chastic simulation of the genetic advancement of multiple traits using sequential culling. N. Z .J. For. Sci. **29**: 25–43 (1999). — JAYAWICKRAMA, K. J. S. and LOW, C. B.: Radiata pine selections from different regions of New Zealand differ in branch habit, form and growth rate. N. Z. J. For. Sci. **29**: 3–24. (1999). — JAYAWICKRAMA, K. J. S., SHELBOURNE, C. J. A. and CARSON, M. J.: New Zealand's long-internode breed of radiata pine. N. Z. J. For. Sci. **27**: 126–141 (1997b). — JOHNSON, G. R. and BURDON, R. D.: Family-site interaction in *Pinus radiata:* implications for progeny testing strategy and regionalised breeding in New Zealand. Silvae Genet. **39**: 55–61 (1990). — JOHNSON, G. R., VOLKER, P. W. and PEDERICK, L. A.: An examination of correlations between New Zealand and Australian sites for radiata pine. Proc. 10th Meeting of Research Working Group No. 1, Australian Forestry Council, Gympie, Queensland (1988) — KING, J. N. and BURDON, R. D.: Time trends in heritabilities and projected efficiencies of early selection in a large 17-year-old progeny test of *Pinus radiata.* Can. J. For. Res. **21**: 1200–1207 (1991). — KING, J. N. , CARSON, M. J. and JOHNSON, G. R.: Analysis of disconnected diallel mating designs. II – Results from a third generation progeny test of the New Zealand radiata pine improvement programme. Silvae Genet. **47**: 80–87 (1998). — LAVERY, P. B.: Plantation forestry with *Pinus radiata.* Paper No. 12, School of Forestry, University of Canterbury, Christchurch, New Zealand. (1986). — LIBBY, W. J.: Native origins of domesticated radiata pine. *In:* BURDON, R. D. and MOORE, J. M. (Ed.): "IUFRO '97 Genetics of Radiata Pine", Proceedings of NZ FRI-IUFRO Conference 1 to 4 December and Workshop 5 December, Rotorua, New Zealand. FRI Bulletin 203, New Zealand Forest Research Institute, Rotorua, New Zealand pp. 9–21 (1997). — LINDREN, D., GEA, L. and JEFFERSON, P.: Loss of genetic diversity monitored by status number. Silvae Genet. **45**: 52–59 (1996). — LOW, C. B.: Interaction of *Cyclaneusma* needle cast with early selection. pp. 113–115. *In:* Proc. FRI/NZFP Forests "Clonal Forestry Workshop". Edited by J. T. MILLER. FRI Bulletin No. 160, Forest Research Institute, Rotorua (1991). — LOW, C. B. and SMITH, T.: Use of the Guadalupe provenance in *Pinus radiata* improvement in New Zealand. *In:* BURDON, R. D. and MOORE, J. M. (Ed.): "IUFRO' 97 Genetics of Radiata Pine", Proceedings of NZ FRI-IUFRO Conference 1 to 4 December and Workshop 5 December, Rotorua, New Zealand. FRI Bulletin 203, New Zealand Forest Research Institute, Rotorua, New Zealand. pp. 57–61 (1997). — MACLAREN, J. P.: Radiata pine grower's manual. FRI Bulletin No. 184. New Zealand Forest Research Institute, Rotorua, New Zealand. (1993). — MCKEAND, S. E. and BRIDGWATER, F. W.: A strategy for the third breeding cycle of lobolly pine in the south-eastern U.S. Silvae Genet. **47**: 223–234 (1998). — MORAN, G. F. and BELL, J. C.: The origin and genetic diversity of *Pinus radiata* in Australia. Theor. Appl. Genet. **73**: 616–622 (1987). — NAMKOONG, G.: A multiple index selection strategy. Silvae Genet. **25**: 199–201 (1976). — PONZONI, R. W. and NEWMAN, S.: Developing breeding objectives for Australian beef cattle production. Anim.Prod. **49**: 35–47 (1989). — SHELBOURNE, C. J. A.: Tree breeding methods. New Zealand Forest Service, Forest Research Institute, Technical paper 55 (1969). — SHELBOURNE, C. J. A.: Genetics of adding value to the end-products of radiata pine. *In:* BURDON, R. D. and MOORE, J. M. (Ed.): "IUFRO '97 Genetics of Radiata Pine", Proceedings of NZ FRI-IUFRO Conference 1 to 4 December and Workshop 5 December, Rotorua, New Zealand. FRI Bulletin 203, New Zealand Forest Research Institute, Rotorua, New Zealand. pp. 129–146 (1997). — SHELBOURNE, C. J. A., BURDON, R. D., CARSON, S. D., FIRTH, A. and VINCENT, T. G.: Development plan for radiata pine breeding. Forest Research Institute, Rotorua, New Zealand (1986). — SHELBOURNE, C. J. A., CARSON, M. J. and WILCOX, M. D.: New techniques in the genetic improvement of radiata pine. Comm. For. Rev. **68**: 191–201 (1989). — SHELBOURNE, C. J. A. and LOW, C. B.: Multi-trait index selection of *P. radiata* progenies at five sites and associated genetic gains. N. Z. J. For. Sci. **10**: 307–324 (1980). — SHEPHERD, R. W.: Early importations of *Pinus radiata* to New Zealand and distribution in Canterbury to 1885: implications for the genetic makeup of *Pinus radiata* stocks. Horticulture in New Zealand **1**(1): 33–38 (1990). — SHEPHERD, W. and COOK, W.: The Botanic Garden Wellington: A New Zealand history 1840 to 1987. Millwood Press, Wellington, NZ (1988). — SORENSSON, C. T., BURDON, R. B., COWN, D. J., JEFFERSON, P. A. and SHELBOURNE, C. J. A.: Incorporating spiral grain into New Zealand's radiata pine breeding programme. *In:* BURDON, R. D. and MOORE, J. M. (Ed.): "IUFRO'97 Genetics of Radiata Pine", Proceedings of NZ FRI-IUFRO Conference 1 to 4 December and Workshop 5 December, Rotorua, New Zealand. FRI Bulletin 203, New Zealand Forest Research Institute, Rotorua, New Zealand. pp. 180–191 (1997). — THULIN, I. J.: Application of tree breeding to forestry in New Zealand. N. Z. J. For. **VII**: 41–46 (1957). — VAN BUIJTENEN, J. P. and BURDON, R. D.: Expected efficiencies of mating designs for advanced-generation selection. Can. J. For. Res. **20**: 1648–1663. — VINCENT, G.: Radiata pine tree breeding. NZ Tree Grower **18** (2): 21–24 (1997). — VINCENT, G. and BUCK, K.: Radiata pine seedlots – GF Plus **TM.** NZ Tree Grower **19**(2): 33–35 (1998). — VINCENT, T. G.: Certification system for forest tree seed and planting stock. FRI Bulletin 134, New Zealand Forest Research Institute, Rotorua, New Zeala (1987). — WHITE, T. L., HODGE, G. R. and POWELL, G. L.: An advanced-generation tree improvement plan for slash pine in the south-eastern United States. Silvae Genet. **42**: 359–371 (1993). — WITEHIRA, D.: The development of relative "economic weights" using STANDPAK. B. For. Sci. thesis, University of Canterbury, New Zealand (1996). — WOOLASTON, R. R. and JARVIS, S. F.: The importance of breeding objectives in forest tree improvement. In: Proc. CRCTHF-IUFRO Conference on Eucalypt Plantations: Improving Fibre Yield and Quality. Hobart, Australia, 19 to 24 February 1995. pp. 184–188 (1995).

Genetic Analysis of Isozyme Variants in Open Pollinated Families of Southern Beech *Nothofagus nervosa* (PHIL.) DIM. et MIL.

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Abstract

Nothofagus nervosa (= N. alpina) represents one of the most important native tree species among the Southern Southamerican Temperate Forest. Its ecological situation in Argentina with a very reduce distribution area, overexploitation in the past, recurrent forest fires and overgrazing makes it a suitable species for conservation and improvement programs.

In order to conduct a complete population genetic study of the species in Argentina, isozyme gene markers were determined. Genetic analysis of the observed phenotypic variation was done applying the method described by GILLET and HATTEMER (1989). Twelve loci were analysed corresponding to six enzyme systems. Four of them were monomorphic, but two had species-specific alleles. Among the remainder, genetic control could be demonstrated in five loci and hypothesis on the mode of

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inheritance of another one has been suggested. Finally, genetic control could not be proved in two loci and discussion on the reliability of these loci as gene markers have been done.

Key words: Isozymes, genetic analysis, *Nothofagus nervosa (= N. alpina), Nothofagaceae, Fagaceae.*

Introduction

Nothofagus nervosa (PHIL.) DIM. et MIL. (LENNON et al., 1987) *(= N. alpina = N. procera)* is one of the most important native forest tree species in Argentina, where it has a small natural distribution area along the Andes Mountains, covering a narrow fringe of about 120 km in length and 40 km in maximum width. Due to its high wood quality, which resembles that of *Fagus sylvatica,* it was overexploited in the past. Some populations were seriously affected by this overexploitation and others disappeared. In addition, the occurrence of forest fires followed by grazing has also affected the species. Some studies on genetics and geographic variation have been done using morphological traits (MARCHELLI and GALLO, 1995, 1999) as well as chloroplast DNA markers (MARCHELLI et al., 1998). However, a complete study of within- and between-population variation to provide a guide for conservation and improvement programs is missing.

Isozyme gene markers have been widely used in forest tree research for population genetics studies. In this kind of studies it is very important to have a previous knowledge about the genetic control of the observed isozymic variation in order to be sure of dealing with gene markers and to identify the different alleles. In angiosperms, genetic analysis of the observed isozyme phenotype variation requires offspring from controlled crosses, which, in forest trees, is technically difficult to succeed. To avoid the need of controlled crosses, an alternative method was developed by GILLET and HATTEMER (1989). It consists on analyses of isozyme phenotypes from single tree tissue and from samples of their offspring from open pollination. The advantage of this method lies in that there is no need of controlled crosses and, besides, in the possibility of using different tissues in parents and offspring.

Among the *Fagaceae,* the mode of inheritance of isozyme gene markers has been analysed using controlled crosses in some species, e.g. in *Fagus sylvatica* (THIEBAUT et al., 1982; MÜLLER-STARCK and STARKE, 1993); in *Quercus petraea* (MÜLLER-STARCK et al., 1996); in *Q. robur* (ZANETTO et al., 1996). In other cases, analysis of single trees and of their offspring has been used, (e.g. in *Castanea sativa,* applying the method of GILLET and HATTEMER (1989): FINESCHI et al.(1990); *F. sylvatica* (MERZEAU et al., 1989). Finally, in many studies, the genetic basis of the observed variation is inferred directly from the zymograms and compared with those of other tree species (e.g. in *Nothofagus nitida, N. betuloides* and *N. dombeyi* (PREMOLI, 1996); in *Nothofagus spp.* from New Zealand (HAASE, 1993); in *Q. cerris, Q. pubescens, Q. petraea* and *Q. robur* (SAMUEL et al., 1995); in *Q. rubra* and *Q. ellipsoidalis* (HOKANSON et al., 1993)).

In *N. nervosa,* genetic control for 6 isozyme gene loci *(Adh, Idh-A, Mdh-B, Mdh-C, Pgi-B* and *Pgm-A)* has been shown (GODOY, 1994). However, hybrid seedlings between this species and *N. obliqua* were observed among the offspring of most of the trees analysed by GODOY (GALLO, 1995) and later on the basis of species-specific isozyme gene markers were found in the parental species (GALLO et al., 1997a and b, in press). This has interfered with the genetic interpretation of some of the observed zymograms analysed by GODOY. For this reason, a genetic analysis of isozymes from trees belonging to a pure *N. nervosa* population, to avoid interspecific hybridisation, was necessary to correctly determine the gene markers.

In the present study, segregation ratios of isozyme variation were analysed in descendants from trees belonging to two pure *N. nervosa* populations following the method described by GILLET and HATTEMER (1989).

Table 1. – Enzyme systems, electrode and gel buffers and electrophoresis conditions.

Enzyme system	Buffer system
Alcohol dehydrogenase (ADH) E.C.1.1.1.1	1
Alkaline phosphatase (ALP) E.C.3.1.3.1	1
Catalase and Peroxsídase (CAT)(PER) E.C.1.11.1.6 and 1.11.1.7	2
Diaphorase (DIA) E.C.1.6.4.3	2
Fumarase (FUM) E.C.4.2.1.2	1
Glutamate dehydrogenase (GDH) E.C.1.4.1.3	2
Glutamate oxalacetate transaminase (GOT) E.C.2.6.1.1	2
Isocitrate dehydrogenase (IDH) E.C.1.1.1.42	1
Leucine-amino peptidase (LAP) E.C.3.4.11.1	2
Malate dehydrogenase (MDH) E.C.1.1.1.37	1
Nicotinamide adenine dinucleotide dehydrogenase (NADHDH) E.C.1.6.99.3	2
Phosphoglucose isomerase (PGI) E.C.5.3.1.9	2
Shikimic acid dehydrogenase (SDH) E.C.1.1.1.25	1

1: Electrode 0.13 M Tris – 0.04 M citric acid pH 7; gel: diluted electrode buffer (1:2.5) 4 hs. 155 mAmp.
2: Electrode 0.3 M Boric acid – 0.06 M NaOH pH 8.2 (POULIK, 1959); gel: 0.07 M Tris – 0.008 M citric acid pH 8.7. 5 hs. 65 mAmp.

Table 2. – Genetic analysis in single-locus codominant mode of inheritance (from GILLET and HATTEMER, 1989).

Proposed maternal genotype	Possible genotypes of offspring	Expected relationship between observed numbers of offspring phenotypes
$A_i A_i$	$A_i A_i$	
	$A_i A_k$ $(k \neq i)$	
$A_i A_j$ $(i \neq j)$	$A_i A_i$	
	$A_i A_j$	$N_{ij} = N_{ii} + N_{ij}$
	$A_i A_j$	
	$A_i A_k$	$N_{ik} = N_{jk}$ $(k \neq i, j)$
	$A_i A_k$ $(k \neq i, j)$	

Materials and Methods

Analysed tissue

Buds from 53 mother trees and their seeds from open-pollination were collected from two pure populations of *Nothofagus nervosa* located one in Tromen Lake Basin and the other in Huechulafquen Lake Basin, both in Argentina. Seeds were kept at 4 °C and buds at –20 °C until electrophoretical analysis was carried out.

Enzyme extraction

To reduce the presence of resins, buds were soaked in acetone for 5 minutes and centrifuged at 10000 rpm for 15 minutes prior to homogenisation. Bud tissue and embryos were homogenised and proteins extracted with the vegetative extraction buffer I from CHELIAK and PITEL (1984) with slight modifications. Other extraction buffers were also assayed, e.g. seed extraction buffer (CHELIAK and PITEL, 1984) and the extraction buffer described by MÜLLER-STARCK (1985), but they were less suitable for both tissues. Electrophoresis was performed the day after protein extraction; samples were stored at 4 °C.

Electrophoresis

The enzymes were separated by horizontal starch gel electrophoresis. Electrode and gel buffers, electrophoresis conditions and enzyme systems are shown in *table 1.* After electrophoresis, gels were sliced horizontally into three pieces. Staining solutions were prepared according to CHELIAK and PITEL (1984) with slight modifications. Omitting enzyme substrates and coenzymes parallel to the regular stains verified enzyme specific activity.

Genetic analysis and isozyme designation

Genetic analysis was carried out following the method described by GILLET and HATTEMER (1989), which is based on three general requirements:

(i) regular meiotic segregation during egg production;

(ii) random fertilization of the eggs by each pollen (haplo) type;

(iii) absence of differential viability selection in the offspring prior to the investigation.

In the frame of a single-locus codominant mode of inheritance (*Table 2*) as well as in the frame of a single-locus with

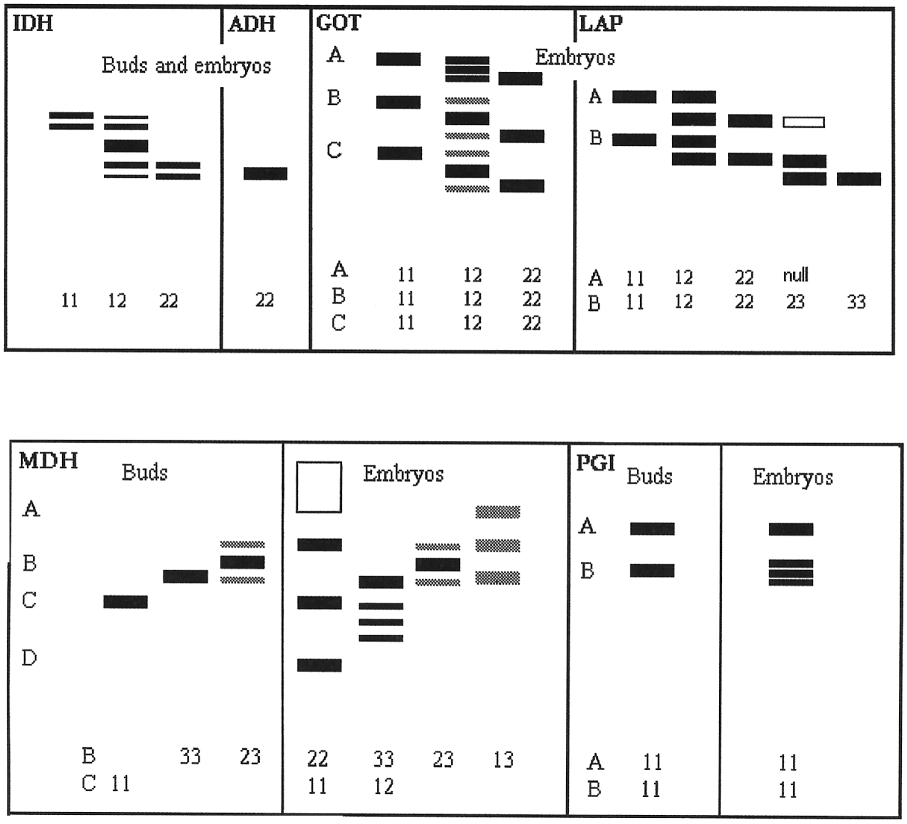

Fig. 1. – Phenotypes observed in buds and embryos from *Nothofagus nervosa.* Intuitive genotype designation is also given.

recessive null allele and codominance between active alleles, hypothesis can be tested qualitatively and quantitatively on each maternal tree and a random sample of its offspring from open pollination.

A screening of buds from the 53 individual trees was done in order to look for those that seemed heterozygous for each enzyme system. From each heterozygous tree, at least 100 seeds from open-pollination were analysed.

Zymograms were divided into zones within which the observed phenotypic variation was supposed to be controlled by one gene locus. Enzyme systems were designated with their conventional abbreviation and zones in decreasing order of relative mobility, both in capital letters. Loci were named by the enzyme and zone designation in italics and alleles with numbers in decreasing order of relative mobility.

Chi-square goodness-of-fit test was applied to test the fulfilment of the monogenic inheritance.

Results and Discussion

Prior to the analysis of buds, different enzymes were assayed in order to look for those that showed a good resolution and were polymorphic. Among the analysed enzymes, some were discarded for different reasons. In CAT (and PER) and NAD-HDH, no activity was observed with the buffer system used. Tiny bands with bad resolution were obtained for GDH, DIA, ALP, PGM and SDH whereas FUM showed a good resolution, but was monomorphic for all analysed seeds and buds (81 seeds and buds from 18 trees). PGI and ADH were also monomorphic in all studied mother trees, but they were not discarded because of the occurrence of species-specific alleles (GALLO et al., 1997a and b, in press) that could help to discriminated the variation caused by the interspecific hybridisation between *Nothofagus nervosa* and *N. obliqua.* Cathodal migration was tested for all the enzymes, but no staining activity was observed. Unspecific activity was not detected when enzyme substrates and coenzymes were omitted.

The enzymes MDH, IDH, ADH, PGI, GOT and LAP had a good resolution in embryos but, in general, weak or no activity was observed in bud tissue (*Fig. 1*). Due to this reason, genetic control of some of the observed zymograms or zones (GOT, LAP, MDH-A and MDH-D) could not be statistically proved and therefore they were inferred from the phenotypes observed in embryos from the open pollinated families. In the other observed systems and zones, bud and embryo tissue showed the same electrophoretic patterns and a genetic analysis was performed.

Description of the phenotypes

The zymograms obtained for bud and embryo tissues are shown in *figure 1.* Prior to the analysis of the offspring, an intuitive interpretation of the mode of inheritance is also shown.

IDH

One zone of activity was observed in this system with two different double-banded and one five-banded patterns, both in buds and embryos. The same mobility of the bands was found in both tissues. This observation agrees with the results obtained by GODOY (1994) who also found another zone with little activity, which could not be observed in the present study. Among the *Fagaceas,* one zone was described for this enzyme system in *Fagus sylvatica* (MÜLLER-STARCK and STARKE, 1993) and two zones in *Castanea sativa* (one invariant) (FINESCHI et al., 1990), *Quercus robur* (ZANETTO et al., 1996) and *Q. petraea* (MÜLLER-STARCK et al., 1996).

MDH

Four zones could be observed on embryos, but only two showed activity on buds: zones B and C. However, activity in zone B was very low and tiny bands were observed only in some of the trees. Two different phenotypes were observed among the buds: one showed a single band and the other three bands. However, two single-banded and three triple-banded phenotypes were found on embryos.

Zone C did not express any variation in the buds of the analysed trees, showing a single-banded phenotype in all of them. But, it showed a tripled-banded phenotype in few seeds.

The most anodal zone, observed only on embryos, MDH-A, was difficult to understand, may be due to overlapping with zone B. The most cathodal zone, MDH-D, was invariant, showing a single band.

Three zones were observed for this enzyme system on leaves of *Nothofagus dombeyi, N. nitida* and *N. betuloides,* two of them with one invariant band and the other with three different single-banded phenotypes and three different triple-banded ones (PREMOLI, 1996). Four zones were observed in *Quercus petraea* (MÜLLER-STARCK et al., 1996) and three in *Q. robur* (ZANETTO et al., 1996) and *Fagus sylvatica* (MÜLLER-STARCK and STARKE, 1993).

ADH

Buds and embryos revealed identical phenotypes showing only one single-banded invariant zone in all the analysed mother trees and their offspring. One zone with an invariant single-banded phenotype was also detected for *N. obliqua* (GALLO et al., 1997a and b), two variable zones were described for *N. dombeyi* and *N. betuloides* and two invariant single-banded phenotypes in *N. nitida* (PREMOLI, 1996).

PGI

This enzyme revealed two invariant zones, both in buds and embryos. However, a single band was observed for zone B on buds, which corresponds to three invariant bands on embryos. Same patterns were described by GODOY (1994). Similar phenotypes were found in *N. obliqua* (GALLO et al., in press) where two zones were also observed. PREMOLI (1996) has also described two invariant zones for this enzyme in *N. nitida* as well as one invariant and one with four different phenotypes for *N. dombeyi,* and *N. betuloides.* Two zones were also observed in New Zealand *Nothofagus* (HAASE, 1993). Among the *Fagaceae,* two zones were detected in *Quercus petraea* (MÜLLER-STARCK et al., 1996), *Q. robur* (ZANETTO et al., 1996), *Fagus sylvatica* (MÜLLER-STARCK and STARKE, 1993) and *Castanea sativa* (FINESCHI et al., 1990).

GOT

Activity was not detected on buds, while three variable zones were observed on embryos (GOT-A, GOT-B and GOT-C), each of them having two single-banded phenotypes and a triple-banded one. Three zones were also described in buds from *Fagus sylvatica* by THIEBAUT et al. (1982), although only one was analysed, and two zones in different tissues (embryos, buds, young leaves) of the same species by MÜLLER-STARCK and STARKE (1993). Three zones showed activity in buds, seeds and young radicles of *Quercus petraea* (MÜLLER-STARCK et al., 1996) and *Q. robur* (ZANETTO et al., 1996), although one of the zones in *Q. petraea* could not be analysed due to problems in the visualisation on one of the studied tissues.

LAP

Activity on buds could not be detected, while two zones were displayed on embryos. The most anodal zone showed two

single-banded phenotypes, one double-banded and another one without any bands. The other zone (LAP-B) presented five different phenotypes: three single-banded and two double-banded. Two zones were also observed for this enzyme in buds, seeds and young radicles of *Quercus robur* (ZANETTO et al., 1996) and *Q. petraea* (MÜLLER-STARCK et al., 1996); in buds, seeds, young leaves and pollen of *Fagus sylvatica* (MÜLLER-STARCK and STARKE, 1993) and in buds of *Populus tremula* and *P. tremuloides* (GALLO and GEBUREK, 1991). Very weakly activity for *Lap-B* in buds and young leaves was reported in beech (MÜLLER-STARCK and STARKE, 1993) and problems in the visualisation of the activity in one of the analysed tissues were also found in *Q. petraea* (MÜLLER-STARCK and STARKE, 1993).

Genetic analysis

IDH

Intuitively, the phenotypes observed on buds (*Fig. 1*), agree with a single-locus codominant mode of inheritance and a dimeric structure. Two alleles were present among the analysed trees. As it is shown in *figure 1,* double-banded patterns were observed for putative homozygous genotypes and five-banded ones for heterozygous. The same was observed by GODOY (1994) in *Nothofagus nervosa.* Artifacts (a tiny band below the principal one) were also reported for this enzyme in *Fagus sylvatica* (MÜLLER-STARCK and STARKE, 1993).

Heterozygous trees were chosen for the genetic analysis. Eight out of the 53 individual trees analysed showed five banded patterns, six from Tromen Lake Basin and two from Huechulafquen Lake Basin population. Offspring from these trees were analysed to determine the mode of inheritance of the enzyme variants. *Table 3* shows the statistical approaches to test the single-locus codominant mode of inheritance proposed. As can be seen there, the qualitative conditions are fulfilled since all the offspring had a maternal allele and, among the offspring of homozygous individuals, no homozygous phenotypes for the other allele were observed. The quantitative analysis between observed and expected ratios could be proved only in four of the analysed trees. This could be due to the high frequency of one of the alleles (allele number 1) in both populations. Analysis of eleven populations from the entire distribution area of this species in Argentina showed that allele 2 was in low frequency in all of them, only few embryos being homozygotes (MARCHELLI and GALLO, submitted).

A single-locus codominant mode of inheritance for this enzyme was reported in *Fagus sylvatica* (MERZEAU et al., 1989; MÜLLER-STARCK and STARKE, 1993), *Quercus robur* L. (ZANETTO et al., 1996) and *Quercus petraea* (MÜLLER-STARCK et al., 1996); in all these cases the analysis were performed on material from controlled crosses. Similar results were described for *Castanea sativa* (FINESCHI et al., 1990) using the same statistical method than here. Two alleles were observed in chestnut, three and four in beech and five in both *Quercus.*

MDH

For this enzyme, activity in bud tissue was only observed for loci B and C. Locus C remained invariant in all the analysed trees, so the genetic control could not be studied. However, a rare allele (p = 0.0085) appeared among the offspring of some trees, always in heterozygous genotypes: 12 out of 1405 embryos were heterozygous. According to this, a single-locus codominant mode of inheritance could be suggested.

For *Mdh-B*, activity in buds was very low and bands could be seen only in some of the trees (*Table 4*). Among these trees only two genotypes were observed, one homozygous and one heterozygous, suggesting a single-locus codominant mode of inheritance with two alleles. Offspring analysis showed additionally the existence of a third allele. Offsprings from four heterozygous trees were analysed, and segregation for alleles 2 and 3 was statistically proved in all of them. Segregation for allele number 1 in the families was proved only in one of them. In addition, offsprings from four other trees supposed to be heterozygous were studied. In all the cases, the hypothesis was qualitatively fulfilled. Except for one tree, segregation could not be proved at the same time for the three alleles, only for two of them in each case. One reason could be the very low frequency of allele 1 in the pollen cloud (p = 0.07). Although zymograms from buds could be observed only in 9 out of 53 trees, none of them had this allele. Besides, among all the analysed embryos, homozygous genotypes for that allele were not observed. This implies that allele number 1 is not only scarce among the mother trees, but in the pollen pool as well. In addition, genotypes 13 were more frequent than genotypes 12 (*Table 4*), suggesting a possible selection against the latter. Using embryos as analysed tissue, postzygotic selection is limited; therefore a prezygotic selection could more likely be acting. Significant deviations from the MENDELian expectations

Table 3. – Genetic analysis of the enzyme IDH. Tested hypothesis: $N_{11} + N_{22} = N_{12}$ Σ: total sample size. H: Population from Huechulafquen Lake Basin; T: Population from Tromen Lake Basin.

Mother tree	Mother genotype	Offspring genotype			Σ	χ^2
		N_{11}	N_{12}	N_{22}		df=1
21 H	12	59	39	2	100	4.84 *
24 H	12	68	49	6	123	5.08*
3 T	12	39	51	10	100	0.04 ns
6 T	12	40	50	15	105	0.24 ns
17 T	12	54	40	6	100	4.00 *
21 T	12	46	44	10	100	1.44 ns
24 T	12	51	46	12	109	2.65 ns
30 T	12	50	40	10	100	4.00 *
7 T	11	41	2	0	43	
10 T	11	43	10	0	53	
25 T	22	0	20	16	36	

Levels of significance: ns: not significant; * significant at the 0.05 level.

Table 4. – Genetic analysis of the enzyme MDH-B. Tested hypothesis: $N_{22} + N_{33} = N_{23}$ and $N_{12} = N_{13}$ Σ: total sample size. H: Population from Huechulafquen Lake Basin; T: Population from Tromen Lake Basin.

Mother tree	Mother genotype	Offspring genotypes					Σ	χ^2_1	χ^2_2	χ^2_{1+2}
		N_{12}	N_{13}	N_{22}	N_{23}	N_{33}		$N_{22}+N_{33}=N_{23}$	$N_{12}=N_{13}$	df=2
21 H	23	0	1	18	72	37	128	2.28 ns	1 ns	3.27 ns
24 H	23	0	11	28	47	40	126	3.83 ns	11 *	14.83 *
3 T	?	10	4	49	25	16	104	17.78 *	2.57 ns	20.35 *
6 T	23	3	22	16	37	19	97	0.06 ns	14.44 *	14.50*
10 T	?	1	3	24	6	11	45	20.51 *	1.00 ns	21.51*
17 T	?	6	8	35	40	4	93	0.01 ns	0.29 ns	0.30 ns
24 T	?	3	1	29	54	2	89	6.22 *	1.00 ns	7.22 *
30 T	23	0	43	5	25	34	107	3.06 ns	43.00 *	46.06*
21 T	?	0	10	0	30	60	100			

Levels of significance: ns: not significant; * significant at the 0.05 level.

were observed for some loci in *Quercus robur* L. (ZANETTO et al., 1996). The authors related this lack of fit with an excess of the most common allele due probably to the occurrence of viability selection in early stages of the seedlings bearing rare alleles. A similar situation was also observed in *Quercus petraea,* where prezygotic and postzygotic viability selection was proposed (MÜLLER-STARCK et al., 1996).

As was mentioned before, four zones were observed for this enzyme in embryos. The fastest one, *Mdh-A,* was not included in the analysis since lecture of the zymograms was difficult and led to inconsistent interpretation. The lowest one, *Mdh-D,* was monomorphic for all the analysed embryos.

The zymograms obtained for MDH were different from those observed by GODOY (1994) in hybrid seeds. The main differences were detected in loci B and C, where the presence of more bands was evident in hybrid seeds and the interpretation on the mode of inheritance was different. These extra bands observed in the hybrid seeds could belong to *N. obliqua* which overlap with the bands of *N. nervosa* (GALLO and MARCHELLI, unpublished data).

ADH and PGI

Both enzymes were monomorphic in all the analysed trees and their offspring (2129 analysed embryos for PGI and 1208 for ADH). Due to the lack of variation, no inferences on the mode of inheritance could be done. However, as was mentioned before, these two enzymes revealed the existence of species-specific alleles. Two alleles are present both in ADH and in PGI-B: *Adh-1* is present in *Nothofagus obliqua,* while *Adh-2* in *N. nervosa* (GALLO et al., 1997). Similarly, *Pgi-1* appears in *N. nervosa* and *Pgi-2* in *N. obliqua* (GALLO et al., 1997a, in press). In both cases, heterozygous genotypes were found in the hybrids. The absence of heterozygous genotypes verified the absence of hybrids among the offspring of the analysed trees and, therefore, the correctness of the genetic analysis.

It is worth noting that GODOY (1994) could not verify the genetic control of the above mentioned enzymes due, in both cases, to the lack of variation among the analysed trees. Among the offspring of these trees, two genotypes appeared: one homozygous (*N. nervosa* like) and one heterozygous.

Species-specific alleles for ADH were also observed in *Prosopis* the hybrids displaying the alleles of the parental species (VERGA, 1995).

For PGI, a single locus codominat mode of inheritance with three alleles was proposed in *Fagus sylvatica* (MERZEAU et al., 1989; MÜLLER-STARCK and STARKE, 1993) and in *Castanea sativa* (FINESCHI et al., 1990). The same, but with five alleles was described for *Quercus robur* (ZANETTO et al., 1996) and *Q. petraea* (MÜLLER-STARCK et al., 1996).

GOT

Although no activity could be observed in bud tissue, seeds from those trees supposed to be heterozygous were analysed. As was mentioned before, in each of the three zones, three genotypes were observed among the offspring of some trees. Each zone seemed to be controlled by a single locus with two codominant alleles. In all cases, the qualitative conditions of our analysis were fulfilled (GILLET and HATTEMER, 1989). For *Got-A*, nine open pollinated families were studied, but expected segregation was met only in four of them very likely due to the higher frequency of one of the alleles in the pollen cloud. Besides this, six progenies without one of the homozygous expected genotypes were also assayed confirming the mode of inheritance proposed (*Table 5a*). Both for *Got-B* and *Got-C*, five trees were supposed to be heterozygous and, in all of them, the expected segregation could be statistically proved. These two loci seemed to be linked because they were found to exhibit in the progeny a high frequency of parental allelic associations. Linkage between *Got* loci was reported in poplars (MÜLLER-STARCK, 1992).

Single-locus codominat mode of inheritance was described for the two loci observed in *Fagus sylvatica* with three and four alleles in each one (MÜLLER-STARCK and STARKE, 1993). The same inheritance pattern was proposed for the two analysed loci in *Quercus robur* and *Q. petraea* where three and five alleles were observed (ZANETTO et al., 1996 and MÜLLER-STARCK and STARKE, 1993, respectively).

LAP

Although no activity could be observed on buds, a possible hypothesis on the mode of inheritance of this enzyme was tested. The two zones seemed to be controlled by different loci. The presence of single and double-banded phenotypes agrees with the monomeric structure of this enzyme, with homozygous genotypes represented by single bands and heterozygous by two bands. A monomeric structure of this enzyme was also reported in other forest trees, (e.g. in *Fagus sylvatica* (MÜLLER-STARCK and STARKE, 1993), in *Quercus robur* (ZANETTO et al., 1996) and in *Q. petraea* (MÜLLER-STARCK et al., 1996). In *Lap-A,* two codominants and a recessive null allele were detected. Although the hypothesis was qualitatively fulfilled, the high

frequency of the null alleles led to its rejection in the statistical test. Most of the analysed embryos were homozygous for the null allele (no visible bands) or had allele number 2, either in homozygotic dotation or as heterozygous with null allele (*Table 6a*). Similar results were obtained for *Lap-B* where three codominant alleles were observed, but expected segregation could not be proved. In this case, the most frequent allele was number 2.

Null alleles were observed in this enzyme system in other forest trees, e.g. in *Fagus sylvatica* (KIM, 1979), in *Quercus robur* (ZANETTO et al., 1996), in *Populus tremula, P. tremuloides* and their hybrids (GALLO and GEBUREK, 1991).

Conclusions

Genetic analysis of the mode of inheritance of observed isozyme phenotypes should always be done to unequivocally determine the gene markers, which only then could later on be applied on genetic variation studies. In our case, if open pollinated families and buds from the corresponding mother trees had not been analysed, discrimination between zones A and B in MDH could not have been done due to overlapping and misinterpretation of the controlling gene would have taken place. The absence of activity of zone A in buds and the analysis of the zymograms obtained for zone B on this tissue allowed the genetic analysis of this locus. Moreover, the complexity

Table 5. – Genetic analysis of the enzyme GOT. Tested hypothesis: $N_{11} + N_{22} = N_{12}$ Σ: total sample size. H: Population from Huechulafquen Lake Basin; T: Population from Tromen Lake Basin. a) *Got-A*; b) *Got-B* and *Got-C*.

a)

Mother tree	Offspring genotypes			Σ	χ^2
	N_{11}	N_{12}	N_{22}		df=1
9 H	24	52	24	100	0.16 ns
10 H	30	56	14	100	1.44 ns
24 H	60	37	3	100	6.76 *
29T	45	54	8	107	0.00 ns
21T	17	47	36	100	0.36 ns
24T	44	40	16	100	4.00*
21 H	67	22	9	98	29.76*
17T	68	29	4	101	18.31*
30T	55	34	11	100	10.24*
3T	0	18	21	39	
13T	83	22	0	105	
16 H	67	27	0	94	
6T	39	25	0	64	
16T	35	17	0	52	
10T	27	12	0	39	

b)

Mother tree	*Got-B* Offspring genotypes			Σ	χ^2	*Got-C* Offspring genotypes			Σ	χ^2
	N_{11}	N_{12}	N_{22}		df=1	N_{11}	N_{12}	N_{22}		df=1
9 H	3	45	56	104	1.88 ns	2	46	56	104	1.38 ns
10 H	8	53	43	104	0.04 ns	8	53	43	104	0.04 ns
16 H	4	44	52	100	1.44 ns	4	44	52	100	1.44 ns
24 H	3	53	46	102	0.16 ns	3	50	49	102	0.04 ns
13T	2	53	45	100	0.36 ns	2	54	45	101	0.49 ns
29T	12	87	1	100	54.76*	12	88	0	100	
6T	0	4	59	63		0	4	55	59	
16T	0	20	33	53		0	20	31	51	
17T	0	1	103	104		0	1	103	104	
21T	0	1	102	102		0	1	102	103	
24T	0	0	86	86		0	0	86	86	
21 H	0	0	92	92		0	0	99	99	
30T	0	0	105	105		0	0	105	105	
3T	0	0	38	38		0	0	38	38	
10T	0	0	37	37		0	0	33	33	
25T	0	1	21	22		0	1	20	21	

Levels of significance: ns: not significant; * significant at the 0.05 level.

Table 6. – Offspring genotypes observed in LAP-A (a) and LAP-B (b).

a)

Mother Tree	Genotype				N
	1-	12	2-	Null	
3T	0	9	19	71	99
6T	0	6	24	58	88
13T	0	3	27	71	101
16T	0	2	27	22	51
17T	0	7	23	53	83
21T	11	10	31	47	99
24T	0	14	28	27	69
29T	2	13	55	42	112
30T	0	3	12	88	103
9BA	13	0	20	75	108
10BA	5	4	28	56	93
16BA	0	1	44	57	102
21BA	0	2	20	86	108
24BA	0	32	78	44	154

b)

Mother tree	Genotypes					N
	11	12	22	23	33	
3T	0	3	86	7	3	96
6T	1	2	79	6	0	88
13T	0	5	80	15	0	100
16T	0	1	49	2	0	52
17T	0	3	74	6	0	83
21T	0	2	67	20	10	89
24T	0		53	14	2	67
29T	1	4	99	7	0	111
30T	0		74	21	8	95
9BA	0	3	89	13	3	108
10BA	0	7	81	10	6	104
16BA	0	1	91	7	1	100
21BA	14	8	77	9	0	108
24BA	1	7	145	1	0	154

of the zymograms observed in embryos would have made it impossible to correctly determine the gene markers just in samples from natural populations. In more than 1200 embryos analysed from eleven populations only few showed the combination of alleles 1 and 2 for *Mdh-B* (MARCHELLI and GALLO, submitted) that could be proved in the genetic analysis. Rare alleles would have not been detected unequivocally without the genetic analysis.

Due to the lack of activity in bud tissue for some enzyme systems, the genetic analysis method described by GILLET and HATTEMER (1989) could not be statistically applied in all segregating zymogram zones. An intuitive mode of inheritance and a description of the observed phenotypes in those zones were proposed from the point of view of the qualitative analysis, as it is done in most of the papers dealing with isozymic genetic markers. Nevertheless, it should be emphasised that in such cases we are not allowed to talk about gene markers and therefore, our population genetic conclusions should be very cautious. For example in LAP-A and LAP-B, where no activity was observed on buds and segregation ratios were significantly different from those expected, no gene markers could be determined and therefore the observed variation should be consider as phenotypic. These two isozymes cannot be used as gene markers in further studies.

In other systems, deviations from the expected proportions of offspring phenotypes were observed in the progenies of some trees, indicating that selection during reproduction could be acting. If this is the case, one of the general requirements of the applied method (number iii) (GILLET and HATTEMER, 1989) is not completely fulfilled.

In all cases where the genetic control could be proved, a monolocus control for the isozymes with codominant alleles was confirmed. Both for MDH and GOT, homozygotes were represented by one band and heterozygotes by three, confirming the dimeric structure expected in these enzymes. The same holds for IDH, although a double band represented the homozygous genotypes and five bands the heterozygous ones. The LAP phenotypes seem to agree with the monomeric structure that was reported for this enzyme, since two bands was observed for putative heterozygotes embryos.

In general, more active zones were detected on embryos compared to buds. The same holds for leaf tissue where fewer zones on the zymograms were observed (GALLO et al., 1997a and b, in press). It can be conclude that seeds constitute a better material for detection of markers.

From the results presented here, it has to be stressed the importance of collecting seeds from pure populations of the species under consideration for a correct determination of gene markers. Hybridisation is a common process among forest trees and it could lead to misinterpretations on the mode of inheritance of the isozymes within a certain species. Material from pure populations can help then to study the occurrence of species-specific markers to allow the determination of hybridisation rates, introgression and gene flow processes.

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References

CHELIAK, W. M. and PITEL, J. A.: Techniques for starch gel electrophoresis of enzymes from forest tree species. Information Report PI-X-42. Petawawa National Forestry Institute, Canadian Forestry Service. Agriculture Canada (1984). — FINESCHI, S., GILLET, E. and MALVOLTI, M. E.: Genetics of Sweet Chestnut (*Castanea sativa* MILL.). III. Genetic Analysis of Zymograms of Single Tree Offspring. Silvae Genetica **39**: 188–194 (1990). — GALLO, L. A.: Supuestos híbridos interespecíficos en progenies de Raulí. Actas XXVI Congreso Argentino de Genética. San Carlos de Bariloche, 23–25 de octubre de 1995 (1995). — GALLO, L. A. and GEBUREK, TH.: Genetics of isozyme variants in *Populus tremula, P. tremuloides* and their hybrids. Euphytica **53**: 225–233 (1991). — GALLO, L. A., MARCHELLI, P. and BREITEMBÜCHER, A.: Allozymic heterozygosity in families with natural hybrids between two southern bechees (*Nothofagus spp.*). II Southern Connection Congress. 6 al 11 de enero de 1997, Valdivia, Chile (1997a). — GALLO, L. A., MARCHELLI, P. and BREITEMBÜCHER, A.: Morphological and allozymic evidence of natural hybridization between two southern bechees (*Nothofagus spp.*) and its relation to heterozygosity and height growth. Forest Genetics **4**(1): 15–23 (1997b). — GALLO, L. A., MARCHELLI, P. and CREGO, P.: Hybridisation dynamic between two southern beeches (*Nothofagus spp.*). XXI IUFRO World Congress, 7 to 12 August 2000, Kuala Lumpur, Malaysia (Acepted). —

GILLET, E. and HATTEMER, H.: Genetic analysis of isoenzyme phenotypes using single tree progenies. Heredity **63**: 135–141 (1989). — GODOY, M.: Determinación de isoenzimas y análisis genéticos en *Nothofagus nervosa* (PHIL.) DIM. et MIL. raulí. Trabajo científico para la obtención del grado "Magister der tropischen Forstwirtschaft" en el área de Ciencias Forestales de la Universidad Georg-August de Göttingen, Alemania (1994). — HAASE, P.: Isozyme studies of New Zealand *Nothofagus* species (Southern beech) using leaf extracts. Silvae Genetica **42**: 46–51 (1993). — HOKANSON, S. C., ISEBRANDS, J. G., JENSEN, R. J. and HANCOCK, J. F.: Isozyme variation in oaks of the Apostle Islands in Wisconsin: Genetic structure and levels of inbreeding in *Quercus rubra* and *Q. ellipsoidalis* (Fagaceae). American Journal of Botany **80**(11): 1349–1357 (1993). — KIM, Z. S.: Inheritance of leucine aminopeptidase and acid phosphatase isoenzymes in beech (*Fagus sylvatica* L.). Silvae Genet. **28**: 68–71 (1979). — LENNON, J. A., MARTIN, E. S., STEVEN, R. A. and WIGSTON, D. L.: *Nothofagus nervosa* (PHIL.) DIM. et MIL. – The correct name for raulí, chilean southern beech, (*N. procera*). Arboricultural Journal **11**: 323–332 (1987). — MARCHELLI, P. and GALLO, L. A.: Variación en el peso de las semillas de 15 procedencias de *Nothofagus nervosa* (PHIL.) DIM. et MIL. IV Jornadas Forestales Patagónicas., San Martín de los Andes, 24 to 27 de Octubre de 1995 (1995). — MARCHELLI, P. and GALLO, L. A.: Annual and geographic variation in seed traits of Argentinean populations of southern beech *Nothofagus nervosa* (PHIL.) DIM. et MIL. Forest Ecology and Management **121**: 239–250 (1999). — MARCHELLI, P., GALLO, L. A., SCHOLZ, F. and ZIEGENHAGEN, B.: Chloroplast DNA markers revealed a geographical divide across Argentinean southern beech *Nothofagus nervosa* (PHIL.) DIM. et MIL. distribution area. Theor. Appl. Genet. **97** (4): 642–646 (1998). — MERZEAU, D., DI GIUSTO, F., THIÉBAUT, B., LETOUZEY, J. and CUGUEN, J.: Genetic control of isozyme systems and heterogeneity of pollen contribution in beech (*Fagus sylvatica* L.). Silvae Genetica **38**: 195–201 (1989). — MÜLLER-STARCK, G.: Genetic differences between "tolerant" and "sensitive" beeches (*Fagus sylvatica* L.) in an environmentally stressed adult forest stand. Silvae Genet. **34**: 241–247 (1985). — MÜLLER-STARCK, G.: Genetic control and inheritance of isoenzymes in poplars of the Tacamahaca section and hybrids. Silvae Genetica **41**(2): 87–95 (1992). — MÜLLER-STARCK, G. and STARKE, R.: Inheritance of isoenzymes in European Beech (*Fagus sylvatica* L.). Journal of Heredity **84**: 291–296 (1993). — MÜLLER-STARK, G., ZANETTO, A., KREMER, A. and HERZOG, S.: Inheritance of isoenzymes in sessile oak (*Quercus petraea* (MATT.) LIEBL.) and offspring from interspecific crosses. Forest Genetics **3**(1): 1–12 (1996). — PREMOLI, A. C.: Allozyme polymorphisms, outcrossing rates, and hybridization of South American *Nothofagus*. Genetica **97**: 55–64 (1996). — SAMUEL, R., PINSKER, W. and EHRENDORFER, F.: Electrophoretic analysis of genetic variation within and between populations of *Quercus cerris, Q. pubescens, Q. petraea* and *Q. robur* (Fagaceae) from Eastern Austria. Bot. Acta **108**: 290–299 (1995). — THIEBAUT, B., LUMARET, R. and VERNET, PH.: The bud enzymes of beech (*Fagus sylvatica* L.) genetic distinction and analysis of polymorphism in several french populations. Silvae Genetica **31**: 51–59 (1982). — VERGA, A.: Genetische Untersuchungen an *Prosopis chilensis* und *P. flexuosa* (Mimosaceae) im trockenen Chaco Argentiniens. Göttingen Research Notes in Forest Genetics, Abteilung für Forstgenetik und Forstpflanzenzüchtung der Universität Göttingen (1995). — ZANETTO, A., KREMER, A., MÜLLER-STARCK, G. and HATTEMER, H. H.: Inheritance of isozymes in Pedunculate Oak (*Quercus robur* L.). The Journal of Heredity **87**: 364–370 (1996).

Performance and Wood Quality of *in Vitro* Propagated Hybrid Curly Birch (*Betula pendula* x *Betula pendula* var. *carelica* SOK.) Clones

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Abstract

Selected adult hybrid curly birch clones were multiplied by tissue culture. The donor trees originated from breeding experiments which sought to combine straight stem form and a strongly curled wood structure. Micropropagated plants were planted by machine in rows in a field trial. After ten years the height and breast height diameter of the clones were measured and the wood quality of two clones was assessed by felling sample trees to observe wood grain and veneer quality. Results show that tissue culture propagation methods are a valuable tool for the „true-to-type" propagation of birch with regards to outstanding wood qualities.

Key words: Betula pendula x *Betula pendula* var. *carelica* SOK., curly birch, micropropagation, field performance, veneer.

Introduction

Different types of valuable birch wood have been described in the literature (SCHRÖCK and SCHOLZ, 1953). Several attempts have been carried out to propagate selected adult trees of birch with certain wood qualities, but with only limited success (SCHOLZ, 1960). Intensive breeding experiments were conducted in the 1950's at the Institute for Forest Tree Breeding at Waldsieversdorf to combine stem straightness from selected white birch (*Betula pendula* ROTH) with curled wood quality of curly birch (*Betula pendula* var. *carelica* SOK.) selected from natural stands in Poland (SCHOLZ, 1963). Valuable curly-grained wood of birch is characterised by the inclusion of bark particles into the wood, visible as brown curls in vertical sections of the trunk or as rings or V-shaped figures in horizontal sections of the trunk.

These experiments showed that the combination of both characters (straight stems and curled wood) in one individual was a rare event. Thus, the production of large numbers of straight stemmed, curled wood plants via seed seems improbable. Although infrequently, some offspring did display the desired combination of stem straightness and curled wood along the whole stem. Cuttings obtained from topped (severely hedged) trees were used to vegetatively propagate the desired specimens with combined straightness and curled wood (MATSCHKE and SCHNECK, 1981). With the availability of suitable tissue culture methods for propagation of birch (CHALUPA, 1981, 1989; RYYNÄNEN and RYYNÄNEN, 1986; MATSCHKE *et al.*, 1987) there was an opportunity to produce large quantities of desirable curly birch if stem straightness and curled wood

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