over Valparai local. However, interplot competition is likely to be exaggerating differences between poor-performing and good-performing provenances.