germination value is concerned, significant differences between 15 and 30 day lengths were not found in all cases as also reported by THAPLIYAL and GUPTA (1980) for the *C. deodara*. However, the germination value was always higher in the 30 day stratification.

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Studies on *in vitro* Clonal Propagation of *Paulownia tomentosa* STEUD. and Evaluation of Genetic Fidelity through RAPD Marker

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Abstract

Nodal explants were used for clonal propagation of *Paulownia tomentosa* by manipulating the cytokinin and auxin. Bud proliferation and multiple shoots were achieved from nodal explants derived from greenhouse grown plants of *Paulownia tomentosa* on Murashige and Skoog (MS) medium supplemented with 2.22–6.66 µM BA. Inclusion of NAA (0.53–1.34 µM) in the culture medium enhanced the rate of multiplication. The shoot length was attained 3–4 cm on MS medium supplemented with 4.44 µM BA + 0.53 µM NAA + 3% (w/v) sucrose within 4 weeks of culture. The rate of multiplication was maintain in subsequent subculture. Excised shoots were rooted on half-strength MS medium supplemented with 0.49 µM IBA after 2 weeks of culture. On an average of 81 plantlets were obtained from each nodal explant within 12-week of subculture. Rooted propagules were acclimatized and successfully transferred to greenhouse. The micropropagated plantlets appeared morphologically similar with the mother plants. No variation was detected among the micropropagated plants by the use of Randomly Amplified Polymorphic DNA (RAPD) markers.

Key words: Growth regulators, nodal explants, RAPD, shoot multiplication, woody tree.

Abbreviations: Ads – adenine sulfate; BA – 6-benzylaminopurine; Kn – kinetin, IAA – indole-3-acetic acid, IBA – indole-3-butyric acid, NAA – 1-naphthaleneacetic acid, MS medium – MURASHIGE and SKOOG (1962) medium.

Introduction

Micropropagation of tree species offers a rapid means of producing clonal planting stock for afforestation, woody biomass production and an effective way to capture genetic gain (PARK and BONGA, 1992). Micropropagation has been applied to many tree species (BONGA and VON ADERKAS, 1992). *Paulownia tomentosa* (Scrophulariaceae) is a fast growing deciduous tree, growing for afforestation and mine site reclamation (ZHU et al., 1988). The wood and bark were reported to have astringent properties. The charcoal made from this wood is used in high class fire works and in the preparation of gun powder (Anonymous, 1988). It is propagated through seed or by root cuttings. Conventional methods of propagation through seed is unreliable because of disease and pest problem, poor germination and also slow growth than root cuttings (BERGMANN and MOON, 1997; BERGMANN, 1998). Application of *in vitro* culture techniques have great potential for clonal propagation of this important forest species. *In vitro* propagation of *Paulownia tomentosa* was achieved through shoot bud regeneration direct-

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ly from leaf explants or via. the callus phase (MARCOTRIGIANO and STIMART, 1983; RAO et al., 1996). Mass multiplication of *Paulownia elongata* through nodal culture has also been reported (IPEKCI et al., 2001). Reports on its mass cultivation programmes is limited. The present communication describes *in vitro* clonal propagation of *P. tomentosa* from axillary meristems and determines the genetic stability of the micropropagated plants through RAPD marker.

Materials and Methods

Plant material and explant source

Semi-mature twig measuring 4–5 cm were collected from greenhouse grown plants of *Paulownia tomentosa* and brought to the laboratory. The explants were washed in 0.1% (v/v) detergent "Teepol" (Qualigen, India) solution for 15 min and subsequently rinsed in running tap water. Further, the explants were disinfested in 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 15 min and then rinsed three times in sterile distilled water. The twig is cut into smaller segments (~ 0.5 cm each) having one node in each segment. Nodal explants were used as explant source.

Culture medium

MURASHIGE and SKOOG (MS) basal medium supplemented with different concentrations of BA (0.0–8.88 µM), Kn (0.0–9.3 µM), Ads (0.0–148.15 µM), NAA (0.0–2.68 µM) and IBA (0.0–2.46 µM) were tested for bud break and shoot multiplication. All media were adjusted to pH 5.7 using 0.1N HCl or 0.1N NaOH before autoclaving. The medium was gelled with 0.8% (w/v) agar (Qualigen, India). Routinely, 20 ml and 100 ml of molten medium were dispensed into a culture tube (25 x 150 mm) and 250 ml conical flask (Borosil, India) respectively and plugged with non-absorbant cotton wrapped in one layer of cheese cloth. The culture vessels were steam sterilized at 1.06 kg. cm^{-2} for 15 min.

For induction of rooting, the *in vitro* raised shoots measuring about 2–3 cm growing on multiplication medium (MS + 4.44 µM BA + 0.53 µM NAA + 3% (w/v) sucrose) were excised and cultured on half-strength basal MS medium supplemented with different concentrations of IAA (0, 0.35, 0.70 and 1.42 µM), IBA (0, 0.30, 0.60 and 1.23 µM) and NAA (0, 0.32, 0.65 and 1.34 µM) and 2% (w/v) sucrose.

Culture condition

All cultures were incubated under 16h photoperiod under light intensity 55 µ mol $m^{-2}s^{-1}$ provided by cool, white fluorescent lamps (Phillips, India) at 25 ± 2°C with 55% relative humidity. The cultures were maintained by regular subculturing at 4-week intervals to fresh medium with the same composition.

DNA extraction

DNA was extracted from fresh leaves derived from both *in vitro* raised plants and field grown mother plants by the CTAB method (BOUSQUET et al., 1990). Approximately, 250 mg of fresh leaves were ground to powder in liquid nitrogen using a mortar and pestle. The ground powder was transferred to a 25 ml tube with 10ml of CTAB (Cetyltrimethyl Amonium Bromide, EMERK) buffer : 2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, and 0.2% (v/v) β-mercaptoethanol. The homogenate was incubated at 60°C for 2h, extracted with an equal volume of chloroform : iso-amyl alcohol (24:1), and centrifuged at 10,000 rpm for 20 min. DNA was precipitated from the aqueous phase by mixing with an equal volume of isopropanol. After centrifugation at 10,000 rpm for 10 min., the DNA pellet was washed with 70% ethanol, air dried, and resuspended in 10 mM Tris pH 8.0, 0.1 mM EDTA buffers. DNA quantity was estimated spectrophotometrically by measuring the absorbance at 260 nm.

PCR amplification

Twenty arbitrary 10-base primers (Operon Technologies Inc., Alameda, California) were used for Polymerase Chain Reaction (PCR). Amplification reactions were performed in 25 µl : 2.0 µl of 1.25 mM each of dNTP's, 15 ng of the primer, 1x Taq polymerase buffer, 0.5 U of Taq DNA polymerase (Genei, India) and 20 ng of genomic DNA. DNA amplification was performed in a PTC 100 DNA Thermal Cycler (M. J. Research, Inc., Watertown, MA, USA) programmed for 45 cycles : 1st cycle of 3.5 min at 94°C, 1 min at 37°C and 2 min at 72°C; then 44 cycles each of 1 min at 94°C, 1 min at 37°C, 2 min at 72°C followed by one final extension cycle of 7 min at 72°C. Amplified products were electrophoresed in a 1.2% (w/v) agarose (Sigma, USA) gels with 1x TAE buffer, stained with ethidium bromide, and photographed under ultraviolet (UV) light. The size of the amplification products were estimated for a 100-bp (100-bp to 3.0 Kb) ladder (M B I. Fermentas Inc.), All the reactions were repeated at least thrice.

Amplified DNA marker scoring

Amplified DNA markers were scored as present or absent both in the regenerated and the mother plants. Electrophoretic DNA bands of low visual intensity that could not be readily distinguished as present or absent were considered ambiguous markers and were not scored.

Acclimatization

Rooted micropropagules were removed from culture vessels, washed gently under running tap water and planted in 5 cm diam. earthen pots containing a mixture of sand, soil and well rotted cowdung manure (1:1:1; v/v/v). The plantlets were kept in the greenhouse for acclimatization before subsequent transfer to the field.

Statistical analysis

Usually, 20 cultures were raised per each treatment and each experiment was conducted at least tree times. The cultures were examined periodically and the morphological changes were recorded on the basis of visual observations. The effects of different treatments were quantified as the percent of explants showing multiplication, mean number of multiple shoots/culture, percent of rooting and number of roots/shoot. The data were statistically analysed by DUNCAN's multiple range test (HARTER, 1960).

Results and Discussion

The present investigation was carried out to explore the morphogenic potential of *Paulownia tomentosa* from nodal explants using growth regulators. Of the different cytokinins tested, BA was the most effective for inducing bud break in axillary meristems (*Table 1*). Axillary bud was developed within 8–10 days of inoculation onto MS basal medium supplemented with 2.22–6.66 µM BA. The maximum bud proliferation was observed in MS medium supplemented with 4.44 µM BA within 4-week of culture, the axillary meristems elongated upto 1.5–2.0 cm height (*Figure 1A*). Prolonged culture on induction medium resulted the elongation of shoots. At concentrations of BA above 4.44 µM, proliferation and growth of axillary shoots were inhibited. Present results corroborate those reported by AMIN and JAISWAL (1993) in *Artocarpus heterophyllum*, LE ROUX

and VAN STADEN (1991) in *Eucalyptus species* and QURAISHI et al. (1997) in *Lagerstroemia parviflora*. Though, kinetin and adenine sulfate at lower concentration induced bud break but the axillary buds became thick and stunted in nature. Combination of two cytokinins did not favour positive response on shoot elongation and multiplication. The results demonstrated that BA was found to be more effective for bud proliferation than kinetin and adenine sulfate. BA was then tested in combination with NAA (0.0, 0.53, 1.34 and 2.68 µM) or IBA (0.0, 0.49, 1.23 and 2.46 µM) to enhance the rate of shoot multiplication (*Figure 2A–B*). The medium containing BA (4.44 µM) with NAA (0.53 µM) proved to be the most effective treatment for promoting shoot multiplication (3–4 shoots per nodal explant, each having 2–3 nodes) in 4-week of culture (*Figure 1B*). Addition of low concentration of auxin promoted shoot multiplication were reported by various authors in *Paulownia elongata* (IPEKCI et al., 2001), *Madhuca longifolia* (ROUT and DAS, 1993), *Dalbergia latifolia* (RAGHAVA SWAMY et al., 1992) and *Pterocarpus santalinus* (LAKSHMI SITA et al., 1992). Newly growing multiple shoots again cut into smaller segments having one node in each segment were cultured in similar medium for multiplication. The result implies that the axillary explants derived from *in vitro* raised shoots promoted higher rate of shoot multiplication than explants derived from greenhouse grown plants (data not shown). Higher rate of multiplication was due to juvenility of the explants (GREENWOOD, 1995; VON ADERKAS and BONGA, 2000). For continuous production of healthy shoots with well developed leaves were achieved on subculturing at every 4 weeks interval on medium having 4.44 µM BA, 0.53 µM NAA and 3% sucrose. The rate of multiplication was varied from 4.22 ± 0.6 to 5.42 ± 0.5 shoots/culture with subsequent subculture (*Figure 2A–B*). The multiplication frequency was maintained for prolonged period without loss of multiplication ability. This might be due to a better balance of the endogenous and exogenous growth regulators and ionic concentration of nutrient salts as reported earlier in other crops (ROUT and DAS, 1994).

Microshoots derived from axillary meristems were separated and transferred to a rooting medium having half-strength basal MS medium supplemented with different concentrations of IAA, IBA or NAA with 2% (w/v) sucrose. The excised shoots

Table 1. – Effect of cytokinins on response of shoot multiplication of *Paulownia tomentosa* after 4 weeks of culture. The data represent the mean of three independent experiments.

MS + Cytokinins (µM)			Percent of cultures showing multiple shoots (Av. Number of shoots/culture ± S.E)*
BA	Kn	Ads	
0	0	0	0
2.22	0	0	32.6 ± 1.0 d (2.52)
4.44	0	0	70.4 ± 1.2 h (3.28)
6.66	0	0	54.8 ± 0.8 g (2.84)
8.88	0	0	c
0	2.32	0	22.6 ± 0.6 b (1.26)
0	4.65	0	34.2 ± 0.8 e (1.52)
0	6.97	0	c
0	9.3	0	c
0	0	37.0	28.4 ± 1.0 c (1.36)
0	0	74.0	36.2 ± 0.8 f (1.42)
0	0	111.1	c
2.22	2.32	148.0	c
4.44	2.32	0	32.6 ± 0.7 d (1.02)
2.22	4.65	0	16.2 ± 0.4 a (1.0)
4.44	0	37.0	c
0	4.65	37.0	c
4.44	0	74.0	c

*20 replicates/treatment; repeated thrice.
c - callusing.
Mean having same letter in a column were not significantly different by Post-Hoc Multiple Comparison test P < 0.05 level.

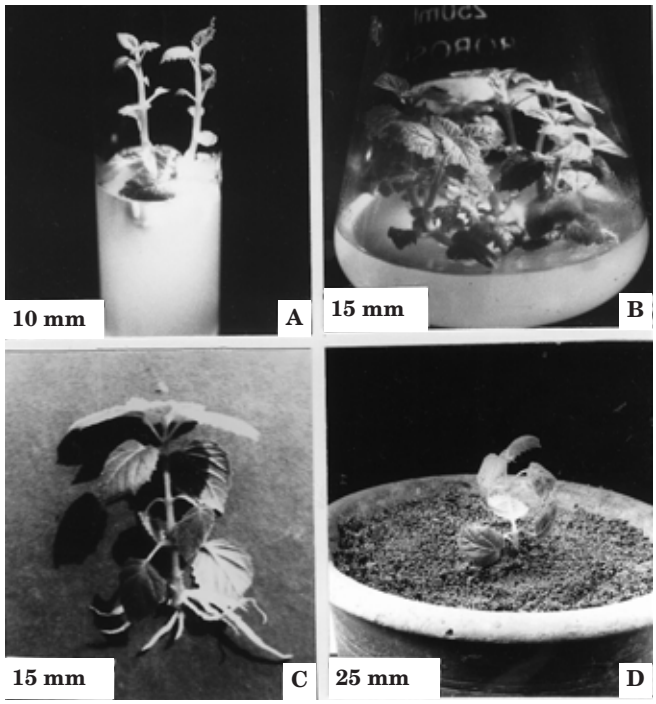

Figure 1 A–D. – Micropropagation of *Paulownia tomentosa.*
Figure 1A. – Proliferation of axillary bud of *Paulownia tomentosa* from a nodal explant cultured on MS medium supplemented with 4.44 µM BA after 4 week of culture. (Bar = 10 mm).
Figure 1B. – Formation of multiple shoots regenerated from axillary buds of a nodal explant cultured on MS medium supplemented with 4.44 µM BA + 0.53 µM NAA after 4 weeks of culture (Bar = 15 mm).
Figure 1C. – Rooting of microshoot on ½ strength MS basal medium supplemented with 0.60 µM IBA + 2% (w/v) sucrose after 2 weeks of culture (Bar = 15 mm).
Figure 1D. – *In vitro* raised plantlet transplanted in soil (Bar = 25 mm).

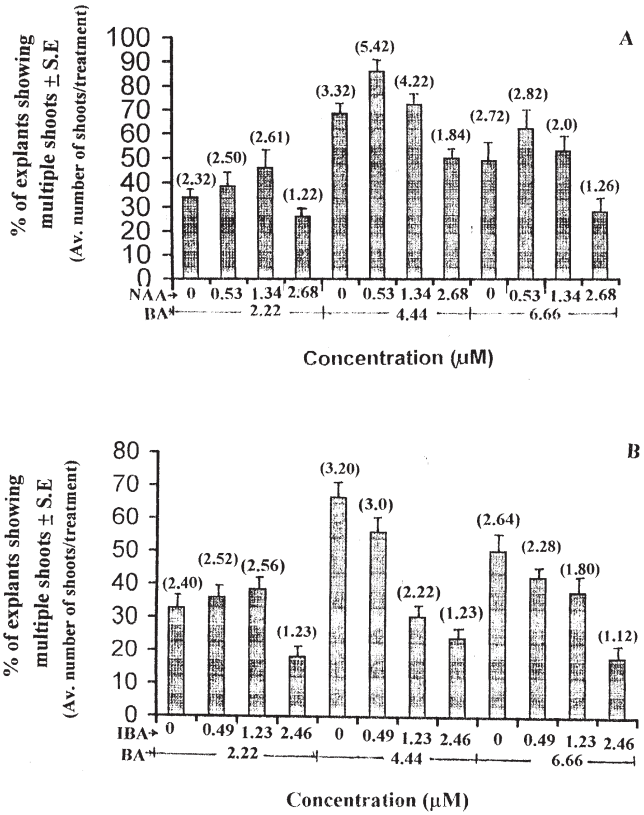

Figure 2 A–B. – Effect of BA in combination with NAA (A) and IBA (B) on shoot multiplication of *P. tomentosa* after 4 weeks of culture (20 replicates/treatment; repeated thrice).

did not rooted on culture medium without growth regulator. Of the three auxins tested, only IBA induced rooting on (0.30–0.60 µM) within 2 weeks of culture (*Table 2*). NAA and IAA promoted basal callusing with thin root hairs. Each of the shoot formed 2–3 roots in the culture having 0.60 µM IBA after 2 weeks of incubation (*Figure 1C*). At higher concentration of IBA (1.23 µM), the rooting was reduced and basal callus formation was observed. About 84.2% of the shoots were rooted on medium containing 0.60 µM IBA within 2 weeks of culture. These observations have been reported earlier in other tree species (IRIONDO et al., 1995; ROUT et al., 1995).

Table 2. – Effect of auxins on rooting of excised shoots of *P. tomentosa* within 2 weeks of culture.

Auxins	Concentration (µM)	Mean percentage of rooting +S.E*	Av. number of roots/shoo+ S.E*
0	0	0	0
IAA	0.35	0	0
	0.70	+	+
	1.42	+	+
IBA	0.30	68.5 ± 1.1	2.2 ± 0.6
	0.60	84.2 ± 1.2	3.2 ± 0.5
	1.23	50.6 ± 1.0 c	1.2 ± 0.4c
NAA	0.32	+	+
	0.65	+	+
	1.34	+	+

*20 replicates/treatment; repeated thrice.
c - callusing at the cut end.; + - basal callusing with root hairs.

Out of the twenty different primers tested, three primers (OPN – 08, OPA – 10 and OPA – 09) produced good amplification products that were monomorphic across all the micropropagated plants (*Figures 3A–C*). The size of the monomorphic fragments, produced by three primers ranged from 1.6 to 0.712 Kb for OPN – 08, 1.6 to 0.545 Kb for OPA – 10 and 2.0 to 0.300 Kb for OPN – 16. There were no polymorphic DNA fragments among the micropropagated plants as well as the mother plants. The pattern of monomorphic bands in the *in vitro* raised plants were reported earlier in other plants using different primers (ANGEL et al., 1996; KUMAR et al., 1999). RANI and RAINA (2000) reported that there was no variation in plants derived from meristem culture.

Rooted propagules were removed from culture vessel and washed throughly with tap water to remove the adhering gel, transplanted to 5 cm earthern containers on sterile pot-compost containing soil:sand: cow-dung (1:1:1) and kept under high humidity for acclimatization. About 60% of the rooted propagules were established in the greenhouse (*Figure 1D*).

Table 3. – Description of the micropropagation steps of *P. tomentosa*.

Step	Time (Weeks)	Medium
(1) *In vitro* bud proliferation	4	MS + 4.44 µM BA + 3% (w/v) sucrose.
(2) Multiplication and shoot multiplication	4	MS + 4.44 µM BA + 0.49 µM NAA + + 3% (w/v) sucrose.
(3) *In vitro* rooting	2	½ MS + 0.60 µM IBA + 2% (w/v) sucrose
(4) Plantlet establishment	4	Sand : soil : cow-dung (1:1:1)

In conclusion, an attempt was made in this investigation in order to develop a rapid clonal propagation of *Paulownia tomentosa*, a multipurpose fast growing tree species by manipulating the cytokinin and auxin. The mass propagation protocol was presented in *Table 3*. Though BA alone promoted in bud break and growth, the presence of NAA promoted higher

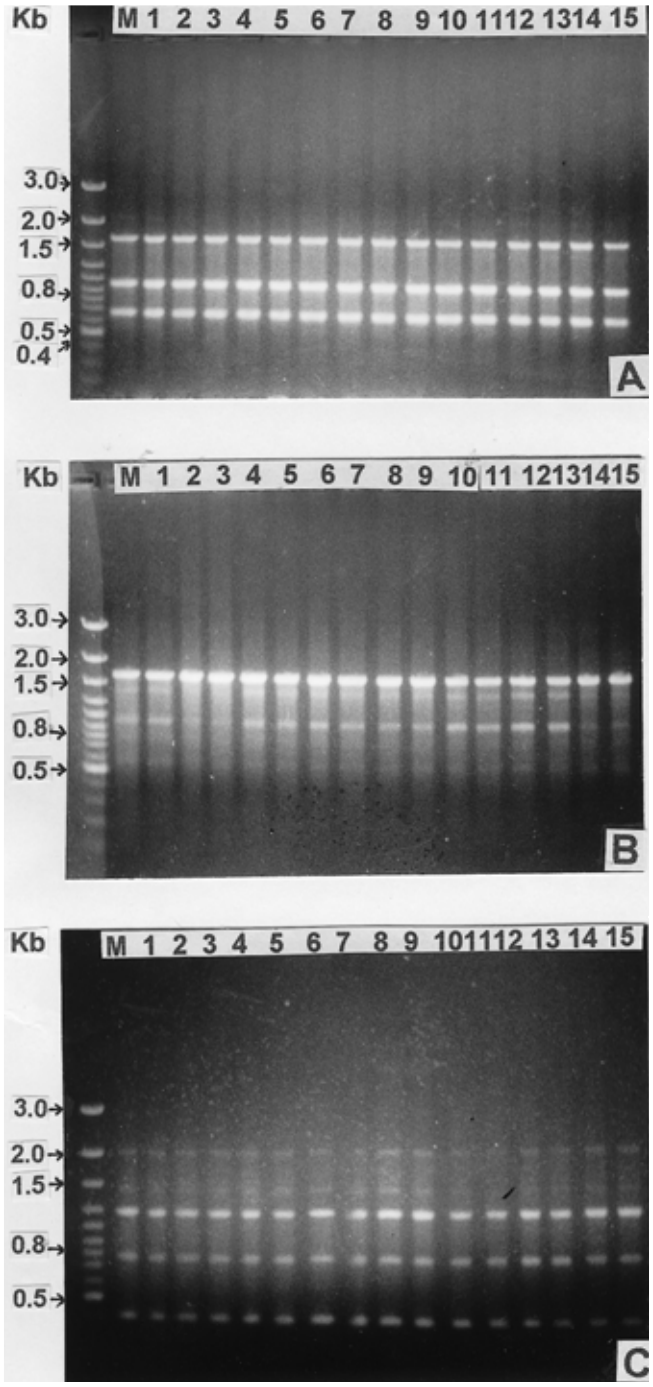

Figure 3A–C. – RAPD profiles of micropropagated plants from meristems of *Paulownia tomentosa* using primers (A) OPN – 08 (5'-ACCTCAGCTC -3'), (B) OPA – 10 (5'-GTGATCGCAG-3') and (C) OPN – 16 (5'-AAGCGACCTG -3'). Lane – M shows RAPD bands from the greenhouse grown mother plant. Arrow indicates the size of the marker. Lane 1 – 15 show RAPD products from micropropagated plants.

frequency of shoot multiplication. The RAPD analysis indicated that the plants raised directly from the meristems were genetically similar with the mother plants. This protocol might be helpful in the clonal production of other forestry species as well as conservation.

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Genetic Characters and Diameter Growth of Provenances of Scots Pine (*Pinus sylvestris* L.)

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Abstract

Correlation between genetic characters and growth parameters was studied with respect to four provenance samples, which are part of a 47-year-old provenance trial of Scots pine. Two samples with superior growth were contrasted with two weakly growing samples. Based on a sample size of 100 trees per provenance, genotypes were monitored at 16 enzyme coding gene loci. Effects between genetic and growth traits were studied by means of two-factorial analysis of variance and linear regression models with respect to diameter classes and subsets of elite and non-elite trees within provenance samples as well as diameter subsets of pooled provenance samples.

Significant deviations are evident among the genetic structures of the four provenance samples. In each sample, deviations from HARDY-WEINBERG structures are indicated at single loci. Inbreeding does not primarily account for such deviations. The study of diameter classes reveals that an increase of stem volume tends to coincide with an increase of the observed heterozygosities in three out of four provenance samples. In the case of two-locus genotypes at 6-PGDH and MDH-C, significant effects of heterozygosity on diameter growth are observed. By focusing the two most frequent alleles at six differentiation effective gene loci, significant relations to diameter growth can be particularly verified for two locus combinations including AAT-A. The genetic comparison of the elite and non-elite trees, the first subset reveals larger values for diversities, differentiation and heterozygosities than the second does. The genetic comparison between the subset of the 50 thinnest and the 50 thickest trees among the 400 individuals indicates higher values for the genetic multiplicity and the gene pool and multilocus diversity for the subsets with superior growth. It is concluded for the present that isoenzyme gene markers reveal an indicative potential for quantitative traits.

Key words: Pinus sylvestris, provenances, isoenzyme gene markers, genetic variation, differentiation, diameter growth.

Introduction

Scots pine (*Pinus sylvestris* L.) covers a climatically divers continuous habitat, which ranges from Central and Northern Europe to Russia and Northern-Central Asia. Additional parts of the habitat are isolated, for instance the Pyrenees, Scotland and Northern Turkey. The vertical dimension of the habitat is extraordinary large: Scots pine is a significant carrier species of various ecosystems from low elevations up to the mountainous regions and in some cases also the sub-alpine vegetation zones.

A great variety with respect to morphological traits, especially habitus and growth, is evident throughout the habitat of Scots pine. The idea of a structuration of the Scots pine habitat into provenances goes back to the 16th century (HAUSRATH, 1982).

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